EDGE HILL UNIVERSITY

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Time-lapse systems: incubation and annotation

Submitted for the degree of Doctor of Philosophy

by

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Declaration

I certify that this thesis does not, to the best of my knowledge:

(i) incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education;

(ii) contain any material previously published or written by another person except where due reference is made in the text;

Abstract

Time-lapse system(s) (TLS) have, potentially, two benefits over standard incubation systems; an undisturbed culture environment and an enormous volume of images of the embryos within them. The current research aimed to determine if a TLS could provide a comparably stable culture environment compared to a standard incubator measured as pH, osmolality and treatment success rates. Second, the hypothesis that patient, treatment and environment specific embryo selection algorithms (ESAs) are required to improve the efficacy of a TLS as an embryo assessment tool was tested.

A TLS was shown to provide a comparably stable environment when compared to a standard incubator in terms of pH and osmolality. In addition, using a strict matched-pair design, embryos cultured in a TLS resulted in a significantly higher implantation, clinical pregnancy and live birth rates. It was also concluded that, of six published ESAs, none performed with clinically relevant predictive capabilities when applied to the same cohort of known implantation embryos. Owing to this, the identification of five abnormal division events as significantly reducing an embryos implantation potential was performed providing an easily adopted, clinically relevant means to deselect embryos cultured in a TLS. A regression analysis found a number of treatment and patient parameters having a significant effect on crucial morphokinetic parameters, although no systemic effect was observed. Finally, an interim analysis of a sibling oocyte study of three, commercially available culture media revealed significant differences in the time of embryo compaction as well as embryo quality and utilisation.

Together, these results highlight that a TLS provides a stable culture environment and leads to increased implantation, clinical pregnancy and live birth rates. It is also likely that the patient, treatment type and environment can significantly alter an embryos morphokinetic profile and specific ESAs are required to unlock the true potential of time-lapse technology.

Key words; morphokinetics, embryo, time-lapse, incubation, annotation

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List of publications

Chapter 3

Barrie A, Homburg R, McDowell G, Brown J, Kingsland C, Troup S. Embryos cultured in a time-lapse system result in superior treatment outcomes: a strict matched pair analysis. Hum Fertil (Camb.) 2016 Nov 24:1-7 (paper 1)

Chapter 4

Barrie A, Homburg R, McDowell G, Brown J, Kingsland C, Troup S. 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. Fertil Steril 2017 Jan 6:pii: S0015-0282 (16) 63014-5.doi: 10.1016/j.fertnstert.2016.11.014 (paper 2)

Chapter 5

Barrie A, Homburg R, McDowell G, Brown J, Kingsland C, Troup S. A preliminary investigation into the prevalence and implantation potential of five abnormal embryonic phenotypes assessed using time-lapse imaging. Reprod Biomed Online (in press) (paper 3)

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List of abbreviations

AC	Absent cleavage
ACE	Association of Clinical Embryologists
aCGH	Array comparative genomic hybridisation
AQB	Average quality blastocyst
AQE	Average quality embryo
ART	Assisted reproductive technologies
AS	Angelman syndrome
AUC	Area under the curve
BFS	British Fertility Society
BPR	Biochemical pregnancy rate
BMI	Body mass index
BWS	Beckwith-Weidemann Syndrome
CC	Chaotic cleavage
CL	Cell lysis
CO_2	Carbon dioxide
CPR	Clinical pregnancy rate
DC	Direct cleavage
DET	Double embryo transfer
D-IVF	Donor *in vitro* fertilisation
D-ICSI	Donor intracytoplasmic sperm injection
DMR	Differentially methylated regions
DNA	Deoxyribonuclease
DOHaD	Developmental origin of health and disease
EDTA	Ethylenediaminetetraacetic acid
Eeva™	Early embryo viability assessment
ESA	Embryo selection algorithm
ESHRE	European society of human reproduction and embryology
F-DC	False direct cleavage
FET	Frozen embryo transfer
fhb	Fetal heartbeat
FISH	Florescent *in situ* hybridisation
FNS	Fertility nurse specialist
GQB	Good quality blastocyst
GQE	Good quality embryo
h	Hours
hCG	Human chorionic gonadotrophin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFC	Hewitt Fertility Centre
HFEA	Human Fertilisation and Embryology Authority
hpi	Hours post insemination
HTF	Human tubal fluid
ICSI	Intracytoplasmic sperm injection
IDEAS	Infertility database for embryology and andrology
IR	Implantation rate
IRAS	Integrated research application system
IVF	*In vitro* fertilisation
Kg	Kilograms
KID	Known implantation data

KSOM	Potassium supplemented simplex optimised medium
LBR	Live birth rate
LR	Likelihood ratio
MI	Metaphase I oocyte
MII	Metaphase II oocyte
MR	Miscarriage rate
MNB	Multinucleated blastomeres
N_2	Nitrogen
NaCl	Sodium chloride
NIRS	Near infrared spectroscopy
NPV	Negative predictive value
O_2	Oxygen
OCC	Oocyte cumulus complex
PGD	Preimplantation genetic diagnosis
PGS	Preimplantation genetic screening
pHe	External pH
pHi	Internal pH
PN	Pronuclei
PPV	Positive predictive value
PQB	Poor quality blastocyst
PQE	Poor quality embryo
RC	Reverse cleavage
rcf	Relative centrifugal force
RCT	Randomised controlled trial
REC	Research ethics committee
S.D	Standard deviation
SET	Single embryo transfer
SPSS	Statistical package for the social sciences
T-DC	True direct cleavage
TLS	Time-lapse system
U-DC	Unconfirmed direct cleavage
VS	Viability score
vs.	versus

CHAPTER 1: General introduction

In vitro (from Latin meaning; in glass) fertilisation (IVF) is a laboratory procedure developed in the last half of the 20th century by noted scientists Robert Edwards and Patrick Steptoe. In 1978 the first birth from assisted reproductive technologies (ART) was seen. Since then, births from ART have reached 5.4 million worldwide with over 200,000 births in the UK (European society of human reproduction and embryology (ESHRE), 2016). In principle, the idea of assisted conception is to perform fertilisation outside of the human body followed by the transfer of embryo(s) into the recipient's uterus to overcome fertility issues and increase the chance of pregnancy. In a normal female reproductive cycle, a single oocyte will be ovulated from the ovary and, following intercourse, will fertilise in the fallopian tube where it will then develop over six to eight days leading to implantation and a pregnancy. By contrast, IVF involves the drug-induced, super-ovulation of the female causing multiple oocytes to be produced. These oocytes are then collected from the female and fertilised *in vitro* following the collection of semen from the male through masturbation. The development of any subsequent embryos is then monitored whilst the environment in which they are contained is controlled at 37°C, 6% carbon dioxide (CO_2) and 5% oxygen (O_2) (at most clinics in the UK). Embryo (s) are then transferred into the uterus after, usually, five days of culture in the laboratory where the embryo would be classed as a blastocyst.

Over the past thirty years there have been numerous developments within assisted conception allowing the treatment of many couples unable to conceive naturally due to an ever-increasing list of infertility diagnoses. Currently, the national average success rate of assisted conception treatments is 33.7% live birth rate (LBR) in good prognosis patients (maternal age under 35) and 13.8% LBR in poorer prognosis patients (maternal age 40-42) (www.hfea.gov.uk, 2017). Assisted conception units, such as the Hewitt Fertility Centre (HFC), are constantly striving for higher success rates whether this is through the employment of new drugs, protocols or novel embryo selection techniques. From a laboratory perspective, the embryo is the best

indicator for success; generally, if the embryo is high quality then the chance of pregnancy is higher than if the embryo were low quality (Cutting *et al*, 2008). However, the patient also affects the chance of success; if the patient is less than 35 years of age then their chance of pregnancy is higher than if the patient is over 40 years of age as is evident from those LBR quoted earlier (www.hfea.gov.uk, 2017). Consequently, efforts for improved success are geared towards producing and choosing better embryos, as well as investigating and treating patient parameters such as recurrent miscarriage and unexplained infertility.

The desirable outcome of assisted conception treatment is the generation of a healthy singleton and, increasingly, the avoidance of multiple pregnancy and its associated complications. Since the birth of assisted conception the maternal and neonatal health implications have been monitored closely and it has been recognised that the number of multiple births following assisted conception is sixteen times higher than the incidence following natural conception (www.hfea.gov.uk, 2017). Clearly, this increase in multiple births is due to the transfer of more than one embryo owing to the lack of reliable embryo selection methods thirty years ago, a situation that persists even today, albeit to a lesser degree. By far the simplest way to achieve a healthy singleton is to perform a single embryo transfer (SET), an approach now widely adopted in the UK. Evidently, this approach necessitates selection of embryo(s) with highest implantation potential, and has led to the development of many novel embryo selection methods. These include both non-invasive (extended embryo culture to the blastocyst stage, metabolomic embryo profiling and morphokinetics) and invasive (preimplantation genetic diagnosis (PGD) and screening (PGS)) methods.

Current embryo selection methods rely on observing the embryos at specific, restricted time points throughout their development facilitated by removing the dishes containing the embryos and viewing them under a microscope; the result being a total of five to six static images over the same number of days. This selection method, simply termed ‘embryo grading’, is one adopted by all assisted conception units in one form or another. It can be argued that, due to

the importance of the quality of a transferred embryo and the limited ability to reliably assess embryos via static observations, this method of embryo grading may not be sophisticated enough. With the advent of time-lapse systems (TLS), embryos can now be monitored continuously throughout their development in a relatively undisturbed *in vitro* environment. The EmbryoScope® incubator (Figure 1, appendices section 9.1) is a commercially available time-lapse imaging incubator, which automatically records an image of the embryo every ten to twenty minutes in five to seven focal planes giving hundreds of images of an embryo's development over the course of its *in vitro* culture. Embryos contained within this sophisticated incubator can be assessed in much greater detail and embryologists are now able to view many more embryological phenomena. Furthermore, it is possible to pinpoint the specific timings in which an embryo progresses through each cell stage, such information being previously unavailable. Therefore, instead of having vague ranges for specific embryo divisions, as is the current case, a more accurate range can be defined. The use of these specific embryo development timings is a method termed morphokinetics. Although a significant amount of information regarding an embryo's development is available to the embryologists, the value of much of the information is, as yet, largely unknown. The use of these incubators present changes to long-standing laboratory practice as well as a new incubation method aside from the time-lapse imaging capacity. The research study presented sought to evaluate both the intricacies of the EmbryoScope® incubation system and the intricacies of the embryos contained within it. With this intention, this research study aimed to test that the EmbryoScope® is a well-matched incubation method for human preimplantation embryos and that the morphokinetic information that a time-lapse enabled incubator provides can become a useful adjunct to existing embryo selection methods.

1.1 Current and alternative embryo selection methods

In order to effectively delineate the research study, it is prudent to first discuss other available methods of embryo selection, both invasive and non-invasive. Currently, there is no consensus for the best approach to embryo selection and although basic embryo grading is considered the gold standard, many

embryo grading schemes exist (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest group of Embryology, 2011) and success rates appear to have reached a plateau for many (www.hfea.co.uk, 2017). In addition, it is reasonable to suggest that the *in vitro* environment is artificial and if there were methods that could be developed or employed to select embryos sooner in their preimplantation development timeline then this would be an added benefit. As such, there is now a growing need for more robust and valid embryo selection methods and as a result, they are continuously researched and developed.

