Time-lapse technology in the IVF laboratory

Assessing safety and human embryo development

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Cover illustration: Time-lapse documentation of human embryos

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To Daniel, Eric and Filippa

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ABSTRACT

Background: Time-lapse monitoring of human embryos is becoming increasingly utilized in clinical IVF laboratories. This technology allows for uninterrupted, continuous observation of embryo development without having to remove embryos from the controlled environment inside the incubator. Additional information about embryo development can be obtained and combined with traditional morphological evaluations. However, few randomized controlled trials have been performed investigating the efficacy and safety of closed culture systems utilizing time-lapse technology.

Aim: To investigate in a randomized controlled trial (RCT) if the number of good quality embryos (GQEs) derived from culture in a closed system (the EmbryoScope^{TM}) was superior compared to culture in a conventional culture system. A further aim was to investigate if one or more morphokinetic variables could predict live birth after day 2 transfer, when analysed in combination with conventional morphology and patient variables.

Materials and Methods: A total of 364 patients were randomized to culture until day 2 in either the EmbryoScope (n=240) or in a conventional incubator (n=124) at atmospheric O_2 and 6% CO_2. Only first cycle patients treated with ICSI (intracytoplasmic sperm injection) were included. In paper I the mean number of GQEs in each group was the primary endpoint. In paper II, time-lapse images of 207 transferred embryos from patients achieving the same number of live born children as transferred embryos, or no live birth, were analysed by logistic regression to determine predictors of live birth among morphological-, morphokinetic- and patient variables.

Results: In Paper I, no significant difference was found in the mean ± SD number of GQEs between the groups cultured for two days in a closed (n=240), compared to a conventional (n=124) culture system (2.41±2.27 vs. 2.19±1.82, p=0.34). In Paper II, early cleavage and fragmentation grade were the only variables that independently

could predict live birth (OR 4.84 (95% CI: 2.14-10.96) p=0.0002) and (OR 0.46 (95% CI: 0.25-0.84) p=0.012), respectively), early cleavage as a positive predictor and fragmentation grade as a negative predictor of live birth. No morphokinetic variables were independently predictive of live birth.
Limitations: The primary outcome of the RCT, number of GQEs on day 2, was a surrogate variable for live birth (paper I). In addition, only ICSI patients were included, and different culture dishes for the time-lapse incubator and the conventional incubator were used.
Conclusion: No benefit was found for the time-lapse system over the conventional system, with regards to the number of GQEs on day 2. None of the included morphokinetic variables were predictive of live birth.

Keywords: IVF, embryo, time-lapse, morphokinetics, morphology, live-birth, embryo selection

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SAMMANFATTNING PÅ SVENSKA

Under de drygt 40 år sedan IVF introducerades har det skett en snabb utveckling inom området; till exempel har introduktion av ICSI (intracytoplasmatisk spermieinjektion), utveckling av förbättrade odlingsmedier och utveckling av nya, mer effektiva frysmetoder för ägg och befruktade embryon lett till både högre födelsefrekvenser och utökade behandlingsmöjligheter för fler patientgrupper. Även på tekniksidan har mycket hänt, bland annat har nya och mer effektiva inkubatorer för odling av embryon tagits fram. Ett exempel är den så kallade time-lapse inkubatorn.

Time-lapse tekniken går numera att återfinna på allt fler IVF-kliniker runtom i världen och har möjliggjort kontinuerlig monitorering och bedömning av embryon under odlingstiden utan att behöva ta ut dem ur inkubatorn. Embryonerna fotograferas automatiskt med ett par minuters mellanrum av en inbyggd kamera, och med hjälp av tillhörande mjukvaruprogram kan embryonerna dokumenteras och analyseras direkt i realtid, och även i form av en film som visas på en datorskärm. En ytterligare fördel med en sluten time-lapse inkubator där man inte behöver ta ut embryonerna under odlingstiden för analys, är att de är skyddade från miljöpåverkan utifrån, framför allt avseende pH och temperatur.

I denna avhandling undersökte vi om andelen embryon av god kvalitet (”good-quality embryos”) skiljde sig efter två dagars odling i antingen en vanlig inkubator utan time-lapse teknologi, jämfört med i en sluten inkubator med ett inbyggt time-lapse system. Resultaten visade att det inte fanns någon statistisk skillnad i antalet embryon av god kvalitet som erhölls då embryon odlades under två dagar i vanlig inkubator jämfört med sluten odling i time-lapse inkubatorn (2.19±1.82 och 2.41± 2.27, p=0.34).

I den andra delen av studien analyserades filmer från odlingsperioden för de embryon som odlats i time-lapse inkubatorn och där det var känt om embryot gett upphov till graviditet eller ej. Vi fann att de variabler som hade störst påverkan på sannolikheten att ge upphov till ett levande fött barn, var om embryot delade sig tidigt till två celler (OR 4.84 (95% CI: 2.14-10.96), p=0.0002) och hur mycket embryot hade fragmenterat på dag 2 (OR 0.46 (95% CI: 0.25-0.84) p=0.012).

Slutsats: Efter två dagar fann vi ingen signifikant skillnad mellan andelen embryon av god kvalitet efter odling i en vanlig inkubator jämfört med i en sluten time-lapse inkubator. Det är möjligt att två dagars odling är för kort tid för att kunna påvisa någon fördel med den mer konstanta odlingsmiljön inuti en time-lapse inkubator. Vi drar även slutsatsen att traditionell morfologisk bedömning av tidig delning och fragmenteringsgrad på dag 2 bör tas hänsyn till vid selektion av embryon som odlats upp till 2 dagar efter befruktning.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Park H, Bergh C, Selleskog U, Thurin-Kjellberg A, Lundin K.**
No benefit of culturing embryos in a closed system compared with a conventional incubator in terms of number of good quality embryos: results from an RCT. Human Reproduction 2015 Feb;30(2):268-75

II. **Ahlström A, Park H, Bergh C, Selleskog U, Lundin K.**
Conventional morphology performs better than morphokinetics for prediction of live birth after day 2 transfer. Reproductive BioMedicine Online (2016) 33, 61-70

CONTENTS

ABBREVIATIONS

AI	Artificial intelligence
ART	Assisted reproductive technology
AUC	Area under the curve
CGH	Comparative genomic hybridization
CI	Confidence interval
DC	Direct cleavage (also known as Rapid cleavage)
DET	Double embryo transfer
eSET	Elective single embryo transfer
FET	Frozen embryo transfer
FISH	Fluorescence in situ hybridization
FSH	Follicle stimulating hormone
GEE	Generalized estimation equations
GnRH	Gonadotrophin releasing hormone
GQE	Good quality embryo
hCG	Human chorionic gonadotropin
ICM	Inner cell mass
ICSI	Intracytoplasmic sperm injection
ITT	Intention to treat
IVF	*In vitro* fertilization
KID	Known implantation data
LH	Luteinizing hormone
MOPS	3-(N-morpholino) propanesulfonic acid
NGS	Next generation sequencing
OR	Odds ratio
PGT-A	Pre-implantation genetic testing for aneuploidies
PGT-M	Pre-implantation genetic testing for monogenic/single gene disorders
PGT-SR	Pre-implantation genetic testing for chromosomal structural rearrangements

PN	Pronuclei
RCT	Randomized controlled trial
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SET	Single embryo transfer
TE	Trophectoderm
TLI	Time-lapse imaging
ZP	Zona pellucida

1 INTRODUCTION

In 1978, following the pioneering work of Edwards and Steptoe, the world´s first baby after *in vitro* fertilization (IVF) was born (Steptoe and Edwards 1978). Since then, IVF has become available worldwide and today it is estimated that more than 9 million babies have been born through IVF (ESHRE ART fact sheet 2020). The development and refinements of techniques such as intracytoplasmic sperm injection (ICSI) and cryopreservation of embryos and gametes has led to improvement of success rates and also made IVF accessible to additional groups such as same-gender couples and single women, as well as for fertility preservation. However, assisted reproductive technology (ART) is still a relatively new and fast developing field of medicine and it is important that continuous follow-up studies are performed regarding efficacy and safety of different treatments, to keep track of potential health risks.

A well-functioning and quality-controlled laboratory is crucial for success. An IVF laboratory must be able to maintain a stable and efficient environment for embryo culture and to select, from a cohort, the embryo(s) that will have the highest probability to give rise to implantation and live birth. All new techniques should be properly validated, preferentially by prospective randomized controlled trials (RCTs), before being introduced in a clinical setting. This is time-consuming and expensive, albeit an important process in order to assure safety, efficacy and reproducibility (Harper et al. 2012; Provoost et al. 2014).

1.1 IN VITRO FERTILIZATION AND BIRTH RATES

In Sweden, almost 20 000 IVF treatments (12 500 fresh cycles and 7 500 frozen-thawed transfers) are performed each year. In 2017, the delivery rate per fresh ET was 29%. Eighty-four percent of all fresh transfers and 98% of all frozen embryo transfers (FET) were single embryo transfers (SET) (Q-IVF Årsrapport 2019) (data from 2017).

Although the transfer of multiple embryos increases the chance of becoming pregnant it also elevates the risks associated with multiple gestations. With the increased efficiency of IVF in the 1980s and 90s, rates of multiple gestations increased. In a large retrospective cohort study, Bergh et al. (1999) analysed the obstetric outcomes of babies born through ART (n=5856) and those spontaneously

conceived (n=1 505724) in Sweden between 1982 and 1995 (Bergh et al. 1999). In the ART group, multiple births occurred in 27% of the pregnancies compared to 1% in the general population. Multiple births were associated with an increased incidence of prematurity in the IVF group compared to the general population (<37 weeks, 30.3% vs. 6.3%) and low birth weight (<2500g, 27.4% vs. 4.6%) respectively. The perinatal mortality was 1.9% in the *in-vitro* fertilization group and 1.1% in the control group. Of the singletons born after IVF, 11.2% were born before week 37, the corresponding figure for the general population was 5.4%. Singleton IVF babies with low birth weight <2500g was shown to be 9.0%, compared to 3.6% of the general population. For IVF singleton babies, the risk ratios, adjusted for year of birth, for very preterm birth (<32 weeks) and very low birthweight (<1500g) were 3.54 (95% CI: 2.90-4.32) and 4.39 (95% CI: 3.62-5.32), respectively. These findings were further supported by Pinborg et al. (2004) in a Danish registry study analysing data from 8 602 babies born between 1995 and 2000 in Denmark (Pinborg et al. 2004). In a large RCT from the Nordic countries, including 661 randomized patients, it was shown that elective single embryo transfer (eSET) in combination with a frozen-thawed SET resulted in live birth rates that were not substantially lower as compared to double embryo transfer (38.8% vs. 42.9%, difference 4.1%, 95% CI: -3.4% to 11.6%). Furthermore, the multiple birth rates decreased dramatically (0.8% for the single embryo transfer group versus 33.1% for the double embryo transfer group, p<0.001) (Thurin et al. 2004). The further implementation of SET has reduced perinatal risks for children conceived by ART (Henningsen et al. 2015).

Traditionally, the success rates during an ART treatment are reported as pregnancy per started IVF cycle or pregnancy per embryo transfer. However, cumulative pregnancy and live-birth per oocyte pick-up or calculated per an entire treatment period and including both fresh and frozen transfers is becoming a more attractive alternative to estimate successful IVF outcome (Olivius et al. 2002; Tiitinen et al. 2004; Lundin and Bergh 2007; Malizia et al. 2009; Malchau et al. 2017; Malchau et al. 2019).

1.2 HANDLING AND CULTURE OF HUMAN EMBRYOS

In vitro culture of human embryos aims to maintain an environment that supports development by mimicking their natural environment as much as possible. However, the handling of embryos such as transferring them from one dish to another, assessing them under the microscope, as well as freezing and thawing, all traditionally involve

removing the embryo from the incubator, potentially causing physiological stress that may compromise development (Swain et al. 2016; Wale and Gardner 2016).

Changes in key parameters such as pH, temperature and oxygen levels, may affect embryo development. It is therefore important to monitor these parameters at regular intervals and to keep the handling time of embryos outside the incubator to a minimum. The introduction of blastocyst culture where the embryos spend an even longer time *in vitro*, poses additional challenges.

1.2.1 THE INCUBATOR

One of the most important pieces of equipment in the IVF laboratory is the incubator; if maintained correctly, it provides a stable and safe environment for the embryos. It regulates gas concentrations (pH, oxygen tension), temperature and humidity; however every opening of the incubator door disrupts the environment for a shorter or longer time (Swain 2014).

Temperature

Control of temperature is crucial during embryo culture. Studies using polarized light microscopy have found that altering the culture temperature affects the stability of the meiotic spindle of preimplantation embryos (Wang et al. 2001; Eichenlaub-Ritter et al. 2002; Wang et al. 2002; Sun et al. 2004) which in turn may have an impact on cell cleavage.

While the optimal temperature for embryo culture is still unknown, it is recommended to culture and handle human embryos at 37°C (De los Santos et al. 2016). Some studies have indicated that culturing embryos closer to 36 °C could improve embryo quality (for example, (Leese et al. 2008)). However, in a RCT from 2014, Hong et al. (2014) showed that culture of human embryos at 36°C compared to 37°C led to a reduced mean number of blastomeres on day 3 (7.0±0.1 vs. 7.7±0.1, respectively, p=0.0001) and a lower rate of good quality blastocysts (41.2% vs. 48.4%, respectively, p=0.03) (Hong et al. 2014). Furthermore, Fawzy et al. (2018) found in an RCT that culture at 36.5°C, despite a significantly higher cleavage rate, resulted in a lower fertilization rate, fewer good quality embryos on day 3, lower blastocyst formation rate on day 5, and fewer total numbers of good quality blastocysts compared with culture at 37°C (Fawzy et al. 2018).

pH

The pH of a cell is important for regulating cell division, protein synthesis, membrane transport, cell communication and other cellular processes (see eg. review by Bavister et al. (1995) (Bavister 1995)). In the laboratory, pH levels are determined by the carbon dioxide (CO_2) concentration inside the incubator and the bicarbonate (HCO_3-) concentration of the culture media. The optimal pH for embryo culture is not specifically known but ranges between 7.0-7.4; fluctuations in both intracellular and extracellular pH outside this range have been shown to have a negative impact on embryo development (Dale et al. 1998; Zander-Fox et al. 2010; Hentemann et al. 2011). Denuded oocytes and cryopreserved/thawed embryos appear to be particularly sensitive to fluctuations in extracellular pH (Dale et al. 1998; Lane et al. 1999; Lane et al. 2000; Swain 2012).

Oxygen

Despite the fact that it has long been known that the oxygen concentration in the mammalian oviduct of different species ranges between 2 and 8% (Fischer and Bavister 1993) and that a high oxygen concentration may promote the generation of reactive oxygen radicals, eg. see Catt and Henman (2000) (Catt and Henman 2000), embryos have traditionally been cultured at an atmospheric oxygen concentration, i.e. approximately 20%. It has been shown that culture at atmospheric oxygen levels has a negative impact on the transcriptome (Rinaudo et al. 2006), the proteome (Katz-Jaffe et al. 2005) and the epigenome (Gaspar et al. 2015; Ghosh et al. 2017; Skiles et al. 2018) of the developing embryo. In fact, in some studies even brief exposure to atmospheric oxygen concentrations during the culture of pronucleate mouse oocytes was shown to delay development to the morula stage (Pabon et al. 1989), resulting in a decrease in blastocyst cell number (Karagenc et al. 2004).

For human embryos, Dumoulin et al. (1999), found no difference in pregnancy and implantation rate for embryos cultured until day 2 and 3 in 20% or 5% oxygen concentration. However, when the embryos were cultured for longer time-periods, up to the blastocyst stage, culture at low oxygen seemed to be beneficial, resulting in a higher rate of blastocysts as well as blastocysts with a higher number of cells (Dumoulin et al. 1999). In more recent studies, culture at low oxygen concentration (approximately 5%) has indicated increased clinical pregnancy rates and birth rates (Kovacic and Vlaisavljevic 2008; Meintjes et al. 2009; Waldenstrom et al. 2009; Kasterstein et al. 2013) and in a meta-analysis by Bontekoe et al. 2012, the difference was found to be significant for blastocyst transfers but not for early transfers, which

is in line with the findings by Dumoulin et al. (1999) (Dumoulin et al. 1999; Bontekoe et al. 2012).
Knowledge obtained from these studies, together with the introduction of long-term (blastocyst) culture, has resulted in the recommendation to culture embryos at reduced oxygen levels (De los Santos et al. 2016).

Light

During conventional culture conditions, gametes and embryos are exposed to light of different wavelengths both from the surroundings (when outside of the incubator) and from the microscope (when assessing gametes/embryos). Embryos may be sensitive to light, especially short wavelength light (Hirao and Yanagimachi 1978), probably due to the generation of reactive oxygen species (ROS), which are harmful to embryos (Goto et al. 1993). Schumacher and Fischer (1988) showed, in rabbits, that the pre-compacted embryo is more sensitive to direct light than the post-compacted embryo (Schumacher and Fischer 1988). Oh et al. (2007) showed that hamster 2-cell embryos exposed to red light (620-750nm) were more likely to develop into blastocysts compared to embryos exposed to blue light (445-500nm) (Oh et al. 2007). In the ART laboratory, effects from blue and near-blue light, which have shown to be the most harmful, can be minimized by limiting the time spent of the embryo outside of the incubator, and by using microscope filters.

1.2.2 CULTURE MEDIA AND EMBRYO DEVELOPMENT

The role of culture media includes being able to support the growth of embryos by providing the nutrients required and to mimic the natural environment found in the oviduct and uterus. As early as 1956, it was shown that Krebs-Ringer bicarbonate solution media supplemented with glucose, antibiotics and bovine serum albumin could support the development of 2- and 8-cell mouse embryos to the blastocyst stage (Whitten 1956). This medium was later used when live birth was obtained in mice (McLaren and Biggers 1958). In 1959, Chang´s experiments in rabbits resulted in live births following IVF and embryo transfer (Chang 1959). However, in humans, the early culture media could not successfully support blastocyst development and therefore, short *in vitro* culture (1-3 days), became the standard. For many years IVF laboratories produced their own media, or purchased ”basic” cell culture media and supplemented it with the patient´s own serum (Chronopoulou and Harper 2015). At the time of the birth of the first IVF baby, Steptoe and Edwards were using Earle´s simple salt solution with pyruvate supplemented with the patient´s serum, (Steptoe and Edwards 1978).

Single- and sequential media

Today, most clinics use highly complex media from biotechnology companies. Increased knowledge about embryo metabolism and consumption of media constituents, and how these differ in a pre-compacted versus a post-compacted embryo, has made it possible to develop media supporting growth up to the blastocyst stage (Gardner et al. 2002). Media used for embryo culture can be categorized as one of two types: sequential or single. Their basic compositions are similar, consisting of pyruvate, glucose, lactate and amino acids (Summers and Biggers 2003; Machtinger and Racowsky 2012) but differ in how they are used. When using sequential media the embryo is cultured in the first medium until day 2 or 3 and then transferred to a second medium of modified composition and kept there until blastocyst stage (Gardner and Lane 1997; Gardner 1998; Pool 2002). This is intended to mimic the changing environment *in vivo* when the fertilized and cleaving embryo is transported through the fallopian tube to the uterus. Thus, sequential media is based on the idea that the embryo needs to be supplied with different nutrients during the different times of its development. Single media, on the other hand, is based on the concept that the embryo can be supplied directly with all that it needs for the first 5-6 days, and that the embryo itself chooses what it needs at a certain time (Biggers and Summers 2008; Machtinger and Racowsky 2012). It is of importance that single media used during long-term culture can provide sufficient nutrients to last throughout the *in vitro* culture period of the embryo (Gardner and Lane 1997; Biggers and Summers 2008; Machtinger and Racowsky 2012; Wale and Gardner 2016). It should also minimize ammonium build-up, which may have a negative effect on blastocyst formation (Virant-Klun et al. 2006) and embryonic gene expression (Gardner et al. 2013).

In a recent systematic review of randomized controlled trials comparing sequential- and single media for blastocyst culture, no difference was found in ongoing pregnancy- (RR: 0.9, 95 % CI: 0.7-1.3, two studies including 246 women), clinical pregnancy- per randomized woman (RR: 1.0, 95 % CI: 0.7-1.4, one study including 100 women) or miscarriage rate per clinical pregnancy (RR: 1.3, 95% CI: 0.4-4.3, two studies including 246 participants) (Sfontouris et al. 2016). This was supported by a meta-analysis performed in 2017 (Dieamant et al. 2017). Both types of media are currently in use, and there is no evidence for higher success rates with either method. However, with the introduction of closed culture of embryos in a time-lapse setting, single media has gained popularity, allowing embryos to remain in culture without interruption for change of media, as would be the case in a sequential media system (Hardarson et al. 2015).

Embryo culture *in vitro*

After the oocytes have been harvested, they are fertilized by either conventional IVF or ICSI and then cultured in an incubator with a strictly controlled environment until embryo transfer and/or cryopreservation.

The formation of one maternal and one paternal pronucleus (PN) indicates that correct fertilization has taken place. At approximately 25-27 hours after fertilization *in vitro*, the oocyte/zygote will enter the first mitotic division which results in two cells (blastomeres). Blastomere division continues and, optimally, compaction takes place four days later, forming cell junctions between the blastomeres. Next, a cavity is formed and the embryo is now known as a blastocyst (Fig 1). In ideal circumstances, this blastocyst will eventually hatch out of its protective zona pellucida (ZP) and implant into the endometrium of the uterus (Magli et al. 2012; Lubis and Halim 2018).

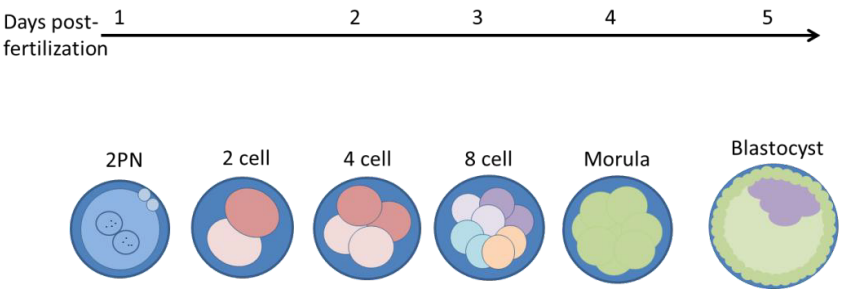


Fig 1. *Optimal timings of in vitro embryo development from 2PN to blastocyst stage (according to Alpha Scientists in reproductive Medicine and ESHRE Special Interest Group Embryology, Istanbul 2011).*

It was shown early *in vitro*, that an embryo that reaches different stages of development at specific times, has an increased chance to implant and result in a pregnancy, compared to an embryo which might develop faster or slower (Giorgetti et al. 1995; Ziebe et al. 1997; Van Royen et al. 1999). During the period of this thesis, at the IVF laboratory at Sahlgrenska University Hospital in Gothenburg, the embryos were mainly cultured until day 2 and were mainly at the 4-cell stage when transferred to the patient. However, blastocyst culture, where the embryo is cultured to blastocyst stage (5-6 days after oocyte retrieval), is now commonly practiced.

Long term culture

With the introduction of more complex culture media, successful long-term culture to the blastocyst stage has increasingly been introduced in IVF laboratories. Historically, *in vitro* culture environments have been considered suboptimal for supporting long-term growth, and the embryo should be returned to its natural *in vivo* environment as soon as possible. Advocates of blastocyst culture argue that the blastocyst better represents the true developmental stage of the *in vivo* embryo when replaced in the uterus, thus leading to a better synchronization between the endometrium and the embryo. Furthermore, it is argued that blastocyst culture allows for the selection of the most viable embryos, ultimately resulting in higher implantation rates (Jones et al. 1998).

There is however the concern of an increase in cancelled cycles where no embryos have developed into blastocysts on the day of transfer (Papanikolaou et al. 2008), explaining why many clinics still choose to transfer at the cleavage stage if only a few number of embryos are available. Potential epigenetic changes arising due to long term *in vitro* culture have also been mentioned as a cause for concern (Maheshwari et al. 2016).

A Cochrane meta-analysis including 27 RCTs (where 13 RCTs reported live birth rate) showed a significant increase in live birth rates after fresh transfer with blastocysts (n=1360 women, OR=1.48, 95% CI: 1.20-1.82) compared to cleavage stage embryos (Glujovsky et al. 2016). Although it was shown that the blastocyst transfer groups had lower rates of embryos cryopreserved per treatment, there was no clear evidence of a difference in cumulative pregnancy rate (fresh and frozen-thawed cycle transfers). It was suggested that the added benefit of a higher cryopreservation rate in the cleavage stage group might cancel out the higher implantation rates of the fresh day 5 to 6 transfers. However, the findings could also be due to the differences in freezing methods, as the only study using vitrification showed an increased cumulative pregnancy rate for the blastocyst transfer group (Glujovsky et al. 2016). A retrospective study by De Vos et al. (2016) compared cumulative results from 377 day 3 fresh + frozen-thawed transfers with 623 day 5 fresh + frozen-thawed transfers. No differences in cumulative live birth rates were found, although the day 3 strategy required higher numbers of transfers in total before reaching a live birth (De Vos et al. 2016).

1.3 EMBRYO SELECTION

Using standardized morphological scoring systems in accordance with the Istanbul consensus (Alpha and ESHRE 2011), it is estimated that between 25-35% of embryos transferred at the cleavage stage implant, while for blastocyst stage transfer the figure is estimated to be up to 60% (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine 2017). A majority of the non-implanting embryos would presumably have been classified as being of morphologically good quality, indicating that one reason for failed implantation may be that a high degree of embryos displaying normal morphology are in fact chromosomally and/or metabolically abnormal (Turner et al. 1994; Magli et al. 2001; Ziebe et al. 2003; Marquard et al. 2010; Alfarawati et al. 2011; Lundin and Ahlstrom 2015; Thompson et al. 2016). Transfer of chromosomally compromised embryos might lead to either failed implantation or miscarriage (Marquard et al. 2010). Although the rate of chromosomal errors decreases during embryo development, around 50% of blastocysts graded as being of good quality have been shown to have chromosomal errors (Alfarawati et al. 2011; Fragouli et al. 2011; Capalbo et al. 2014; Fragouli et al. 2014). Chromosomal abnormalities are highly correlated to maternal age, with younger women having a lower aneuploidy rate both for cleavage stage embryos and blastocysts compared with older women (Fragouli et al. 2014; Minasi et al. 2016; La Marca et al. 2017). It has been shown that morphologically good quality blastocysts are more often euploid compared to those graded as being of lower quality (Capalbo et al. 2014; Wang et al. 2018; Yoshida et al. 2018). Furthermore, La Marca et al. (2017) showed that levels of serum AMH had a positive, statistically significant, correlation to the rate of euploid blastocysts in a patient cohort (La Marca et al. 2017).

With single embryo transfer increasingly becoming the golden standard to reduce the risk of multiple pregnancies, more reliable methods to identify the embryos most suitable for transfer are needed. Clearly, there is room for improvement regarding embryo selection.

1.3.1 NON-INVASIVE EMBRYO SELECTION

The concept of non-invasive embryo selection is that the embryo itself is not manipulated. This term mainly implies assessment of embryo morphology and cleavage rates, and more recently includes the use of time-lapse documentation. However attempts to include analysis of the surrounding culture media have also been investigated but none so far successfully implemented eg. see (Lundin and Ahlstrom 2015).

Traditional morphological grading

The assessment of embryos for transfer and cryopreservation is mainly based on scoring of morphological criteria by visualization through light microscopy. Time of culture varies between laboratories, being dependent upon the day of transfer, which ranges from day 2 post-insemination up to the blastocyst stage, and on time of cryopreservation (up to 6 days in culture). Early stage embryos are assessed mainly by cell numbers and cell size, grade of fragmentation and number of nuclei within each blastomere. The blastocyst stage is graded according to the expansion grade, the appearance of the inner cell mass (ICM) and the trophectoderm (TE) cells (Alpha and ESHRE 2011).