1.1.1 Non-invasive: basic embryo grading

Current morphological methods for embryo selection have been utilised for many years and are thought to be useful for effective embryo selection involving a number of different parameters; pronuclear morphology (z-scoring) (Tesarik and Greco, 1999; Scott, 2003); polar body alignment and appearance (Payne *et al,* 1997; De Placido *et al,* 2002); appearance of cytoplasm and zona pellucida (Palmstierna *et al,* 1998); early cleavage (Lundin *et al,* 2001; Isiklar *et al,* 2002); multinucleation (Pickering *et al,* 1995; Jackson *et al,* 1998; Yakin *et al,* 2005); cell (blastomere) morphology (Shapiro *et al,* 2000; Hardarson *et al,* 2001; Johansson *et al,* 2003); and fragmentation (van Royen *et al*, 2003; Munne, 2006). Specifically, for cleavage stage embryos (up to day three of preimplantation development), the number of blastomeres the embryo has, the evenness of the blastomeres and the amount of fragmentation in the embryo are most commonly considered. For blastocyst stage embryos, the level of expansion, the inner cell mass and the trophectoderm are commonly considered. In a recently published, evidence-based, scheme (Cutting *et al,* 2008, Figure 2, appendices section 9.1) embryos are assigned a grade that broadly corresponds to their ability implant represented as a series of numbers and letters. The embryologist then uses this information to select the best embryo(s) for transfer. Basic embryo grading was the inaugural method of embryo selection and remains the gold standard however, a clear drawback of this basic embryo selection method is the translation of one embryologist's static visualisation of an embryo into a series of numbers and letters for interpretation by other embryologists. In

addition, removing embryos from the incubator, in order to visualise their morphology, causes the environmental parameters (temperature and gas concentrations), to which the embryo is exposed, to become sub-optimal and, potentially, cause stress to the embryo. Therefore, embryos are removed from the incubator no more than twice per day meaning embryo selection, using conventional incubation and basic embryo grading, must rely on five or six observations made by numerous embryologists that are then translated onto paper for interpretation by others. These shortcomings obviously impinge on effective embryo selection; the first premise for undertaking this research study.

1.1.2 Non-invasive: extended embryo culture

Extended embryo culture, introduced in the late 1990's (Gardner *et al*, 1998a, b; Schoolcraft *et al*, 1999), is a method of embryo selection that involves keeping embryos *in vitro* for an additional two to three days (a total of five or six days) allowing the most competent embryos to progress. Historically, embryos would be kept *in vitro* until they reached four to eight cells (day two or three of embryo development, respectively) and those of the highest quality would subsequently be transferred. Those embryos less functionally competent are much less likely to develop to the blastocyst stage, thereby facilitating the selection of embryos with the highest implantation potential. In essence, extended culture employs a 'survival of the fittest' self-selection approach. Basic embryo grading as a selection method is still relied upon when multiple blastocysts are formed and, as described, numerous grading schemes for blastocysts have been established (Figure 3, appendices section 9.1), (Dokras *et al,* 1993; Shoukir *et al,* 1998; Balaban *et al,* 2000; Yoon *et al,* 2001; Kovacic *et al,* 2004; Cutting *et al,* 2008) and many centres also develop their own.

Extended embryo culture is a highly successful method of embryo selection that has led to an increase in pregnancy rates in many centres (Shapiro *et al,* 2002; Frattarelli *et al,* 2003; Criniti *et al,* 2005; Khalaf *et al,* 2008; Zander-Fox *et al,* 2011). This development in the assisted conception field has been one

of the most important for many years, however, there is evidence to suggest that extended culture can cause unnecessary stress to the embryo which can potentially lead to an increase in specific disorders in resulting children (Doherty *et al,* 2000; Khoshla *et al,* 2001; DeBaun *et al,* 2003; Gicquel *et al,* 2003; Maher *et al,* 2003; Halliday *et al,* 2004; Allen and Reardon, 2005; Fauque *et al,* 2007; Lim *et al,* 2009; Manipalvitari *et al,* 2009). These disorders have been termed imprinting disorders and result from genetic aberrations in an embryo's natural genomic imprinting process.

Genomic imprinting is an epigenetic phenomenon whereby methylation of deoxyribonucleic acid (DNA) causes mono-allelic expression of genes throughout the human genome. A small proportion of the human genome is thought to be subject to imprinting with at least 80 known genes subject to imprinting (Turnpenny and Ellard, 2007). During gametogenesis, the imprinted regions from the previous generation are erased and new imprints are established using the mechanism of methylation (Gomes *et al,* 2009). These imprints are then maintained through preimplantation development and sustained through a *de novo* methylation that occurs in early preimplantation development. Different clinical features result depending on how the genes are inherited and how they are methylated (Turnpenny and Ellard, 2007). There are a number of disorders associated with imprinting defects such as Prader-Willi Syndrome and Angelman Syndrome (AS) resulting from aberrant methylation patterns on chromosome 15. The mechanisms by which ART induce methylation aberrations is largely unclear, however it is well known that there are two critical points in epigenetic reprogramming occurring during gametogenesis and preimplantation development (Morgan *et al,* 2005). It has been theorised that manipulation and exposure to *in vitro* environments at these crucial times could cause instability of the maintenance of the methylation patterns (Fauque *et al,* 2007). In particular it has been concluded that the maternally expressed H19 gene, as well as specific loci, appear to be more sensitive to environmental manipulations (Doherty *et al,* 2000; Li *et al,* 2005a; Fauque *et al,* 2007; Gomes *et al,* 2009). It has also been suggested that ART could generate a deficiency in the uptake of substrates essential for

accurate methylation leading to the aberrations seen in some imprinting disorders (Fauque *et al,* 2007). From an Australian population, it was concluded that if a child has Beckwith-Weidemann syndrome (BWS), the chance that the child was conceived through ART is 18 times higher than for a child without BWS (Halliday *et al,* 2004). In addition to this, imprinting disorders, specifically AS, have been linked to intra-cytoplasmic sperm injection (ICSI), an additional laboratory manipulation used for couples where the male patient has a particularly low sperm concentration, morphology or motility (Cox *et al,* 2002; Orstavik *et al,* 2003). Many studies have reported up to a nine-fold increase in the incidence of imprinting disorders in children conceived by ART (DeBaun *et al,* 2003; Gicquel *et al,* 2003; Halliday *et al,* 2004; Allen and Reardon, 2005; Ludwig, 2005; Maher *et al,* 2003; Orstavik *et al,* 2003; Sutcliffe *et al,* 2006; Fauque *et al,* 2007).

Along with the retrospective analysis studies outlined above, differentially methylated regions (DMRs) have been examined. Mouse embryos have been shown to have aberrant expression of the DMR H19 when cultured *in vitro* (Li *et al,* 2005a). Numerous studies have revealed that patients with BWS conceived by ART consistently show isolated loss of methylation at the maternal KvDMR loci (DeBaun *et al,* 2003; Gicquel *et al,* 2003; Maher *et al,* 2003). In conjunction with this, a recent study revealed that abnormal methylation of the KvDMR region is also found in clinically normal individuals conceived by ART (Gomes *et al,* 2009). This could be attributed to a level of mosaicism, a natural phenomenon where the chromosome complement in each blastomere is slightly different, but supports the theory that ART can cause methylation aberrations. Similarly, 25 patients with BWS conceived through ART showed, predominantly, hypomethylation of the KvDMR loci but also had an increased chance of other loci being methylated when compared to those patients who were naturally conceived (Lim *et al,* 2009) also confirmed by others (Manipalvitari *et al,* 2009). Further studies, using mouse embryos, have concluded a higher level of methylation in *in vitro* embryos when compared to *in vivo* counterparts (Fulka and Fulka, 2006; Zaitseva *et al,* 2007).

However, there have been numerous surveys in many countries (Denmark, Europe, USA, Australia) all finding a relatively small increase in imprinting disorders associated with ART suggesting that while the risk is increased, it is not significant enough to be attributed solely to the process of ART. A very extensive investigation determined the level of methylation of ten DMRs including H19 and KvDMR in 113 clinically normal patients conceived through ART compared to 73 naturally conceived patients. This study found no difference in the level of methylation in any of the DMRs investigated (Tierling *et al,* 2010). Although this study did not investigate those patients with clinically proven imprinting disorders and used relatively low numbers, this is contrasting evidence to that outlined above. This investigation did however find a link between a DMR methylation pattern, birth weight and length. Nevertheless, it should be noted here, that a power calculation was conducted for this study revealing that over 4000 ART cases and 4000 control cases would be needed to gain conclusive answers. A further study found no difference in methylation patterns in three DMRs (IGF2, Cdkn1c and Slc221L) between *in vivo* and *in vitro* mouse embryos, although, as previously mentioned, an aberrant methylation pattern was noted in this study in KvDMR for those embryos cultured *in vitro* (Li *et al,* 2005a). A large survey conducted in 2005 in Denmark analysing the health of all children born between 1995 and 2001 by both ART and natural conception assessed a total of 442,349 naturally conceived and 6052 ART conceived patients (Lidegaard *et al,* 2005). After a follow-up for an average of four years, no significant increase in the incidence of imprinting disorders was found.

It is clear that there is conflicting evidence for the argument that extended culture, or that of the *in vitro* environment as a whole, causes methylation aberrations however there may be explanations for these discrepancies. Super ovulation, a mandatory part of an IVF/ICSI treatment cycle, has been associated with aberrant methylation of H19, therefore, if different methods for super ovulation are used, this may cause differences in the methylation patterns (Fauque *et al,* 2007; Guens *et al,* 2007). Furthermore, it has been suggested that the common bisulphite analysis method used to determine the

methylation status of DMRs could lead to bias amplification when a single analysis is relied upon. With this in mind, Tierling *et al* (2010) repeated any abnormal scores found in their experiment that may indicate changes to methylation, and these were predominantly amplification errors rather than true results. Most interestingly, a phenomenon where embryos possess a level of flexibility and are capable of compensating for *in vitro* impedes leading to methylation deformities could explain the variation seen in experiments, however, this is merely a theory (Fauque *et al,* 2007). Interestingly, it has also been suggested that the differences seen between studies could be attributed to ethnic variations (Tierling *et al,* 2010).

Although contradictory evidence is presented, the basic principle, that the *in vitro* environment is artificial and the early stages of embryo development are crucial, is clear. It is therefore logical to assume that any method that could reduce these potential *in vitro* insults would be beneficial. Extended embryo culture is an embryo selection method employed by most assisted conception units, including the HFC, and an increase in success rates is evident. Further research is required regarding methylation patterns in relation to extended embryo culture and the effect on the preimplantation embryo to draw firmer conclusions. However, the use of time-lapse technology provides an environment more reflective of *in vivo* thus reducing *in vitro* stress as well as the capacity to, potentially, negate the need for extended culture whilst maintaining, if not improving, the current success rates; the second premise for undertaking the research study presented here.

1.1.3 Non-invasive: metabolomic embryo profiling

As early as 1987, it was known that embryos utilised different concentrations of substrates and that this could be related to their viability (Gardner and Leese, 1987). Further to this, it was then discovered that embryos utilised different substrates at different stages of preimplantation development (Gardner and Leese, 1988). If these molecular profiles could be defined then a non-invasive method to predict embryo viability could be developed. With this prediction in mind, the metabolomics, one of the four “omic” technologies,

of an embryo was investigated. Metabolomics is defined as the systematic analysis of the inventory of metabolites, as small molecular biomarkers, that represent the functional phenotype at a cellular level used to explain the change in metabolic regulation as a function of abnormal development (Botros *et al,* 2008).