Many years of traditional morphological grading has resulted in a substantial amount of knowledge regarding the behaviour and appearance of the human embryo when cultured *in vitro*. It has been shown that embryos with unevenly sized blastomeres result in lower implantation and pregnancy rates (Hardarson et al. 2001), that embryos displaying ≤20% fragmentation have a higher pregnancy rate and that embryos with 4 cells on day 2 implant more frequently than those with fewer or more cells (Giorgetti et al. 1995; Ziebe et al. 1997; Thurin et al. 2005). Early cleavage has been shown to predict embryo quality, implantation frequency and birth rate (Lundin et al. 2001; Salumets et al. 2003; Rienzi et al. 2005). In addition, early cleaving embryos cleave more evenly which has been shown to correlate with a lower incidence of chromosomal errors (Hardarson et al. 2001).

Prediction models have been developed to rank embryos according to implantation potential (Steer et al. 1992; Giorgetti et al. 1995; Desai et al. 2000; Van Royen et al. 2001; Sjoblom et al. 2006; Holte et al. 2007; Rhenman et al. 2015). Using these models, cleavage rate, fragmentation, presence or absence of multinucleation, uniformity in blastomere size and symmetry of cleavage, have all been shown to have prognostic value regarding pregnancy-, implantation- and/or live birth rate. However, variations in scoring models makes it difficult to perform comparisons between clinics. In addition, the many variations in protocols for scoring embryo morphology as well as the subjective nature of embryo scoring makes this process prone to inter- and intra-observer variability (Arce et al. 2006; Baxter Bendus et al. 2006; Paternot et al. 2009; Paternot et al. 2011; Storr et al. 2017; Martinez-Granados et al. 2018). In order to harmonize embryo assessments and allow for benchmarking between clinics, Alpha and ESHRE have in collaboration proposed a standardization of how to grade embryos, including the timing of observations (Table 1) (Alpha and ESHRE 2011). Furthermore, many laboratories participate in external quality control

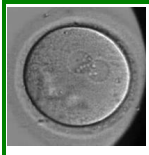
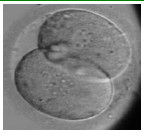
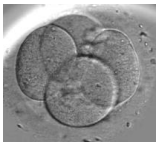
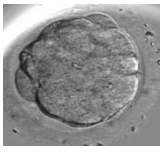
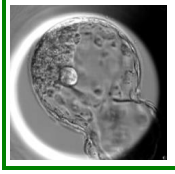
programs for embryo evaluation, which has improved inter-observational agreement (Arce et al. 2006; Paternot et al. 2009; Castilla et al. 2010).

Embryo metabolism

Despite being able to identify embryos with good morphology, normal development, and with euploid status, we still have very little knowledge of the metabolic status of the embryo. In 2000, the Human Genome Project was completed (Venter et al. 2001), and with that, several new fields in molecular biology developed, collectively known as the "Omics". The term includes the study of genes (genomics), epigenetics (epigenomics), gene expression (transcriptomics), proteins (proteomics) and metabolites (metabolomics) (Egea et al. 2014; Lundin and Ahlstrom 2015). Research is currently being conducted applying these new techniques in order to identify novel biomarkers secreted or taken up by the embryo, for prediction of early embryo development and for embryo implantation. However, none of these methods have yet been clinically applied or validated (Lundin and Ahlstrom 2015; Thompson et al. 2016).

Recently it has been reported that the level of mitochondrial DNA (mtDNA) in the embryo negatively correlates to the implantation rate, possibly indicating that embryos with a higher mtDNA content are under metabolic stress (Diez-Juan et al. 2015; Ravichandran et al. 2017). The measurement of mitochondrial concentrations could be a possible future candidate for selection of the embryo with the highest potential for implantation and live birth.

Table 1. *Proposed timings of observations during embryo culture according to the Istanbul consensus (Alpha and ESHRE 2011).*

Embryo developmental stage	Action	Time post-insemination
	**Check for fertilization** *two pronuclei and two polar bodies should be visible*	17±1h
	**Check for early cleavage** *division to two blastomeres containing one nuclei each*	26±1 h post ICSI, 28±1 h post IVF
	**Day 2 embryo assessment** *the embryo should preferably consist of 4 blastomeres, each containing a single nuclei*	44±1 h
	**Day 3 embryo assessment** *the embryo should preferably consist of 8 blastomeres each containing a single nuclei*	68±1 h
	**Day 4 embryo assessment** ***c**ompaction, morula stage. All blastomeres are involved in the compaction process*	92±2 h
	**Day 5 embryo assessment** *blastocyst development. Expansion has occurred and an ICM and TE can be distinguished*	116±2 h Can be used for fresh transfer or cryopreservation
 *	**Day 6 embryo assessment** *blastocyst development. Expansion has occurred and an ICM and TE can be distinguished*	139±2 h Can be used for cryopreservation and subsequent transfer on day 5 post LH surge

* *Day 6 grading is not included in the ESHRE Alpha recommendations, but is performed at the above time in our laboratory.*

Time-lapse imaging

When culturing embryos in conventional incubators, the culture environment (pH, temperature) is disrupted each time the embryo is removed from the incubator for assessment under the microscope. In addition, no information regarding embryo development is obtained between viewings. Important events such as internalization of fragments (Hardarson et al. 2002) and abnormal cell divisions, including direct cleavage (defined by Rubio et al. (2012) as embryos dividing from two to three blastomeres in less than 5 hours (Rubio et al. 2012)), and reverse cleavage (defined as blastomere fusion by (Liu et al. 2014)) may be missed.

By going through time-lapse sequences before and after the established times for morphological grading (Alpha and ESHRE 2011), Montag et al. (2011) showed that pronuclear morphology and day 2/3 embryo morphology could change within short time-intervals. Furthermore, when analysing the time of the first cleavage (defined as early cleavage) for 59 embryos showing normal fertilization, it was found that this event ranged from 22 to 36 hours post- insemination (Montag et al. 2011).

Time-lapse monitoring systems take images of embryos at specific time intervals ranging from 5 to 20 minutes. The system, i.e. either a moving camera, or a fixed camera with a moving embryo dish holder, is placed inside an existing incubator or is built in as part of an incubator. The embryo(s) can thereby be assessed via a screen outside of the incubator, both in "real-time" and ultimately as a film sequence covering the entire developmental period. A more stable culture environment is provided and the logistics of the IVF laboratory is made easier. Furthermore, it also allows for training of embryologists and can be used for quality control when validating scoring systems and culture methods (Apter et al. 2020).

In the beginning of the 1980´s, the time-lapse technique was used to study the hatching of bovine blastocysts (Massip and Mulnard 1980; Massip et al. 1982). Several years later, Payne et al. (1997) were the first to use time-lapse to show the events that occurred in the human oocyte up to 17-20 h post-injection, including second polar body extrusion and pronuclei appearance (Payne et al. 1997). Since then, an increasing number of studies have been published with the aim of finding correlations between time-lapse parameters and the ability of the embryo to reach blastocyst stage or implant and subsequently give rise to a healthy baby.

Today, several time-lapse systems are available in clinical IVF laboratories; including the EmbryoScope™ (Ottosen et al. 2007; Scott et al. 2008) Vitrolife, Sweden, Primo-Vision™ (Pribenszky et al. 2010), Vitrolife, Eeva™ (Conaghan et

al. 2013), Auxogyn, US and Miri®, Esco, DK. Figure 2 provides a brief overview of different features of the time-lapse systems. The EmbryoScope and Miri are integrated systems, whereas the PrimoVision and Eeva are placed within conventional incubators. All are equipped with a system-specific software system, capable of presenting images on a computer screen, enabling analysis of the images from a remote location.

In those cases where the time-lapse system is placed within a conventional incubator, the culture environment will be affected by the opening and closing of incubator doors, which in turn might affect embryo development, as previously shown by Fujiwara et al. (2007) (Fujiwara et al. 2007). In addition, there are differences in the design of the culture dishes and in the software systems; some are delivered with pre-developed algorithms and others provide user-defined variables for morphokinetic assessment (see Fig 2 for overview of different time-lapse systems).

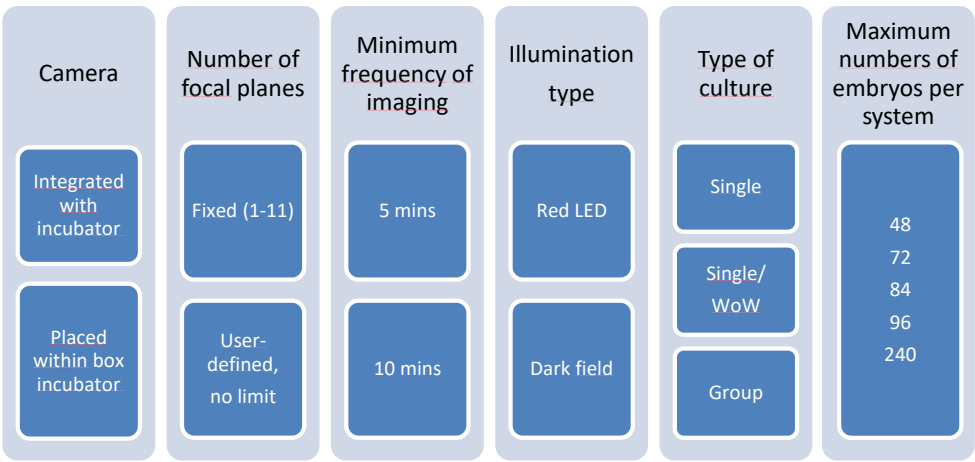


Fig 2. *Comparison of the various features of different commercial time-lapse systems.*

Morphokinetics and algorithms

The assessment of embryonic developmental events at specific time points using time-lapse imaging (TLI), is referred to as "morphokinetics" (Meseguer et al. 2011). This enables digitalization of the embryonic timing data. The "time-stamps" given for an exact morphokinetic variable are referred to as "annotations". Guidelines have

been developed to help facilitate and to harmonize the use of time-lapse variables in the IVF laboratory (Ciray et al. 2014), see table 2 and Fig 3.

Table 2. *Morphokinetic variables and their definition according to Ciray et al. (2014) (Ciray et al. 2014). Variables correspond to images in Fig 3.*

Variable	Definition
t0	Time of insemination (insemination (IVF) or when half of the patient´s oocytes have been injected (ICSI))
tPB2	Time of second polar body extrusion
tPNa	Time of appearance of the pronuclei
tPNf	Time of pronuclei fading
t2-t9	Time of division to 2-9 blastomeres, respectively
ECC1	Duration of first cell cycle (t2-tPB2)
ECC2	Duration of second cell cycle (t4-t2)
cc2a	Duration of single blastomere cell cycle (t3-t2)
cc2b	Duration of single blastomere cell cycle (t4-t2)
ECC3	Duration of third cell cycle (t8-t4)
cc3a	Duration of single blastomere cell cycle (t5-t4)
cc3b	Duration of single blastomere cell cycle (t6-t4)
cc3c	Duration of single blastomere cell cycle (t7-t4)
cc3d	Duration of single blastomere cell cycle (t8-t4)
tSC	Time of initiation of compaction
tM	Time of compaction (morula formation)
tSB	Time of initiation of blastulation
tB	Time of full blastocyst (last frame before the zona starts to thin)
tEB	Time of expanded blastocyst where zona thickness has been reduced to half
tHB	Time of hatching of the blastocyst

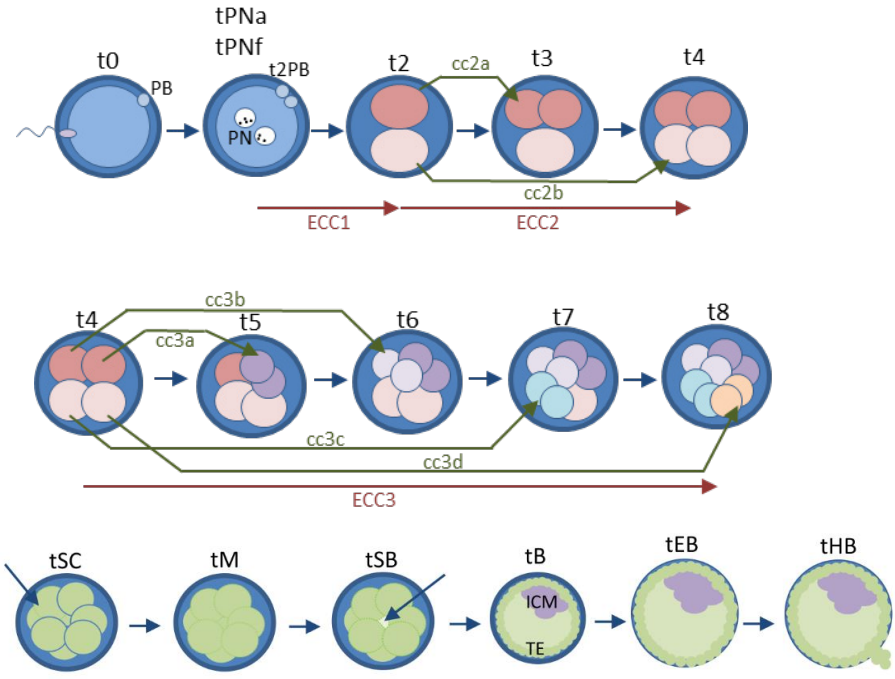


Fig 3. *Schematic representation of the morphokinetic variables in Table 2.*

With time-lapse documentation, a substantial amount of novel information about embryo development is acquired. The challenge has been how to use this information, and a large number of studies have been performed trying to determine which particular morphokinetic parameters/variables that may be more important for identifying the embryo with the highest chance of achieving success.

Using 247 embryos with known implantation data (KID), Meseguer and colleagues (2011) were the first to publish an algorithm to predict implantation (Meseguer et al. 2011). By deselecting embryos with morphological abnormalities and "negative" morphokinetic characteristics, such as direct cleavage, multinucleation and uneven blastomere size, the authors used a classification tree model to group the remaining embryos into different (hierarchal) categories.

The same group tested the above hierarchic classification system in a retrospective observational cohort study, and compared pregnancy rates derived from embryos selected by conventional culture and morphologic grading or by time-lapse

incubation including morphokinetic selection. The results were in favour of the time-lapse group; the fully corrected OR in the model for clinical pregnancy per cycle with oocyte retrieval (n=7,305) was 1.201 (95% CI: 1.059–1.363, p=0.0043) when comparison was made between treatments made with time-lapse selection and standard incubation. The OR for the 6,961 cycles receiving ET was 1.157 (95% CI: 1.018-1.315, p=0.0254) (Meseguer et al. 2012).

Following this, a study by Rubio et al. (2014), including 843 patients, was the first randomized controlled trial conducted where a benefit in terms of an increase in implantation- and pregnancy rates from time-lapse culture could be shown. The ongoing pregnancy rate (per started cycle) for the time-lapse group was 51.4% (95% CI: 46.7-56.0) compared to the standard incubator group at 41.7% (95% CI: 37.0-46.6), p=0.005 and when analysing per transfer, the results continued to be in favour of the time-lapse group (54.5% ongoing pregnancy rate, 95% CI: 49.6-59.2) compared to the standard incubator group (45.3% ongoing pregnancy rate, 95% CI: 40.3-50.4), p=0.01. (Rubio et al. 2014).

Since then, several morphokinetic models have been developed (Basile et al. 2014; Kramer et al. 2014; Basile et al. 2015; Goodman et al. 2016; Liu et al. 2016; Petersen et al. 2016). All algorithms developed so far, have however shown to be sensitive to differences in overall embryo culture conditions. For example, Storr et al. (2017) applied 7 different pre-developed algorithms (Meseguer et al. 2011; Conaghan et al. 2013; VerMilyea et al. 2014; Basile et al. 2015; Goodman et al. 2016; Liu et al. 2016; Petersen et al. 2016) on a cohort of their cycles for selection of day 5 embryos for transfer (Storr et al. 2017). The agreement between algorithms turned out to be poor. Similar results have been demonstrated by other groups when pre-defined models were applied to their clinical settings (Kirkegaard et al. 2013; Yalcinkaya et al. 2014; Freour et al. 2015; Barrie et al. 2017) showing poor prognostic ability of these models when applied to a different clinical setting. Liu et al. (2019) retrospectively applied four different day 3 algorithms to two types of datasets called “KID” (n=270) and “SET” (n=144) with endpoints implantation and live birth. The SET subgroup consisted of only single transfers and patients were only included once. It was shown that the level of prediction, measured as area under the curve (AUC), decreased considerably when the algorithms were applied to the SET dataset, compared to the KID dataset. The authors advised that a dataset comprised of only single embryo transfers with live birth as endpoint is preferable for the development and validation of algorithms (Liu et al. 2019).

A majority of studies are based on embryos with KID, i.e. where the fate (implanted/non-implanted) of the transferred embryo is known. Positive (or

negative) morphokinetic markers associated with a positive or negative outcome (implantation or not) respectively, were used to construct the algorithms (see Tables 3 and 4).

Table 3. *Observational studies evaluating time-lapse technology.*

Author	Type of study, number of included subjects	Outcome	Main finding	Time-lapse setting
Lemmen et al. 2008	Retrospective cohort n=102 fertilized oocytes	Day 2 cell number and ongoing pregnancy	Early PN disappearance and first cleavage correlated with higher number of blastomeres on day 2	Own/non-commercial system
Wong et al. 2010	Experimental, non-clinical n=242 fertilized oocytes	Blastocyst formation	Second cell cycle length (cc2), synchrony of the second and third cell divisions (s2) and the duration of the second cell division can predict blastocyst development	Own/non-commercial with cell tracking algorithm
Meseguer et al. 2011	Retrospective cohort n=285 couples, 247 transferred embryos with known outcome	Implantation, day 3 transfer	Time of division to 5 cells (t5), time between division to 3 cells and subsequent division to 4 cells (s2) and duration of cell cycle two (cc2) correlate with implantation	EmbryoScope/integrated TL system with algorithm
Cruz et al. 2011	Prospective, randomly allocated TL incubation and conventional selection vs. conventional incubation and selection n=60 couples, 478 embryos (n=238 cultured in the EmbryoScope, n=240 in the conventional incubator) 108 embryos transferred	Blastocyst development rate and ongoing pregnancy rate. Day 3 and day 5 transfers	No differences in outcome for embryos cultured in a time- lapse incubator (closed system) versus a conventional incubator	EmbryoScope/integrated TL system
Rubio et al. 2012	Retrospective multicenter cohort n=979 IVF cycles, 1659 embryos transferred	Implantation, day 3 transfer	Embryos with direct cleavage from two cells to three (DC2–3) have a significantly lower implantation rate than embryos with a normal cleavage pattern	EmbryoScope/integrated TL system with algorithm
Meseguer et al. 2012	Retrospective cohort study (multicenter, heterogeneous data set) 7305 IVF cycles, 1390 with time-lapse and 5915 in conventional incubator	Clinical pregnancy rates, day 3 or day 5 transfers, fresh or vitrified embryos	The analysis of retrospective data indicated that the time-lapse system/algorithm significantly improved clinical pregnancy rate	EmbryoScope/integrated TL system with algorithm

Cruz et al. 2012	Retrospective cohort n=165 oocyte donation recipients, 834 2PN embryos	Blastocyst formation and quality	Embryos that cleave earlier have a significantly improved chance of continuing development to day 5	EmbryoScope/integrated TL system with algorithm
Dal Canto et al. 2012	Retrospective cohort n=71 IVF cycles, 459 fertilized oocytes, 134 transferred embryos with known outcome	Blastocyst formation and expansion Implantation day 3 or day 5 transfer	Cleavage from 2- to 8-cell stage occurs earlier in embryos with the ability to develop to blastocyst, expand and implant	EmbryoScope/integrated TL system with algorithm
Azzarello et al. 2012	Prospective cohort n=130 couples, 159 transferred embryos with known outcome	Live birth, day 2 transfer	No embryo with PN breakdown (PNB) earlier than 20 h 45 min resulted in live birth	EmbryoScope/ integrated TL system
Aguilar et al. 2014	Retrospective cohort n=842 patients, 899 transferred embryos with known outcome	Implantation, day 3 transfer	The timings at which second polar body extrusion, pronuclear fading and length of S-phase occurred were linked to embryo implantation	EmbryoScope/integrated TL system with algorithm
Wirka et al. 2014	Retrospective multicenter cohort study n=67 patients, 649 fertilized oocytes	Embryo development and blastocyst formation	Identification of four groups of atypical embryo phenotypes showing significantly lowered blastocyst developmental rates: abnormal syngamy (AS), abnormal first cytokinesis (A1cyt), abnormal cleavage (AC), and chaotic cleavage (CC)	Eeva/TL system placed inside conventional incubator with algorithm

Table 4. *Randomized controlled trials evaluating time-lapse technology. For explanation of annotation codes, see Table 2.*

Author	Type of study, number of included subjects	Outcome	Main finding	Time-lapse setting
Kirkegaard et al. 2012	**RCT** TL incubation and conventional selection vs. conventional incubation and selection. n=59 patients, (297 embryos cultured in the EmbryoScope, 303 in the conventional incubator)	Proportion of 4-cell embryos on day 2. Secondary outcomes: proportion of 7–8 cell embryos on day 3 and proportion of blastocysts on day 5	No significant differences in embryo development for embryos cultured in a time- lapse incubator versus a conventional incubator. No differences in implantation and clinical pregnancy rates	EmbryoScope/integrated TL system
Kahraman et al. 2013	**RCT** TL incubation and selection vs. conventional incubation and selection. n=64 patients (33 in the EmbryoScope and 31 in the conventional incubator) n=846 oocytes	Proportion of good and top quality blastocysts on day 5. Secondary outcome: implantation and pregnancy rates. Single blastocyst transfer.	No significant differences in outcome for blastocyst developmental rate, implantation or clinical pregnancy rates when comparing culture in a conventional incubator vs. a time-lapse incubator	EmbryoScope/integrated TL system
Rubio et al. 2014	**RCT** TL incubation and selection vs. conventional incubation and selection. n=843 patients (438 in the EmbryoScope and 405 in the conventional incubator)	Ongoing pregnancy rates. Secondary outcomes: fertilization rates, embryo development , implantation rates and early pregnancy loss	Addition of time-lapse morphokinetic data resulted in a statistically significantly increased ongoing pregnancy rate for the TL group The TL group had a statistically significantly increased implantation rate and a statistically significantly decreased early pregnancy loss	EmbryoScope/integrated TL system with algorithm
Goodman et al. 2016	**RCT** TL incubation and TL selection vs. TL incubation and conventional selection n=235 patients (119 with time-lapse morphokinetic monitoring, 116 with conventional screening) 296 embryos with known outcome	Clinical pregnancy rates. Secondary outcomes: implantation and miscarriage rates. Day 3 or day 5 transfers	Addition of time-lapse morphokinetic data did not significantly improve overall clinical outcomes Timing of blastulation and morphokinetic score were associated with implantation rate for day 5 transfers	EmbryoScope/integrated TL system with algorithm

Wu et al. 2016	**RCT** TL incubation and selection vs. conventional incubation and selection n=31 patients (16 in the EmbryoScope and 15 in the conventional incubator)	Clinical pregnancy rates per started cycle Day 3 embryo transfer	No significant differences in day 3 embryo scores, implantation and clinical pregnancy rates when comparing culture in a conventional incubator vs. a time-lapse incubator	EmbryoScope/ integrated TL system
Yang et al. 2018	**RCT** Day 3 embryo transfer (n=290) using a TL hierarchical classification model vs day 5 transfer (n=295) using conventional morphological selection	Ongoing pregnancy rates. Day 3 or day 5 single embryo transfers	Pregnancy rates after day 3 SET using hierarchical classification time-lapse selection was significantly lower compared with day 5 blastocyst SET using conventional morphology	Primo Vision with algorithm
Barberet et al. 2018	**RCT** n=386 patients (191 in time-lapse incubator and 195 in benchtop incubator)	Implantation rates (primary outcome). Embryo morphology grades. Day 2, 3 or day 5 transfers	No difference in implantation rate between the two systems. The proportion of good quality day 2 embryos and total proportion of cryopreserved embryos were higher for the ES system	TLI systems EmbryoScope and G185 K-System

Of the 18 studies presented in Tables 3 and 4, seven are RCTs. None of them have live birth as primary outcome. Four of them have pregnancy as primary outcome (Rubio et al. 2014; Goodman et al. 2016; Wu et al. 2016; Yang et al. 2018). As described in more detail above, Rubio et al. (2014) found a significantly increased ongoing pregnancy rate (per started cycle, n=843 patients) for the time-lapse group compared to the standard incubator group and when analysing per transfer, the results continued to be in favour of the time-lapse group compared to the standard incubator group. For the time-lapse group, there was a statistically significant decrease in early pregnancy loss (16.6%, 95% CI: 12.6-21.4) compared to the standard incubator group (25.8%, 95% CI: 20.6-31.9), p=0.01. Furthermore, the implantation rate was significantly increased for the time-lapse group, showing a 44.9% implantation rate (95% CI: 41.4-48.4) versus 37.1% for the standard incubator group (95% CI: 33.6-40.7), p=0.02 (Rubio et al. 2014).

In a trial by Goodman et al. (2016), including 235 patients, no significant differences were found in clinical pregnancy- and implantation rate when comparing the time-lapse group where the selection was based on time-lapse parameters vs. the group where conventional morphological selection was performed (Goodman et al. 2016).

In their two-part prospective randomized study, Wu et al. (2016) found no difference in day 3 embryo quality, implantation- and pregnancy rates for poor-prognosis patients (n=31), when culturing embryos in a standard incubator compared to a time-lapse incubator (Wu et al. 2016).

Yang et al. (2018) compared the use of an algorithm for choosing embryos for transfer on day 3 (n=290) versus conventional morphological selection after extended culture until day 5 (n=295) in a non-inferiority trial. They found a difference in the ongoing pregnancy rate when comparing patients who received embryo transfer on day 3 using a time-lapse algorithm for selection, vs. those receiving transfer on day 5 based on morphological selection (56.6% vs. 64.1%, relative risk 0.88, 95% CI: 0.77, 1.01, p=0.06). The results per protocol (PP) indicated a lower pregnancy rate for the day 3 transfer + algorithm group (59.4% vs. 68.4%, relative risk 0.87, 95% CI: 0.76, 0.99, p=0.03) (Yang et al. 2018). Two other RCTs having embryo or blastocyst development as main outcomes, were unable to find any statistically significant differences between TL and conventional culture (Kirkegaard et al. 2012; Kahraman et al. 2013). Barberet et al. (2018) compared two different incubator systems (EmbryoScope versus K-System benchtop incubator). They found no difference in implantation rates, but a significantly higher proportion of good quality embryos on day 2 (40.4% vs. 35.2%, p=0.025) and total proportion

of cryopreserved embryos (29.5% vs. 24.8%, p=0.027) in the EmbryoScope compared to the benchtop incubator. This difference in morphology between incubators remained significant in a multivariate analysis (TLI vs. G185: OR: 1.27, 95% CI: 1.04–1.55, p=0.02) (Barberet et al. 2018).

The other 11 cited studies are retrospective or prospective non-randomized studies, analysing morphokinetic data, with or without the use of algorithms, in relation to embryo development and/or clinical outcomes.

In the earlier studies, which to a large extent laid the foundation for the construction of future algorithms, a number of findings were published regarding specific cleavage timings and information about embryo development and embryo quality (Lemmen et al. 2008; Wong et al. 2010; Meseguer et al. 2011; Cruz et al. 2012; Rubio et al. 2012). Since the establishment of certain "promising" timing criteria, several studies looking at possible correlations with implantation and/or pregnancy rates or live birth were published ((Cruz et al. 2011; Azzarello et al. 2012; Dal Canto et al. 2012; Meseguer et al. 2012; Aguilar et al. 2014; Athayde Wirka et al. 2014). However, no consistent predictors were found between them, see Tables 3 and 4 for overview.

A common finding in several of these studies is the correlation between certain atypical blastomere cleavage patterns and blastocyst development and implantation (Wong et al. 2010; Meseguer et al. 2011; Cruz et al. 2012; Dal Canto et al. 2012; Rubio et al. 2012). For example, "direct cleavage" (also defined as rapid cleavage by Ciray et al. (2014) (Ciray et al. 2014)) – a term introduced by Rubio et al. (2012) where embryos cleave within 5h from two to three cells, was associated with a lower implantation rate than embryos not showing this phenomenon (1.2% vs. 20.2%, respectively) (Rubio et al. 2012). Additional types of adverse cleavage patterns have been identified and studied using time-lapse monitoring, such as "reverse cleavage" where two blastomeres fuse and become one, and "chaotic cleavage" where the embryo appears to cleave "chaotically", without forming distinctive blastomeres. Embryos displaying these adverse cleavage patterns have been shown to have a decreased developmental and implantation potential (Athayde Wirka et al. 2014; Liu et al. 2014). It is advised that embryos displaying these types of cleavage patterns should not be transferred if there are other embryos available (Barrie et al. 2017).