It is attractive to be able to quantify an embryos' substrate usage as well as their waste production thus, the whole metabolome is of interest to many research groups. There have been a number of experiments utilising Raman spectroscopy to correlate embryos' metabolomic profiles with implantation potential by assessing the spent culture media. A series of experiments totalling 490 spent culture media samples found a significant correlation between implantation potential and an embryo's 'viability score' (VS) (Seli *et al,* 2006; Nagy and Posillico, 2007; Scott *et al,* 2007; Seli *et al,* 2007; Scott *et al,* 2008). Another popular technology for the determination of the whole metabolome of an embryo is near infrared spectroscopy (NIRS). There have been a number of studies on prominent embryo developmental stages; day two, three and five (Kato *et al*, 2007; Vergouw *et al,* 2007a; Seli *et al,* 2008b). These studies all found a significant correlation between the implantation potential of embryos and their respective VS produced from the NIRS. Interestingly, these experiments also concluded that there was no correlation between VS and an embryo's morphology grade with striking results such as 98% of embryos being classed as top quality morphologically but only 29% of these implanting (Vergouw *et al,* 2007a). The results of this particular experiment support the first premise of this research study; basic embryo grading methods may not effectively reflect an embryo's ability to create a pregnancy. A further experiment analysing the whole metabolome of 228 day three or day five spent culture media samples, using nuclear magnetic resonance spectroscopy, found a significant positive correlation between the implantation potential of embryos producing a high VS (Nagy and Posillico, 2007).

Rather than trying to analyse the whole metabolome of embryos, specific

markers have been identified that could give a similarly useful indication of the embryos viability. Between 2007 and 2008 there were three experiments by the same group analysing a total of 129 samples of spent culture media from day three embryos. This analysis concluded that there was a marked difference in biomarkers such as lactate, alanine and glycine between spent culture media of embryos that resulted in pregnancy when compared to those that did not. Specifically, an increase in glutamate and a decrease in alanine, pyruvate and glucose were significantly correlated with viable pregnancies and finally, the ratio between glutamate and alanine/lactate was higher for implanted embryos (Seli *et al,* 2007a; Seli *et al,* 2008a; Seli *et al,* 2008c) supported by others (Brison *et al,* 2004; Scott *et al,* 2008).

Oxidative metabolism as a potential marker for a non-invasive selection method has also been studied. Two studies, analysing a total of 425 spent culture media samples of day two, three and five embryos using NIRS, found a significant correlation between five oxidative metabolism biomarkers and implantation potential (Hardarson *et al,* 2007; Vergouw *et al,* 2007b). In addition to these results it was concluded that morphology scores did not correlate with either the oxidative metabolic activity or pregnancy outcome with 86% of transferred embryos being classed as good quality morphologically but only 27% resulting in pregnancy (Vergouw *et al,* 2007b); further supporting evidence of the shortcoming of basic embryo grading selection methods.

As the principle aim of assisted conception is to achieve pregnancies for as many patients as possible, any available technology that claims to help with this is well received. Promisingly, there appears to be very little contradictory evidence suggesting that metabolomic profiling as a method of embryo selection will not be clinically relevant and, as a result of experiments performed to define embryo metabolomics, various technologies have been developed that claim to be able to determine an embryo's viability from the non-invasive analysis of metabolites in a clinical setting. One of these, Viametrics-E™, began pilot studies in 2009. No clinical benefit of the

instrument was found and it was withdrawn from use. There have been no significant developments for its use clinically and so the goal of utilising embryo metabolomics in a clinical setting appears to be distant; the third premise to support the undertaking of the presented research study.

1.1.4 Invasive: embryo biopsy

Preimplantation genetic diagnosis (PGD) and PGS are laboratory procedures used to identify the chromosomal complement of cellular material taken from either oocytes or embryos. The basis for the use of these techniques is that, in over half of all embryos produced *in vitro,* the chromosome complement is abnormal (termed aneuploid) and the likelihood of these embryos creating a pregnancy is reduced (Donoso *et al,* 2007). This has been partly attributed to the reduction in fertility that occurs with increasing maternal age (Hassold and Chiu, 1985). At birth, females have a finite number of oocytes and the initial stages of meiosis of these oocytes take place during fetal development. However, the developing oocytes enter a period of arrest until after puberty during which time the chromosomes are suspended in alignment on the spindle. The completion of meiosis only occurs upon fertilisation and it has been suggested that this extended suspension could damage the spindle and decrease its segregation ability (Fragouli *et al,* 2006) thus causing aneuploidy. It therefore stands to reason that the higher the maternal age, the longer the spindle must uphold this strenuous confirmation thus increasing the incidence of aneuploidy.

Using laboratory techniques such as florescent *in situ* hybridisation (FISH) (Zamora *et al*, 2011), polymerase chain reaction (Sermon and De Rycke, 2007) and more recently, array or microarray comparative genomic hybridisation (aCGH) (Geraedts and Sermon, 2016) PGD and PGS have been used to assess an embryos genetic competence. Although similar embryo selection tools, PGD and PGS have a single major difference; PGD offers a diagnostic result for many specific chromosomal loci for, usually, fertile patients whereas PGS gives a result for the number of whole chromosomes for infertile patients. Preimplantation genetic diagnosis was first

performed on rabbit embryos in 1968 (Edwards and Gardner, 1968) and first used in human embryos in 1989 (Grady, 1995). Much debate surrounds the methodology of cell collection and the optimal developmental stage for biopsy, with some highlighting the benefits of polar body biopsy (Fragouli *et al,* 2010; Chang *et al,* 2011) and others trophectoderm biopsy (Dokras *et al,* 1990; McArthur *et al,* 2005) although it is now largely accepted that trophectoderm biopsy (performed at the blastocyst stage) is the most favoured (Geraedts and Sermon, 2016). In addition to this, figures from the PGD consortium revealed a 19.9% LBR (1152 babies born from 5780 cycles) when PGD was applied (De Rycke *et al*, 2015). A LBR significantly lower than the national average for good prognosis patients (33.7%) in a fertile population with the transfer of an embryo that has been quantified as chromosomally normal throws the efficacy of such an invasive procedure into question. Preimplantation genetic diagnosis does, however, have a clear place for the detection of inherited disorders.

Preimplantation genetic screening is a more recent addition to the embryo selection arsenal. In PGS, usually a single blastomere (in some cases two) or multiple trophectoderm cells are aspirated from the developing embryo and the chromosome number analysed. This process assesses that the number of chromosomes in the cells is correct, as described previously, a major contributing factor to pregnancy failure in infertile patients. Large cohort studies sought to assess the effectiveness of PGS as an embryo selection technique where few found that PGS could increase the chance of pregnancy and effectively identify those embryos with the highest implantation potential (Verlinsky and Kuliev, 2004, Milan *et al,* 2010). Most reported that PGS does not help in embryo selection and can drastically decrease implantation rates (Staessen *et al,* 2004; Mastenbroek *et al,* 2007; Sermon *et al,* 2007). More recently, reviews have concluded that PGS is yet to be proven to be clinically effective (Gleicher *et al*, 2014; Mastenbroek and Repping, 2014). It would therefore seem that PGS is not as a successful selection technique as the ART community would have hoped. For basic embryo selection the application of this invasive technique seems unnecessary, if not brutal.

Aside from the differing reports of success, these invasive methods have one significant drawback termed mosaicism. Mosaicism describes a natural phenomenon when one or more of the cells within an embryo have differing chromosome numbers. As a result, when a single cell or biopsy is used to define the chromosomal complement of a whole embryo, it may not be a true reflection of the whole due to the possibility that the embryo is exhibiting mosaicism. Some sought to overcome this problem by taking two biopsies from an embryo. However, when, for example, an eight-cell embryo has a quarter of its cells removed, it informs a level of damage that may prevent the embryo from continuing with development. This theory could, partially, explain the reduction in success rates seen when using PGS (as described above) (Cohen *et al*, 2007).

Regardless of the glaringly obvious drawbacks of these methods (high invasive nature, mosaicism, lack of increase in pregnancy rates) many more exist; allele dropout, false positive results, false negative results, expense; the reason why these invasive methods are not employed by all fertility units. To this end, the research study was supported by this further premise that invasive methods of embryo selection are not reliable, require further scrutiny of the biopsy type and stage and are largely inaccessible due to expense, specialised materials required for diagnostic tests and the essentially high skill level.

1.2 Time-lapse imaging as a novel method of embryo selection

It is now possible to defend the use of time-lapse imaging as a novel, reliable method of embryo selection in a clinical setting. As previously described, in order to select embryos, they are currently removed from the incubator and visualised under a microscope for a matter of seconds, providing the observer with a snapshot of the embryo's development. This method has two limitations; a restricted overview of an embryo's development and the exposure of the embryo to suboptimal conditions such as reduced temperature and gas concentrations. However, with the introduction of time-lapse imaging, where an image of each embryo is taken every five to ten minutes, more intricate embryo parameters can be viewed whilst leaving the

embryos in a completely undisturbed environment. As the availability of these TLS increased, attention was first focused on assessing their clinical safety. Once this had been established and the available technologies (Primovision™, EmbryoScope®, Early Embryo Viability Assessment (Eeva™)) validated for clinical use (Freour *et al,* 2012; Nakahara *et al,* 2010; Basile *et al,* 2011; Cruz *et al,* 2011; Kirkegaard *et al,* 2012) research then focussed on determining how the TLS could be used to increase pregnancy rates through in-depth embryo analysis and an undisturbed culture system.

1.2.1 Morphokinetics and embryo viability

Many morphokinetic parameters have been identified that correlate with the embryo's ability to create a pregnancy both in humans and animals; the appearance and disappearance of pronuclei (PN) and nuclei at each cell stage (Payne *et al,* 1997; Lemmen *et al,* 2008; Scott, 2010; Azzarello *et al,* 2012) the length of time between early cytokineses (Gonzales *et al,* 1995; Ramsing and Cellesen, 2006; Ramsing *et al,* 2007; Lechniak *et al,* 2008; Herrero *et al,* 2011; Cruz *et al,* 2012; Hlinka *et al,* 2012; Meseguer *et al,* 2011), and start times of blastulation (Campbell *et al,* 2013a).

Additional embryological phenomena have been observed using TLS including the reabsorption of fragments. Hardarson *et al* (2002) provided the first evidence that cellular fragments are able to ‘disappear’ during *in vitro* culture. This is an important observation especially when it has been suggested that a separate grading system be introduced to reflect fragmentation position and distribution as it was found that larger fragments may impinge on implantation to a greater degree than those that are localised or small and scattered (Alikani *et al,* 1999). As fragmentation has been included in current embryo grading methods for many years and has been strongly correlated with pregnancy rate (Alikani *et al,* 1999; Fujimoto *et al,* 2011) the use of TLS could illuminate fragment behaviour to aid in future embryo selection.

A further embryological feature that could be illuminated using TLS is the direct cleavage of embryos from one to three cells (DC). A large multicenter

analysis of DC including 5225 embryos found that 13.68% of these elicited DC. From those embryos transferred (1659) 6.6% elicited DC and of these 1.21% implanted. This research demonstrated that embryos that undergo DC have extremely reduced implantation potential and it is thought that this is due to a “short cell cycle” where the cells have not taken enough time to undergo the correct DNA replication which should take between 10-12 hours (h) (Rubio *et al,* 2012). In conjunction with unusual embryo division patterns, that of reverse cleavage (RC) has been described (Hickman *et al,* 2012) where a cell undergoes a division and then reabsorbs the resulting cell. The phenomenon of RC has been shown to have no effect on the embryos continued development however time-lapse imaging may be used to better inform users of the reasons why this might occur and what it could truly mean for an embryo’s viability.