1.3.2 INVASIVE EMBRYO SELECTION

In contrast to non-invasive embryo selection, invasive embryo selection involves manipulating the embryo itself, commonly through biopsy of polar bodies or single blastomeres before compaction, or by extracting multiple cells from the blastocyst. One concern, apart from the potential harmful effect of removing cells from the embryo, is at which embryo developmental stage the biopsy should be performed to best identify genetic and/or metabolic anomalies (Scott et al. 2013).

Preimplantation testing

Preimplantation genetic testing (PGT) involves the biopsy of cells from the embryo, usually on day 3 or day 5/6. The method is differentiated into PGT-A (PGT for aneuploidy), PGT-M (PGT for monogenic/single genes), and PGT-SR (PGT for structural rearrangements) (Zegers-Hochschild et al. 2017). PGT-A is mainly used in order to try to enhance pregnancy/live birth rates by excluding embryos with aneuploidic cells, while PGT-M and PGT-SR are mainly used for screening of embryos from parents with a known hereditary disease.

In the early years of PGT-A, blastomere biopsy on day 3 and fluorescent *in situ* hybridization (FISH) were used for analysis of chromosomal anomalies (Munne et al. 1995; Verlinsky et al. 1995; Hardarson et al. 2008). However, this method was never shown to increase live birth rates, presumably due to a high rate of mosaicism at this cell stage, and the low number of cells and chromosomes being analysed (Mastenbroek et al. 2011). To overcome these problems, PGT-A is now increasingly being carried out at the blastocyst stage (TE biopsy) where a higher number of cells can be biopsied. Furthermore, in combination with new techniques, such as array comparative genomic hybridization (aCGH) and next generation sequencing (NGS), all 24 chromosomes could efficiently be screened for aneuploidies (Yang et al. 2015).

The efficiency of PGT-A is however still being debated (Practice Committees of ASRM and SART 2018; Sciorio et al. 2020). In the most recent and largest RCT where the modern techniques were used, the STAR trial, which included 661 treatment cycles, no difference in ongoing pregnancy rate was found between the study group and the control group per ITT (41.8% vs. 43.5%, p=0.65) or per embryo transfer (50% vs. 45,7%, p= 0.32) (Munne et al. 2019). It was shown that 12.7% of the patients in the study group had no euploid embryos available for transfer. The frequency of not having any euploid embryos increased with maternal age: 8.9% in women aged 25-34 years of age and 17.2% in women aged 35-40.

2 AIM

The overall objective of this thesis was to investigate whether culture of human embryos in a closed time-lapse system resulted in an improved outcome regarding embryo quality and embryo selection.

The specific aims for each study were:

Paper I:
To investigate if culture of human embryos in a closed culture system, where the embryos were assessed without removing them from the incubator, was superior in terms of number of good quality embryos on day 2 post-insemination compared to culture in a conventional "open" culture system.

Paper II:
To assess whether morphokinetic variables, when analysed together with patient characteristics and conventional morphological variables, could independently predict live birth after day 2 transfer.

3 PATIENTS AND METHODS

A more detailed description of the different methods used can be found in the respective papers.

The first study (Paper I) was a prospective randomized controlled trial whereby patients were randomized to either having their embryos cultured in a conventional incubator culture system or in a closed culture system (the EmbryoScope™). The primary end-point was the number of good quality embryos on day 2, defined as consisting of 4-6 blastomeres, ≤20% fragmentation and the absence of multiple nuclei. Secondary end-points included fertilization rate, number of 4-cell embryos on day 2, implantation-, pregnancy-, miscarriage- and ongoing pregnancy rates.
Paper II constituted a retrospective analysis of patients randomized to the study group: "closed culture system" to identify independent predictors of live birth outcome from patient-, cycle- and embryo variables.

Setting

Both studies were carried out at Reproductive Medicine, Sahlgrenska University Hospital, Gothenburg. Embryo culture and time-lapse image analysis were performed by embryologists at the IVF laboratory.

Study population

Patients taking part in the IVF program were asked to participate in the study. Recruitment took place between May 2010 and February 2014. Inclusion criteria were; ≤40 years of age, patients undergoing their first fresh IVF cycle using ICSI and having at least one oocyte retrieved at ovum pick up. Patients undergoing egg donation or pre-implantation genetic diagnosis were excluded.

In total, 364 patients were randomized. One cycle per patient was included. Analysis was performed according to the intention-to-treat (ITT) approach (Fig 5).
In paper II, only cycles from the study group where the number of live births matched the number of transferred embryos or where no live birth occurred were included (199 patients, 207 transferred embryos) (Fig 5). Exclusion criteria included patients who gave birth to a singleton child after double embryo transfer (n=1), patients not receiving embryo transfer due to all embryos cryopreserved (n=11), no mature oocytes (n=1), failed fertilization (n=6), or non-optimal embryo development (n=4). Also excluded were patients where no time-lapse images were available due to technical failure (n=19).

Randomization

Randomization was carried out by a web-based randomization program. Patients were randomized by the embryologist directly after oocyte retrieval; all the patients´ oocytes were allocated to culture in either a conventional incubator culture system (control group) or in a closed system, the EmbryoScope (study group), with a 1:2 allocation ratio. In total, 364 patients (365 cycles) were randomized: 240 patients (241 cycles, 2280 oocytes) to the study group and 124 patients (124 cycles, 1180 oocytes) to the control group. Patients as well as the treating physician and the person performing the statistical analyses were blinded to group allocation until the outcome of transfer (pregnant versus not pregnant) was known. Embryologists were not possible to blind.

Ethical approval

The study was approved by the Ethical Committee of the University of Gothenburg on December 9, 2009 (Dnr: 666-09) and all patients signed an informed consent.

Stimulation, oocyte retrieval and ICSI

Ovarian stimulation was performed using a long protocol down-regulation with a gonadotrophin releasing hormone (GnRH) agonist followed by stimulation with recombinant follicle stimulating hormone (FSH) or urinary-derived gonadotrophins. In a few cases (n=28), a GnRH antagonist short protocol was used. Follicular development was monitored by serum estradiol levels and vaginal ultrasound. Oocyte retrieval was scheduled 36±2h after human chorion gonadotrophin (hCG) injection. Luteal support with vaginal progesterone was given.

Oocyte-cumulus complexes were collected using transvaginal ultrasound guided puncture, rinsed in MOPS and placed in a culture dish containing G-IVF medium. The oocyte-cumulus complexes were denuded using hyaluronidase and ICSI was performed within 5 hours of oocyte retrieval on mature (MII) oocytes.

Embryo culture

Immediately after the ICSI procedure, the embryos were placed into their respective culture systems, and were cultured, according to randomization, in either a conventional incubator (control) or in the EmbryoScope time-lapse incubator (study group) for two days.

All oocytes were cultured at 37°C, 6% CO_2 and atmospheric O_2 concentration until embryo transfer on day 2. For the control group, the embryos were taken out of the

incubator at given time points (see below) and assessed under a Olympus inverted microscope with a 20 x 1.5 Hoffman Modulation contrast objective, and for the study group the oocytes were cultured in the EmbryoScope until time of transfer.

In contrast to the control group, the embryos in the study group were not removed from the incubator (the EmbryoScope) during assessment. Instead, images present on the monitor of the EmbryoScope were viewed at the exact same time points as for the control group (times given below).

The EmbryoScope ES-D2 with accompanying software version 4.0.2 was used for this study (Unisense, Fertilitech, Århus, Denmark), Fig 4. The EmbryoScope is a benchtop incubator with an integrated camera with a Leica 20 × 0.40 LWD Hoffman Modulation contrast objective. Air is continuously purified through a HEPA/VOC filter to ensure that the embryos are not exposed to any contaminants. It is capable of acquiring images for up to 72 oocytes/embryos simultaneously. During image acquisition, the embryos were illuminated with low intensity red light at 635 nm for <0.5 seconds per image. For this study, images were acquired every 20 minutes, at seven focal planes.

Fig 4. *The EmbryoScope^{TM} ES-D2 time-lapse incubator. Published with permission from Vitrolife^{AB}. The EmbryoScope may be used either as a "closed, non-disturbed" incubator or in combination with morphokinetic grading using the Viewer software program (Vitrolife ^{AB}).*

Embryo selection and transfer

In the first part of the thesis (Paper I), a minimum of two embryologists were involved in assessing the quality of each embryo. Morphological assessment and selection of embryos for transfer were made at equal time points using the same criteria for the control group and the study group.

Individual time-lapse images were used for assessment of the embryos cultured in the EmbryoScope, however, recordings and morphokinetic parameters were not taken into consideration when assessing and selecting embryos in Paper I.

Confirmation of fertilization, i.e. the presence of two pronuclei, was performed at 16-18 hours post-injection. At 25-27 hours post injection, early cleavage was assessed and on day 2 (at 43-45 hours post-injection), embryos were graded according to blastomere number and size, degree of fragmentation, and presence of multinucleation. A good quality embryo was eligible for transfer if it had four to six blastomeres, less than 20% fragmentation and no observed multinucleation.

When selecting embryos with otherwise equal quality on day 2 for transfer, early cleavage and the presence of nuclei in the cells were also taken into account. If no GQEs were available, embryos with an increased fragmentation rate could be transferred but would not be cryopreserved.

Single embryo transfer was performed in all cycles except 12 where DET was carried out.

Time-lapse monitoring and annotations

In Paper II, the embryos cultured in the EmbryoScope and used for transfer were assessed and annotated retrospectively.

Two senior embryologists simultaneously performed manual annotations. Time of injection was used as starting time for all developmental events annotated in the EmbryoViewer image analysis software program (Vitrolife, Sweden). All times were recorded in hours post-insemination (hpi). The following morphokinetic variables were annotated according to the definitions described by Ciray et al. (2014) (Ciray et al. 2014), see table 5.

Table 5. *Morphokinetic variables included in annotation analysis for Paper II*

Morphokinetic parameter	Definition
tPB2	Appearance of second polar body
tPNa	Time of pronucleus (PN) appearance
tPNf	Time of PN fading
t2, t3, t4	Time of division to 2-cell (t2), 3-cell (t3) and 4-cell (t4) stages

From these (static) time variables, a number of identified morphokinetic (duration) variables could be calculated.
Calculated variables in Paper II included:

- tPNa-tPB2: time from polar body extrusion to PN appearance
- tPNf-tPNa: PN duration
- t4-t2: duration of the second cell cycle (cc2)
- t4-t3: synchrony of blastomere divisions in the second cleavage cycle (s2)
- direct cleavage observed during first or second cleavage cycle where one blastomere divides directly into three or more daughter cells

A number of morphological characteristics were also documented for each cell stage. These included fragmentation grade, bi- and multinucleation and blastomere size. In addition, patient characteristics and cycle variables were included in the analyses (Table I, Paper II).

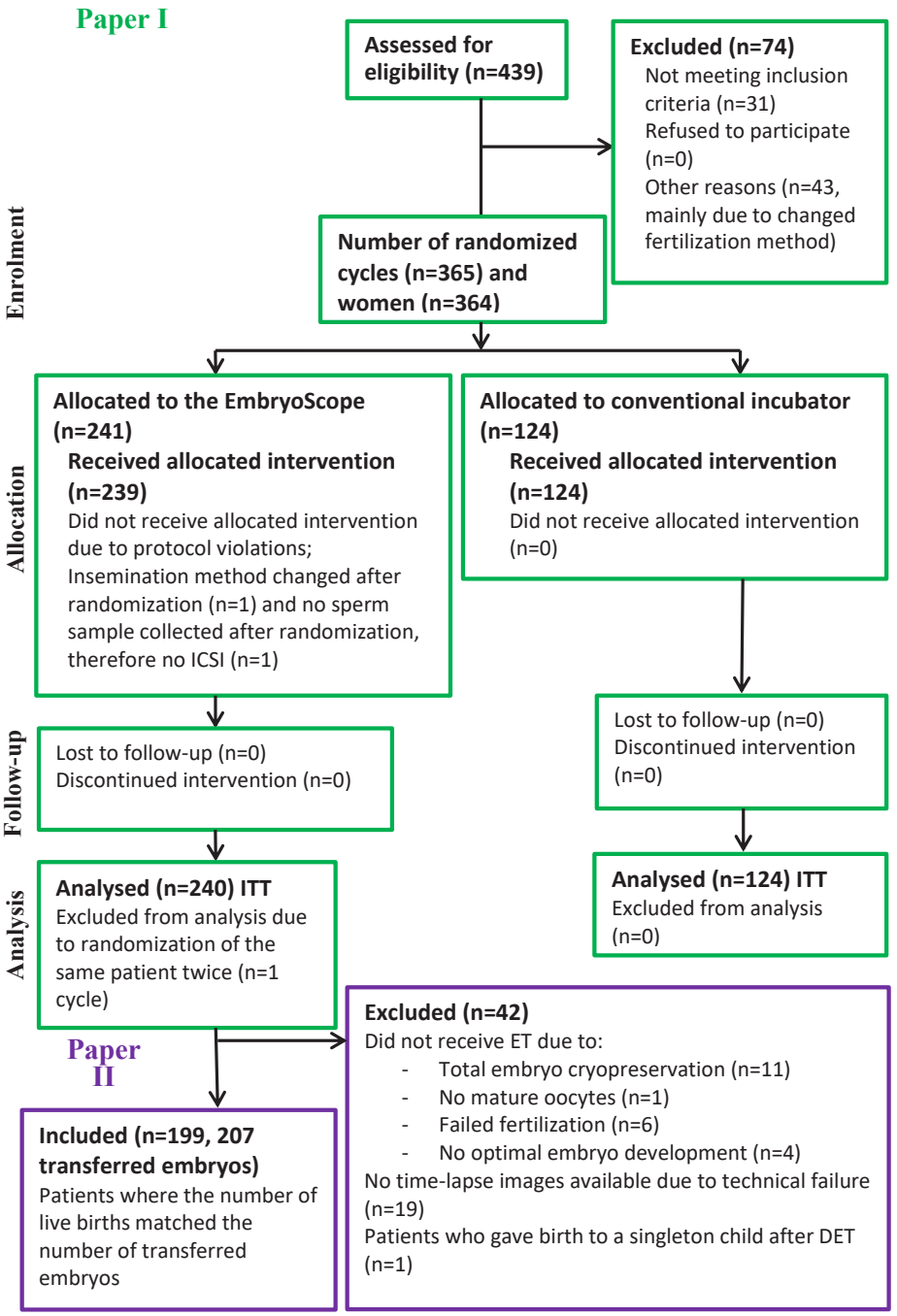


Fig 5. *Flowchart of the patients included in Paper I and Paper II.*

Statistical analysis

An overview of the design, endpoint, statistical analysis and results from the two papers are presented in Table 6.

Paper I

The study was designed as a superiority trial. Primary end-point was number of good quality embryos on day 2. Secondary end-points were fertilization rate, number of 4-cell embryos on day 2, implantation-, pregnancy-, miscarriage- and ongoing pregnancy rates.

Sample size calculations prior to study start showed that 357 patients were needed (average number of GQEs=3.9 and SD=3.1) in order to show an increase with 1.0 GQEs in the study group (α-value 0.05, power 80%), if randomization into the study versus control groups was performed with a ratio of 2:1, i.e. 238 patients in the study group and 119 in the control group. The patients were randomized by a web-based computer program. Stratification was performed by minimizing for age and mean number of aspirated oocytes.

Descriptive statistics are given by mean and SD for continuous variables and by numbers and percentages for categorical variables. For comparison between the groups, Fisher´s exact test was used for dichotomous variables and Mann-Whitney U-test was used for continuous variables. A p-value of <0.05 was considered significant. To select independent predictors of the dependent variables ongoing pregnancy rates and miscarriage rates, univariate logistic regression analysis was first performed for each of the baseline variables. Variables with $p < 0.25$ were then entered into a stepwise multiple logistic regression analysis.

Statistical analyses were performed using SAS software version 9.3 (SAS Institute, Inc., NC, USA), and SPSS software version 22, 2013 (SPSS, Chicago, IL, USA).

Paper II

The primary end-point was live birth. Logistic regression was used to analyse the association between patient and treatment variables, embryo morphological variables and morphokinetic variables and live birth. Since a few patients received two embryos at transfer, the models were estimated with generalized estimation equations (GEE) to account for dependencies within individuals.

Univariate logistic regression models were used to identify the predictors that significantly affected live birth. Prediction models for live birth or models for ranking of live birth potential were developed and evaluated by splitting the data into two parts, using half of the observations for model development and the other half for model evaluation. Stepwise logistic regression was used for selection of variables to be included in the prediction models, adding and removing the most/least significant variables one at a time. All variables significant at 10% level in the univariate models were considered for inclusion in the prediction models. Prediction models were developed for the following three categories of variables separately: i) patient and treatment variables, ii) embryo morphological variables, and iii) morphokinetic variables. Models with all three categories tested simultaneously were also constructed. The prediction performance was evaluated by means of area under the curve (AUC) on the evaluation data set.

The use of a classification tree model for prediction of live birth was also investigated. With classification trees, classification models are built in the form of a tree structure and data is broken down into increasing numbers of subsets in a hierarchical manner. Continuous variables are dichotomized sequentially in pre-defined categories in order to find regions that separate the outcome in an optimal way.

Our data was also applied to another published model, the Eeva™ (Early Embryo Viability Assessment, Auxogyn, USA) Test - a commercially available model for good quality blastocyst prediction. This model automatically measures the time between first and second mitosis and also between the second and third. Ultimately, a score is provided to reflect the probability of blastocyst formation. In our study, data were used to generate a receiver operating characteristic (ROC) curve and to calculate AUC for live birth.

All available data was used for each variable in univariate analyses, and all observations with complete data on the variables selected by the stepwise selection procedure were used in the prediction models.

All analyses were done with SAS v9.4 (SAS, Cary, NC, USA). All tests were two-tailed and conducted at 5% significance level.

Table 6. *Study design and results obtained in the two papers*

	Paper I	**Paper II**
Analysis	RCT Study group: embryo culture in EmbryoScope (n=240) Control group: embryo culture in conventional incubator (n=124)	Analysis of TL subgroup All patients from study group with known outcome of all transferred embryos (n=199)
Primary end-point	No. of GQEs on day 2	Prediction of live birth
Statistical analysis	Fisher´s exact test Mann-Whitney U-test	Logistic regression analysis Fisher´s exact test Classification trees Prediction models
Main finding	No statistical difference between groups in numbers of GQEs on day 2	Early cleavage and fragmentation grade independent predictors of live birth

4 RESULTS

The results presented in this thesis are based on a randomized controlled trial resulting in two publications. In Paper I, the results in terms of number of good quality embryos after closed incubation in a time-lapse system versus conventional incubation are presented. In Paper II, morphokinetic data of the transferred embryos were analysed.

The main findings in this thesis were:

- Culture of embryos until day 2 in a closed culture system compared to a conventional culture system did not increase the number of good quality embryos
- No morphokinetic variables were selected as independent predictors of live birth. The strongest predictors of live birth after day 2 transfer were the variables early cleavage in combination with fragmentation grade at 43- 45 hours post-insemination, both assessed by conventional morphology

PAPER I

364 ICSI patients (365 cycles) were randomized 2:1 for culture in either the time-lapse incubator (241 cycles, 2280 oocytes allocated, 1979 oocytes injected) or a conventional incubator (124 cycles, 1180 oocytes allocated, 1000 oocytes injected). No significant difference was found between the two groups regarding patient demographics (Table 1, Paper I).

For the primary end-point: "number of GQEs on day 2" no significant differences were found (2.41±2.27 for the TL incubator group and 2.19±1.82 for the conventional incubator group; p=0.34, difference 0.23, 95% CI: 0.69; -0.24), nor for any of the other embryo variables (Table 2, Paper I).

No significant differences were found in ongoing pregnancy rate, defined as fetal heartbeat at ≥8 weeks of gestation, (30.0% vs. 31.5% per randomized woman (p=0.87) and 33.5% vs. 34.2% per embryo transfer (p=0.99), respectively), between the study and the control group. However, the miscarriage rate was significantly higher in the study group (33.3%) compared with the control group (10.2%), p=0.011, resulting in a numerically lower ongoing pregnancy rate per transfer for the study group (22.3%) compared to the control group (30.7%), although this was not significant (p=0.13). (Table 3, Paper I).

In the stepwise multiple logistic regression analysis, the baseline variables "smoking" and "number of embryos transferred" were independently associated with the variable "ongoing pregnancy" (adjusted OR 0.329, 95% CI: 0.112-0.967, adjusted p=0.035 and adjusted OR 3.351, 95% CI: 1.393-13.699, adjusted p=0.0075, respectively).

PAPER II

Of the 199 patients included, 191 (96.0%) received single embryo transfer and eight received double embryo transfer. The live birth rate was 21.1% (42/199) (Table 1, Paper II). No live births resulted from the double embryo transfers.

Univariate analysis

In the univariate logistic regression of the cycle variables, it was found that the probability of live birth increased significantly for each additional 4-cell embryo available on day 2 of development (OR 1.27, 95 % CI: 1.07-1.50, p=0.005), (Fig 1, Paper II). For the conventional morphology variables, early cleavage was found to significantly increase the odds of live birth (OR 4.84, 95% CI: 2.14-10.96, p=0.0002) and each increase in grade of fragmentation at 43-45 h significantly decreased the likelihood of live birth (OR 0.46, 95% CI: 0.25-0.84, p=0.012), (Fig 2, Paper II) Fig 6.

For the morphokinetic variables, it was found that time to PN fading (OR 0.80, 95% CI: 0.70-0.91, p=0.0009), time to 2-cell (OR 0.80, 95% CI: 0.71-0.90 p=0.0003), 3-cell (OR 0.90, 95% CI: 0.84-0.97, p=0.0083) and 4-cell stage (OR 0.91, 95% CI: 0.83-0.99, p=0.032), duration from second polar body extrusion to 2-cell stage (OR 0.86, CI 0.77-0.95, p=0.0039) and duration from PN appearance to disappearance (OR 0.89, 95% CI: 0.80-0.99, p=0.026) were significant predictors of live birth, (Fig 3, Paper II).

Fig 6. *Embryos showing an increasing degree of fragmentation as seen from left to right. In Paper II it was shown that an increase in grade of fragmentation at 43-45 h post-insemination decreased the likelihood of live birth.*

Stepwise logistic regression

When entering the category of patient- and treatment variables into a stepwise logistic regression model, only the number of 4-cell embryos at 43–45 h post-insemination was selected as a predictor (Table I, Paper II). When analysing the category of embryo morphological variables only, early cleavage was first selected. A significant interaction of early cleavage with fragmentation grade at 43–45 h was also found. It was shown that the live birth rate was 26.7% for embryos showing early cleavage in combination with high fragmentation grade, and 37.1% when embryos displaying early cleavage also had a low fragmentation grade on day 2 (AUC=0.74). When analysing the morphokinetic variables category separately, only time to first cleavage (t2) was selected. (Summary in Table 2, Paper II).

When all above categories (variables) were entered simultaneously for selection into a predictive model, early cleavage (scored by conventional assessment) together with fragmentation grade was selected.

Furthermore, the model constructed from the 104 observations (model development), fitted nicely with the 103 observations used for the evaluated model data, showing similar ROC AUC values at 95% CI for patient- and treatment-, morphological- and morphokinetic variables, respectively (Table 2, Paper II).

Considering the morphokinetic variables tested with the classification tree model, the best model was found for tPNf within ranges 2.33-2.67, AUC=0,61 on model evaluation data, compared to AUC=0,83 on model development data (Table 2, Paper II).

Test of a commercially available model

When the Eeva test (Conaghan et al. 2013) was applied to our data set to predict live birth outcome, 23% of the embryos categorized as Low, 24.1% categorized as Medium and 17.2% categorized as High, resulted in a live birth.

When looking at the group of patients who obtained live birth, 45% had embryos classified as "low" according to Eeva, meaning they had a low potential for blastocyst development, according to the model.

5 DISCUSSION

Main findings

The main findings of the individual studies were:

Paper I: No significant differences were found in terms of number of GQEs on day 2 between the embryos cultured in the conventional incubator versus the EmbryoScope. Neither were there any significant differences in the number of obtained 4-cell embryos, implantation-, pregnancy- or ongoing pregnancy rates. This shows that in our clinical setting, culture during two days in the EmbryoScope time-lapse incubator is not superior to culture in a standard incubator, in terms of the above outcomes. This is in line with previous studies (Nakahara et al. 2010; Cruz et al. 2011; Kirkegaard et al. 2012; Goodman et al. 2016).

Paper II: Using stepwise logistic regression, embryos cultured in the EmbryoScope and with known outcome regarding live birth were analysed. When including patient-, cycle-, embryo morphology- and morphokinetic variables, only the morphological static variables early cleavage and fragmentation grade were found to independently predict live birth. No morphokinetic variables were selected as predictors of live birth. Thus, in the settings and culture conditions used, early cleavage assessed as a static (dichotomous) variable at 26±1h post-insemination was an independent predictor of live birth following day 2 embryo transfer, but not the timing of the first cell division assessed as a continuous morphokinetic variable (t2).

The safety of using time-lapse

Back in 1997, Payne and colleagues used a time-lapse imaging system to record images of 38 microinjected oocytes. During the recordings, which lasted for up to 20h after ICSI, the researchers could follow several early events in the oocyte; cytoplasmic waves within the ooplasm, extrusion of the second polar body, the formation of the pronuclei and the movement of nucleoli within the pronuclei. These observations were taken into account when assessing the same embryos on day 3 and it could be shown that good quality embryos generally developed from oocytes with a more uniform and narrow timing of cellular events between injection and pronuclear formation (Payne et al. 1997).

In 2008, Mio and Maeda developed a time-lapse system that enabled the recording of embryos up to the blastocyst stage and were thereby able to observe and document

events that occurred after fertilization had taken place, such as cell divisions, morula formation and the hatching of blastocysts (Mio and Maeda 2008). In the same year, Lemmen and co-workers, with the aid of time-lapse imaging, studied the timing of events occurring after fertilization up to day 2 in 102 2PN oocytes. Observations were made of the timings of pronuclei fading, onset of cell cleavages and the appearance and disappearance of nuclei within the blastomeres (Lemmen et al. 2008).

The above studies showed that incubation in specialized time-lapse units, including handling, culture in specially designed dishes, image capturing etc., did not seem to impair embryo development when compared to sibling oocytes cultured in a conventional setup. Based on these promising observational studies, time-lapse systems soon became commercially available and several RCTs were conducted. These studies showed similar results in terms of fertilization rate, embryo quality at cleavage- and blastocyst stage and ongoing pregnancy rates when incubating embryos in a closed time-lapse system compared to incubation in a conventional incubator (Nakahara et al. 2010; Cruz et al. 2011; Kirkegaard et al. 2012).

Paper I in this thesis was designed to examine the potential benefit of the closed culture environment provided by the time-lapse system. Therefore, the embryo selection software tools were not utilized and conventional morphology assessment was carried out by viewing still images from the time-lapse incubator.

In a large Cochrane meta-analysis including nine RCTs (2955 couples) by Armstrong et al. (2019) (Armstrong et al. 2019), three RCTs had, in a similar manner to our study as described in Paper I, compared embryo development in a time-lapse incubator versus a conventional incubator ((Kahraman et al. 2013; Wu et al. 2016; Barberet et al. 2018), see also Table 4). The morphological assessment in the time-lapse incubator was carried out by viewing still images while for the conventional incubator the embryos were taken out and viewed under a microscope.

Kahraman et al. (2013), in their prospective randomized study, analysed the number of good quality embryos on day 5 from good prognosis patients (n=64) when cultured in a conventional incubator (n=31) versus a time-lapse system (the EmbryoScope, n=33). No differences were found in blastocyst development, biochemical-, clinical- or ongoing pregnancy rates between the groups (Kahraman et al. 2013).

Wu et al. (2016) performed a two-part prospective randomized study which included poor prognosis patients (n=31) and egg donor-recipient cycles (n=17), respectively. Comparison was made between culture in a conventional incubator versus a time-lapse system (the EmbryoScope). Embryo quality and pregnancy rates were compared for day 3. No differences were found when comparing culture in the EmbryoScope vs. conventional incubation in embryo quality for number of good, fair or poor quality embryos on day 3, clinical pregnancy rate per randomized patient or implantation rates. However, there was a difference in the egg donor-recipient part of the study, where the number of good quality embryos in the EmbryoScope was found to be lower compared to the conventional incubator (55.8 ± 6.4% vs. 81.2 ± 4.1%, p=0.005) (Wu et al. 2016).