With these parameters in mind, many used implantation rate (IR), clinical pregnancy rate (CPR) and LBR as indicators of embryo viability (Lemmen *et al,* 2008; Meseguer *et al,* 2011; Kirkegaard *et al,* 2012), however some used an embryos ability to create a blastocyst to indicate viability (Wong *et al,* 2010; Dal Canto *et al,* 2012; Hlinka *et al*, 2012; Cruz *et al*, 2012). Owing to the evidence previously presented regarding imprinting disorders, if an embryo’s viability could be determined earlier in development then the benefits of extended culture could remain without the potentially harmful side effects. In relation to this, Cruz *et al* (2012) examined 834 embryos and suggested that early embryo development was strongly correlated with blastocyst formation rate (BFR). An earlier study suggested that it is possible to predict blastocyst formation with a 93% specificity based on the first few cell divisions of preimplantation embryo development (Wong *et al,* 2010). Of the 242 embryos analysed, 100 were cultured to the blastocyst stage and 14 were analysed to test the parameters set out. It was predicted that, out of the 14 embryos, nine would reach the blastocyst stage and five would arrest all of which, except one, were correct. These data used low numbers and the incorrect prediction of an embryo to arrest is a fundamental failure. Nevertheless, these data suggest, with a 93% specificity (the proportion of arrested embryos that were correctly identified), that BFR can be predicted based on the first few cell

divisions in an embryo, also supported by others (Dal Canto *et al,* 2012; Hlinka *et al,* 2012). Although, independent morphokinetic parameters such as time to two-cells have, thus far, been well studied (Gonzales *et al,* 1995; Ramsing and Cellesen, 2006; Ramsing *et al,* 2007; Lechniak *et al,* 2008; Herrero *et al,* 2011; Meseguer *et al,* 2011; Cruz *et al,* 2012; Hlinka *et al,* 2012), there have been few investigations linking an embryo's morphokinetics to parameters such as patient age, culture media and treatment type.

1.2.2 Development and use of embryo selection algorithms

Single morphokinetic parameters have been used to develop embryo selection algorithms (ESAs). Embryo selection algorithms outline a set of questions for the user where, depending on the answers to the questions asked, a result is given that will aid in the selection of the best embryo(s) for utilisation. There are various types of ESAs; hierarchical, additive and multiplicative. Most algorithms published thus far are hierarchical containing a maximum of three parameters. These algorithms are classed as hierarchical because the first question asked is deemed to be more predictive of implantation (or other appropriate end-point) than the last. Additive and multiplicative models can involve more than three parameters and assign each question with a weight. The weights are then either added (in an additive model) or multiplied (in a multiplicative model) to give a continuous rather than a discrete score, as in a hierarchical.

Multiple ESAs have been published each with different parameters, optimum timings and end points (Wong *et al,* 2010; Meseguer *et al,* 2011; Azzarello *et al,* 2012; Cruz *et al,* 2012; Chavez *et al,* 2012; Campbell *et al,* 2013a; Chamayou *et al,* 2013; Dal Canto *et al,* 2012). However, as the development of ESAs was pursued, it was recognised that centres were annotating in different ways and a consensus was required. After deliberation regarding possible annotations, a consensus was published (Table 1, appendices section 9.1).

An ESA, published in 2011 (Meseguer *et al,* 2011, Figure 4, appendices section 9.1), is one that was employed at the HFC to aid in embryo selection

upon the introduction of the time-lapse enabled incubator, the EmbryoScope®. This ESA, like all those published, uses morphokinetic parameters to identify embryos with the highest chance of creating a pregnancy and has been demonstrated to achieve 86% specificity. This hierarchical algorithm places importance on the time at which the embryo reaches the five-cell stage (t5), the time between the third and fourth cell divisions (s2) and the time between the second and third cell divisions (cc2), respectively. The algorithm then assigns a grade (A+ to E) to each embryo where A+ indicates the most likely to implant and E the least likely to implant. A retrospective cohort study using over 7000 cycles was then undertaken where a significant increase in CPR was found, attributed mainly to the use of morphokinetic parameters and the developed ESA (Meseguer *et al,* 2012). Due to the strength that this publication posed, the ESA was employed at the HFC as an adjunct to established embryo-grading protocols. After a period of approximately nine months, the ESA was validated using HFC data. Between 22nd October 2011 and 14th July 2012, 173 embryos cultured in the EmbryoScope® at the HFC with known implantation were identified. Known implantation data (KID) relates to those patients having a SET resulting in either a fetal heartbeat (fhb) at ultrasound scan (six to seven weeks post embryo transfer) or a negative urinary human chorionic gonadotrophin (hCG) test, and those patients having a double embryo transfer (DET) resulting in either a negative urinary hCG test or two fhb identified at ultrasound scan (six to seven weeks post embryo transfer). This ensures that the transferred embryos fate is known, allowing analysis of embryo quality and its relation to treatment outcome. The ESA score (A+ - D-) that each of the 173 embryos had attained was then correlated with IR (Figure 5, appendices section 9.1). Those embryos assigned an A+, supposedly those most capable of implanting, had a comparable implantation rate (40%) to those assigned a D- (37%). Consequently, the development of an HFC-specific ESA began. This involved the assessment of the same 173 embryos, and the optimum time frames for each of the morphokinetic parameters of interest. Seven parameters were assessed for the development of the HFC ESA; t2, t3, t4, t5, t2-t3 (cc2), t3-t4 (s2) and t4-t5 (cc3). The timings of each parameter for each embryo were correlated with the IR and the point at which the IR was highest

was classed the optimum time frame for this parameter to occur. Once the optimum timings had been identified for each parameter, the predictive capability of each was then assessed using a logistic regression analysis. This analysis revealed two parameters that were significantly predictive of implantation (Table 2, appendices section 9.1). The hierarchical HFC ESA (Figure 6, appendices section 9.1) was then developed with the parameter of highest predictive power as the first tier (time between three and four-cells (s2)) followed by the next most predictive parameter (time between four and five-cells (cc3)) ending with the time to five-cells (t5). Although the influence of t5 was statistically insignificant in this analysis, it was added to the ESA by virtue of its importance in the published ESA. The resulting difference in IR between A+ and D- embryos was then much greater (68% vs. 9%, p=0.001, chi-square test) (Figure 7, appendices section 9.1). Before the application of the HFC ESA clinically, it first required validation. After a further period of approximately 12 months, 511 KID embryos were identified, eliminating the original 173 used for model development. Both the published ESA and the HFC ESA were applied to this cohort of embryos and the implantation rates assessed (Figure 8, appendices section 9.1). These analyses revealed a difference in each of the ESAs ability to detect both high and low implantation capacity embryos with the HFC ESA able to detect these embryos more effectively than the published ESA. However, limitations to the HFC ESA were identified; a high number of embryos were being classed in the B categories with very few being classed in the C categories. This result highlights the imperative for the evolution of the HFC ESA.

1.3 Research aims

The ultimate aims of this research were to support that i) the EmbryoScope® can provide a comparable incubation environment when compared to standard incubators employed at the HFC and ii) the information that a TLS provides can contribute to effective embryo selection in an IVF laboratory in the form of patient, treatment and environment specific ESAs. Although this area of research is currently prominent in the assisted conception field, the current research sought to undertake that which had not yet been addressed and improve on that which had. To this effect, firstly, the incubator was

assessed. This involved an evaluation of basic environmental parameters between the two incubation systems; osmolality and pH. Further to this a strict, matched-pair analysis comparing the treatment outcomes of embryos cultured in a time-lapse enabled incubator when compared to a standard incubator employed at the HFC was performed. Secondly, the annotation aspect of time-lapse enabled incubators was scrutinised. This involved the validation of six current published ESAs on a maximised number of KID embryos to determine their efficacy thus highlighting the need for the development of more specific ESAs. Further interrogation of the data that the EmbryoScope® gives access to informed the analysis of five abnormal embryo development events and how these can be used as deselection criteria; another step towards more specific methods of embryo selection or deselection. Finally, patient, treatment and environmental factors were assessed to further aid in the development of specific ESAs. The first part of this section involved a sibling oocyte design where patient's oocytes/embryos were randomised to three commercially available culture media. The aim of this was to determine if there was a need for the development of culture-specific ESAs. Second, a regression analysis was performed to determine the effects of patient (maternal age, infertility diagnosis, maternal body mass index (BMI)) and treatment (suppression protocol, treatment type) parameters on embryo morphokinetics in line with one of the overall aims of this research; to highlight the need for the development of specific ESAs.

CHAPTER 2: General materials and methods

Unless stated otherwise the below materials and methods were adhered to throughout. In cases where either/or is stipulated below, the relevant methods will be stated within each chapter.

2.1 Research governance

All investigations were approved by the NHS Haydock research ethics committee (REC) (ref: 14/NW/1043) and the institutional REC (appendices section 9.2). All procedures and protocols (appendices section 9.3) complied with UK regulation (Human Fertilisation and Embryology Act, 1990, 2008).

2.2 Ovarian stimulation

Pituitary down regulation was achieved using either a gonadotrophin releasing hormone agonist (buserelin, Suprecur®, Sanofi Aventis, UK) or antagonist (cetrorelix acetate, Cetrotide®, Merck Serono, Germany). Ovarian stimulation was performed using urine derived or recombinant follicle stimulating hormone (Progynova (Bayer, Germany), Fostimon, Merional (IBSA, Switzerland), Menopur® (Ferring Fertility, Switzerland), Gonal f® (Merck Serono). Doses were adjusted based on patient demographic and response. Patients were given 5000IU of subcutaneous hCG (Gonasi® HP, IBSA Pharmaceuticals, Italy) 36h prior to oocyte collection. Luteal support was provided using 400mg of progesterone pessaries twice daily (Cyclogest®, Actavis, UK) until the pregnancy test was performed.

2.3 Oocyte retrieval and embryology

Ultrasound guided oocyte collection was performed transvaginally under sedation (Diprivan, Fresenius Kabi, USA). Collected oocyte-cumulus complexes were cultured in 4-well dishes (Nunc™, Thermo Scientific, USA) containing 0.65ml G-IVF™ (Vitrolife) overlayed with 0.35ml OVOIL™ (Vitrolife) in a standard incubator. Sperm preparation was performed using a standard gradient separation (for ejaculated sperm only) at 0.3 relative centrifugal force (rcf) for ten minutes (ISolate®, Irvine Scientific, USA) followed by two washes at 0.6rcf for ten minutes using G-IVF™. Those oocytes destined for ICSI were prepared using enzymatic (HYASE-10X™,

Vitrolife) and mechanical digestion. ICSI was performed on all metaphase II (MII) oocytes approximately four hours following oocyte collection after which time all injected oocytes were placed in individual culture drops of either G-1™ or G-TL™ (Vitrolife). Embryos were cultured in either an EmbryoSlide® in the EmbryoScope® (Vitrolife) or a 4-well dish in a standard incubator (Sanyo Multigas Incubator MCO-18M). Those oocytes destined for standard insemination had this performed approximately four hours after collection and replaced into a standard incubator until fertilisation check the following day. Oocytes were then checked for fertilisation approximately 16-18 hours post-insemination (hpi) and all fertilised oocytes along with all unfertilised MII oocytes were placed in individual culture drops of G-1™ or G-TL™ and cultured in either the an EmbryoSlide® in the EmbryoScope® or a 4-well dish in a standard incubator. Where culture to day five was undertaken, and sequential culture media was used, a media change was performed on day three. For those embryos cultured in the EmbryoScope®, 20µl from each well was aspirated and replaced with 20µl of G-2™ (Vitrolife). For those embryos cultured in standard incubation, all embryos were moved to a new 4-well culture dish comprising individual 20µl drops of G-2™. Embryo selection for those cultured in the EmbryoScope® was performed using the national grading scheme (Association of Clinical Embryologists (ACE)/ British Fertility Society (BFS) guidelines, Cutting *et al*, 2008) with an internally derived embryo selection, time-lapse algorithm as an adjunct if a decision could not be made between embryos of similar morphology. Where applicable, embryo selection for those cultured in the standard incubator was performed using the national grading scheme only. The internally derived embryo selection algorithm used three morphokinetic parameters; the time between three-cell and four-cell (s2), the time between four-cell and five-cell (cc3), the time to five-cell (t5) (Figure 6, appendices section 9.1). The national grading scheme combines three parameters for day three embryos; cell number (n), blastomere evenness (1-4) and fragmentation (1-4). For day five embryos, the national grading scheme includes the level of expansion (1-6), quality of the inner cell mass (A-E) and quality of the trophectoderm (A-C). Embryo transfer was performed using the highest-grade embryo(s) either three or five days post collection depending on the number of good quality embryos (GQE) the

patient had on day three as well as how many were to be transferred. Selected embryos were cultured in EmbryoGlue® (Vitrolife) prior to embryo transfer. Embryos were cultured at 37°C, 6% CO_2, 5% O_2, 89% nitrogen (N_2) throughout.

2.4 Analysis of time-lapse information

The image interval on the EmbryoScope® was set to ten minutes with seven focal planes. Images were collected for the duration of culture immediately following ICSI or fertilisation check (for IVF derived embryos) to utilisation. Annotation was performed manually as part of the clinical workload in the embryology laboratory using the published annotation consensus (Ciray *et al,* 2014). Accuracy of annotation was corroborated by the participation of the annotating embryologists in an internal quality assurance scheme for morphokinetic analysis. Unless stated otherwise, t0 was defined as the time of insemination/injection for pronuclear fading and pronuclear fading for all other morphokinetic parameters.

2.5 Statistical analysis

Various statistical analyses were performed based on the datasets available and are outlined in each chapter. A University statistician was consulted on the use of appropriate methods for analysis throughout.

CHAPTER 3: The EmbryoScope® as an incubator; fundamental measurements of pH and osmolality

3.1 Introduction

Osmolality and pH are pivotal in the culture of human preimplantation embryos and must be appropriately maintained for effective development and subsequent pregnancy in assisted reproductive technologies (ART). The investigation of these environmental factors will be addressed here as 'stressors' to the preimplantation embryo and represent two of many. Stress can be defined broadly as anything that affects homeostasis. Various forms have been extensively researched including culture media composition, oxygen tension, method of fertilisation and even culture dish rigidity all having been shown to impact embryo development, specifically morphology and gene expression (Rinaudo and Schultz, 2004; Rinaudo *et al,* 2006; Giritharan *et al,* 2007; 2010; 2012; Kolahi *et al,* 2012). There is little literature detailing the effects of pH and osmolality, most likely due to their 'unseen' nature however, the author speculates that research in this area might spike when children born from assisted conception reach an age where pathologies could manifest.

Osmolality and pH of culture media will be put into context by considering each separately, describing their physiological background, importance in preimplantation embryo development and finally, existing literature that helps delineate the effects of deviations in them. Experiments were performed to assess the ability of a time-lapse enabled incubator, the EmbryoScope®, to maintain the pH and the osmolality of culture media compared to standard incubation. The aims of these experiments were to confirm the stable culture conditions of the EmbryoScope® and demonstrate that it may enable a reduction in the exposure of the embryos contained within it to *in vitro* stressors. There have been a number of publications on the superiority of the EmbryoScope® in terms of increasing success rates in an ART laboratory (Meseguer *et al,* 2012; Rubio *et al,* 2014) along with promising results at the HFC. Many attribute this to the stable culture conditions however, as far as

the author is aware, this is the first series of experiments to investigate the culture conditions, *per se,* of the EmbryoScope® compared to a standard incubator.

3.2 Osmolality

3.2.1 Definition

Osmolality is defined as the amount solute per kilogram (kg) of solvent and is recorded as mOsm/kg. This, although similar, is different to the less accurate measure of osmolarity, which is defined using the solvent volume, and is recorded as mOsm/l (Swain *et al,* 2012). Osmolality is directly related to the process of osmosis and, simply, is a measure of the level of water in a given environment. Osmolality is regulated in cells primarily by the movement of water across a semi-permeable membrane from an area of low solute concentration (hypoosmotic) to an area of high solute concentration (hyperosmotic) and thus, solutes exert osmotic pressure and can alter cell volume. These solutes are often referred to as osmolytes; compounds affecting osmolality (Goodman, 2007).

3.2.2 Osmolality in the context of the embryo

The culture of preimplantation embryos began in 1912 with rabbit embryos (Brachet, 1912). During the development of preimplantation embryo culture, many struggled to overcome developmental blocks exhibited by different species at various cell stages. However, culture media was then developed that could sustain human embryos from oocyte to blastocyst. It was not until 1956 that preimplantation embryos were cultured successfully to the blastocyst stage (Whitten, 1956). This break-through has been attributed to particular differences in the culture media used today; the addition of glutamine and ethylenediaminetetraacetic acid (EDTA) and the reduction in osmolality from 290mOsm/kg to 250mOsm/kg. The reduction in osmolality was deemed the most likely contributing factor to the successful development of preimplantation embryos, the basis for which has two theories. Firstly, it was thought that the reduction in osmolality mimicked the *in vivo* environment more closely. However, this theory had been disproved by previous

experiments finding that the *in vivo* environment had the same salt concentrations as serum elsewhere in the system and also indicated that, if anything, it may be slightly higher in the reproductive tract in comparison to other areas (Williams *et al,* 1972; Collins and Baltz, 1999). This was confirmed with experiments revealing the *in vivo* reproductive environment to be as high as 360mOsm/kg (Borland *et al,* 1977; Van Winkle *et al,* 1990). The second theory was linked to the knowledge that preimplantation embryos required organic osmolytes to develop effectively. In earlier culture media compositions, these organic osmolytes were not present and so preimplantation embryos may have been unable to develop effectively (Baltz and Tartia, 2010). However, it has been suggested that embryos might not require these organic osmolytes at lower osmolality's thus the absence of these osmolytes did not negatively affect embryo development.

As a reduction is osmolality led to successful embryo development *in vitro* yet the *in vivo* osmolality level has been shown to be higher, an embryo must possess mechanisms for alleviating the effect of these higher osmolality's in the reproductive tract that cannot be activated *in vitro*; a potential caveat of commercially available media (Dawson and Baltz, 1997). A range of osmolality's have been employed by commercially available culture media with most between 260mOsm/kg and 280mOsm/kg (Table 1). Even a brief exposure to osmolality's outwith the desirable ranges can cause impaired development (Van Winkle *et al,* 1990; Biggers *et al,* 1993, Dawson and Baltz, 1997) with osmolality's over 300mOsm/kg causing severe developmental retardation (Van Winkle *et al,* 1990; Hay-Schmidt, 1993; Miyoshi *et al,* 1994).

It is well known that cell volume homeostasis is a key factor in successful embryo development (Baltz and Tartia, 2010) and preimplantation embryos contain various mechanisms to ensure cell volume is regulated effectively including the activation of appropriate transporters (for example Na+/K+ ATPase's and glycine transporters), increased expression of various genes such as CCM2 which codes for a protein involved in the regulation of cell junctions and p38 MAPK, a class of protein kinases that are responsive to stress stimuli (Fong *et al,* 2007). When an osmotic imbalance is experienced,

Table 1. Osmolality ranges of various commercial media (adapted from Quinn, 2014)

Media Type	Osmolality range (mean) (mOsm/kg)
IRVINE	
P1	282-298
ECM	282-295
Single-step	280-295
Multiblast	281-291
HTF	272-288
SAGE	
Fert media	265±8
Cleavage media	265±8
Blastocyst media	265±8
IVM	265±8
VITROLIFE	
GIVF	279±5
G1.5	261±5
G2.5	260±5
LIFE GLOBAL	
Global	260-270 (265)
Global Fert	280-292 (285)
Blastocyst	260-270 (265)
HTF	280-292 (285)
HTFxtra	280-292 (285)
MEDICULT	
Universal IVF	272-288
ISM1	272-288
ISM2	272-288
EmbryoAssist	272-288
BlastAssist	272-288
COOK	
Sydney IVF fert	285-295
Sydney IVF cleavage	285-295
Sydney IVF blastocyst	285-295

cells have been shown to regulate the level of osmolytes in their cytoplasm through the export (where cell swelling occurs) and import (where cell shrinkage occurs) of solutes. Usually, in situations where solutes must be imported into the cells, inorganic ions are selected, however these can cause protein destabilisation and metabolic disruptions (Dawson and Baltz, 1997). Cells can also utilise organic compounds for cell volume regulation and these have been suggested to reduce the negative impact of their inorganic counterparts (Dawson and Baltz, 1997). Known organic osmolytes include small zwitterionic amino acids, methylammonium compounds and sugars including, glycine and betaine (Dawson and Baltz, 1997). Cells are able to withstand higher osmolality's than they would be exposed to physiologically as long as the medium in which they are cultured contains organic osmolytes such as those described above (Van Winkle *et al,* 1990; Dawson and Baltz, 1997; Baltz, 2001).

Hammer *et al* (2000) demonstrated that embryos possess processes to counteract increases in osmolality. When the osmolality was increased from 250 to 310mOsm/kg the amount of glycine increased indicating that it was being accumulated to provide osmotic support. This has also been linked to the viability of preimplantation embryos using metabolomic experiments where a high glycine turnover was associated with higher viability (Brison *et al,* 2004) demonstrating that embryos that have activated glycine transporters are more able to overcome cell blocks and progress beyond certain cell stages. In line with this, Baltz and Tartia (2010) have also concluded that if an embryo's ability to use glycine for cell volume control is disrupted then fertilised oocytes fail to develop to blastocysts. Incidentally, this has interesting connotations for the assessment of single blastomeres arresting and consequently lysing within a cleavage stage embryo as is regularly seen when using a TLS thus posing another consideration for further research in this area; do single cells that arrest and lyse lack glycine transporters?

3.2.3 How osmolality affects the embryo

Once the successful culture of blastocysts was achieved, focus on osmolality waned. A small number of experiments were conducted to determine optimum

osmolality's but much of the research has been conducted on porcine, murine and bovine embryos therefore extrapolations to the human, as always, must be made with caution.