Barberet et al. (2018) randomized 386 patients to culture in either the EmbryoScope (n=195) or a benchtop incubator (n=191) with implantation rate as primary end-point. No significant difference was found for implantation rate, or for the secondary endpoints clinical pregnancy rate, miscarriage or ongoing pregnancy rates. There was however a statistically significant increase in the proportion of good quality embryos per patient on day 2, 3 and 5/6 when cultured in the EmbryoScope compared with the conventional incubator, 40.3% vs. 34.6% respectively, p=0.037, and the proportion of frozen embryos was significantly higher in the time-lapse group (29,5% versus 24.8% , p=0.027) (Barberet et al. 2018).

As in our study, the above three studies have all relied on a computer-based randomization programme and all have utilized the EmbryoScope for their study groups. However, the conventional incubators differ - both Kahraman et al. (2013) and Wu et al. (2016) used box incubators whereas Barberet et al. (2018) used a benchtop incubator. They also differ in terms of types of culture dishes being used for the control group. Similar to our study, Wu et al. cultured at atmospheric O_2 tension for the control group, while Barberet and Kahraman used lowered oxygen levels. Day of transfer ranges from day 2 to day 5. Furthermore, two of the studies were small (Kahraman et al. 2013; Wu et al. 2016) and the number of transferred embryos per woman was not described by Wu et al. 2016.

The conclusion of the Cochrane meta-analysis mentioned above (Armstrong et al. 2019) was that the evidence was insufficient to prove that embryo culture using time-lapse systems with or without embryo selection software would provide any difference in live birth or ongoing pregnancy (OR 0.91, 95% CI: 0.67 - 1.23, 3 RCTs, n=826, low-quality evidence), miscarriage rate (OR 1.90, 95% CI: 0.99 - 3.61, 3 RCTs, n=826, low-quality evidence) stillbirth (OR 1.00, 95% CI: 0.13 - 7.49, 1 RCT,

n=6, low-quality evidence) or clinical pregnancy (OR 1.06, 95% CI: 0.79 to 1.41, 4 RCTs, n=875, low-quality evidence) compared to culture in conventional incubators. The authors emphasized that the studies were of low or very low quality and were at high risk of bias for randomization and allocation concealment. In addition, despite similar study designs, there were many variations between the studies, for example different patient populations, different days of transfer and type of time-lapse system used. The conclusion was that further randomized controlled trials need to be carried out, using similar patient groups, similar culture conditions (open vs closed, type of culture dishes etc.), day of transfer (day 5), number of transferred embryos (one) and type of time-lapse system.

In another systematic review and meta-analysis from 2019 (Magdi et al. 2019), 6 RCTs with a total of 2057 patients were included. With only two studies including live birth data, a significant increase in live birth was found for the time-lapse group, (OR 1.43; 95% CI: 1.10 – 1.85, p=0.007) although of low quality evidence. No difference was shown for implantation-, ongoing pregnancy- or clinical pregnancy rates, but selecting embryos based on morphokinetics appeared to be associated with a lower early miscarriage rate (OR 0.71; 95% CI: 0.52-0.97, p=0.03) compared to when the embryo selection was based on conventional morphological assessment. However, when performing subgroup analysis including only studies using the same incubator and culture conditions in the groups, the difference was not significant any longer. The authors concluded that routine implementation of time-lapse technology is costly and premature, and should be offered in a research based setting and free of charge.

In our RCT, we also found a significantly increased miscarriage rate for women who had their embryos cultured in the time-lapse incubator. Although a worrying finding, the study was not powered to detect differences in miscarriage rate and should therefore be taken with caution. To our knowledge, no other studies to date have linked time-lapse incubation to a higher miscarriage rate or other detrimental outcomes.

Time-lapse technology as a tool for embryo selection

Time-lapse studies have emphasized the fact that the traditional morphological evaluation of embryos only gives us “snap-shot” images of the dynamic processes involved in embryo development. When documented by such traditional “snap-shot” images only, aberrant cleavage patterns and cleavages taking place outside the established time ranges, (as defined by the Istanbul consensus (Alpha and ESHRE 2011)), associated with a lower implantation and live birth rate (Rubio et al. 2012;

Zhan et al. 2016; Azzarello et al. 2017) will be missed. For example, it has been observed that on day 2, the morphology score may change within a few hours; the embryo could go from being assessed as a low morphology grade embryo, displaying multiple fragments, to a high morphology grade embryo with no/low fragmentation, or vice versa (Montag et al. 2011). Thereby, using only "snap-shot" image assessment, the embryo may be incorrectly classified and would not be deselected and/or receive a correct ranking within the available embryo cohort.

In addition to studies designed to demonstrate the safety of time-lapse systems, many studies have been performed to identify morphokinetic parameters that could predict success in terms of blastocyst formation, implantation- or live birth rates. When utilizing the time-lapse system to its fullest extent, including morphokinetic assessments and taking into account the exact timings of cellular divisions and the time between such events (see Table 2), a substantial amount of novel data becomes available. Compiling morphokinetic data from a large number of datasets including documented timings and correlating to outcome, it is hypothesized that algorithms can be calculated to aid in the identification of what is considered to be an embryo with the highest potential for implantation and live birth (Wong et al. 2010; Meseguer et al. 2011; Chavez et al. 2012; Campbell et al. 2013; Basile et al. 2014; Kramer et al. 2014; Petersen et al. 2016). Furthermore, algorithms have been proposed to be able to distinguish between euploid and aneuploid embryos (Chavez et al. 2012; Basile et al. 2013; Campbell et al. 2013; Basile et al. 2014; Patel et al. 2016; Reignier et al. 2018). However, all of these algorithms have been shown to be sensitive to differences in the overall embryo culture conditions (Ciray et al. 2012; Basile et al. 2013) and so far, no single morphokinetic parameter has been found to be able to universally predict implantation potential.

In addition, a question of concern/interest is whether the morphokinetic variables will perform better than the traditional ones, and if abandoning the traditional morphological parameters will actually lead to an improvement of embryo selection or if morphokinetics should be used as an additional parameter (Gardner and Balaban 2016).

Therefore, in Paper II, we wanted to test the predictive power of conventional morphological grading and patient variables in combination with morphokinetic variables. Only the embryos cultured in the time-lapse incubator were included in this analysis, utilizing the embryo documentation software. We found that the morphological variables early cleavage combined with fragmentation grade on day 2 was independently predictive of live birth. When assessing morphology alone, both

early cleavage and fragmentation grade have previously been shown to be associated with blastocyst development, implantation and ongoing pregnancy/live birth (Lundin et al. 2001; Van Montfoort et al. 2004; Rhenman et al. 2015).

In a similar manner to our analysis, Goodman et al. (2016) performed a study where they combined morphology and morphokinetics by retrospectively adding time lapse parameters to a group of embryos cultured in the EmbryoScope and initially scored using morphology only. They found no significant improvement in clinical reproductive outcomes when adding the morphokinetic variables to embryo selection. By reviewing the embryos with time-lapse, they detected significantly more multinucleation (35.3% vs 7.0%) and this was shown to be negatively associated with implantation and pregnancy rates (Goodman et al. 2016). Liu et al. (2016), developed a deselection model using time-lapse variables. However, a following study using this time-lapse deselection model, could not show the same predictive power/efficacy of this model on a new dataset (Liu et al. 2019).

Adamson et al. (2016) found, in a prospective study, a significant increase in implantation- (30.2% for the study group and 19% for the control group, p=0.003) and clinical pregnancy rates (46% for the study group and 32.1% for the control group, p=0.02) in patients (n=319 in total) who underwent transfer of day 3 embryos selected based on the combination of morphokinetic data along with traditional morphology, compared to those selected based exclusively on morphological assessment (Adamson et al. 2016).

We also applied our data to another prediction model; a tree classification model, similar to the hierarchical classification model used by Meseguer et al. (2011), where an algorithm was proposed for embryo selection based on morphological and morphokinetic characteristics classifying embryos into 10 categories (A to F) that were associated with decreasing implantation rates (Meseguer et al. 2011). Liu et al (2016), in their time-lapse deselection model used a combination of morphological score on day 3, morphokinetic parameters, and cleavage patterns to categorize embryos into 7 grades (from A+ to F) of decreasing implantation potential (Liu et al. 2016). In our analysis, the classification model developed was found to be promising on the model developmental data set (AUC: 0.83) but performed less satisfactorily on the model evaluation data set (AUC: 0.61).

In our analysis of the time-lapse subgroup, we have included morphokinetic variables as described by Ciray et al. (2014) (Ciray et al. 2014) that can be observed in the time

allowed for embryo culture and indicated in previous studies as potential predictors. In order to see how our annotated time-lapse data would fit into a different algorithm, we tested the Eeva (Early Embryo Viability Assessment, Auxogyn, USA) algorithm (Conaghan et al. 2013). In short, this algorithm is constructed from three main time points; P1 (duration of first cytokinesis), P2 (time interval between cytokinesis 1 and 2) and P3 (time interval between cytokinesis 2 and 3). The algorithm has previously been used to predict the development of blastocysts by categorizing the embryos into Low, Medium or High probability, depending on whether they fell into the predefined time limits for cell division (Conaghan et al. 2013). When applying the Eeva algorithm which classifies the embryos according to blastocyst development, to our time-lapse study group data, it was found to be non-predictive in our clinical setting.

Strengths and limitations

The strength of this study is that it is a large, single-center RCT. We have applied computer-based randomization and SET. Statisticians, patients and clinicians were blinded. In addition, we compared assessment by ordinary microscopy and by the TL images, finding that of the 146 transferred embryos that were assessed both in the EmbryoScope and in the Olympus inverted microscope, 134 (91.8%) were scored equally in both systems.

The limitations are that only ICSI cycles were included and that embryos were only cultured until day 2 post-insemination. Two days of culture may not be enough time to show a difference in terms of embryo quality and to draw benefits of the closed culture environment (Dumoulin et al. 1999; Alhelou et al. 2018). However, as mentioned above, several studies performed on embryos cultured for longer periods of time also have failed to show an improvement in embryo quality (Table 3 and 4).

In our RCT the endpoint was number of good quality embryos on day 2, which is a surrogate outcome to live birth. An alternative, and in retrospect perhaps a more relevant outcome, might have been numbers of good quality blastocysts, since many clinics, including our own, today extend the culture for a majority of the embryos to the blastocyst stage (day 5 or 6). However, at the time of this RCT, fresh embryo transfer at our clinic, as well as in most clinics of the Nordic countries, took place almost exclusively on day 2.

An unexpected finding in this thesis (Paper I) was that the miscarriage rate was significantly higher for the patients who had their embryos cultured in the

EmbryoScope. However, this finding - although a source of concern- must be treated with caution since the study was not powered to detect differences in miscarriage rate.

Pros and cons of time-lapse technology in ART

Although there are no conclusive studies showing that culturing embryos in a closed system results in improved implantation-, pregnancy- or live birth rates, there are advantages with this system. It provides live image tracking and documentation of the embryos, which makes it possible to assess the morphology and timing of development at any time, and without having to handle the embryo or expose it to changes in the environment.

It also creates the possibility to learn more about "embryo behaviour" (eg. timing, irregular cleavages) and for future studies of correlation to implantation and live birth as well as to aneuploidy and/or metabolics and environmental issues (oxygen levels, temperature, pH).

However, it is important to remember that, in the time-lapse systems, embryo assessment is based on manual annotations. We still face the same problems with individual differences, both regarding embryo morphology and the exact timing of certain events. In addition, the algorithms have not yet been shown to improve embryo selection or clinical outcome.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, the work of this thesis has shown, in a RCT, culture of human embryos within a closed incubator system for the first two days of embryo development did not increase the number of good quality embryos.

In addition, in an analysis including only the time-lapse study group the impact of continuous imaging and morphokinetic evaluation of the development was added and tested in a statistical analysis. When including morphokinetic variables and traditional static assessment variables in a regression analysis, it was found that only the static variable of 2 cells at 25+/-1 hour (early cleavage) and fragmentation grade at 43-45 hours post-insemination, independently predicted live birth.

One perspective of this thesis was to evaluate the possibilities of the developing time-lapse systems, including closed culture methodology, to improve culture environment and embryo selection. At this point of time, it is evident that further randomized controlled trials are needed, preferably using similar conditions for the culture of embryos as well as regarding patients groups, day of transfer (day 5), and number of embryos for transfer (SET), before we can state the potential of these systems.

It is important that all new methodology is properly validated before being put into clinical use (Harper et al. 2012; Provoost et al. 2014; Kamath et al. 2019; Lensen et al. 2019). There is an ongoing discussion about treatment "add-ons" within assisted reproduction, implying that the introduction of new treatments is not always sufficiently based on evidence, but more on a commercial and competitive basis, and "sold" to the patients. Time-lapse systems are expensive, and in the end, it is the society and the patients that have to pay for what could be ineffective or in a worst case scenario, even detrimental treatments.

Today, no conclusive evidence exists that the use of "time-lapse technology", whether as a closed culture system or as an embryo selection model, improves either embryo quality or clinical outcome in IVF. However, the imaging technology provides a useful tool for the laboratory, and the closed environment provides stable culture conditions. Furthermore, the large amounts of data generated may provide more targeted and stable algorithms in the future, aiding in embryo assessment.

It is anticipated that the TL monitoring systems will develop quite rapidly, enabling more automatization regarding annotations, grading and probably also for embryo

selection through machine learning and artificial intelligence (AI). Applying an algorithm based on machine learning instead of manual embryo assessment of embryos would provide consistency and also remove inter- and intra-observer variations which will always be present otherwise. Recently, such algorithms have been developed for prediction of blastocyst quality, showing an at least as good accuracy as the blastocyst grading performed by human embryologists (Khosravi et al. 2019; Kragh et al. 2019).

In another recent study by Tran et al (2019), sets of TL data for all transferred embryos (n=8836) were used in a so-called deep learning project. The complete time-lapse film sequences were used to train the computer program to create an algorithm for prediction of the outcome fetal heartbeat. The system had no presumptions as to which embryo was of "good" or "poor" quality, but analysed all data repeatedly through multiple layers, until a model was found that fitted as close to the known outcome as possible. In the study, the model created was able to predict fetal heart pregnancy from time-lapse videos with an AUC of 0.93 (95% CI: 0.92–0.94) (Tran et al. 2019).

In the light of this development, it may be assumed that in the near future, subjective assessment by the embryologists may no longer be necessary or the standard. Additional refinements to the time-lapse technology and its combination with other techniques (such as genetic or metabolic) may aid in the presently ongoing search for improved markers of embryo viability.

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REFERENCES

*Adamson GD, Abusief ME, Palao L, Witmer J, Palao LM, Gvakharia M. 2016. Improved implantation rates of day 3 embryo transfers with the use of an automated time-lapse–enabled test to aid in embryo selection. Fertility and sterility **105**(2): 369-375. e366.*

*Aguilar J, Motato Y, Escriba MJ, Ojeda M, Munoz E, Meseguer M. 2014. The human first cell cycle: impact on implantation. Reproductive biomedicine online **28**(4): 475-484.*

*Alfarawati S, Fragouli E, Colls P, Stevens J, Gutierrez-Mateo C, Schoolcraft WB, Katz-Jaffe MG, Wells D. 2011. The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender. Fertility and sterility **95**(2): 520-524.*

*Alhelou Y, Mat Adenan NA, Ali J. 2018. Embryo culture conditions are significantly improved during uninterrupted incubation: A randomized controlled trial. Reproductive biology **18**(1): 40-45.*

Alpha and ESHRE. 2011. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting.

*Apter S, Ebner T, Freour T, Guns Y, Kovacic B, Le Clef N, Marques M, Meseguer M, Montjean D, Sfontouris I et al. 2020. Good practice recommendations for the use of time-lapse technology(†). Human reproduction open **2020**(2): hoaa008.*

*Arce JC, Ziebe S, Lundin K, Janssens R, Helmgaard L, Sorensen P. 2006. Interobserver agreement and intraobserver reproducibility of embryo quality assessments. Human reproduction (Oxford, England) **21**(8): 2141-2148.*

*Armstrong S, Bhide P, Jordan V, Pacey A, Marjoribanks J, Farquhar C. 2019. Time-lapse systems for embryo incubation and assessment in assisted reproduction. The Cochrane database of systematic reviews **5**: CD011320.*

*Athayde Wirka K, Chen AA, Conaghan J, Ivani K, Gvakharia M, Behr B, Suraj V, Tan L, Shen S. 2014. Atypical embryo phenotypes identified by time-lapse microscopy: high prevalence and association with embryo development. Fertility and sterility **101**(6): 1637-1648 e1631-1635.*

*Azzarello A, Hoest T, Hay-Schmidt A, Mikkelsen AL. 2017. Live birth potential of good morphology and vitrified blastocysts presenting abnormal cell divisions. Reproductive biology **17**(2): 144-150.*

*Azzarello A, Hoest T, Mikkelsen AL. 2012. The impact of pronuclei morphology and dynamicity on live birth outcome after time-lapse culture. Human reproduction (Oxford, England) **27**(9): 2649-2657.*

*Barberet J, Chammas J, Bruno C, Valot E, Vuillemin C, Jonval L, Choux C, Sagot P, Soudry A, Fauque P. 2018. Randomized controlled trial comparing embryo culture in two incubator systems: G185 K-System versus EmbryoScope. Fertility and sterility **109**(2): 302-309 e301.*

*Barrie A, Homburg R, McDowell G, Brown J, Kingsland C, Troup S. 2017. Examining the efficacy of six published time-lapse imaging embryo selection algorithms to predict implantation to demonstrate the need for the development of specific, in-house morphokinetic selection algorithms. Fertility and sterility **107**(3): 613-621.*

*Basile N, Morbeck D, Garcia-Velasco J, Bronet F, Meseguer M. 2013. Type of culture media does not affect embryo kinetics: a time-lapse analysis of sibling oocytes. Human reproduction (Oxford, England) **28**(3): 634-641.*

*Basile N, Nogales Mdel C, Bronet F, Florensa M, Riqueiros M, Rodrigo L, Garcia-Velasco J, Meseguer M. 2014. Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetics analysis. Fertility and sterility **101**(3): 699-704.*

*Basile N, Vime P, Florensa M, Aparicio Ruiz B, Garcia Velasco JA, Remohi J, Meseguer M. 2015. The use of morphokinetics as a predictor of implantation: a multicentric study to define and validate an algorithm for embryo selection. Human reproduction (Oxford, England) **30**(2): 276-283.*

*Bavister BD. 1995. Culture of preimplantation embryos: facts and artifacts. Human reproduction update **1**(2): 91-148.*

*Baxter Bendus AE, Mayer JF, Shipley SK, Catherino WH. 2006. Interobserver and intraobserver variation in day 3 embryo grading. Fertility and sterility **86**(6): 1608-1615.*

*Bergh T, Ericson A, Hillensjo T, Nygren KG, Wennerholm UB. 1999. Deliveries and children born after in-vitro fertilisation in Sweden 1982-95: a retrospective cohort study. Lancet (London, England) **354**(9190): 1579-1585.*

*Biggers JD, Summers MC. 2008. Choosing a culture medium: making informed choices. Fertility and sterility **90**(3): 473-483.*

Bontekoe S, Mantikou E, van Wely M, Seshadri S, Repping S, Mastenbroek S. 2012. Low oxygen concentrations for embryo culture in assisted reproductive technologies. The Cochrane database of systematic reviews(7): CD008950.

*Campbell A, Fishel S, Bowman N, Duffy S, Sedler M, Thornton S. 2013. Retrospective analysis of outcomes after IVF using an aneuploidy risk model derived from time-lapse imaging without PGS. Reproductive biomedicine online **27**(2): 140-146.*

*Capalbo A, Rienzi L, Cimadomo D, Maggiulli R, Elliott T, Wright G, Nagy ZP, Ubaldi FM. 2014. Correlation between standard blastocyst morphology, euploidy and implantation: an observational study in two centers involving 956 screened blastocysts. Human reproduction (Oxford, England) **29**(6): 1173-1181.*

*Castilla JA, Ruiz de Assin R, Gonzalvo MC, Clavero A, Ramirez JP, Vergara F, Martinez L. 2010. External quality control for embryology laboratories. Reproductive biomedicine online **20**(1): 68-74.*

*Catt JW, Henman M. 2000. Toxic effects of oxygen on human embryo development. Human reproduction (Oxford, England) **15 Suppl 2**: 199-206.*

*Chang MC. 1959. Fertilization of rabbit ova in vitro. Nature **184(Suppl 7)**: 466-467.*

*Chavez SL, Loewke KE, Han J, Moussavi F, Colls P, Munne S, Behr B, Reijo Pera RA. 2012. Dynamic blastomere behaviour reflects human embryo ploidy by the four-cell stage. Nature communications **3**: 1251.*

*Chronopoulou E, Harper JC. 2015. IVF culture media: past, present and future. Human reproduction update **21**(1): 39-55.*

*Ciray HN, Aksoy T, Goktas C, Ozturk B, Bahceci M. 2012. Time-lapse evaluation of human embryo development in single versus sequential culture media--a sibling oocyte study. Journal of assisted reproduction and genetics **29**(9): 891-900.*

*Ciray HN, Campbell A, Agerholm IE, Aguilar J, Chamayou S, Esbert M, Sayed S. 2014. Proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group. Human reproduction (Oxford, England) **29**(12): 2650-2660.*

*Conaghan J, Chen AA, Willman SP, Ivani K, Chenette PE, Boostanfar R, Baker VL, Adamson GD, Abusief ME, Gvakharia M et al. 2013. Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: results from a prospective multicenter trial. Fertility and sterility **100**(2): 412-419 e415.*

*Cruz M, Gadea B, Garrido N, Pedersen KS, Martinez M, Perez-Cano I, Munoz M, Meseguer M. 2011. Embryo quality, blastocyst and ongoing pregnancy rates in oocyte donation patients whose embryos were monitored by time-lapse imaging. Journal of assisted reproduction and genetics **28**(7): 569-573.*

*Cruz M, Garrido N, Herrero J, Perez-Cano I, Munoz M, Meseguer M. 2012. Timing of cell division in human cleavage-stage embryos is linked with blastocyst formation and quality. Reproductive biomedicine online **25**(4): 371-381.*

*Dal Canto M, Coticchio G, Mignini Renzini M, De Ponti E, Novara PV, Brambillasca F, Comi R, Fadini R. 2012. Cleavage kinetics analysis of human embryos predicts development to blastocyst and implantation. Reproductive biomedicine online **25**(5): 474-480.*

*Dale B, Menezo Y, Cohen J, DiMatteo L, Wilding M. 1998. Intracellular pH regulation in the human oocyte. Human reproduction (Oxford, England) **13**(4): 964-970.*

De los Santos MJ, Apter S, Coticchio G, Debrock S, Lundin K, Plancha CE, Prados F, Rienzi L, Verheyen G, Woodward B et al. 2016. Revised guidelines for good practice in IVF laboratories (2015). Human reproduction (Oxford, England) ***31****(4): 685-686.*

De Vos A, Van Landuyt L, Santos-Ribeiro S, Camus M, Van de Velde H, Tournaye H, Verheyen G. 2016. Cumulative live birth rates after fresh and vitrified cleavage-stage versus blastocyst-stage embryo transfer in the first treatment cycle. Human reproduction (Oxford, England) ***31****(11): 2442-2449.*

Desai NN, Goldstein J, Rowland DY, Goldfarb JM. 2000. Morphological evaluation of human embryos and derivation of an embryo quality scoring system specific for day 3 embryos: a preliminary study. Human reproduction (Oxford, England) ***15****(10): 2190-2196.*

Dieamant F, Petersen CG, Mauri AL, Comar V, Mattila M, Vagnini LD, Renzi A, Petersen B, Ricci J, Oliveira JBA et al. 2017. Single versus sequential culture medium: which is better at improving ongoing pregnancy rates? A systematic review and meta-analysis. JBRA assisted reproduction ***21****(3): 240-246.*

Diez-Juan A, Rubio C, Marin C, Martinez S, Al-Asmar N, Riboldi M, Diaz-Gimeno P, Valbuena D, Simon C. 2015. Mitochondrial DNA content as a viability score in human euploid embryos: less is better. Fertility and sterility ***104****(3): 534-541 e531.*

Dumoulin JC, Meijers CJ, Bras M, Coonen E, Geraedts JP, Evers JL. 1999. Effect of oxygen concentration on human in-vitro fertilization and embryo culture. Human reproduction (Oxford, England) ***14****(2): 465-469.*

Egea RR, Puchalt NG, Escriva MM, Varghese AC. 2014. OMICS: Current and future perspectives in reproductive medicine and technology. Journal of human reproductive sciences 7(2): 73-92.

Eichenlaub-Ritter U, Shen Y, Tinneberg HR. 2002. Manipulation of the oocyte: possible damage to the spindle apparatus. Reproductive biomedicine online ***5****(2): 117-124.*

ESHRE ART fact sheet. 2020. https://www.eshre.eu/Press-Room/Resources.

ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine. 2017. The Vienna consensus: report of an expert meeting on the development of ART laboratory performance indicators. In Reproductive biomedicine online, Vol 35, pp. 494-510.

Fawzy M, Emad M, Gad MA, Sabry M, Kasem H, Mahmoud M, Bedaiwy MA. 2018. Comparing 36.5 degrees C with 37 degrees C for human embryo culture: a prospective randomized controlled trial. Reproductive biomedicine online ***36****(6): 620-626.*

Fischer B, Bavister BD. 1993. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. Journal of reproduction and fertility ***99****(2): 673-679.*

Fragouli E, Alfarawati S, Daphnis DD, Goodall NN, Mania A, Griffiths T, Gordon A, Wells D. 2011. Cytogenetic analysis of human blastocysts with the use of FISH, CGH and aCGH: scientific data and technical evaluation. Human reproduction (Oxford, England) ***26****(2): 480-490.*

Fragouli E, Alfarawati S, Spath K, Wells D. 2014. Morphological and cytogenetic assessment of cleavage and blastocyst stage embryos. Molecular human reproduction ***20****(2): 117-126.*

Freour T, Le Fleuter N, Lammers J, Splingart C, Reignier A, Barriere P. 2015. External validation of a time-lapse prediction model. Fertility and sterility ***103****(4): 917-922.*

Fujiwara M, Takahashi K, Izuno M, Duan YR, Kazono M, Kimura F, Noda Y. 2007. Effect of micro-environment maintenance on embryo culture after in-vitro fertilization: comparison of top-load mini incubator and conventional front-load incubator. Journal of assisted reproduction and genetics ***24****(1): 5-9.*

Gardner DK. 1998. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. Theriogenology ***49****(1): 83-102.*

Gardner DK, Balaban B. 2016. Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and 'OMICS': is looking good still important? Molecular human reproduction ***22****(10): 704-718.*

Gardner DK, Hamilton R, McCallie B, Schoolcraft WB, Katz-Jaffe MG. 2013. Human and mouse embryonic development, metabolism and gene expression are altered by an ammonium gradient in vitro. Reproduction (Cambridge, England) ***146****(1): 49-61.*

Gardner DK, Lane M. 1997. Culture and selection of viable blastocysts: a feasible proposition for human IVF? Human reproduction update ***3****(4): 367-382.*

Gardner DK, Lane M, Schoolcraft WB. 2002. Physiology and culture of the human blastocyst. Journal of reproductive immunology ***55****(1-2): 85-100.*

Gaspar RC, Arnold DR, Correa CA, da Rocha CV, Jr., Penteado JC, Del Collado M, Vantini R, Garcia JM, Lopes FL. 2015. Oxygen tension affects histone remodeling of in vitro-produced embryos in a bovine model. Theriogenology ***83****(9): 1408-1415.*

Ghosh J, Coutifaris C, Sapienza C, Mainigi M. 2017. Global DNA methylation levels are altered by modifiable clinical manipulations in assisted reproductive technologies. Clinical epigenetics ***9****: 14.*

Giorgetti C, Terriou P, Auquier P, Hans E, Spach JL, Salzmann J, Roulier R. 1995. Embryo score to predict implantation after in-vitro fertilization: based on 957 single embryo transfers. Human reproduction (Oxford, England) ***10****(9): 2427-2431.*

Glujovsky D, Farquhar C, Quinteiro Retamar AM, Alvarez Sedo CR, Blake D. 2016. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. The Cochrane database of systematic reviews(6): CD002118.