In 1994, the effects of osmolality were assessed using rat embryos (Miyoshi *et al,* 1994). This experiment was simple in design and effective in achieving it's aims. The experiments involved six groups of 44 embryos each. Sodium chloride (NaCl) was used to achieve osmolality's of 212, 244, 256, 264, 276 and 304mOsm/kg. The percentage of two-cell, four-cell, morula and blastocyst embryos were observed for each group. It appeared that embryos were able to progress to the two-cell stage at any of the tested osmolality's however only 11% embryos reached the four-cell stage when cultured in 304mOsm/kg compared to 98% at 256mOsm/kg. No embryos cultured in 304mOsm/kg reached the morula or blastocyst stage compared to 80% and 61% at 256mOsm/kg, respectively. These results were statistically significant and the optimum osmolality was defined as 244-264mOsm/kg. The authors of this experiment noted that their method for varying the osmolality may have caused the change in embryo development and they were therefore unable to attribute the changes to osmolality definitively. Three years later, Dawson and Baltz (1997) conducted an experiment to test the effects of osmolality on the development of murine embryos and used two methods to vary the osmolality; NaCl and an organic osmolyte. One conclusion from this experiment was that embryonic development was identically inhibited by raised osmolality using NaCl or an organic osmolyte therefore, although authors of the previous study were justified in their identification of a potential flaw in their experiment, this was disproved by their successors. In this experiment, murine embryos were cultured in modified culture media (potassium-supplemented simplex optimised medium (KSOM)) at 230, 250, 290, 310 and 340mOsm/l each with or without 1mM glutamine (acting as the organic osmolyte). Of note is that the measure of osmotic pressure in this case was osmolarity as opposed to osmolality thus an increased degree of inaccuracy is to be expected due to the sensitivity of solvent volume to temperature. From this experiment, raising the osmolarity of the medium with additional NaCl had a significant negative effect on the development of embryos past the four-cell stage and the

optimum osmolarity was identified as 290mOsm/l in the absence of glutamine. At osmolarity's above 310mOsm/l almost no blastocysts developed in the absence of glutamine. In addition, at osmolarity's above 310mOsm/l, the most marked difference in embryo development was seen in the absence and presence of glutamine with over 70% of embryos reaching the blastocyst stage when glutamine was present but only 10% of embryos reaching the blastocyst stage in the absence of glutamine, compounding the need for organic osmolytes in any commercial culture media with a high osmolality.

A further experiment assessed the impact of osmolality on embryo development by altering the osmolality of human tubal fluid (HTF) using NaCl to give varying osmolality's (270, 290, 310, 330mOsm/kg) (Swain *et al,* 2012). This experiment found that, compared to 310mOsm/kg, 330mOsm/kg yielded significantly lower rates of development to eight-cells at 48hpi and total blastocyst formation at 96hpi. Nevertheless, no significant differences in embryo development were observed between embryos cultured in 310 and 330mOsm/kg at any other time point or between 270 and 290mOsm/kg at any time point. These experiments indicate that increased osmolality can have an effect on embryo development, which has been confirmed by others demonstrating a negative impact of increased osmolality (>310mOsm/kg) on embryo development (Brinster, 1965; Hay-Schmidt, 1993; Liu and Foote, 1996; Richards *et al,* 2010).

Experiments conducted in 2006 used porcine embryos to determine the effects of nine different culture systems on embryo development (Ozawa *et al,* 2006). The oocytes were exposed to a short insemination time following *in vitro* maturation with 40 embryos assigned to each group. All embryos were cultured to day six of development with two media changes on days two and four. The osmolality was adjusted to 285mOsm/kg using pure water for all culture systems and the media used in experimental groups was pre-equilibrated in 5% CO_2. Six repeated measurements were taken of each group. The nine groups included a combination of various culture systems that are routinely adopted clinically; open or closed incubation, with or without a buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)) and

with or without oil (1 – (control) no HEPES, no oil, open; 2 – no HEPES, no oil, open; 3 – no HEPES, oil, open; 4 – HEPES, no oil, open; 5 – HEPES, oil, open; 6 – no HEPES, no oil, closed; 7 – no HEPES, oil, closed; 8 – HEPES, no oil, closed; 9 – HEPES, oil, closed). An open system describes a system where no lid is used on the carrier (i.e. culture dish). A closed system describes a system where the lid is used on the carrier. HEPES buffer is one that aids in the maintenance of the pH of the culture media contained in the carrier. Oil is often used to cover the culture media contained in the carrier and aims to reduce gaseous exchange and thus fluctuations in pH. These culture systems have both benefits and drawbacks and are adopted in different variations between units. The total number of cells in the resulting blastocysts was lower in all closed groups compared to open groups and gradually increased within the closed groups (group 6 to 9). In addition, the total number of blastocysts on day six was lower in groups 6 and 7. These results indicate that a closed embryo culture system may not be as conducive to embryo cleavage as an open system. The authors concluded that there seemed to be no association between embryo development and osmolality because the osmolality remained unchanged in the groups where changes in embryo development were seen, contrary to evidence discussed previously.

3.2.4 How osmolality can be affected

Having discussed how osmolality effects embryos it is prudent to discuss what can cause these changes in osmolality. Clearly, the conditions or techniques used during media preparation could impact osmolality. Culture media that was not used for the culture of any gametes or embryos (unused) was tested in a series of observations in 2012 that elucidated interesting features of the ART laboratory that could affect the osmolality of culture media. The volume of media (10, 20, 40µl), drop type (wash or micro-drop), temperature of preparation surface and time to prepare were all assessed. It was shown that the osmolality was significantly higher when the surface temperature was 37°C as opposed to 23°C, micro-drops were prepared as opposed to wash drops and when the size of micro-drop was smaller. This was the first report demonstrating that the way in which culture media is prepared can have a significant effect on osmolality (Swain *et al,* 2012).

Experiments have also been conducted assessing the optimum culture conditions in standard incubation. In one set of experiments different dish types, oil overlays, micro-drop sizes, with or without lids and in a humidified and non-humidified incubator were assessed. When using 4-well culture dishes, the micro-drop and oil overlay volume was varied and cultured with both lids on and lids off. No change in osmolality was seen when an oil overlay of 0.7ml was used regardless of the micro-drop size (20µl or 30µl) with both the lids on and off. When the oil overlay was decreased from 0.7ml to 0.5ml, however, a gradual increase in osmolality was seen over 72h up to 420mOsm/kg with the lids off and 320mOsm/kg with the lids on. This indicates that adequate oil overlay is crucial for culture systems and the addition of a lid where the oil overlay is not sufficient does not prevent an increase in osmolality. When using 60mm round dishes where the lid was present, the osmolality remained relatively stable. For those experiments involving 60mm round dishes where the lid was removed the osmolality significantly increased to 458mOsm/kg when 50µl of media and 7ml of oil were used. The osmolality also steadily increased when 200µl of media and 8ml oil was used illustrating insufficient oil overlay for the volume of media used. The presence of humidity was also considered in this series of experiments and although an increase in osmolality was evident, it was seen in both humidified and non-humidified environments so the change in osmolality was not a result of humidity, or lack of. The conclusion of this was that the presence of a humidified environment did not have an effect on the osmolality of the culture media (Barrie *et al,* 2012).

The manufacturers of the TLS in question also performed a series of experiments to support the clinical application of the incubator. These experiments were performed in order to assess the level of medium evaporation over the course of five days using a standard EmbryoSlide® set-up (25µl in each of the 12 wells covered with 1.2ml oil overlay). A total of 4.15% of medium was lost over the course of five days and amounted to a 0.014g reduction in weight of the EmbryoSlide® (FertiliTechNote, 2013). However, the results do not translate this to a quantifiable change in osmolality, therefore, although an overall loss of 4.15% appears to be low, in

the context of osmolality, it is difficult to ascertain it's significance. Crudely, if this 4.15% loss were extrapolated to osmolality then, with a starting osmolality of 280mOsm/kg, this change would increase the osmolality to approximately 292mOsm/kg (FertiliTechNote, 2013); considered by some to be an acceptable level (Table 1). In addition, the EmbryoScope® incubator, unlike most standard incubators, is not humidified. However, as discussed, humidity may not be critical for maintenance of osmolality. The experiments outlined here aim to determine the effectiveness of the EmbryoScope® incubator at maintaining an osmolality conducive to human preimplantation embryo development and highlight it's ability to reduce the amount of stress that an embryo, cultured *in vitro,* experiences.

3.3 pH

3.3.1 Definition

pH is a measure of acidity or alkalinity of a solution and is an abbreviation meaning 'power of hydrogen'. It is represented on a logarithmic scale where a difference in one pH unit is equivalent to a tenfold difference in hydrogen ion concentration meaning that a strongly acidic solution can have one-hundred million, million times more hydrogen ions than a strongly basic solution (Swain, 2010).

During human embryo culture pH is maintained through the interaction of CO_2 supplied by the incubator and bicarbonate in the media (Swain, 2010). Carbon dioxide is relatively small and uncharged; it can diffuse readily through the cell membrane where it combines with water to form carbonic acid, which rapidly dissociates into a bicarbonate ion and a hydrogen ion (McLiman, 1972). pH is often described using the Henderson-Hasselbalch equation that denotes pH as a measure of acidity (Henderson, 1908).

$pH = pK_a + \log_{10} ([A^-]/[HA])$

Where;

$pK_a = -\log_{10}(K_a) = -\log_{10} (([H3O^+][A^-])/[HA])$

$[A^-]$ = molar concentration of the acid's conjugate base

[HA] = molar concentration of the undissociated weak acid

Broadly, as long as the CO_2 concentration is kept at the required level, the pH should fall within physiological ranges for preimplantation embryo development (Mortimer and Mortimer, 2004).

3.3.2 pH in the context of the embryo

As with osmolality, commercial companies have provided suggested optimal pH levels for their culture medium (Table 2) that are all attained through a variation in CO_2 concentration provided by the incubator. There are very few publications on this requirement of embryo culture because the maintenance of pH at a physiological range simply requires the stabilisation of CO_2. As with osmolality, it appears that an educated guess was made as to the optimum

Table 2. pH ranges of various commercial media (adapted from Swain, 2010)

Media Type	Recommended pH range
IRVINE	
P1	7.27-7.32
ECM	7.2-7.25
Single-step	7.28-7.32
Multiblast	7.3-7.4
HTF	7.2-7.3
SAGE	
Fert media	7.3±0.1
Cleavage media	7.2±0.1
Blastocyst media	7.3±0.1
IVM	7.2±0.1
VITROLIFE	
G5 Series	7.27±0.07
LIFE GLOBAL	
Global	7.2-7.4
Global Fert	7.2-7.4
Blastocyst	7.2-7.4
HTF	7.2-7.4
HTFxtra	7.2-7.4
MEDICULT	
Universal IVF	7.3-7.4
ISM1	7.2-7.3
ISM2	7.35-7.45
EmbryoAssist	7.2-7.3
BlastAssist	7.35-7.45
COOK	
Sydney IVF fert	7.3-7.5
Sydney IVF cleavage	7.3-7.5
Sydney IVF blastocyst	7.3-7.5

pH for culture of human embryos and the standard for blood chemistry was adopted; 7.4. However, the concentration of bicarbonate was found to be higher in the reproductive tract than the blood serum in the rabbit (Vishwakarma, 1962), rhesus monkey (Maas *et al,* 1977) and hamster (Lyman and Hastings, 1951). In addition to this, some have shown that different stages of embryos require different pH's with fertilisation events seeming to require a more alkaline pH (Dale *et al,* 1998, Hentemann *et al,* 2011), cleavage stage embryos a slightly lower pH and blastocysts a slightly higher pH again; this has been come to known as the high-low-high paradigm (Swain, 2010). Also, the pH of the fallopian tubes has been shown to be alkaline in several species, ranging from 7.7 – 8.2 (Maas *et al,* 1987; Ben-Yousef *et al,* 1996; Zhao and Baltz, 1996; Phillips *et al,* 2000). However, when the pH of the uterine environment was assessed, it was found to be markedly reduced and more towards an acidic level (6.96) suggestive of a stage-specific pH which has been corroborated by a study where enhanced embryo development was evident when embryos were exposed to higher pH in the early cleavage stages then a lower pH after compaction (Hentemann *et al,* 2011). In relation to this, the environment *in vivo* has been shown to adapt when specific events occur. For example, the pH of uterine fluid has been shown to increase by 0.2-0.95 units after intercourse for approximately 30 minutes; a reaction thought to be to protect ejaculated spermatozoa from the acidic vaginal secretions and to aid in capacitation (Fox *et al,* 1973). pH variations are also seen alongside the menstrual cycle signifying a role in pH regulation in optimising conception events (Pommerenke and Breckenridge, 1952; Macdonald and Lumley, 1970; Maas *et al,* 1976; 1977).

It has been suggested that embryos must possess internal processes in order to regulate their pH as has been shown with cell volume regulation and osmolality. The resting pH of embryos has been shown to be around 7.0-7.4 however this represents a considerable range in hydrogen ion concentration. Phillips *et al* (2000) reported a resting pH of 7.0-7.1 whereas Dale *et al* (1998) reported the resting pH to be 7.4. The authors of the former speculate that this is due to the culture media used and the compositions of components such as pyruvate and lactate all affecting the pH.

pH is regulated by at least two mechanisms in the preimplantation embryo; the HCO_3^-/Cl^- exchanger that relieves alkalosis and the Na+/H+ antiporter that relieves acidosis. The ability of an embryo to regulate its pH within a narrow range requires the presence of HCO_3^- and CO_2. In absence of HCO_3^-/CO_2 human embryos have been shown to be less able to regulate their intracellular pH (pHi). Recovery from alkalosis by the HCO_3^-/Cl^- exchanger requires intracellular HCO_3^-. This has implications for handling human embryos in buffered medium with low HCO_3^- concentrations in atmospheric CO_2. It has been predicted that exposure to this environment would impair the ability of embryos to maintain their pHi since the low CO_2 would result in very low intracellular HCO_3^- which would inhibit the HCO_3^-/Cl^- exchange. In addition, low external HCO_3^- would slow any HCO_3^- dependent mechanism for alleviating acidosis thus media containing sufficient HCO_3^- with appropriate CO_2 tension is preferable (25mmol/l HCO_3^- / 5% CO_2) (Phillips *et al,* 2000).

3.3.3 How pH affects the embryo

Little is known about human embryo pH regulation however there have been numerous experiments on other mammalian embryos such as the mouse and hamster (Zhao *et al,* 1995; Lane *et al,* 1998; 1999a; 1999b; Phillips and Baltz, 1999). One of the first experiments to investigate the recovery of human embryos from fluctuations in pH found that embryos were able to recover from alkalosis effectively but the researchers were unable to show recovery from acidosis (Dale *et al,* 1998). The pH of both the external environment (pHe) and the pHi have been linked to sustained embryo development (see comprehensive review by Swain, 2010). Raising the pH in the environment harbouring murine embryos to 7.4 or lowering to 6.8 for as little as three hours disrupts localisation of mitochondrial and actin filaments (Squirrell *et al,* 2001) and even minor rises in pH can have dramatic effects on embryo metabolism through the destabilisation of enzymes (Swain, 2010). The effects of pH have also been extended to fetal outcomes where a disruption in pH has been shown to reduce the fetal birth weight of mice (Zander-Fox *et al,* 2008).

In one set of experiments, there was a marked difference in murine embryo development at varying pH levels. Sodium bicarbonate was used to vary the

pH to 7.0, 7,15 and 7.3. At a pH of 7.0 the embryo formation rate at each developmental stage remained constant at 30%. At a pH of 7.15 the embryo formation rate for each developmental stage remained constant but was higher than that of 7.0 (50% vs. 30%). When the pH was increased to 7.3 the embryo formation rate at the early cleavage stage was 70% but decreased to 25% at the blastocyst stage. The authors concluded that a pH of 7.3 up to day two then 7.15 thereafter was optimum (Hentemann *et al,* 2011). The aims of an earlier investigation were to examine the effects of increasing the bicarbonate concentration of culture media and the CO_2 concentration of the incubation atmosphere on eight-cell hamster embryos. When embryos were cultured from the eight-cell stage in 5% CO_2, a significantly higher proportion of embryos developed to the blastocyst stage when compared to ambient CO_2 concentration (approximately 0.04%). When the bicarbonate concentration alone was altered, no difference in embryo development was observed but, when it was altered in conjunction with the CO_2 concentration, embryo development was affected where the authors concluded that a combination of 25nM of bicarbonate at a concentration of 10% CO_2 resulted in a significantly higher proportion of blastocysts formed. The authors also concluded that the difference in embryo development was a result of the changed CO_2 concentration rather than the effect that this has on the pH of the culture media. Interestingly, this series of experiments also identified that the hatching of embryos from the zona pellucida was pH dependent (Carney and Bavister, 1987).

A study conducted in 2000 (Phillips *et al*) found a mean intracellular pH of 7.12 from 199 embryos and concluded that embryo morphology was not affected by pHi however embryos rated 2 or 3 had a significantly more acidic pHi when compared to embryos rated 5 (where 5 denotes a higher quality embryo). The authors comment that the numbers in this study were small; grade 1 (n=11), grade 2 (n=53), grade 3 (n=122), grade 4 (n=21), grade 5 (n=6), therefore it would be difficult to conclude that pHi had a significant effect on embryo morphology. Several have also reported that changes in pHe correlates with metabolic changes, specifically glycolytic activity (Gardner

and Leese, 1988; Edwards *et al,* 1998a; 1998b; Lane and Gardner, 2000; 2003).

However, some have found that the effects of pH on the preimplantation embryo are not immediately obvious when considering embryo morphology. Mouse embryos fertilised and cultured in either optimal or suboptimal conditions *in vitro* have been shown to exhibit similar morphologies and implantation rates when compared to *in vivo* counterparts however the miscarriage rate (MR) was significantly higher in the *in vitro* cultured embryos and highest after suboptimal culture (Holm *et al,* 1996; Khosla *et al,* 2001; Fernandez-Gonzalez *et al,* 2004; Block *et al,* 2010; 2011; Bermejo-Alvarez *et al,* 2012; for a comprehensive review see Feuer and Rinaudo, 2013).

3.3.4 How pH can be affected

The culture environment is the most prominent source of pH variation. However, as previously mentioned, the presence or absence of specific compounds required for pH regulation in the embryo, such as HCO_3^- could also lead to unnecessary pH variations.

pH is notoriously difficult to measure *in situ*, partly due to the availability of effective probes for its measurement under normal culture conditions but also because of the rapid shift in pH when the surrounding environment is changed. A series of simple experiments represented the rapid shift in pH experienced when the external environment changes using two different culture systems; 50µl media drops covered with oil or 5ml of medium (Mortimer and Mortimer, 2004). In the former, the pH began to rise as soon as the dish was removed from the incubator and the pH increased to around 7.65 within two minutes. Once replaced back into the incubator, the media required 35 minutes to re-equilibrate. When considering the latter culture system (5ml medium only) the pH increased to approximately 7.8 within two minutes from dish removal and required 15 minutes to re-equilibrate. A further experiment assessed the pH of nine different culture systems, as described previously with regards to osmolality (Ozawa *et al,* 2006). In these experiments, the pH was shown to be significantly higher in a closed culture system not supplemented with the pH buffer, HEPES. However, the pH of culture medium

in a closed system supplemented with HEPES and covered with oil did not significantly differ from the control. This experiment highlights the need to consider the culture systems used to ensure a stable environment for embryo culture.

3.3.5 Research Aims

The evidence presented above indicates that embryos are highly reactive to their external environment. Despite embryos having been shown to be able to withstand a range of pH and osmolality's there is an argument against exposing embryos to these variations as they act as stressors to embryonic development. These stressors can affect the ability of an embryo to continue with development normally and could result in sub-optimal embryo development. This relates to the quiet embryo hypothesis (Leese, 2002) where embryos that have a low turnover of substrates are more viable than those that have a high turnover. This hypothesis is based on the rationale that the use of substrates can be attributed to repair processes such as DNA repair pathways. Varying pH and osmolality could cause errors in DNA replication, thus the embryo would need to undertake more metabolic processes to repair any damage caused. In addition to the immediate *in vitro* effects, one hypothesis exists that has been corroborated beyond doubt that the environment under which critical developmental events occur, for example, preimplantation development, can have an effect on the health of the individual much later in life (Barker and Osmond, 1986). This hypothesis is known as the Barker hypothesis or the developmental origin or health and disease (DOHaD) and should be acknowledged when considering optimum culture conditions for preimplantation embryos.

Optimum osmolality appears to be defined in the literature as between 260-290mOsm/kg and pH appears to be approximately 7.4, however, commercially available media still have variable levels (Table 1 and 2). It appears that development of culture media has largely been experimental with little scientific reasoning for changes to components of media resulting in differences in embryo development. The exact components of commercially available culture media were, until recently, not publically available but after

experiments on seven types they were found, not surprisingly, to vary considerably (Morbeck *et al,* 2014).

Although the aims of the experiments presented here are not to determine the optimum pH and osmolality for embryo culture, the confirmation that embryo culture in a TLS, the EmbryoScope®, is maintained at stable and physiological levels, are. To this end, the aim of this series of experiments was to demonstrate that the EmbryoScope® provides a stable culture system, maintaining a constant osmolality and pH and thus a potential reduction in the level of stress embryos contained within it are exposed to.

3.4 Materials and methods

This laboratory experiment involved repeated measurements of the osmolality (mOsm/kg) and pH of media not used for gamete or embryo culture (unused) incubated in either a standard incubator (Sanyo Multigas Incubator MCO-18M, 37°C, 5% O_2, 6% CO_2) or a TLS (EmbryoScope®, Vitrolife, 37°C, 5% O_2, 6% CO_2). Target population, participant recruitment and inclusion/exclusion criteria were not applicable due to the use of unused culture media and lack of patient involvement.

3.4.1 Osmolality assessment

Culture dishes were set-up to allow repeated measurements of 60µl of media every 24h for a total of 144h in each system (n (standard) = 14, n (EmbryoScope®) = 14). Culture dishes (4-well) containing three 20µl micro-drops of G-TL™ with 0.7ml oil overlay (OVOIL™) in each of the first three wells and 0.65ml G-TL™ with 0.35ml oil overlay in the fourth well. The fourth well was not used for sampling as this served as a reservoir of media for the micro-drops used during dish preparation. This dish configuration was adopted to represent standard culture conditions as it was used routinely before the introduction of TLS at the HFC. EmbryoSlides® were prepared including 12 micro wells of 25µl G-TL™ with 1.4ml oil overlay. Both dish types were placed in their respective incubators and the time recorded as time zero. The micro-digital osmometer was calibrated to ensure effective measurement

immediately before use using sterile water (0.00mOsm/kg) and a calibration solution of 300mOsm/kg.

A 60µl sample of uncultured G-TL™ was taken immediately using an air displacement pipette (Gilson p100) and the osmolality determined to act as a reference. The culture media was sampled every 24h thereafter up to 144h. Each sample was taken by placing pipette directly into the culture drops and removing the required volume of media (15µl) from each micro-drop in the 4-well dishes and 20µl from each micro well in the EmbryoSlide®. The pipette tip was cleaned with a lint-free tissue to remove oil residue, which could interfere with the osmolality measurement, and the sample expelled into a pre-labeled Eppendorf® tube. This process was repeated until the Eppendorf® tube contained 60µl; a sufficient volume for analysis. Osmolality was measured using a micro-digital osmometer, which uses the principle that the measurement of a freezing point of a solution is directly related to its osmotic concentration.