Goodman LR, Goldberg J, Falcone T, Austin C, Desai N. 2016. Does the addition of time-lapse morphokinetics in the selection of embryos for transfer improve pregnancy rates? A randomized controlled trial. Fertility and sterility ***105****(2): 275-285 e210.*

Goto Y, Noda Y, Mori T, Nakano M. 1993. Increased generation of reactive oxygen species in embryos cultured in vitro. Free radical biology & medicine ***15****(1): 69-75.*

Hardarson T, Bungum M, Conaghan J, Meintjes M, Chantilis SJ, Molnar L, Gunnarsson K, Wikland M. 2015. Noninferiority, randomized, controlled trial comparing embryo development using media developed for sequential or undisturbed culture in a time-lapse setup. Fertility and sterility ***104****(6): 1452-1459*

Hardarson T, Hanson C, Lundin K, Hillensjö T, Nilsson L, Stevic J, Reismer E, Borg K, Wikland M, Bergh C. 2008. Preimplantation genetic screening in women of advanced maternal age caused a decrease in clinical pregnancy rate: a randomized controlled trial. Human reproduction (Oxford, England) ***23****(12): 2806-2812.*

Hardarson T, Hanson C, Sjogren A, Lundin K. 2001. Human embryos with unevenly sized blastomeres have lower pregnancy and implantation rates: indications for aneuploidy and multinucleation. Human reproduction (Oxford, England) ***16****(2): 313-318.*

Hardarson T, Lofman C, Coull G, Sjogren A, Hamberger L, Edwards RG. 2002. Internalization of cellular fragments in a human embryo: time-lapse recordings. Reproductive biomedicine online ***5****(1): 36-38.*

Harper J, Magli MC, Lundin K, Barratt CL, Brison D. 2012. When and how should new technology be introduced into the IVF laboratory? Human reproduction (Oxford, England) ***27****(2): 303-313.*

Henningsen AA, Gissler M, Skjaerven R, Bergh C, Tiitinen A, Romundstad LB, Wennerholm UB, Lidegaard O, Nyboe Andersen A, Forman JL et al. 2015. Trends in perinatal health after assisted reproduction: a Nordic study from the CoNARTaS group. Human reproduction (Oxford, England) ***30****(3): 710-716.*

Hentemann M, Mousavi K, Bertheussen K. 2011. Differential pH in embryo culture. Fertility and sterility ***95****(4): 1291-1294.*

Hirao Y, Yanagimachi R. 1978. Detrimental effect of visible light on meiosis of mammalian eggs in vitro. The Journal of experimental zoology ***206****(3): 365-369.*

Holte J, Berglund L, Milton K, Garello C, Gennarelli G, Revelli A, Bergh T. 2007. Construction of an evidence-based integrated morphology cleavage embryo score for implantation potential of embryos scored and transferred on day 2 after oocyte retrieval. Human reproduction (Oxford, England) ***22****(2): 548-557.*

*Hong KH, Lee H, Forman EJ, Upham KM, Scott RT, Jr. 2014. Examining the temperature of embryo culture in in vitro fertilization: a randomized controlled trial comparing traditional core temperature (37 degrees C) to a more physiologic, cooler temperature (36 degrees C). Fertility and sterility **102**(3): 767-773.*

*Jones GM, Trounson AO, Lolatgis N, Wood C. 1998. Factors affecting the success of human blastocyst development and pregnancy following in vitro fertilization and embryo transfer. Fertility and sterility **70**(6): 1022-1029.*

*Kahraman S, Çetinkaya M, Pirkevi C, Yelke H, Kumtepe Y. 2013. Comparison of blastocyst development and cycle outcome in patients with eSET using either conventional or time lapse incubators. A prospective study of good prognosis patients. Journal of Reproductive and Stem Cell Biotechnology **3**(2): 55-61.*

*Kamath MS, Mascarenhas M, Franik S, Liu E, Sunkara SK. 2019. Clinical adjuncts in in vitro fertilization: a growing list. Fertility and sterility **112**(6): 978-986.*

*Karagenc L, Sertkaya Z, Ciray N, Ulug U, Bahceci M. 2004. Impact of oxygen concentration on embryonic development of mouse zygotes. Reproductive biomedicine online **9**(4): 409-417.*

*Kasterstein E, Strassburger D, Komarovsky D, Bern O, Komsky A, Raziel A, Friedler S, Ron-El R. 2013. The effect of two distinct levels of oxygen concentration on embryo development in a sibling oocyte study. Journal of assisted reproduction and genetics **30**(8): 1073-1079.*

*Katz-Jaffe MG, Linck DW, Schoolcraft WB, Gardner DK. 2005. A proteomic analysis of mammalian preimplantation embryonic development. Reproduction (Cambridge, England) **130**(6): 899-905.*

*Khosravi P, Kazemi E, Zhan Q, Malmsten JE, Toschi M, Zisimopoulos P, Sigaras A, Lavery S, Cooper LAD, Hickman C et al. 2019. Deep learning enables robust assessment and selection of human blastocysts after in vitro fertilization. NPJ digital medicine **2**: 21.*

*Kirkegaard K, Hindkjaer JJ, Grondahl ML, Kesmodel US, Ingerslev HJ. 2012. A randomized clinical trial comparing embryo culture in a conventional incubator with a time-lapse incubator. Journal of assisted reproduction and genetics **29**(6): 565-572.*

*Kirkegaard K, Kesmodel US, Hindkjaer JJ, Ingerslev HJ. 2013. Time-lapse parameters as predictors of blastocyst development and pregnancy outcome in embryos from good prognosis patients: a prospective cohort study. Human reproduction (Oxford, England) **28**(10): 2643-2651.*

*Kovacic B, Vlaisavljevic V. 2008. Influence of atmospheric versus reduced oxygen concentration on development of human blastocysts in vitro: a prospective study on sibling oocytes. Reproductive biomedicine online **17**(2): 229-236.*

*Kragh MF, Rimestad J, Berntsen J, Karstoft H. 2019. Automatic grading of human blastocysts from time-lapse imaging. Computers in biology and medicine **115**: 103494.*

*Kramer YG, Kofinas JD, Melzer K, Noyes N, McCaffrey C, Buldo-Licciardi J, McCulloh DH, Grifo JA. 2014. Assessing morphokinetic parameters via time lapse microscopy (TLM) to predict euploidy: are aneuploidy risk classification models universal? Journal of assisted reproduction and genetics **31**(9): 1231-1242.*

*La Marca A, Minasi MG, Sighinolfi G, Greco P, Argento C, Grisendi V, Fiorentino F, Greco E. 2017. Female age, serum antimullerian hormone level, and number of oocytes affect the rate and number of euploid blastocysts in in vitro fertilization/intracytoplasmic sperm injection cycles. Fertility and sterility **108**(5): 777-783 e772.*

*Lane M, Baltz JM, Bavister BD. 1999. Bicarbonate/chloride exchange regulates intracellular pH of embryos but not oocytes of the hamster. Biology of reproduction **61**(2): 452-457.*

*Lane M, Lyons EA, Bavister BD. 2000. Cryopreservation reduces the ability of hamster 2-cell embryos to regulate intracellular pH. Human reproduction (Oxford, England) **15**(2): 389-394.*

*Leese HJ, Baumann CG, Brison DR, McEvoy TG, Sturmey RG. 2008. Metabolism of the viable mammalian embryo: quietness revisited. Molecular human reproduction **14**(12): 667-672.*

*Lemmen JG, Agerholm I, Ziebe S. 2008. Kinetic markers of human embryo quality using time-lapse recordings of IVF/ICSI-fertilized oocytes. Reproductive biomedicine online **17**(3): 385-391.*

*Lensen S, Shreeve N, Barnhart KT, Gibreel A, Ng EHY, Moffett A. 2019. In vitro fertilization add-ons for the endometrium: it doesn't add-up. Fertility and sterility **112**(6): 987-993.*

*Liu Y, Chapple V, Feenan K, Roberts P, Matson P. 2016. Time-lapse deselection model for human day 3 in vitro fertilization embryos: the combination of qualitative and quantitative measures of embryo growth. Fertility and sterility **105**(3): 656-662 e651.*

*Liu Y, Chapple V, Roberts P, Matson P. 2014. Prevalence, consequence, and significance of reverse cleavage by human embryos viewed with the use of the Embryoscope time-lapse video system. Fertility and sterility **102**(5): 1295-1300 e1292.*

*Liu Y, Feenan K, Chapple V, Matson P. 2019. Assessing efficacy of day 3 embryo time-lapse algorithms retrospectively: impacts of dataset type and confounding factors. Human fertility (Cambridge, England) **22**(3): 182-190.*

Lubis HP, Halim B. 2018. Human Blastocyst Formation and Development. In Embryogenesis. IntechOpen.

*Lundin K, Ahlstrom A. 2015. Quality control and standardization of embryo morphology scoring and viability markers. Reproductive biomedicine online **31**(4): 459-471.*

*Lundin K, Bergh C. 2007. Cumulative impact of adding frozen-thawed cycles to single versus double fresh embryo transfers. Reproductive biomedicine online **15**(1): 76-82.*

*Lundin K, Bergh C, Hardarson T. 2001. Early embryo cleavage is a strong indicator of embryo quality in human IVF. Human reproduction (Oxford, England) **16**(12): 2652-2657.*

*Machtinger R, Racowsky C. 2012. Culture systems: single step. Methods in molecular biology (Clifton, NJ) **912**: 199-209.*

*Magdi Y, Samy A, Abbas AM, Ibrahim MA, Edris Y, El-Gohary A, Fathi AM, Fawzy M. 2019. Effect of embryo selection based morphokinetics on IVF/ICSI outcomes: evidence from a systematic review and meta-analysis of randomized controlled trials. Archives of gynecology and obstetrics **300**(6): 1479-1490.*

*Magli MC, Gianaroli L, Ferraretti AP. 2001. Chromosomal abnormalities in embryos. Molecular and cellular endocrinology **183 Suppl 1**: S29-34.*

*Magli MC, Jones GM, Lundin K, Van den Abbeel E. 2012. Atlas of human embryology: from oocytes to preimplantation embryos. Human reproduction (Oxford, England) **27**(Suppl 1): i1.*

*Maheshwari A, Hamilton M, Bhattacharya S. 2016. Should we be promoting embryo transfer at blastocyst stage? Reproductive biomedicine online **32**(2): 142-146.*

*Malchau SS, Henningsen AA, Forman J, Loft A, Nyboe Andersen A, Pinborg A. 2019. Cumulative live birth rate prognosis based on the number of aspirated oocytes in previous ART cycles. Human reproduction (Oxford, England) **34**(1): 171-180.*

*Malchau SS, Henningsen AA, Loft A, Rasmussen S, Forman J, Nyboe Andersen A, Pinborg A. 2017. The long-term prognosis for live birth in couples initiating fertility treatments. Human reproduction (Oxford, England) **32**(7): 1439-1449.*

*Malizia BA, Hacker MR, Penzias AS. 2009. Cumulative live-birth rates after in vitro fertilization. The New England journal of medicine **360**(3): 236-243.*

*Marquard K, Westphal LM, Milki AA, Lathi RB. 2010. Etiology of recurrent pregnancy loss in women over the age of 35 years. Fertility and sterility **94**(4): 1473-1477.*

*Martinez-Granados L, Serrano M, Gonzalez-Utor A, Ortiz N, Badajoz V, Lopez-Regalado ML, Boada M, Castilla JA. 2018. Reliability and agreement on embryo assessment: 5 years of an external quality control programme. Reproductive biomedicine online **36**(3): 259-268.*

*Massip A, Mulnard J. 1980. Time-lapse cinematographic analysis of hatching of normal and frozen-thawed cow blastocysts. Journal of reproduction and fertility **58**(2): 475-478.*

Massip A, Mulnard J, Vanderzwalmen P, Hanzen C, Ectors F. 1982. The behaviour of cow blastocyst in vitro: cinematographic and morphometric analysis. Journal of anatomy ***134****(Pt 2): 399-405.*

Mastenbroek S, Twisk M, van der Veen F, Repping S. 2011. Preimplantation genetic screening: a systematic review and meta-analysis of RCTs. Human reproduction update ***17****(4): 454-466.*

McLaren A, Biggers JD. 1958. Successful development and birth of mice cultivated in vitro as early as early embryos. Nature ***182****(4639): 877-878.*

Meintjes M, Chantilis SJ, Douglas JD, Rodriguez AJ, Guerami AR, Bookout DM, Barnett BD, Madden JD. 2009. A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program. Human reproduction (Oxford, England) ***24****(2): 300-307.*

Meseguer M, Herrero J, Tejera A, Hilligsoe KM, Ramsing NB, Remohi J. 2011. The use of morphokinetics as a predictor of embryo implantation. Human reproduction (Oxford, England) ***26****(10): 2658-2671.*

Meseguer M, Rubio I, Cruz M, Basile N, Marcos J, Requena A. 2012. Embryo incubation and selection in a time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: a retrospective cohort study. Fertility and sterility ***98****(6): 1481-1489 e1410.*

Minasi MG, Colasante A, Riccio T, Ruberti A, Casciani V, Scarselli F, Spinella F, Fiorentino F, Varricchio MT, Greco E. 2016. Correlation between aneuploidy, standard morphology evaluation and morphokinetic development in 1730 biopsied blastocysts: a consecutive case series study. Human reproduction (Oxford, England) ***31****(10): 2245-2254.*

Mio Y, Maeda K. 2008. Time-lapse cinematography of dynamic changes occurring during in vitro development of human embryos. American journal of obstetrics and gynecology ***199****(6): 660 e661-665.*

Montag M, Liebenthron J, Koster M. 2011. Which morphological scoring system is relevant in human embryo development? Placenta ***32 Suppl 3****: S252-256.*

Munne S, Kaplan B, Frattarelli JL, Child T, Nakhuda G, Shamma FN, Silverberg K, Kalista T, Handyside AH, Katz-Jaffe M et al. 2019. Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: a multicenter randomized clinical trial. Fertility and sterility ***112****(6): 1071-1079 e1077.*

Munne S, Sultan KM, Weier HU, Grifo JA, Cohen J, Rosenwaks Z. 1995. Assessment of numeric abnormalities of X, Y, 18, and 16 chromosomes in preimplantation human embryos before transfer. American journal of obstetrics and gynecology ***172****(4 Pt 1): 1191-1199; discussion 1199-1201.*

Nakahara T, Iwase A, Goto M, Harata T, Suzuki M, Ienaga M, Kobayashi H, Takikawa S, Manabe S, Kikkawa F et al. 2010. Evaluation of the safety of time-lapse observations for human embryos. Journal of assisted reproduction and genetics ***27****(2-3): 93-96.*

Oh SJ, Gong SP, Lee ST, Lee EJ, Lim JM. 2007. Light intensity and wavelength during embryo manipulation are important factors for maintaining viability of preimplantation embryos in vitro. Fertility and sterility ***88****(4 Suppl): 1150-1157.*

Olivius K, Friden B, Lundin K, Bergh C. 2002. Cumulative probability of live birth after three in vitro fertilization/intracytoplasmic sperm injection cycles. Fertility and sterility 77(3): 505-510.

Ottosen LD, Hindkjaer J, Ingerslev J. 2007. Light exposure of the ovum and preimplantation embryo during ART procedures. Journal of assisted reproduction and genetics ***24****(2-3): 99-103.*

Pabon JE, Jr., Findley WE, Gibbons WE. 1989. The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. Fertility and sterility ***51****(5): 896-900.*

Papanikolaou EG, Kolibianakis EM, Tournaye H, Venetis CA, Fatemi H, Tarlatzis B, Devroey P. 2008. Live birth rates after transfer of equal number of blastocysts or cleavage-stage embryos in IVF. A systematic review and meta-analysis. Human reproduction (Oxford, England) ***23****(1): 91-99.*

*Patel DV, Shah PB, Kotdawala AP, Herrero J, Rubio I, Banker MR. 2016. Morphokinetic behavior of euploid and aneuploid embryos analyzed by time-lapse in embryoscope. Journal of human reproductive sciences **9**(2): 112-118.*

*Paternot G, Devroe J, Debrock S, D'Hooghe TM, Spiessens C. 2009. Intra- and inter-observer analysis in the morphological assessment of early-stage embryos. Reproductive biology and endocrinology : RB&E **7**: 105.*

*Paternot G, Wetzels AM, Thonon F, Vansteenbrugge A, Willemen D, Devroe J, Debrock S, D'Hooghe TM, Spiessens C. 2011. Intra- and interobserver analysis in the morphological assessment of early stage embryos during an IVF procedure: a multicentre study. Reproductive biology and endocrinology : RB&E **9**: 127.*

*Payne D, Flaherty SP, Barry MF, Matthews CD. 1997. Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. Human reproduction (Oxford, England) **12**(3): 532-541.*

*Petersen BM, Boel M, Montag M, Gardner DK. 2016. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on Day 3. Human reproduction (Oxford, England) **31**(10): 2231-2244.*

*Pinborg A, Loft A, Nyboe Andersen A. 2004. Neonatal outcome in a Danish national cohort of 8602 children born after in vitro fertilization or intracytoplasmic sperm injection: the role of twin pregnancy. Acta obstetricia et gynecologica Scandinavica **83**(11): 1071-1078.*

*Pool TB. 2002. Recent advances in the production of viable human embryos in vitro. Reproductive biomedicine online **4**(3): 294-302.*

*Practice Committees of ASRM and SART. 2018. The use of preimplantation genetic testing for aneuploidy (PGT-A): a committee opinion. Fertility and sterility **109**(3): 429-436.*

*Pribenszky C, Losonczi E, Molnar M, Lang Z, Matyas S, Rajczy K, Molnar K, Kovacs P, Nagy P, Conceicao J et al. 2010. Prediction of in-vitro developmental competence of early cleavage-stage mouse embryos with compact time-lapse equipment. Reproductive biomedicine online **20**(3): 371-379.*

*Provoost V, Tilleman K, D'Angelo A, De Sutter P, de Wert G, Nelen W, Pennings G, Shenfield F, Dondorp W. 2014. Beyond the dichotomy: a tool for distinguishing between experimental, innovative and established treatment. Human reproduction (Oxford, England) **29**(3): 413-417.*

Q-IVF Årsrapport. 2019. https://www.medscinet.com/givf/arsrapporter.aspx.

*Ravichandran K, McCaffrey C, Grifo J, Morales A, Perloe M, Munne S, Wells D, Fragouli E. 2017. Mitochondrial DNA quantification as a tool for embryo viability assessment: retrospective analysis of data from single euploid blastocyst transfers. Human reproduction (Oxford, England) **32**(6): 1282-1292.*

*Reignier A, Lammers J, Barriere P, Freour T. 2018. Can time-lapse parameters predict embryo ploidy? A systematic review. Reproductive biomedicine online **36**(4): 380-387.*

*Rhenman A, Berglund L, Brodin T, Olovsson M, Milton K, Hadziosmanovic N, Holte J. 2015. Which set of embryo variables is most predictive for live birth? A prospective study in 6252 single embryo transfers to construct an embryo score for the ranking and selection of embryos. Human reproduction (Oxford, England) **30**(1): 28-36.*

*Rienzi L, Ubaldi F, Iacobelli M, Romano S, Minasi MG, Ferrero S, Sapienza F, Baroni E, Greco E. 2005. Significance of morphological attributes of the early embryo. Reproductive biomedicine online **10**(5): 669-681.*

*Rinaudo PF, Giritharan G, Talbi S, Dobson AT, Schultz RM. 2006. Effects of oxygen tension on gene expression in preimplantation mouse embryos. Fertility and sterility **86**(4 Suppl): 1252-1265, 1265 e1251-1236.*

*Rubio I, Galan A, Larreategui Z, Ayerdi F, Bellver J, Herrero J, Meseguer M. 2014. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the EmbryoScope. Fertility and sterility **102**(5): 1287-1294 e1285.*

*Rubio I, Kuhlmann R, Agerholm I, Kirk J, Herrero J, Escriba MJ, Bellver J, Meseguer M. 2012. Limited implantation success of direct-cleaved human zygotes: a time-lapse study. Fertility and sterility **98**(6): 1458-1463.*

*Salumets A, Hyden-Granskog C, Makinen S, Suikkari AM, Tiitinen A, Tuuri T. 2003. Early cleavage predicts the viability of human embryos in elective single embryo transfer procedures. Human reproduction (Oxford, England) **18**(4): 821-825.*

*Schumacher A, Fischer B. 1988. Influence of visible light and room temperature on cell proliferation in preimplantation rabbit embryos. Journal of reproduction and fertility **84**(1): 197-204.*

*Sciorio R, Tramontano L, Catt J. 2020. Preimplantation genetic diagnosis (PGD) and genetic testing for aneuploidy (PGT-A): status and future challenges. Gynecological endocrinology : the official journal of the International Society of Gynecological Endocrinology **36**(1): 6-11.*

*Scott KL, Hong KH, Scott RT, Jr. 2013. Selecting the optimal time to perform biopsy for preimplantation genetic testing. Fertility and sterility **100**(3): 608-614.*

*Scott L, Berntsen J, Davies D, Gundersen J, Hill J, Ramsing N. 2008. Symposium: innovative techniques in human embryo viability assessment. Human oocyte respiration-rate measurement--potential to improve oocyte and embryo selection? Reproductive biomedicine online **17**(4): 461-469.*

*Sfontouris IA, Martins WP, Nastri CO, Viana IG, Navarro PA, Raine-Fenning N, van der Poel S, Rienzi L, Racowsky C. 2016. Blastocyst culture using single versus sequential media in clinical IVF: a systematic review and meta-analysis of randomized controlled trials. Journal of assisted reproduction and genetics **33**(10): 1261-1272.*

*Sjoblom P, Menezes J, Cummins L, Mathiyalagan B, Costello MF. 2006. Prediction of embryo developmental potential and pregnancy based on early stage morphological characteristics. Fertility and sterility **86**(4): 848-861.*

*Skiles WM, Kester A, Pryor JH, Westhusin ME, Golding MC, Long CR. 2018. Oxygen-induced alterations in the expression of chromatin modifying enzymes and the transcriptional regulation of imprinted genes. Gene expression patterns : GEP **28**: 1-11.*

Steer CV, Mills CL, Tan SL, Campbell S, Edwards RG. 1992. The cumulative embryo score: a predictive embryo scoring technique to select the optimal number of embryos to transfer in an in-vitro fertilization and embryo transfer programme. Human reproduction (Oxford, England) 7(1): 117-119.

*Steptoe PC, Edwards RG. 1978. Birth after the reimplantation of a human embryo. Lancet (London, England) **2**(8085): 366.*

*Storr A, Venetis CA, Cooke S, Kilani S, Ledger W. 2017. Inter-observer and intra-observer agreement between embryologists during selection of a single Day 5 embryo for transfer: a multicenter study. Human reproduction (Oxford, England) **32**(2): 307-314.*

*Summers MC, Biggers JD. 2003. Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. Human reproduction update **9**(6): 557-582.*

*Sun XF, Wang WH, Keefe DL. 2004. Overheating is detrimental to meiotic spindles within in vitro matured human oocytes. Zygote (Cambridge, England) **12**(1): 65-70.*

*Swain JE. 2012. Is there an optimal pH for culture media used in clinical IVF? Human reproduction update **18**(3): 333-339.*

*Swain JE. 2014. Decisions for the IVF laboratory: comparative analysis of embryo culture incubators. Reproductive biomedicine online **28**(5): 535-547.*

*Swain JE, Carrell D, Cobo A, Meseguer M, Rubio C, Smith GD. 2016. Optimizing the culture environment and embryo manipulation to help maintain embryo developmental potential. Fertility and sterility **105**(3): 571-587.*

*Thompson JG, Brown HM, Sutton-McDowall ML. 2016. Measuring embryo metabolism to predict embryo quality. Reproduction, fertility, and development **28**(1-2): 41-50.*

Thurin A, Hardarson T, Hausken J, Jablonowska B, Lundin K, Pinborg A, Bergh C. 2005. Predictors of ongoing implantation in IVF in a good prognosis group of patients. Human reproduction (Oxford, England) ***20****(7): 1876-1880.*

Thurin A, Hausken J, Hillensjo T, Jablonowska B, Pinborg A, Strandell A, Bergh C. 2004. Elective single-embryo transfer versus double-embryo transfer in in vitro fertilization. The New England journal of medicine ***351****(23): 2392-2402.*

Tiitinen A, Hyden-Granskog C, Gissler M. 2004. What is the most relevant standard of success in assisted reproduction?: The value of cryopreservation on cumulative pregnancy rates per single oocyte retrieval should not be forgotten. Human reproduction (Oxford, England) ***19****(11): 2439-2441.*

Tran D, Cooke S, Illingworth PJ, Gardner DK. 2019. Deep learning as a predictive tool for fetal heart pregnancy following time-lapse incubation and blastocyst transfer. Human reproduction (Oxford, England) ***34****(6): 1011-1018.*

Turner K, Martin KL, Woodward BJ, Lenton EA, Leese HJ. 1994. Comparison of pyruvate uptake by embryos derived from conception and non-conception natural cycles. Human reproduction (Oxford, England) ***9****(12): 2362-2366.*

Van Montfoort AP, Dumoulin JC, Kester AD, Evers JL. 2004. Early cleavage is a valuable addition to existing embryo selection parameters: a study using single embryo transfers. Human reproduction (Oxford, England) ***19****(9): 2103-2108.*

Van Royen E, Mangelschots K, De Neubourg D, Laureys I, Ryckaert G, Gerris J. 2001. Calculating the implantation potential of day 3 embryos in women younger than 38 years of age: a new model. Human reproduction (Oxford, England) ***16****(2): 326-332.*

Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, Eestermans W, Gerris J. 1999. Characterization of a top quality embryo, a step towards single-embryo transfer. Human reproduction (Oxford, England) ***14****(9): 2345-2349.*

Venter JC Adams MD Myers EW Li PW Mural RJ Sutton GG Smith HO Yandell M Evans CA Holt RA et al. 2001. The sequence of the human genome. Science (New York, NY) ***291****(5507): 1304-1351.*

Verlinsky Y, Cieslak J, Freidine M, Ivakhnenko V, Wolf G, Kovalinskaya L, White M, Lifchez A, Kaplan B, Moise J et al. 1995. Pregnancies following pre-conception diagnosis of common aneuploidies by fluorescent in-situ hybridization. Human reproduction (Oxford, England) ***10****(7): 1923-1927.*

VerMilyea MD, Tan L, Anthony JT, Conaghan J, Ivani K, Gvakharia M, Boostanfar R, Baker VL, Suraj V, Chen AA et al. 2014. Computer-automated time-lapse analysis results correlate with embryo implantation and clinical pregnancy: a blinded, multi-centre study. Reproductive biomedicine online ***29****(6): 729-736.*

Virant-Klun I, Tomazevic T, Vrtacnik-Bokal E, Vogler A, Krsnik M, Meden-Vrtovec H. 2006. Increased ammonium in culture medium reduces the development of human embryos to the blastocyst stage. Fertility and sterility ***85****(2): 526-528.*

Waldenstrom U, Engstrom AB, Hellberg D, Nilsson S. 2009. Low-oxygen compared with high-oxygen atmosphere in blastocyst culture, a prospective randomized study. Fertility and sterility ***91****(6): 2461-2465.*

Wale PL, Gardner DK. 2016. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. Human reproduction update ***22****(1): 2-22.*

Wang A, Kort J, Behr B, Westphal LM. 2018. Euploidy in relation to blastocyst sex and morphology. Journal of assisted reproduction and genetics ***35****(9): 1565-1572.*

Wang WH, Meng L, Hackett RJ, Odenbourg R, Keefe DL. 2001. Limited recovery of meiotic spindles in living human oocytes after cooling-rewarming observed using polarized light microscopy. Human reproduction (Oxford, England) ***16****(11): 2374-2378.*

Wang WH, Meng L, Hackett RJ, Oldenbourg R, Keefe DL. 2002. Rigorous thermal control during intracytoplasmic sperm injection stabilizes the meiotic spindle and improves fertilization and pregnancy rates. Fertility and sterility 77(6): 1274-1277.

*Whitten WK. 1956. Culture of tubal mouse ova. Nature **177**(4498): 96.*

*Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, Reijo Pera RA. 2010. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. Nature biotechnology **28**(10): 1115-1121.*

*Wu YG, Lazzaroni-Tealdi E, Wang Q, Zhang L, Barad DH, Kushnir VA, Darmon SK, Albertini DF, Gleicher N. 2016. Different effectiveness of closed embryo culture system with time-lapse imaging (EmbryoScope(TM)) in comparison to standard manual embryology in good and poor prognosis patients: a prospectively randomized pilot study. Reproductive biology and endocrinology : RB&E **14**(1): 49.*

*Yalcinkaya E, Ergin EG, Caliskan E, Oztel Z, Ozay A, Ozornek H. 2014. Reproducibility of a time-lapse embryo selection model based on morphokinetic data in a sequential culture media setting. Journal of the Turkish German Gynecological Association **15**(3): 156-160.*

*Yang L, Cai S, Zhang S, Kong X, Gu Y, Lu C, Dai J, Gong F, Lu G, Lin G. 2018. Single embryo transfer by Day 3 time-lapse selection versus Day 5 conventional morphological selection: a randomized, open-label, non-inferiority trial. Human reproduction (Oxford, England) **33**(5): 869-876.*

*Yang Z, Lin J, Zhang J, Fong WI, Li P, Zhao R, Liu X, Podevin W, Kuang Y, Liu J. 2015. Randomized comparison of next-generation sequencing and array comparative genomic hybridization for preimplantation genetic screening: a pilot study. BMC medical genomics **8**: 30.*

*Yoshida IH, Santos M, Berton CZ, Chiarella CL, Tanada MS, E BC, Carvalho WP, Barbosa CP. 2018. Can trophectoderm morphology act as a predictor for euploidy? JBRA assisted reproduction **22**(2): 113-115.*

*Zander-Fox DL, Mitchell M, Thompson JG, Lane M. 2010. Alterations in mouse embryo intracellular pH by DMO during culture impair implantation and fetal growth. Reproductive biomedicine online **21**(2): 219-229.*

*Zegers-Hochschild F, Adamson GD, Dyer S, Racowsky C, de Mouzon J, Sokol R, Rienzi L, Sunde A, Schmidt L, Cooke ID et al. 2017. The International Glossary on Infertility and Fertility Care, 2017. Fertility and sterility **108**(3): 393-406.*

*Zhan Q, Ye Z, Clarke R, Rosenwaks Z, Zaninovic N. 2016. Direct Unequal Cleavages: Embryo Developmental Competence, Genetic Constitution and Clinical Outcome. PloS one **11**(12): e0166398.*

*Ziebe S, Lundin K, Loft A, Bergh C, Nyboe Andersen A, Selleskog U, Nielsen D, Grondahl C, Kim H, Arce JC. 2003. FISH analysis for chromosomes 13, 16, 18, 21, 22, X and Y in all blastomeres of IVF pre-embryos from 144 randomly selected donated human oocytes and impact on pre-embryo morphology. Human reproduction (Oxford, England) **18**(12): 2575-2581.*

*Ziebe S, Petersen K, Lindenberg S, Andersen AG, Gabrielsen A, Andersen AN. 1997. Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization. Human reproduction (Oxford, England) **12**(7): 1545-1549.*

PAPER I

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ORIGINAL ARTICLE *Embryology*

I

No benefit of culturing embryos in a closed system compared with a conventional incubator in terms of number of good quality embryos: results from an RCT

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STUDY QUESTION: Does culture in a closed system result in an increased number of good quality embryos (GQE) on Day 2 compared with culture in a conventional system?

SUMMARY ANSWER: Culture in a closed system up to 2 days after microinjection results in similar embryo development and morphological quality compared with culture in a conventional incubation system.

WHAT IS KNOWN ALREADY: Time-lapse imaging (TLI) incubators are rapidly being introduced into IVF laboratories worldwide, despite the lack of large prospective randomized trials demonstrating improvement in embryo development or pregnancy rates.

STUDY DESIGN, SIZE, DURATION: A randomized controlled trial including 364 patients (365 cycles) was conducted between May 2010 and February 2014. After oocyte collection, randomization was carried out and all of a patients' oocytes were allocated to culture in either a conventional incubator or a closed incubator system in proportion 1:2 until embryo transfer on Day 2. A total of 1979 oocytes were injected and cultured in the closed system, and 1000 in the standard incubator. The primary end-point was the number of GQE in the two groups.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: In total, 364 patients undergoing their first IVF cycle using ICSI, where at least one oocyte was retrieved, were randomized in a university hospital setting. Two hundred and forty patients were randomized for culture in a closed system and 124 patients for culture in the conventional incubator system (control group). Embryo assessments and final morphological scoring before transfer and cryopreservation were carried out at the same time points for embryos cultured in the conventional incubator and in the closed system.

MAIN RESULTS AND THE ROLE OF CHANCE: There was no significant difference in the mean ± SD number of GQEs between groups: 2.41 ± 2.27 for the closed system group and 2.19 ± 1.82 for the control group ($P = 0.34$, difference 0.23, 95% confidence interval 0.69; −0.24). No significant differences were found in the number of 4-cell embryos, implantation-, pregnancy- or ongoing pregnancy rates. A significantly higher miscarriage rate was found in the TLI group compared with the control group (33.3 and 10.2%, $P = 0.01$).

LIMITATIONS, REASONS FOR CAUTION: Culture media, temperature and gas levels were similar in the open and closed incubator systems, but different culture dishes were used. Culturing embryos for longer time period (to the blastocyst stage) may give different results. Only ICSI patients were included, which may limit the generalizability of the results. Finally, the number of GQEs on Day 2 was used as a surrogate outcome for live birth.

WIDER IMPLICATIONS OF THE FINDINGS: The results are consistent with other, smaller randomized trials showing no difference in embryo quality when comparing culture in a conventional incubator with that of a closed TLI incubator system.

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Key words: assisted reproduction / time-lapse / embryo culture / safety

Introduction

A number of developments with the aim to improve embryo culture have taken place in the assisted reproductive technology (ART) laboratory over the years, for example, the transition to more complex culture media (Gardner and Lane, 1997; Summers and Biggers, 2003; Lane and Gardner, 2007; Mantikou *et al.*, 2013), prolongation of culture from 2–3 to 5–6 days (Marek *et al.*, 1999; Nilsson *et al.*, 2005; Papanikolaou *et al.*, 2006; Glujovsky *et al.*, 2012), and culture at reduced oxygen tension (Waldenström *et al.*, 2008; Kovacic *et al.*, 2010; Bontekoe *et al.*, 2012; Kirkegaard *et al.*, 2013a). The ART sector is rapidly moving forward and many new techniques are being introduced without proper validation of the safety or potential benefits (Vajta *et al.*, 2010; Harper *et al.*, 2012).

Most procedures in the ART laboratory are still performed manually, but new, more automated technologies are now being developed (Meseguer *et al.*, 2012a). Recently, a new type of system for culturing human embryos has been introduced; the time-lapse imaging (TLI) system, where assessment of key events during embryo development can be performed without removing the embryos from the incubator. Although TLI systems can be more or less closed, the possible advantage of these systems is minimization of environmental fluctuations in temperature, pH and humidity, which could impair embryo quality (Fujiwara *et al.*, 2007; Zhang *et al.*, 2010).

When assessing embryos only during short intervals outside the incubator, as in conventional culture systems, important morphological and development events which could have an impact on implantation might be missed. TLI overcomes some of these problems by gaining information of embryo development through continuous image recordings. Several retrospective observational studies using this new methodology have indicated a number of early timing variables, mainly concerning the duration and synchrony of the first cell cycles that may predict blastocyst formation (Wong *et al.*, 2010; Cruz *et al.*, 2012; Dal Canto *et al.*, 2012; Chamayou *et al.*, 2013; Conaghan *et al.*, 2013; Kirkegaard *et al.*, 2013b), implantation (Meseguer *et al.*, 2011; Chamayou *et al.*, 2013) and pregnancy (Lemmen *et al.*, 2008; Meseguer *et al.*, 2012b). However, Kirkegaard *et al.* (2013b) showed, in a prospective cohort study, that although blastocyst formation could be predicted by a few time-lapse variables, there was no difference in the timing between implanted and non-implanted blastocysts. Only a few small studies have looked at the possible advantages of culturing in a TLI system *per se*. Nakahara *et al.* (2010) showed in a prospective study of 292 oocytes from 84 patients that the fertilization rate and number of good quality embryos (GQE) were similar when compared with culture in a standard incubator. Cruz *et al.* (2011), in an oocyte donation programme including 60 patients and 478 oocytes, found no differences in blastocyst and ongoing pregnancy rates for embryos cultured in a conventional incubator versus a TLI incubator, and Kirkegaard *et al.* (2012), in a small randomized controlled trial (RCT) (59 patients), found similar embryo development rates between the two systems, as well as similar pregnancy and implantation rates.

The aim of this RCT was to analyse in a large population whether culture of human embryos in a closed system with TLI is superior to culture in a standard incubator in terms of number of GQEs on Day 2.

Materials and Methods

Clinical setting/patient group

An RCT was conducted at Reproductive Medicine, Sahlgrenska University Hospital, Gothenburg. Patients were recruited between May 2010 and February 2014. Patients were eligible if they were $\leq$40 years of age, undergoing their first IVF cycle using ICSI and at least one oocyte was retrieved. Patients undergoing egg donation were excluded.

In total, 364 patients were included and randomized. Only one cycle per patient was included. Randomization was carried out by the embryologist after oocyte retrieval by a web-based randomization programme and all the patients' oocytes were allocated to culture in either a conventional incubator or in a closed system, in proportion 1:2. The patients as well as the treating physician and the person performing the statistical analyses were blinded to which type of procedure was used until the outcome of transfer (pregnant versus not pregnant) was known. Embryologists were not possible to blind.

The study was approved by the Ethical Committee of the University of Gothenburg (Dnr: 666-09) and all patients signed an informed consent.

Stimulation, oocyte retrieval and ICSI

Ovarian stimulation was performed using down-regulation with a GnRH agonist (Suprecur, Sanofi, Paris, France) in a long protocol, followed by stimulation with recombinant FSH (Gonal-F, Merck Serono, Darmstadt, Germany, or Puregon, MSD, NJ, USA), or urinary-derived gonadotrophins (Menopur, Ferring, Copenhagen, Denmark). In a few cases ($n = 28$), a GnRH antagonist (Orgalutran, MSD) was used in a short protocol. Follicular development was monitored by serum estradiol levels and vaginal sonography. When two or more follicles reached $\geq$18 mm diameter, hCG (Pregnyl 5000 or 10 000 IU, MSD or Ovitrelle 6500 IU, Merck Serono) was administered. Oocyte retrieval was scheduled 36 $\pm$ 2 h after hCG injection. Crinone gel (Merck Serono) or progesterone MIC by vaginal route was given as luteal support after embryo transfer.

The oocyte–cumulus complexes were collected using transvaginal sonographically guided puncture, rinsed in MOPS (Vitrolife, Gothenburg, Sweden) and placed in a culture dish (Falcon, VWR, NJ, USA) containing G-IVF medium (Vitrolife).

The oocyte–cumulus complexes were denuded using hyaluronidase (Vitrolife). ICSI was performed within 5 h of oocyte retrieval on mature (metaphase II: MII) oocytes in pre-equilibrated culture dishes (Falcon) with droplets of Gamete (Vitrolife) with an overlay of 6 ml mineral oil (Ovoil, Vitrolife) under an inverted microscope.

Standard culture system

For the control (standard incubator) group, the oocytes were rinsed in G-1 media directly after the ICSI procedure and then transferred to pre-equilibrated culture dishes (Falcon), with 20 μl droplets of G-1 media (Vitrolife) under mineral oil (Ovoil). The oocytes were cultured in a standard incubator at 37°C, 6% CO_2 and atmospheric O_2 concentration until embryo transfer on Day 2. pH and temperature were monitored on a weekly basis for the standard incubator, while CO_2 was monitored less frequently.

For the control group, the embryos were taken out of the standard incubator at 16–18 h after injection in order to check for fertilization, at 25–27 h post-ICSI for early cleavage screening, and finally at 43–45 h post-ICSI for assessment of quality prior to transfer and cryopreservation. Scoring was performed using an Olympus inverted microscope with a 20 × 1.5 Hoffman Modulation contrast objective.

Closed culture system

The EmbryoScope™ (Unisense Fertilitech, Århus, Denmark) is an incubator with a built-in microscope with a Leica 20 × 0.40 LWD Hoffman Modulation contrast objective. It is capable of acquiring images for up to 72 oocytes/embryos simultaneously. During image acquisition, the embryos are illuminated with low intensity red light at 635 nm for <0.5 s per image. For this study, images were acquired every 20 min, at seven focal planes.

For the study group (closed system), the oocytes were washed in G1 media after injection, transferred with a Cook Flexipet (Cook, Limerick, Ireland) to EmbryoSlides prepared as described below, and cultured in the EmbryoScope™ until time of transfer. The embryos in the EmbryoScope™ were incubated under the same culture conditions as the control group, i.e. at 37°C, 6% CO_2 and atmospheric O_2 concentration. CO_2 and temperature were monitored on a weekly basis for the EmbryoScope™.

According to the manufacturer's recommendations, EmbryoSlides (Unisense, Fertilitech) were prepared in the afternoon the day before oocyte retrieval with 25 μl culture medium (G1). The EmbryoSlides were covered with a 1.2 ml layer of oil (Ovoil) and pre-equilibrated in a standard incubator. In the study group, embryos were not removed from the EmbryoScope™ during assessments.

Embryo selection and transfer

At least two embryologists were involved in assessing embryo quality. For embryos cultured in the EmbryoScope™ as well as for embryos cultured in a conventional incubator, morphological assessment and selection for transfer were made at the same time points using the same criteria.

Additional information available from time-lapse sequences was not used for embryo assessment or selection.

Fertilized oocytes were scored for pronuclei on Day 1 at 16–18 h after ICSI and for early cleavage at 25–27 h after ICSI.

On Day 2, at 43–45 h post-ICSI, embryos were graded according to blastomere number, blastomere size and degree of fragmentation. An embryo was defined as a GQE on Day 2 when having 4–6 blastomeres and <20% fragmentation, with no multinucleation. When choosing embryos for transfer with otherwise equal quality on Day 2, early cleavage and the presence of nuclei in the cells were also taken into account. If no GQEs were available, embryos with an increased fragmentation rate could be transferred but were not cryopreserved.

One embryo (in a few cases two embryos, $n = 12$) of good quality or in some cycles of less good quality ($n = 27$) was transferred on Day 2 and supernumerary GQEs were frozen on the same day using a slow freezing protocol (Cook Medical, Ireland).

End-points

The primary end-point was the number of GQEs. Secondary end-points were fertilization rate, number of 4-cell embryos on Day 2, implantation, pregnancy, miscarriage and ongoing pregnancy rates. Ongoing pregnancy rate was defined as the presence of a gestational sac with fetal heartbeat ≥8 weeks.

Sample size determination and statistical analysis

The study was a superiority trial. The sample size was based on the primary outcome of the study; the number of GQEs. With an average number of GQEs of 3.9 and an SD of 3.1 (Lundin and Bergh, 2007), a total of 357 patients were needed to show an increase with 1.0 GQEs in the intervention group (α-value 0.05, power 80%) if the randomization into the study versus control groups is performed 2:1, i.e. 238 patients in the intervention group and 119 patients in the control group.

The patients were randomized by a web-based computer program. Stratification was performed by minimizing for age and mean number of aspirated oocytes (Pocock, 1983). For descriptive statistics, continuous variables are presented as mean ± SD and ranges. Categorical variables are presented as *n* (%).

For comparison between the groups, Fisher's exact test was used for dichotomous variables and the Mann–Whitney *U*-test was used for continuous variables. For main variables, 95% confidence intervals (CI) were presented for differences in estimates. A *P*-value of <0.05 was considered significant. In order to select independent predictors of the dependent variables ongoing pregnancy rate and miscarriage rate, univariable logistic regression analysis was first performed for each of the baseline variables. Variables with $P < 0.25$ were then entered into a stepwise multiple logistic regression analysis.

Statistical analyses were performed using SAS software version 9.3 (SAS Institute, Inc., NC, USA), and SPSS software version 22, 2013 (SPSS, Chicago, IL, USA).

Results

A flow-chart of patients included in the study is shown in Fig. 1.

In total, 364 patients (365 cycles) were randomized between May 2010 and February 2014. Their oocytes were allocated to culture in either the TLI incubator (241 cycles, 2280 oocytes) or a standard incubator (124 cycles, 1180 oocytes). Analysis was performed by intention-to-treat (Fig. 1), but excluding one cycle prior to analysis, due to this patient having been randomized twice.

Patient demographics are presented in Table I. No significant differences were found between the two groups.

A total of 1979 oocytes were injected and cultured in the EmbryoScope™, and 1000 in the standard incubator. No significant difference was found between culture in the EmbryoScope™ and standard incubator regarding the number of GQEs on Day 2 (2.41 ± 2.27 for the EmbryoScope™ group and 2.19 ± 1.82 for the standard incubator group; $P = 0.34$, difference 0.23, 95% CI 0.69; −0.24), nor for any other embryo variables (Table II).

The pregnancy rate per randomized woman was 30.0% in the EmbryoScope™ and 31.5% in the standard incubator ($P = 0.87$) and per embryo transfer 33.5 and 34.2%, respectively ($P = 0.99$). The ongoing pregnancy rate was 20.0% in the EmbryoScope™ and 28.2% in the standard incubator ($P = 0.10$) per randomized cycle and 22.3 and 30.7% ($P = 0.13$) per embryo transfer, respectively (Table III). The miscarriage rate was 33.3% in the EmbryoScope group and 10.2% in the control group ($P = 0.011$).

In the stepwise multiple logistic regression analysis, the baseline variables 'smoking' [adjusted odds ratio (OR) 0.329; 95% CI 0.112–0.967, adjusted $P = 0.035$] and 'number of embryos transferred' (adjusted OR 3.351; 95% CI 1.447–7.759, adjusted $P = 0.0036$) were independently correlated to the variable ongoing pregnancy.

For the variable miscarriage, only the baseline variable 'group' (adjusted OR 4.367; 95% CI 1.393–13.699, adjusted $P = 0.0075$) was independently correlated to miscarriage.

For comparison, a proportion of transferred embryos ($n = 146$) was scored in both the EmbryoScope and in the Olympus microscope. We found that 134 (91.8%) were scored equally in both systems. Ten

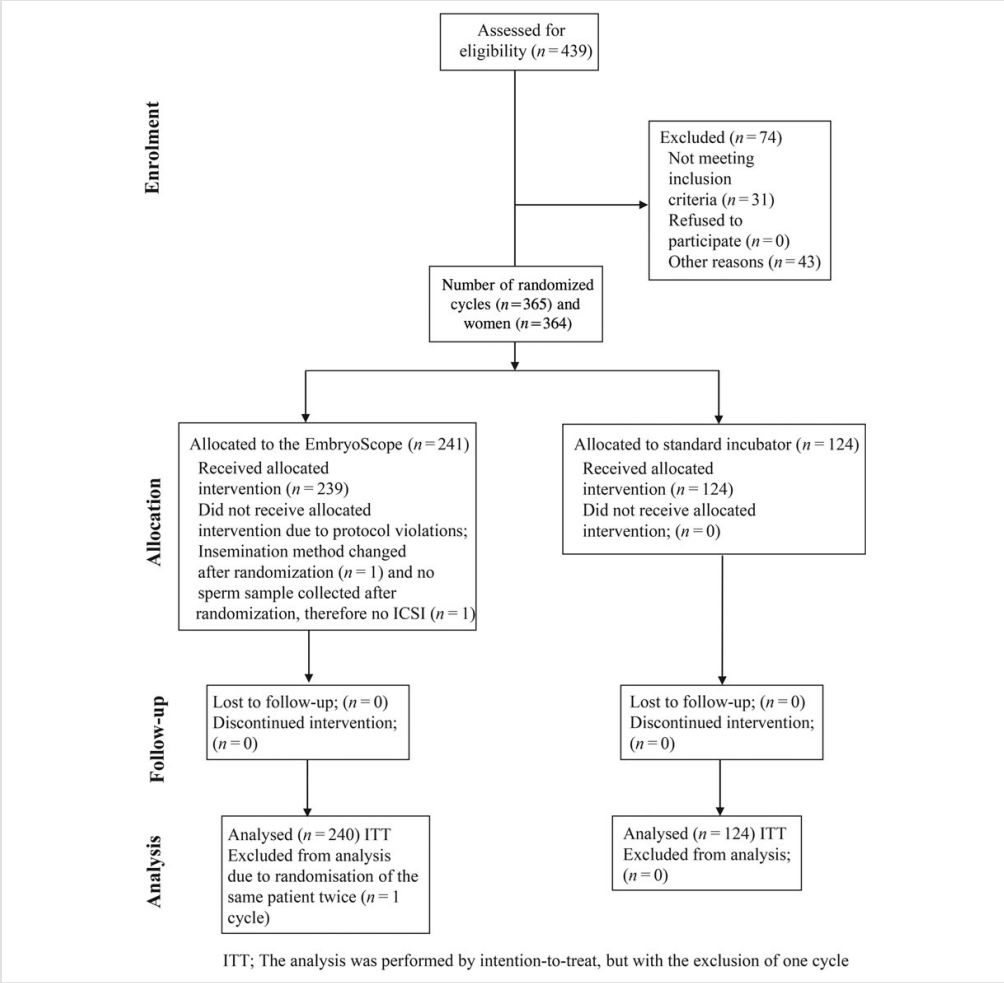

Figure 1 CONSORT statement flow diagram.

embryos were scored as GQE in the EmbryoScope™ but not in the Olympus microscope, and two as GQE in the Olympus microscope but not in the EmbryoScope™. In four embryos, the number of cells differed by maximum one cell, in three embryos, the cell size symmetry was not equally scored and in five embryos, the percentage of fragmentation differed. The outcome of this comparison did not influence which embryo was selected for transfer.

Discussion

There are two key questions when comparing the culture of human embryos in a closed TLI system with a conventional incubator: (i) Is the closed culture system superior to the conventional incubator concerning embryo development? (ii) Is the TLI system, when using new embryo variables identified from the TLI system, superior to conventional embryo morphology when selecting embryos for transfer? In the present study, we have addressed only the first question.

The main finding was that no significant difference between the two groups was found in the number of GQEs on Day 2. Neither were any significant differences in the number of 4-cell embryos, implantation-, pregnancy- or ongoing pregnancy rates detected, while the miscarriage rate was significantly higher in the TLI group.

The main results are in agreement with a recent RCT by Kirkegaard *et al.* (2012), where a closed incubator system was compared with a

Table I Baseline characteristics of patients for the two groups.

	EmbryoScope (n = 240)	Control (n = 124)	P-value
Age, years	31.8 ± 4.3 (21.4–39.7)	31.8 ± 4.1 (22.3–39.7)	0.90
BMI, kg/m^2	24.4 ± 3.9 (16.8–36.1)	24.3 ± 4.0 (16.5–34.0)	0.70
No. of smokers	30 (12.5)	11 (8.9)	0.39
No. of chew (oral tobacco) users	4 (1.7)	5 (4.0)	0.31
Cause of infertility, male factor	239 (99.6)	123 (99.2)	1.00
Cause of infertility, female factor	56 (23.3)	24 (19.4)	0.46
Duration of infertility, years	2.77 ± 1.5 (1.0–11.0)	2.79 ± 1.7 (1.0–12.0)	0.34
Pregnancies in previous relation	44 (18.3)	23 (18.5)	1.00
Pregnancies present relation	28 (11.7)	7 (5.6)	0.09
Miscarriages in previous relation	11 (4.6)	8 (6.5)	0.60
Miscarriages in present relation	20 (8.3)	5 (4.0)	0.18
Parous, previous relation	15 (6.3)	8 (6.5)	
Parous, present relation	0	0	

For categorical variables, n (%) is presented. For continuous variables, mean (SD) and range is presented. For comparison between the groups, Fisher's exact test was used for dichotomous variables and the Mann–Whitney U-test was used for continuous variables.

Table II Comparison of embryology data for the two groups.

	EmbryoScope (n = 240)	Control (n = 124)	P-value	Difference (95% confidence interval)
No. of oocytes retrieved per patient	9.50 ± 5.5 (1–32)	9.52 ± 4.5 (1–23)	0.47	
No. of injected (metaphase II) oocytes	8.25 ± 4.8 (0–27)	8.06 ± 4.0 (1–20)	0.69	
No. of fertilized (2 pronuclei) oocytes	4.70 ± 3.2 (0–21)	4.73 ± 3.1 (0–15)	0.92	
No. of 4-cell embryos Day 2	2.61 ± 2.2 (0–17)	2.65 ± 2.1 (0–10)	0.82	
No. of good quality embryos	2.41 ± 2.3 (0–16)	2.19 ± 1.8 (0–8)	0.34	0.227 (0.690; −0.236)
No. of frozen embryos	1.58 ± 2.3 (0–16)	1.30 ± 1.8 (0–8)	0.33	
No. of ET per randomized woman	215 (89.6)	114 (91.9)	0.47	
No. of transferred embryos, per ET	1.04 ± 0.2 (1–2)	1.03 ± 0.2 (1–2)	0.71	
No. of SET, per ET	206 (95.8)	111 (97.4)	0.71	
Reason for no ET (no. of cycles)				
OHSS, freezing of all embryos	13	3		
Failed fertilization	3	2		
Failed cleavage	9	4		

Continuous variables are presented as mean ± SD and ranges. Categorical variables are presented as n (%). For comparison between the groups, Fisher's exact test was used for dichotomous variables and the Mann–Whitney U-test was used for continuous variables.
ET, embryo transfer; SET, single embryo transfer; OHSS, ovarian hyperstimulation.

standard system. In that study, 676 oocytes from 59 patients were randomized between the two systems. The primary outcome was the number of 4-cell embryos on Day 2. No difference in the number of 4-cell embryos on Day 2, number of 7- to 8-cells on Day 3 or proportion of blastocysts on Day 5 was found. In addition, no differences in clinical pregnancy rates or implantation were found. In another controlled cohort study by Cruz *et al.* (2011), 478 oocytes from 60 egg donation cycles were randomly allocated to the two different incubator systems. No significant differences in the rate of GQEs, calculated as the proportion of blastocysts per cultured embryo, the number of transferred and frozen embryos or the pregnancy rate, were found between the closed TLI system and the traditional culture system. It is important to acknowledge however, that in both these studies—like in our own study—the additional information provided by TLI was not used for selecting embryos for transfer, and only static images were used for assessment. Also, both these studies were powered for the evaluation of embryo quality, and not for pregnancy or live birth.

In the present study, the miscarriage rate was significantly higher in the EmbryoScope™ group. This is a worrying observation, although this finding has to be treated with caution since the study was not powered to detect differences in miscarriage rate. If the finding is true, one possible reason might be that the scoring of embryos based on traditional

Table III Comparison of implantation and pregnancy rates.

	EmbryoScope (n = 240)	Control (n = 124)	*P*-value	Difference (95% CI)
Implantation rate (%)	63/226 (27.9)	37/117 (31.6)	0.32	
No. of pregnancies per randomized woman (%)	72/240 (30.0)	39/124 (31.5)	0.87	−1.5 (−12.1; 9.2)
No. of pregnancies per ET (%)	72/215 (33.5)	39/114 (34.2)	0.99	
Biochemical pregnancies	9	2		
No. of deliveries/ongoing pregnancies per randomized woman (%)	48/240 (20.0)	35/124 (28.2)	0.10	−8.2 (−18.2; 1.8)
No. of deliveries/ongoing pregnancies per ET (%)	48/215 (22.3)	35/114 (30.7)	0.13	
No. of miscarriages (%)	24/72 (33.3)	4/39 (10.2)	0.011	23.1 (3.6; 41.4)

For categorical variables, *n* (%) is presented. For comparison between the groups, Fisher's exact test was used for dichotomous variables.

morphological criteria is more difficult in the Embryoscope™ compared with a high resolution inverted microscope and thus affect selection of embryos for transfer negatively. In fact, we did experience some limitations when using the EmbryoScope™ for monitoring. The images on the monitor were not as sharp and clear as when visualized in the standard inverted microscope, and the focusing levels were limited. Also, embryos in the EmbryoScope™ tended to, despite placing them in the centre of a microwell, migrate to one side of the well, thus scoring them may be challenging at times. We also found it more challenging scoring nuclei in the EmbryoScope™ compared with the standard inverted microscope. It is important to emphasize that in this study, we used the traditional scoring of embryos in both systems. In the Embryoscope system, we evaluated only a single picture. The full potential of the TLI to see additional morphological features was thus not utilized. However, it was found in the subgroup analysis that morphology evaluation correlated well between the EmbryoScope and the standard inverted microscope (91.8%).

In the present study, the failure to observe any beneficial effects of the closed culture system could be explained by the short incubation time (2 days) before assessment and transfer, and extending the culture time to 5 days might have given results indicating a benefit of a closed system. However, in many countries/clinics, the most common practice is still Day 2 transfer and it is important to determine if any potential benefit from investment into these new culture systems can be attained. Initial studies by Kirkegaard *et al.* (2012) and Cruz *et al.* (2011) did not find culturing embryos to Day 5 using TLI systems to be superior to standard culture regarding proportion of blastocysts or pregnancy outcome.

Until now, the aim of most published TLI studies has been to find timing variables for selecting embryos with a high potential for blastocyst development, implantation and pregnancy, while less attention has been given to comparisons between the different culture systems *per se*. Meseguer *et al.* (2012b) retrospectively analysed 7305 treatments from both TLI and non-TLI culture. They found that embryos cultured in the closed TLI system as well as being selected by a hierarchal grading system had a significantly increased clinical pregnancy rate compared with embryos cultured in standard incubators and selected by conventional morphology only. In a recent RCT by Rubio *et al.* (2014), analysing 843 couples, a significantly increased ongoing pregnancy rate was found when the TLI system was compared with a standard incubator system. In this study, only good prognosis patients (≥6 MII oocytes, no recurrent miscarriages, no endometriosis) or donor recipients (with young donors) were included, the embryos were cultured to Day 3 or 5, and the embryos from the TLI system were selected not only by morphology but also by the hierarchical classification described by Meseguer *et al.* (2011). These factors may at least partially explain the difference in results compared with our study. In the RCT by Rubio *et al.* (2014), similar mean number of blastomeres, similar mean rate of embryo symmetry and a significantly higher mean embryo fragmentation rate on Day 3 were found in the TLI system, compared with the standard incubator system. In total, a slightly but significantly higher number of optimal embryos on Day 3 (46.2 versus 43.1%) was obtained in the TLI group.

Certainly, culture in the EmbryoScope™ provides a more stable environment for the embryos in terms of minimal fluctuations in pH, humidity and temperature. In addition, during image acquisition in the EmbryoScope™, the embryos are illuminated with long wavelength light and are subjected to lower light intensities (low intensity red light, 635 nm) than for embryos that are evaluated using a standard microscope. In an animal model, it was demonstrated that light in the range of 445–500 nm appears to be detrimental to blastocyst development (Oh *et al.*, 2007). In a standard IVF microscope, ~15% of light is <550 nm (Meseguer *et al.*, 2011). Furthermore, it has been shown that, for embryos in culture for 3 days, the total light exposure time in the EmbryoScope™ was 57 s compared with 167 s for an IVF treatment, using a standard microscope (Ottosen *et al.*, 2007; Meseguer *et al.*, 2011). These data would suggest a potential benefit when using a TLI system for longer incubation times.

It is clear that when scoring embryos at limited time points, important morphological events might be missed. Several such morphological events have been identified by TLI and suggested to be of predictive value for IVF success. In a study by Rubio *et al.* (2012) of 1659 transferred embryos, it was shown that embryos with a so-called direct cleavage from 2 to 3 cells (i.e. with a 2-cell stage shorter than 5 h) resulted in a significantly lower implantation rate compared with embryos with a 2-cell stage longer than 5 h (1.2 versus 20.2%). Hlinka *et al.* (2012) also found that out of 18 embryos showing a direct cleavage to 3 cells, none developed into blastocyst. Further, in both studies, it was noticed that these 'extra' cells could fuse at a later time, i.e. the embryos would appear to have cleaved in a normal, synchronized manner. Such anomalies would thus only be possible to detect in a TLI system. In a

recent study, retrospectively analysing 651 embryos using TLI, Wirka *et al.* (2014) identified four groups of atypical embryo behaviours, involving syngamy and cleavage patterns, that resulted in decreased embryo development.

Despite the lack of improvement of embryo development and pregnancy rate found in this present and other studies, the TLI systems may be of advantage from other perspectives. Important for the laboratory are the logistic reasons; since the whole developmental process is documented, important events can be analysed retrospectively at any time before selection for transfer. It also allows for more accurate analysis concerning timing and for the possibility of deselecting embryos with atypical cleavages. In combination with the use of single culture media and short insemination with sperm (1–4 h), the oocytes can be transferred to the TLI system on the day of oocyte retrieval, and be kept there until the time of transfer.

Only ICSI patients were included in this study due to the possibility to record the time of fertilization precisely (i.e. time of sperm injection), and because embryos can be kept in the EmbryoScope^{TM} continuously from Day 0, in contrast to IVF embryos which have to be removed from the incubator for denudation at some stage post-fertilization.

The strengths of the present study are that it is an RCT in an area where few RCTs have been published, and that it is blinded to the patients, the physicians and the statistician. In addition, the randomization is performed per patient, instead of per cycles or oocytes, with concealed allocation using a web-based RCT program. Only ICSI patients were included, which could be seen both as a strength (more homogenous) and as a limitation (less generalizability).

The main limitations are having number of GQEs on Day 2 (a surrogate outcome to live birth) as the primary outcome and that the embryos have been cultured in different types of culture dishes.

Apart from the culture dishes and the open versus closed system, the culture conditions were similar regarding oxygen tension, culture medium, temperature and pH.

In conclusion, this large RCT comparing embryo development and morphology between embryos cultured in a closed TLI incubator with those cultured in a standard incubator showed no significant difference in the number of GQEs, implantation- or pregnancy rates, while a significantly higher miscarriage rate was found in the TLI system group. Further prospective and well-designed trials are needed to see if these new culture systems can identify predictive variables for pregnancy and live birth which are of additional importance to conventional morphology assessment when selecting embryos for transfer.

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Authors' roles

K.L. and C.B. designed the study. H.P. and U.S. performed the laboratory work together with the laboratory staff at Reproductive Medicine, Sahlgrenska University Hospital. A.T.-K. was in charge of patients' information and consent. All authors have taken part in the writing, reviewing and approval of the final version of the manuscript.

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Conflict of interest

An unconditional grant was received from Ferring. Ferring had no influence in design of the study, analyses or writing of the manuscript. Unisense provided the EmbryoScope^{TM} free of charge during the study. The manuscript was sent to Unisense but the company had no influence in design of the study, analyses or writing of the manuscript.

References

Bontekoe S, Mantikou E, van Wely M, Seshadri S, Repping S, Mastenbroek S. Low oxygen concentrations for embryo culture in assisted reproductive technologies. *Cochrane Database Syst Rev* 2012;CD008950.

Chamayou S, Patrizio P, Storaci G, Tomaselli V, Alecci C, Ragolia C, Crescenzo C, Guglielmino A. The use of morphokinetic parameters to select all embryos with full capacity to implant. *J Assist Reprod Genet* 2013;**5**:703–710.

Conaghan J, Chen AA, Willman SP, Ivani K, Chenette PE, Boostanfar R, Baker VL, Adamson GD, Abusief ME, Gvakharia M *et al.* Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: results from a prospective multicenter trial. *Fertil Steril* 2013;**2**:412–419.

Cruz M, Gadea B, Garrido N, Pedersen KS, Martinez M, Perez-Cano I, Munoz M, Meseguer M. Embryo quality, blastocyst and ongoing pregnancy rates in oocyte donation patients whose embryos were monitored by time-lapse imaging. *J Assist Reprod Genet* 2011;**7**:569–573.

Cruz M, Garrido N, Herrero J, Perez-Cano I, Munoz M, Meseguer M. Timing of cell division in human cleavage-stage embryos is linked with blastocyst formation and quality. *Reprod Biomed Online* 2012;**4**:371–381.

Dal Canto M, Coticchio G, Mignini Renzini M, De Ponti E, Novara PV, Brambillasca F, Comi R, Fadini R. Cleavage kinetics analysis of human embryos predicts development to blastocyst and implantation. *Reprod Biomed Online* 2012;**5**:474–480.

Fujiwara M, Takahashi K, Izuno M, Duan YR, Kazono M, Kimura F, Noda Y. Effect of micro-environment maintenance on embryo culture after in-vitro fertilization: comparison of top-load mini incubator and conventional front-load incubator. *J Assist Reprod Genet* 2007;**1**:5–9.

Gardner DK, Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? *Hum Reprod Update* 1997;**4**:367–382.

Glujovsky D, Blake D, Farquhar C, Bardach A. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst Rev* 2012;CD002118.

Harper J, Magli MC, Lundin K, Barratt CL, Brison D. When and how should new technology be introduced into the IVF laboratory? *Hum Reprod* 2012; **27**:303–313.

Hlinka D, Kalatova B, Uhrinova I, Dolinska S, Rutarova J, Rezacova J, Lazarovska S, Dudas M. Time-lapse cleavage rating predicts human embryo viability. *Physiol Res* 2012;**5**:513–525.

Kirkegaard K, Hindkjaer JJ, Grondahl ML, Kesmodel US, Ingerslev HJ. A randomized clinical trial comparing embryo culture in a conventional incubator with a time-lapse incubator. *J Assist Reprod Genet* 2012; **6**:565–572.

Kirkegaard K, Hindkjaer JJ, Ingerslev HJ. Effect of oxygen concentration on human embryo development evaluated by time-lapse monitoring. *Fertil Steril* 2013a;**3**:38–744.

Kirkegaard K, Kesmodel US, Hindkjaer JJ, Ingerslev HJ. Time-lapse parameters as predictors of blastocyst development and pregnancy outcome in embryos from good prognosis patients: a prospective cohort study. *Hum Reprod* 2013b;**10**:2643–2651.

Kovacic B, Sajko MC, Vlaisavljevic V. A prospective, randomized trial on the effect of atmospheric versus reduced oxygen concentration on the outcome of intracytoplasmic sperm injection cycles. *Fertil Steril* 2010; **2**:511–519.

Lane M, Gardner DK. Embryo culture medium: which is the best? *Best Pract Res Clin Obstet Gynaecol* 2007;**1**:83–100.

Lemmen JG, Agerholm I, Ziebe S. Kinetic markers of human embryo quality using time-lapse recordings of IVF/ICSI-fertilized oocytes. *Reprod Biomed Online* 2008;**3**:385–391.

Lundin K, Bergh C. Cumulative impact of adding frozen-thawed cycles to single versus double fresh embryo transfers. *Reprod Biomed Online* 2007; **1**:76–82.

Mantikou E, Youssef MA, van Wely M, van der Veen F, Al-Inany HG, Repping S, Mastenbroek S. Embryo culture media and IVF/ICSI success rates: a systematic review. *Hum Reprod Update* 2013;**3**: 210–220.

Marek D, Langley M, Gardner DK, Confer N, Doody KM, Doody KJ. Introduction of blastocyst culture and transfer for all patients in an in vitro fertilization program. *Fertil Steril* 1999;**6**:1035–1040.

Meseguer M, Herrero J, Tejera A, Hilligsoe KM, Ramsing NB, Remohi J. The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod* 2011;**10**:2658–2671.

Meseguer M, Kruhne U, Laursen S. Full in vitro fertilization laboratory mechanization: toward robotic assisted reproduction? *Fertil Steril* 2012a; **6**:1277–1286.

Meseguer M, Rubio I, Cruz M, Basile N, Marcos J, Requena A. Embryo incubation and selection in a time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: a retrospective cohort study. *Fertil Steril* 2012b;**6**:1481–1489.

Nakahara T, Iwase A, Goto M, Harata T, Suzuki M, Ienaga M, Kobayashi H, Takikawa S, Manabe S, Kikkawa F *et al.* Evaluation of the safety of time-lapse observations for human embryos. *J Assist Reprod Genet* 2010; **2–3**:93–96.

Nilsson S, Waldenstrom U, Engstrom AB, Hellberg D. Promising results with 306 single blastocyst transfers. *Fertil Steril* 2005;**6**:1849–1851.

Oh SJ, Gong SP, Lee ST, Lee EJ, Lim JM. Light intensity and wavelength during embryo manipulation are important factors for maintaining viability of preimplantation embryos in vitro. *Fertil Steril* 2007; **4**(Suppl):1150–1157.

Ottosen LD, Hindkjaer J, Ingerslev J. Light exposure of the ovum and preimplantation embryo during ART procedures. *J Assist Reprod Genet* 2007;**2–3**:99–103.

Papanikolaou EG, Camus M, Kolibianakis EM, Van Landuyt L, Van Steirteghem A, Devroey P. In vitro fertilization with single blastocyst-stage versus single cleavage-stage embryos. *N Engl J Med* 2006;**11**:1139–1146.

Pocock SJ. *Clinical Trials: A Practical Approach*. Chichester, England: John Wiley, 1983.

Rubio I, Kuhlmann R, Agerholm I, Kirk J, Herrero J, Escriba MJ, Bellver J, Meseguer M. Limited implantation success of direct-cleaved human zygotes: a time-lapse study. *Fertil Steril* 2012;**6**:1458–1463.

Rubio I, Galán A, Larreategui Z, Ayerdi F, Bellver J, Herrero J, Meseguer M. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the EmbryoScope. *Fertil Steril* 2014;**102**:1287–1294.

Summers MC, Biggers JD. Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. *Hum Reprod Update* 2003;**6**:557–582.

Vajta G, Rienzi L, Cobo A, Yovich J. Embryo culture: can we perform better than nature? *Reprod Biomed Online* 2010;**4**:453–469.

Waldenström U, Engstrom AB, Hellberg D, Nilsson S. Low-oxygen compared with high-oxygen atmosphere in blastocyst culture, a prospective randomized study. *Fertil Steril* 2009;**6**:2461–2465.

Wirka KA, Chen AA, Conaghan J, Ivani K, Gvakharia M, Behr B, Suraj V, Tan L, Shen S. Atypical embryo phenotypes identified by time-lapse microscopy: high prevalence and association with embryo development. *Fertil Steril* 2014;**6**:1637–1648.

Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, Reijo Pera RA. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol* 2010;**10**:1115–1121.

Zhang JQ, Li XL, Peng Y, Guo X, Heng BC, Tong GQ. Reduction in exposure of human embryos outside the incubator enhances embryo quality and blastulation rate. *Reprod Biomed Online* 2010;**4**:510–515.

PAPER II

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II

ARTICLE

Conventional morphology performs better than morphokinetics for prediction of live birth after day 2 transfer

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Aisling Ahlström completed her Bachelor of Science and Honours Degree at the University of Adelaide, Australia in 1997. Thereafter, she worked as a research assistant at the Department of Obstetrics and Gynaecology focusing on reproductive immunology. From the start of the year 2000 until 2013 she worked at a private IVF clinic in Gothenburg and during this time obtained her PhD in Medical Science at the University of Gothenburg, Sweden. Currently she is working at the Unit of Reproductive Medicine, Sahlgrenska University Hospital. Her main interests are basic and clinical embryology, including methods for prediction of embryo viability, blastocyst culture and cryopreservation.

Abstract Numerous studies have reported on the potential value of time-lapse variables for prediction of embryo viability. However, these variables have not been evaluated in combination with conventional morphological grading and patient characteristics. The aim of this study was to assess the ability of patient characteristics and embryo morphology together with morphokinetic variables to predict live birth after day 2 transfer. This retrospective analysis included 207 transferred embryos from 199 couples cultured in a time-lapse system up to day 2 of development. Good prediction of live birth or ranking of embryos with respect to live birth potential was achieved with early cleavage combined with fragmentation grade at 43–45 h. These variables were selected as the strongest predictors of live birth, as assessed by stepwise logistic regression, and additional inclusion of morphokinetic variables did not improve the model significantly. Also, neither logistic regression models nor classification tree models with morphokinetic variables were able to achieve equally good prediction of live birth, as measured by AUC on an external data set not used for model development. In conclusion, for fresh day 2 transfers early cleavage in combination with fragmentation grade at 43–45 h should be considered when selecting between good quality embryos.

KEYWORDS: early cleavage, embryo selection, IVF, live birth, time-lapse

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Introduction

A large number of studies have been published regarding the potential value of time lapse. It was first introduced on a larger scale into IVF laboratories starting around 2010, and has become increasingly popular since then. The technique is used in different ways; as an embryo selection/deselection tool and/or as a logistic tool for the laboratory in order to be able to plan the work in a more efficient way. Divergent results concerning the potential value of time lapse to increase blastocyst, implantation, and/or pregnancy rates have been published and reviewed (Kirkegaard et al., 2012a, 2015). In one randomized controlled trial (RCT) (Rubio et al., 2014) it was found that, using the time-lapse system, ongoing pregnancy rates were significantly improved. Another RCT also showed a significant difference in ongoing pregnancy rate when one or two euploid embryos from preimplantation genetic screening (PGS) patients were selected using the morphokinetic criteria by Meseguer (Meseguer et al., 2011) compared with conventional morphological scoring and a standard incubator system (Yang et al., 2014). However, other published RCT (Goodman et al., 2016; Kirkegaard et al., 2012b; Park et al., 2015) have failed to show any benefit in implantation or pregnancy/live birth by using time lapse, and a recent Cochrane review concluded that there was insufficient evidence of differences in live birth, miscarriage, stillbirth or clinical pregnancy between the time-lapse system and conventional incubation (Armstrong et al., 2015). Furthermore, the efficacy of time-lapse variables assessed in combination with other known predictors of live birth, such as to conventional grading and patient characteristics, has not been studied.

The aim of this study was to determine in a retrospective data set whether morphokinetic variables when analysed in combination with conventional morphology and other patient variables could be used to predict live birth.

Materials and methods

Patients

This is a retrospective analysis of patients allocated to embryo culture in a time-lapse system as part of an RCT conducted at Reproductive Medicine, Sahlgrenska University Hospital, in Gothenburg between May 2010 and February 2014 (Park et al., 2015). In total, 364 patients (365 cycles) were randomized between May 2010 and February 2014. Their oocytes were cultured in either the TLI incubator (241 cycles, 2280 oocytes) or a standard incubator (124 cycles, 1180 oocytes). Patients were eligible if they were ≤40 years of age, undergoing their first fresh IVF cycle, with own gametes using intracytoplasmic sperm injection (ICSI) and where at least one oocyte was retrieved. In this retrospective analysis only patients where the number of live births matched the number of transferred embryos or where no live birth occurred ($n = 199$ patients, 207 transferred embryos) were included. Excluded from this analysis were patients who gave birth to a singleton child after double embryo transfer ($n = 1$), not receiving embryo transfer ($n = 11$ total embryo cryopreservation, $n = 1$ no mature oocytes, $n = 6$ failed fertilization, $n = 4$ no optimal embryo development) and when no time-lapse images were available due to technical failure ($n = 19$).

The study was approved by the Ethical committee of the University of Gothenburg on 9 December 2009 (reference number 666–09).

Ovarian stimulation, IVF and embryo culture

Stimulation protocols were performed as previously described (Park et al., 2015). Briefly, patients were down-regulated with gonadotrophin-releasing hormone (GnRH) agonists (Suprecur, Sanofi, Paris, France) in a long protocol and ovarian stimulation was achieved with either recombinant FSH (Gonal-F, Merck Serono, Darmstadt, Germany or Puregon, MSD, USA) or urinary-derived gonadotrophins (Menopur, Ferring, Copenhagen, Denmark). In a few cases ($n = 16$), patients were down-regulated in a short protocol using a GnRH antagonist (Orgalutran, MSD, NJ, USA). Human chorionic gonadotrophin (HCG; Pregnyl 5000 or 10,000 IU, MSD or Ovitrelle 6500 IU, Merck Serono) was administered when two or more follicles reached ≥18 mm diameter. Follicles were aspirated using vaginal ultrasonography 36 ± 2 h after HCG injection. Retrieved cumulus–oocyte–complexes were rinsed in MOPS (Vitrolife, Gothenburg, Sweden) and placed in G-IVF medium (Vitrolife). Mature oocytes were fertilized using conventional ICSI procedures. Directly after injection oocytes were placed in pre-equilibrated EmbryoSlides® (Vitrolife) containing 25 μl G1 media under 1.2 ml Ovoil (Vitrolife) and cultured in the Embryoscope® (Vitrolife) at 37°C, 6% CO_2 and atmospheric O_2 concentration until embryo transfer on day 2.

Embryo assessment and transfer

Conventional morphological embryo assessments were performed during embryo culture by at least two embryologists without removing the embryos from the EmbryoScope and in accordance with the Istanbul consensus (ALPHA Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011). Fertilized oocytes were confirmed by the presence of two pronuclei at 16–18 h and early cleavage was assessed at 25–27 h post-ICSI. On day 2 (43–45 h) embryos were graded according to blastomere number and size, degree of fragmentation and presence of multinucleation. Good quality embryos, defined as an embryo with four to six blastomeres, less than 20% fragmentation and no observed multinucleation, were primarily selected for transfer. Early cleavage was also considered when selecting between embryos of equal quality. Selections of embryos to transfer were based solely on morphological criteria. Time-lapse recordings and morphokinetic parameters were not annotated during the RCT study period or used for embryo selection. Validations of embryo scoring are performed annually to assess variations between individuals at our clinic and between eight different IVF clinics in Sweden. A high level of agreement for all morphological parameters graded was maintained, both within the group of embryologists at our clinic and in relation to other clinics.

Time-lapse monitoring and annotations

In the present study, annotations were performed simultaneously by two senior embryologists after the study period was completed. The time point of the ICSI procedure was used as the start time for all developmental events annotated manually in the EmbryoViewer image analysis software (Vitrolife, Sweden). All times were recorded in hours post-insemination (hpi). Images were acquired every 20 min, at seven focal planes (15 μm intervals). Morphokinetic variables were documented according to definitions described by Ciray et al. (2014). Kinetic parameters included in the analysis were: appearance of second polar body (tPB2), time of pronucleus (PN) appearance (tPNa) and PN fading (tPNf), time of division to 2-cell (t2), 3-cell (t3), and 4-cell (t4) stages. Calculated variables included; time from polar body extrusion to PN appearance (tPNa-tPB2), PN duration (tPNf-tPNa), duration of the second cell cycle (cc2, t4-t2), synchrony (s2) of blastomere divisions in second cleavage cycle (t4–t3) and so-called direct cleavage observed during first or second cleavage cycle, defined as rapid division of one blastomere into three or more daughter cells. Additionally, PN fading was used as an alternative start time to calculate time to each cell stage, i.e. t2-PNf, t3-PNf and t4-PNf. A number of morphological characteristics were also graded during interphase of each cell stage. Blastomere symmetry was defined as blastomeres not more than 25% larger than siblings. Degree of fragmentation (0–10, 11–20, 21–50, and 51–100% intervals) during each cell stage was annotated at the last frame before the next round of cleavage and presence of binucleation (BNB) or multinucleation (MNB) were also annotated after reviewing the entire sequence of images captured at the 2-cell and 4-cell stages.

End-point and statistical analysis

The primary end-point was live birth. The association between patient and treatment variables, embryo morphological variables, morphokinetic variables and live birth was analysed with logistic regression. The models were estimated with generalized estimation equations (GEE) with compound symmetric covariance structure in order to account for dependencies within individuals, since some patients received two embryos at transfer. Univariate logistic regression models were used to identify the predictors that significantly affected live birth, presenting odds ratios (OR) and area under receiver operating characteristic curve (AUC) with 95% confidence interval (CI). Fisher's exact test was used for variables with non-estimable odds ratios due to complete separation between the groups). Patient characteristics in the two outcome groups are described with mean and standard deviation for continuous variables and number and percentage for categorical variables.

Prediction models for live birth or models for ranking of live birth potential were developed and evaluated by splitting data into two parts, using 104 observations for model development and 103 observations for model evaluation. Stepwise logistic regression was used for selection of variables to be included in the prediction models, adding and removing the most/least significant variables one at a time. The procedure started from a model with an intercept only and stopped when no more significant variables could be added or non-significant variables could be removed. All variables significant at 10% level in the univariate models were considered for inclusion in the prediction models. Instead of constructing binary rules for prediction of live birth, the predicted probabilities of live birth as a continuous measure of live birth potential were considered.

The use of classification trees or hierarchical models for prediction of live birth was also investigated. With classification trees, continuous variables are dichotomized sequentially in order to find regions that separate the outcome in an optimal way. The geometric mean of the true positive rate and true negative rate was used as optimization criterion when defining splits for dichotomization of continuous variables, since the geometric mean is large only when both outcomes are predicted with high accuracy.

Prediction models were developed for the following three categories of variables separately: patient and treatment variables, embryo morphological variables, and morphokinetic variables. Models with all variable types together were also tested. The prediction performance was evaluated by means of AUC on the evaluation data set.

In addition, a commercially available model for good quality blastocyst prediction known as the Eeva™ (Early Embryo Viability Assessment, Auxogyn, USA) Test that categorizes embryos into three categories, High, Medium and Low, was tested on our data set (Conaghan et al., 2013) Embryos are categorized into these categories depending on whether certain time points were within predefined ranges for duration between first and second cytokinesis (t3–t2, 9.33–11.45 h) and for time between second and third cytokinesis (t4–t3, 0–1.73 h). Data were used to generate a receiver operating characteristic curve and to calculate area under the curve for live birth.

For some embryos technical problems during image acquisition inhibited annotation and measurement of specific events, resulting in missing data. All available data was used for each variable in univariate analyses, and all observations with complete data on the variables selected by the stepwise selection procedure were used in the prediction models.

All analyses were done with SAS® v9.4 (SAS, Cary, NC, USA). All tests are two tailed and conducted at 5% significance level.

Results

Of the 199 patients included in the analysis, 191 (96.0%) received single embryo transfer and eight received double embryo transfer. For these double embryo transfers, no live births were obtained. No transfers resulted in twin births. The live birth rate was 20.3% (42/207). Patient and cycle characteristics compared by live birth are summarized in **Table 1**, and morphological and kinetic variables in **Table S1**.

Univariate analyses found that probability of live birth significantly increased for each additional 4-cell embryo available on day 2 of development (OR 1.27, CI 1.07–1.50, $P = 0.005$) (**Figure 1**). Early cleavage was found to significantly increase the odds of live birth by almost fivefold (OR 4.84, CI 2.14–10.96, $P = 0.0002$) and each increase in grade of fragmentation at 43–45 h significantly decreased the likelihood of live birth (OR 0.46, CI 0.25–0.84, $P = 0.012$)

Table 1 Patient and treatment variables according to live birth outcome.

Variable	*No live birth* (n = *165*)	*Live birth* (n = *42*)
Maternal age, years	32.1 ± 4.2 (21.4; 39.6)	31.8 ± 4.1 (24.3; 39.1)
BMI	24.6 ± 4.0 (16.8; 34.6)	24.2 ± 3.8 (18.9; 36.1)
Chew tobacco user	2 (1.2)	1 (2.4)
Smoker	23 (13.9)	1 (2.4)
Female reason for infertility	39 (23.6)	8 (19.0)
Duration of infertility, years	2.8 ± 1.4 (1.0; 10.0)	2.7 ± 2.0 (1.0; 11.0)
Pregnancy in present relationship	18 (10.9)	6 (14.3)
Pregnancy in previous relationship	33 (20.0)	11 (26.2)
Previous miscarriage	20 (12.1)	5 (11.9)
Number of live births in previous relationship(s)		
0	151 (91.5)	40 (95.2)
1	9 (5.5)	2 (4.8)
2	4 (2.4)	0 (0.0)
3	1 (0.6)	0 (0.0)
FSH total dose, IU	1610.1 ± 809.6 (600.0; 6000.0)	1585.5 ± 762.3 (825.0; 3675.0)
FSH dose, IU/oocyte retrieved	264.6 ± 299.4 (32.0; 2057.0)	239.6 ± 204.9 (39.0; 1200.0)
Number of aspirated oocytes	9.0 ± 4.7 (1.0; 32.0)	9.0 ± 4.8 (3.0; 25.0)
Number of fertilized oocytes	4.4 ± 2.6 (1.0; 14.0)	5.0 ± 2.8 (1.0; 13.0)
Number of embryos transferred		
1	149 (90.3)	42 (100.0)
2	16 (9.7)	0 (0.0)
Time between HCG and oocyte retrieval (hours)	35.9 ± 0.3 (35.0; 37.0)	36.1 ± 0.3 (35.8; 37.0)
Time between HCG and and ICSI (hours)	40.8 ± 1.0 (36.0; 43.1)	40.9 ± 0.9 (39.0; 42.5)
Number of 2-cell embryos at 25–27 h	1.4 ± 1.5 (0.0; 7.0)	1.8 ± 1.5 (0.0; 6.0)
Number of 4-cell embryos at 43–45 h	2.2 ± 1.7 (0.0; 9.0)	3.1 ± 2.1 (0.0; 11.0)
Number of good quality embryos	2.1 ± 1.8 (0.0; 10.0)	2.6 ± 2.0 (0.0; 9.0)

For categorical variables *n* (%) is presented. For continuous variables, mean ± SD (min.; max.) is presented.
BMI = body mass index; HCG = human chorionic gonadotrophin; ICSI = intracytoplasmic sperm injection.

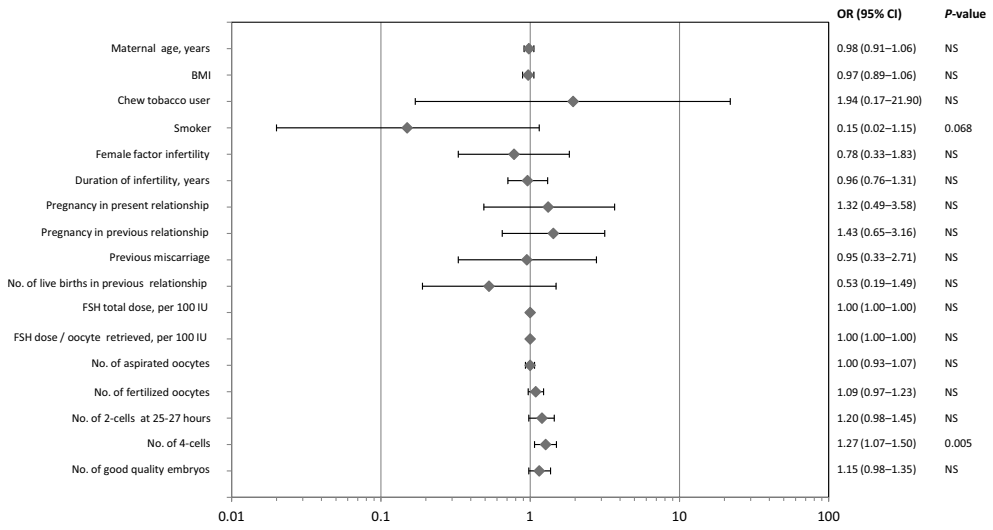

Figure 1 Univariate logisitc regression analysis for prediction of live birth. Patient and treatment variables. NS = non-significant.

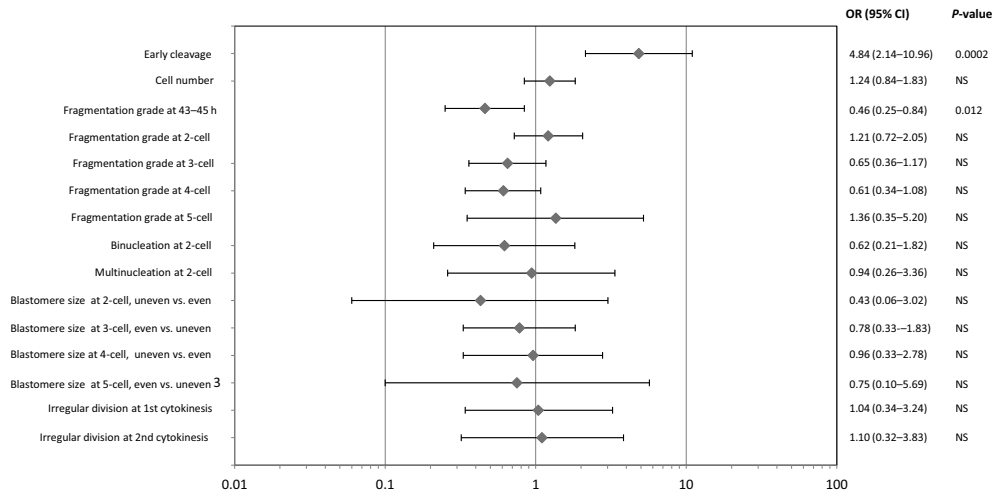

Figure 2 Univariate logistic regression analysis for prediction of live birth. Conventional morphological variables. NS = non-significant.

(Figure 2). Of interest, univariate analysis of morphokinetic variables found that time to PN fading (OR 0.80, CI 0.70–0.91, $P = 0.0009$), time to 2-cell (OR 0.80, CI 0.71–0.90 $P = 0.0003$), 3-cell (OR 0.90, CI 0.84–0.97, $P = 0.0083$) and 4-cell stage (OR 0.91, CI 0.83–0.99, $P = 0.032$), duration from second polar body extrusion to 2-cell stage (OR 0.86, CI 0.77–0.95, $P = 0.0039$) and duration from PN appearance to disappearance (OR 0.89, CI 0.80–0.99, $P = 0.026$) were significant predictors of live birth (Figure 3). Embryos completing these events in shorter times had significantly increased chance of live birth, although, poor AUC_{ROC} values were obtained for all significant predictors (Table S2).

Variables bi- and multinucleation at 4-cell stage (Yes/No) and male reason for infertility (Yes/No) were excluded from logistic regression analysis due to complete separation between the groups, so no odds ratio could be estimated. None were significant, however, tested with Fisher's exact test.

Prediction models

Logisitic regression models

With stepwise logistic regression, only the number of 4-cell embryos at 43–45 h was selected when considering patient and treatment variables only with AUC = 0.65 on model development data and AUC = 0.61 on model evaluation data, summarized in Table 2. Among embryo morphological variables, early cleavage was first selected. A significant interaction with fragmentation grade at 43–45 h was also found, meaning that fragmentation grade adds information about live birth potential for embryos with early cleavage (AUC 0.73, CI 0.62–0.83, $P = 0.028$). In addition to these variables, no more variable was selected by stepwise selection. In the model development data set, 10.2% of embryos without early cleavage resulted in live birth and 30.9% of embryos with early cleavage resulted in live birth. In addition, embryos with early cleavage and high fragmentation grade (>10) had 20.0% live birth rate, while embryos with early cleavage and low fragmentation grade (≤10) had 39.5% live birth rate. The AUC on model development data set was 0.73. On the model evaluation data set the corresponding figures were 6.3% live births among embryos without early cleavage and 30.9% of embryos with early cleavage resulting in live birth. Early cleavage in combination with high and low fragmentation grade resulted in 26.7% and 37.1% live birth rate respectively on the evaluation data set, AUC = 0.74. Among morphokinetic variables only t2 was selected. AUC was 0.67 on model development data set and AUC = 0.65 on model evaluation data set. When all variable types were considered simultaneously for selection into a predictive model, early cleavage together with fragmentation grade was again selected, summarized in Table 2.

Classification tree models

Classification tree models were constructed for morphokinetic variables and for all variable types simultaneously. The same variables selected in the logistic regression models from patient and treatment variables and from embryo morphological variables were also selected in the classification tree models. For morphokinetic variables t2-tPNf, tPNf and tPNf-tPNa were selected with optimal ranges found to be t2-tPNf in 2.33–2.67, tPNf <21.96 and tPNf-tPNa <12.67 or >14.67. In the selected model, data were first split by t2-tPNf (in our inside or outside optimal range) and then by tPNf if t2-tPNf was outside the optimal range and by tPNf-tPNa if t2-tPNf was inside the optimal range, resulting in a four level live birth potential score with AUC = 0.83 and AUC = 0.61 on model development and model evaluation data, respectively. A sensitivity analysis of the splitting criterion used as well as of the proportion of data used for model development and model evaluation was carried out for the classification tree model, showing similar results. Models consisting of a single

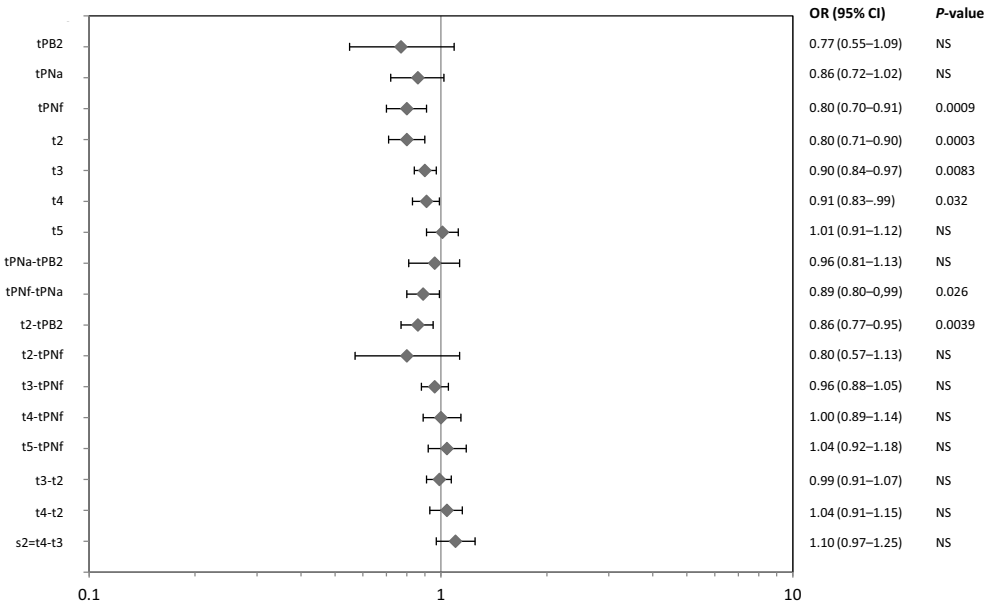

Figure 3 Univariate logistic regression analysis for prediction of live birth. Morphokinetic variables. Time from insemination (hours) to appearance of second polar body (tB2), PN appearance (tPNa), PN fading (tPNf); division to 2-cell (t2), 3-cell (t3), 4-cell (t4) and 5-cell (t5) stages. Calculated variables include time from polar body extrusion to PN appearance (tPNa-tPB2) and to 2-cell (t2-tPB2), PN duration (tPNf-tPNa), duration of the second cell cycle (t4-t2), time between 2-cell and 3-cell stages (t3-t2), synchrony (s2) of blastomere divisions in second cleavage cycle (t4–t3). Additionally, PN fading was used as an alternative start time to calculate time to each cell stage, t2-PNf, t3-PNf, t4-PNf and t5-PNf. NS = non-significant.

dichotomized variable only, giving a two level live birth potential score, were also considered. The best result was obtained with tPNf, with the ranges defined as above, with AUC = 0.60 on model evaluation data.

When the commercially available blastocyst prediction model, the Eeva test (Conaghan et al., 2013) was tested on our data set to predict live birth outcome, 23% (17/74) of embryos categorized as Low, 24.1% (13/54) categorized as Medium, and 17.2% (10/58) categorized as High, resulted in a live birth. The odds of live birth did not significantly increase by one step change in category (OR 0.84, CI 0.57–1.25).

Discussion

The main finding of this study was that embryo morphological variables successfully predicted or ranked embryos in terms of live birth potential after day 2 transfer. A prediction model with morphokinetic variables of high predictive performance was not found. In terms of AUC on the evaluation data set the prediction model with early cleavage in combination with fragmentation grade at 43–45 h outperformed the logistic regression model with selected continuous morphokinetic variables as well as the classification tree model with dichotomized morphokinetic variables. Also, no morphokinetic variables were selected by the stepwise selection procedure when all variable types were considered simultaneously.

Both early cleavage and fragmentation grade have previously been found to be associated with good blastocyst development and significantly higher pregnancy and implantation rates (Edwards et al., 1984; Hesters et al., 2008; Luke et al., 2014; Lundin et al., 2001; Racowsky et al., 2009; Rhenman et al., 2015; Sakkas et al., 1998; Shoukir et al., 1997; Stylianou et al., 2012; Van Montfoort et al., 2004; Van Royen et al., 2001). Similar to the present findings, Lundin et al. (2001) found early cleavage to be an important predictor of live birth for ICSI embryos, and in the study by Van Montfoort et al. (2004) logistic regression analysis found early cleavage to be an important predictor of pregnancy. Likewise, the impact of fragmentation grade is supported by many large studies using regression analyses to construct models based solely on morphological parameters. In these studies, degree of fragmentation was incorporated into the final multivariate models (Luke et al., 2014; Racowsky et al., 2009; Rhenman et al., 2015; Stylianou et al., 2012; Van Royen et al., 2001). Uniquely, the present study showed that when compared with morphokinetic variables, early cleavage and fragmentation grade were better predictors of live birth outcome, and no morphokinetic predictor improved prediction.

Table 2 Summary of models for prediction of live birth. AUC is presented for model development and model evaluation data.

Logistic regression models			*ROC AUC (95% CI)*	
Variable category	*Model to predict live birth*		*Model development data*	*Model evaluation data*
Patient and treatment	Linear predictor =−2.19 + 0.32* No. of 4 cell embryos at 44–46 h		0.65 (0.54; 0.77)	0.61 (0.47; 0.76)
Morphological	Linear predictor =−2.17 + 1.78* Early Cleavage−1.39*(Early Cleavage*Fragmentation grade at 43–45 h)		0.73 (0.62; 0.83)	0.74 (0.63; 0.85)
Morphokinetic	Linear predictor = 4.58 − 0.23*t2		0.67 (0.55; 0.78)	0.65 (0.53; 0.78)
All variables	Linear predictor =−2.17 + 1.78* Early Cleavage−1.39*(Early Cleavage*Fragmentation grade at 43–45 h)		0.73 (0.62; 0.83)	0.74 (0.63; 0.85)
Fragmentation grade is considered as ordered categorical with levels corresponding to 5–10%, 11–20%, 21–50%, 51–100% respectively.				
Predicted probability of live birth = 1/(1 + exp[−linear predictor])				
The coefficients of the linear predictor are the estimated regression coefficients and exp (linear predictor) is the odds of live birth.				
Classification Tree model				
Dichotomized morphokinetic variables	t2-tPNf outside (2.33; 2.67)	tPNf ≥21.96 - > live birth ranking score1 tPNf < 21.96 - > live birth ranking score 2	0.83 (0.75; 0.92)	0.61 (0.48: 0.73)
	t2-tPNf in (2.33; 2.67)	tPNf-tPNa in (12.67; 14.67) - > live birth ranking score 3 tPNf-tPNa outside (12.67; 14.67) - > live birth ranking score 4		

AUC = area under receiver operating characteristic curve; PN = pronucleus; ROC = receiver operating characteristic; t2-tPNf = time from PN fading to 2-cell embryo; tPNf = time from insemination (hours) to PN fading; tPNf-tPNa = time from PN appearance to PN fading.

Interestingly, the present study also showed that early cleavage assessed as a dichotomous variable was a stronger predictor of live birth compared with timing of the first cell division assessed as a continuous morphokinetic variable, t2. In this study, t2 occurred on average 1–2 h earlier for embryos resulting in live birth (median 24.7, min. 19.2, max. 30.6, mean 24.7 ± 2.2 h) compared with those that failed (median 25.9, min. 20.8, max. 44.1, mean 26.8 ± 3.9 h). Several time-lapse studies also found earlier timing of first cell division to be associated with embryo development and implantation (Chamayou et al., 2013; Conaghan et al., 2013; Lemmen et al., 2008; Meseguer et al., 2011). These studies suggested that more precise evaluations of timing of morphological events would improve prediction of embryo viability. However, these studies did not evaluate time-lapse variables in predictive models together with conventional morphology for prediction of outcome. As such, the present findings suggest that it is sufficient to observe early cleavage as an event to gain predictive information.

It is plausible that using logistic regression for evaluating prediction models with morphokinetic variables is inappropriate due to the assumption of a monotone increase or decrease in live birth rate as the predictor changes. It might well be that the probability of live birth as a function of a morphokinetic predictor is U-shaped, so that both low and high values correspond to high live birth rate, or the other way around. This was confirmed in the data in this study as dichotomization of morphokinetic variables t2-tPNf and tPNf-tPNa into ranges with high and low live birth rate were found to be inside or outside of certain intervals. However, the classification tree constructed using these optimal ranges was only found to be promising on the model development data set and performed less satisfactorily on new data. Models with a single split on a single variable did not perform better, either. Furthermore, the sensitivity analysis did not indicate that a better classification tree model could be developed with another splitting criterion or with other sizes of model development and evaluation data.

The shortcoming of time-lapse models in general is their inability to be directly transferable to other IVF clinics (Best et al., 2013; Kirkegaard et al., 2014). In the present study a blastocyst prediction model currently being used clinically to select embryos for transfer was applied retrospectively to the data (Conaghan et al., 2013). This model was unable to significantly predict the odds of live birth, and strikingly 45.2% of live births arose from embryos categorized as having low probability of developing to usable blastocysts. In agreement with Kirkegaard et al. 2014, the risk of classifying embryos with high reproductive potential as non-usable would be detrimentally high when using this model in our clinic. In the Eeva system the annotations are performed automatically, and whether this inaccuracy is a result of manual annotations compared with automated annotations is not known.

Another marker of implantation described by many time-lapse studies is the occurrence of irregular cleavage events during the first or second cytokinesis, referred to as rapid cleavage or trichotomous mitosis (Ciray et al., 2014; Rubio et al., 2012). In the present study, embryos undergoing trichotomous mitosis (t3-t2 = 0) at the first cytokinesis, whereby no 2-cell stage was observed, did not result in a live birth. However, in contrast to the definitions described by Rubio et al. (2012), live births did result from embryos with duration of 2-cell stage <5 h (four live births out of 20 embryos, t3-t2 >1.33 h, 20%). Furthermore, live births also resulted from embryos undergoing rapid cleavage during the second cytokinesis (three out of 14 embryos, 21%). Studies suggest that completion of a cell cycle should take between 10 and 12 h in order to allow ideal DNA replication and karyokinesis before cytokinesis, and that cells displaying an accelerated cycle will incur chromosomal aberrations in daughter cells (Aguilar et al., 2014; Cummins et al., 1986; Lemmen et al., 2008; Rubio et al., 2012). However, it is apparent in the present study that embryos containing accelerated blastomeres can be viable. It is possible that these abnormal cells may be targeted for removal from the cell cycle and/or may fail to proliferate later in development, upon differentiation (Ambartsumyan and Clark, 2008). It would seem that further clinical evidence is needed before some conclusions arising from time-lapse studies are implemented.

A major strength of this study is that the primary endpoint is live birth. The majority of time-lapse studies to date have built predictive models based on associations to surrogate outcomes, primarily blastocyst development and implantation rate. Another strength of this study is that it compares the predictive strength of conventional morphology variables to morphokinetic variables. Most studies focus solely on the performance of morphokinetic variables and design algorithms to replace conventional morphological selection criteria, presuming that these variables will perform better. There is an obvious lack of comparative/combinatorial studies that simultaneously assess all developmental variables that can be used in embryo selection. These studies are necessary before recommendations for a change of routine practice can be justified.

A number of limitations should be mentioned regarding this study. A drawback is the amount of data available, which we consider sufficient but not optimally dimensioned for developing and evaluating models for prediction of live birth. More data could have allowed for inclusion of more predictors in the models as well as for construction of more robust classification trees, possibly with improved prediction as a consequence. However, the aim was primarily to investigate whether addition of morphokinetic variables to conventional morphology increased prediction of live birth, not to construct a predictive model. Another limitation is the number of days embryos were cultured, which in turn restricted the number of morphokinetic and morphological variables evaluated. As such, the present findings cannot be generalized to embryos cultured for more than 2 days and up to blastocyst stage. Finally, a potential source of variation for timings of morphokinetic variables that has not been evaluated in this or other morphokinetic studies is the time between HCG administration and oocyte retrieval or ICSI procedure. Prior to ICSI, the time between HCG and ICSI procedure varied up to 2 h between patients in the present study (**Table 1**). The possible influence of this time difference on timings calculated after ICSI is not known. However, in this study, time of ICSI procedure was selected as the starting point from which all timings were calculated. This was chosen to enable comparisons with published studies and because when measuring the development of ICSI-generated zygotes it can be more accurately recorded.

In conclusion, for fresh day 2 transfers, early cleavage and degree of fragmentation should be primarily used to select

between good quality embryos. No morphokinetic variables up to day 2 were found to improve prediction of live birth further. In order to fully validate these findings, additional prospective time-lapse studies with live birth as the primary end-point are required.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2016.03.008.

References

Aguilar, J., Motato, Y., Escriba, M.J., Ojeda, M., Munoz, E., Meseguer, M., 2014. The human first cell cycle: impact on implantation. Reprod. Biomed. Online 28, 475–484.

ALPHA Scientists in Reproductive Medicine, ESHRE Special Interest Group Embryology, 2011. Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Reprod. Biomed. Online 22, 632–646.

Ambartsumyan, G., Clark, A.T., 2008. Aneuploidy and early human embryo development. Hum. Mol. Genet. 17, R10–R15.

Armstrong, S., Arroll, N., Cree, L.M., Jordan, V., Farquhar, C., 2015. Time-lapse systems for embryo incubation and assessment in assisted reproduction. Cochrane Database Syst. Rev. (2), CD011320.

Best, L., Campbell, A., Duffy, S., Montgomery, S., Fishel, S., 2013. Does one model fit all? Testing a published embryo selection algorithm on independent time-lapse data. Hum. Reprod. 28, i87–i90.

Chamayou, S., Patrizio, P., Storaci, G., Tomaselli, V., Alecci, C., Ragolia, C., Crescenzo, C., Guglielmino, A., 2013. The use of morphokinetic parameters to select all embryos with full capacity to implant. J. Assist. Reprod. Genet. 30, 703–710.

Ciray, H.N., Campbell, A., Agerholm, I.E., Aguilar, J., Chamayou, S., Esbert, M., Sayed, S., 2014. Proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group. Hum. Reprod. 29, 2650–2660.

Conaghan, J., Chen, A.A., Willman, S.P., Ivani, K., Chenette, P.E., Boostanfar, R., Baker, V.L., Adamson, G.D., Abusief, M.E., Gvakharia, M., Loewke, K.E., Shen, S., 2013. Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: results from a prospective multicenter trial. Fertil. Steril. 100, 412–419, e5.

Cummins, J.M., Breen, T.M., Harrison, K.L., Shaw, J.M., Wilson, L.M., Hennessey, J.F., 1986. A formula for scoring human embryo growth rates in in vitro fertilization: its value in predicting pregnancy and in comparison with visual estimates of embryo quality. J. In Vitro Fert. Embryo Transf. 3, 284–295.

Edwards, R.G., Fishel, S.B., Cohen, J., Fehilly, C.B., Purdy, J.M., Slater, J.M., Steptoe, P.C., Webster, J.M., 1984. Factors influencing the success of in vitro fertilization for alleviating human infertility. J. In Vitro Fert. Embryo Transf. 1, 3–23.

Goodman, L.R., Goldberg, J., Falcone, T., Austin, C., Desai, N., 2016. Does the addition of time-lapse morphokinetics in the selection of embryos for transfer improve pregnancy rates? A randomized controlled trial. Fertil. Steril. 105, 275–285.

Hesters, L., Prisant, N., Fanchin, R., Mendez Lozano, D.H., Feyereisen, E., Frydman, R., Tachdjian, G., Frydman, N., 2008. Impact of early cleaved zygote morphology on embryo development and in vitro fertilization-embryo transfer outcome: a prospective study. Fertil. Steril. 89, 1677–1684.

Kirkegaard, K., Agerholm, I.E., Ingerslev, H.J., 2012a. Time-lapse monitoring as a tool for clinical embryo assessment. Hum. Reprod. 27, 1277–1285.

Kirkegaard, K., Hindkjaer, J.J., Grondahl, M.L., Kesmodel, U.S., Ingerslev, H.J., 2012b. A randomized clinical trial comparing embryo culture in a conventional incubator with a time-lapse incubator. J. Assist. Reprod. Genet. 29, 565–572.

Kirkegaard, K., Campbell, A., Agerholm, I., Bentin-Ley, U., Gabrielsen, A., Kirk, J., Sayed, S., Ingerslev, H.J., 2014. Limitations of a time-lapse blastocyst prediction model: a large multicentre outcome analysis. Reprod. Biomed. Online 29, 156–158.

Kirkegaard, K., Ahlstrom, A., Ingerslev, H.J., Hardarson, T., 2015. Choosing the best embryo by time lapse versus standard morphology. Fertil. Steril. 103, 323–332.

Lemmen, J.G., Agerholm, I., Ziebe, S., 2008. Kinetic markers of human embryo quality using time-lapse recordings of IVF/ICSI-fertilized oocytes. Reprod. Biomed. Online 17, 385–391.

Luke, B., Brown, M.B., Stern, J.E., Jindal, S.K., Racowsky, C., Ball, G.D., 2014. Using the Society for Assisted Reproductive Technology Clinic Outcome System morphological measures to predict live birth after assisted reproductive technology. Fertil. Steril. 102, 1338–1344.

Lundin, K., Bergh, C., Hardarson, T., 2001. Early embryo cleavage is a strong indicator of embryo quality in human IVF. Hum. Reprod. 16, 2652–2657.

Meseguer, M., Herrero, J., Tejera, A., Hilligsoe, K.M., Ramsing, N.B., Remohi, J., 2011. The use of morphokinetics as a predictor of embryo implantation. Hum. Reprod. 26, 2658–2671.

Park, H., Bergh, C., Selleskog, U., Thurin-Kjellberg, A., Lundin, K., 2015. No benefit of culturing embryos in a closed system compared with a conventional incubator in terms of number of good quality embryos: results from an RCT. Hum. Reprod. 30, 268–275.

Racowsky, C., Ohno-Machado, L., Kim, J., Biggers, J.D., 2009. Is there an advantage in scoring early embryos on more than one day? Hum. Reprod. 24, 2104–2113.

Rhenman, A., Berglund, L., Brodin, T., Olovsson, M., Milton, K., Hadziosmanovic, N., Holte, J., 2015. Which set of embryo variables is most predictive for live birth? A prospective study in 6252 single embryo transfers to construct an embryo score for the ranking and selection of embryos. Hum. Reprod. 30, 28–36.

Rubio, I., Kuhlmann, R., Agerholm, I., Kirk, J., Herrero, J., Escriba, M.J., Bellver, J., Meseguer, M., 2012. Limited implantation success of direct-cleaved human zygotes: a time-lapse study. Fertil. Steril. 98, 1458–1463.

Rubio, I., Galan, A., Larreategui, Z., Ayerdi, F., Bellver, J., Herrero, J., Meseguer, M., 2014. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the EmbryoScope. Fertil. Steril. 102, 1287–1294, e5.

Sakkas, D., Shoukir, Y., Chardonnens, D., Bianchi, P.G., Campana, A., 1998. Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. Hum. Reprod. 13, 182–187.

Shoukir, Y., Campana, A., Farley, T., Sakkas, D., 1997. Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. Hum. Reprod. 12, 1531–1536.

Stylianou, C., Critchlow, D., Brison, D.R., Roberts, S.A., 2012. Embryo morphology as a predictor of IVF success: an evaluation of the

proposed UK ACE grading scheme for cleavage stage embryos. Hum. Fertil. (Camb.) 15, 11–17.
Van Montfoort, A.P., Dumoulin, J.C., Kester, A.D., Evers, J.L., 2004. Early cleavage is a valuable addition to existing embryo selection parameters: a study using single embryo transfers. Hum. Reprod. 19, 2103–2108.
Van Royen, E., Mangelschots, K., De Neubourg, D., Laureys, I., Ryckaert, G., Gerris, J., 2001. Calculating the implantation potential of day 3 embryos in women younger than 38 years of age: a new model. Hum. Reprod. 16, 326–332.
Yang, Z., Zhang, J., Salem, S.A., Liu, X., Kuang, Y., Salem, R.D., Liu, J., 2014. Selection of competent blastocysts for transfer by combining time-lapse monitoring and array CGH testing for patients undergoing preimplantation genetic screening: a prospective study with sibling oocytes. BMC Med. Genomics 7, 38.

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