3.4.2 pH assessment

A pH meter (Research Instruments Ltd, Cornwall) was cleaned and calibrated using deoinised water and potassium chloride. The probe holder was filled with 0.6ml pre-equilibrated G-TL™ using a syringe to ensure the probe was completely submerged. The media was then covered with 0.2ml oil to both mimic the culture conditions used in the laboratory and also to prevent evaporation of the culture media during the measurement period. The probe was then inserted into the probe holder and placed inside a standard incubator. The probe was allowed to reach temperature and a reading was taken every 30 minutes for five hours. The above process was then repeated for the EmbryoScope® incubator.

As pH is notoriously difficult to measure, the variation in pH in both the standard and EmbryoScope® incubators was also determined using the CO_2 concentration as an indicator. Incubator traces were taken from both the standard and EmbryoScope® incubators for a normal working day using the independent monitoring system (XiltriX, IKS international) and the

EmbryoScope® itself, respectively. Readings of CO_2 were taken from these traces at hour intervals over ten hours.

3.4.3 Statistical analyses

All analyses were conducted with the statistical analysis package, Prism® 5 (GraphPad Software©, USA). To inform the type of statistical test to be used, normality was determined using Shapiro-Wilk and Kolmogorov-Smirnov normality tests. Where normality was confirmed, a paired t-test was used to determine significance between measurements. Where normality was not confirmed the Mann-Whitney U test was used to determine significance between measurements. Differences were considered significant at $p<0.05$.

3.5 Results

3.5.1 Osmolality

Measurements taken reflect no significant change in osmolality for either culture system with mean ± standard deviation for standard and EmbryoScope® incubation systems as 292.9 ± 11.98 and 294.1 ± 11.81, respectively (Tables 3, 4, Figure 1A, 1B). Analysis intervals were consistent for each measurement. Over the course of the experimental period the osmolality remained relatively unchanged in both the standard incubator and the EmbryoScope® at approximately 295mOsm/kg. The standard deviations for the standard incubator and the EmbryoScope® indicate that the variation was similar between the two culture systems, although high. Statistical analyses for the osmolality measurements indicate that there was no statistically significant difference within culture systems demonstrating accuracy of the replicate measurements. Furthermore, there was no statistically significant difference between the culture systems indicating that the EmbryoScope® maintains a consistent culture environment. When comparing the average osmolality between standard and EmbryoScope® incubation there were no significant differences (Table 5 and Figure 1C).

Table 3. Standard incubation osmolality results		
Expected sample time (Actual sample time)	**Measurement 1 (mOsm/kg)**	**Measurement 2 (mOsm/kg)**
0h	275.0	281.0
24h (23h 5m)	297.0	282.0
48h (47h 38m)	308.0	286.0
72h (71h 38m)	329.0	291.0
96h (95h 36m)	284.0	284.0
120h (119h 38m)	325.0	288.0
144h (143h 38m)	288.0	283.0
The repeated measurements of osmolality over 144h in a standard incubation system (n=14) p=0.0528 (two-tailed paired t-test)		

Table 4. EmbryoScope® incubation osmolality results		
Expected sample time (Actual sample time)	**Measurement 1 (mOsm/kg)**	**Measurement 2 (mOsm/kg)**
0h	275.0	281.0
24h (23h 2m)	286.0	284.0
48h (47h 38m)	326.0	304.0
72h (71h 35m)	288.0	312.0
96h (95h 33m)	291.0	289.0
120h (119h 35m)	296.0	293.0
144h (143h 35m)	296.0	297.0
The repeated measurements of osmolality over 144h in an EmbryoScope® incubation system (n=14) p=0.8725 (two-tailed paired t-test)		

Table 5. Standard vs. EmbryoScope® osmolality results		
Sample time	**Standard Incubation Average Osmolality (mOsm/kg)**	**EmbryoScope® Incubation Average Osmolality (mOsm/kg)**
0h	278.0	278.0
24h	289.5	285.0
48h	297.0	315.0
72h	310.0	300.0
96h	284.0	290.0
120h	306.5	294.5
144h	285.5	296.5
Mean	292.9	294.1
S.D	11.98	11.81
A comparison of the means of the repeated measurements (taken from Table 3 and Table 4) from the standard and EmbryoScope® incubation systems. p= 0.8517 (two-tailed unpaired t-test). S.D; standard deviation.		

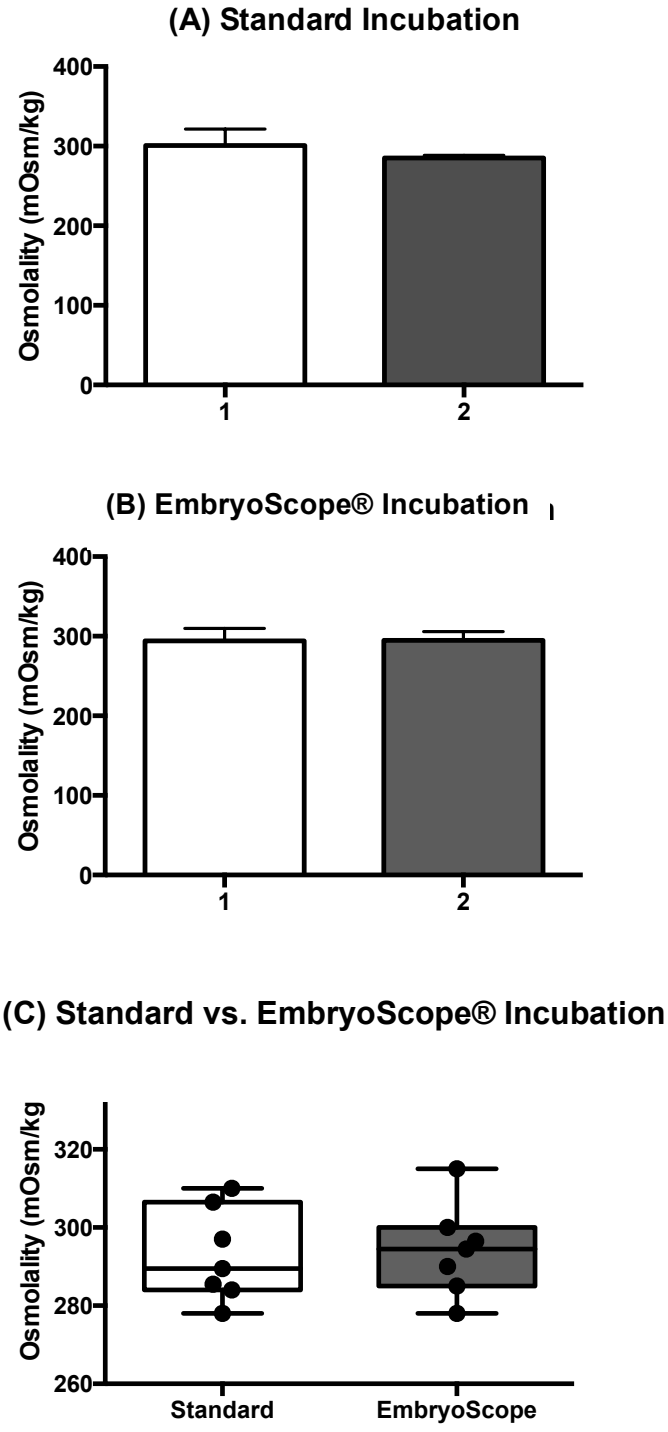


Figure 1. Standard vs. EmbryoScope® osmolality results graphs. A; repeated osmolality measurements ('1' and '2') for the standard incubator ($p>0.05$). B; repeated measurements ('1' and '2') for the EmbryoScope® incubator ($p>0.05$). C; comparison of standard and EmbryoScope® incubation systems ($p>0.05$).

3.5.2 pH

The pH measurements taken using a pH meter revealed mean ± standard deviation for standard and EmbryoScope® incubation systems of 7.64 (±0.18) and 7.75 (±0.12), respectively, shown to be statistically significant (p=0.00027) (Table 6, Figure 2). However, the culture media was not maintained in the probe holder effectively and was seen to evaporate considerably after five hours. Carbon dioxide incubator traces taken of each incubation system on a normal working day in the laboratory are shown in Figure 3. The percent CO_2 was taken every hour for ten hours from both the standard incubator trace (Figure 3A) and the EmbryoScope® incubator trace (Figure 3B). These analyses revealed significantly different results of a mean ± standard deviation for standard and EmbryoScope® incubation systems of 5.61 ± 0.16 and 6.09 ± 0.03 ($p<0.0001$), respectively (Table 7, Figure 4).

Table 6. Standard vs. EmbryoScope® incubation measured pH results				
Sample n	**Standard Measurement 1**	**Standard Measurement 2**	**EmbryoScope® Measurement 1**	**EmbryoScope® Measurement 2**
1	7.5	7.6	7.8	7.5
2	7.4	7.6	7.7	7.6
3	7.6	7.5	7.7	7.9
4	7.7	7.5	7.7	7.9
5	8.0	7.5	7.8	7.9
6	8.2	7.5	7.7	7.9
7	7.7	7.6	7.7	7.9
8	7.6	7.6	7.6	7.7
9	7.6	7.7	7.7	7.6
10	7.6	7.7	7.7	7.9
The repeated measurements of pH using a pH meter in standard and EmbryoScope® incubation systems (n=20), p=0.00027 (Mann-Whitney U test).				

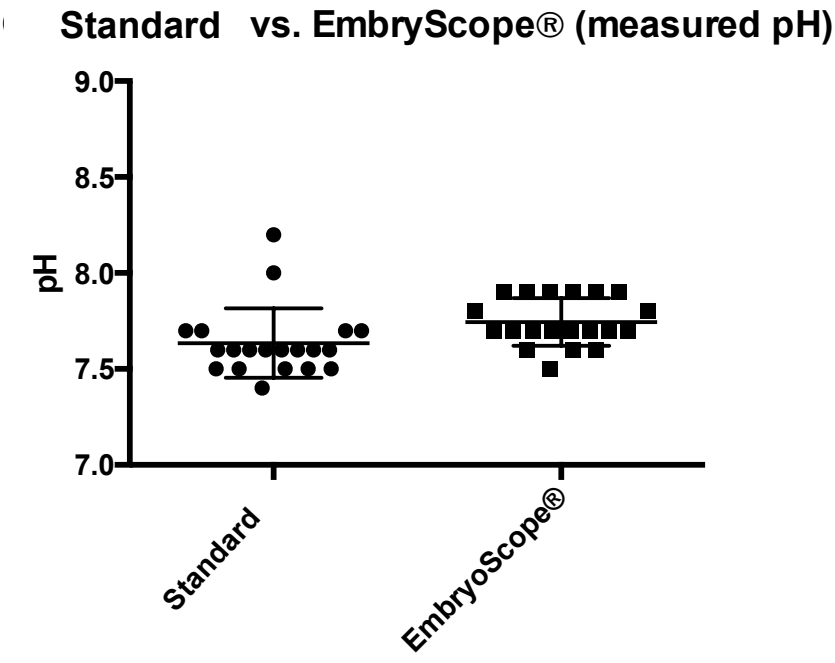


Figure 2. Standard vs. EmbryoScope® measured pH results. Repeated pH measurements taken over five hours every 30 minutes in standard and EmbryoScope® incubation systems using a pH meter (n=20, p=0.00027, Mann-Whitney U test).

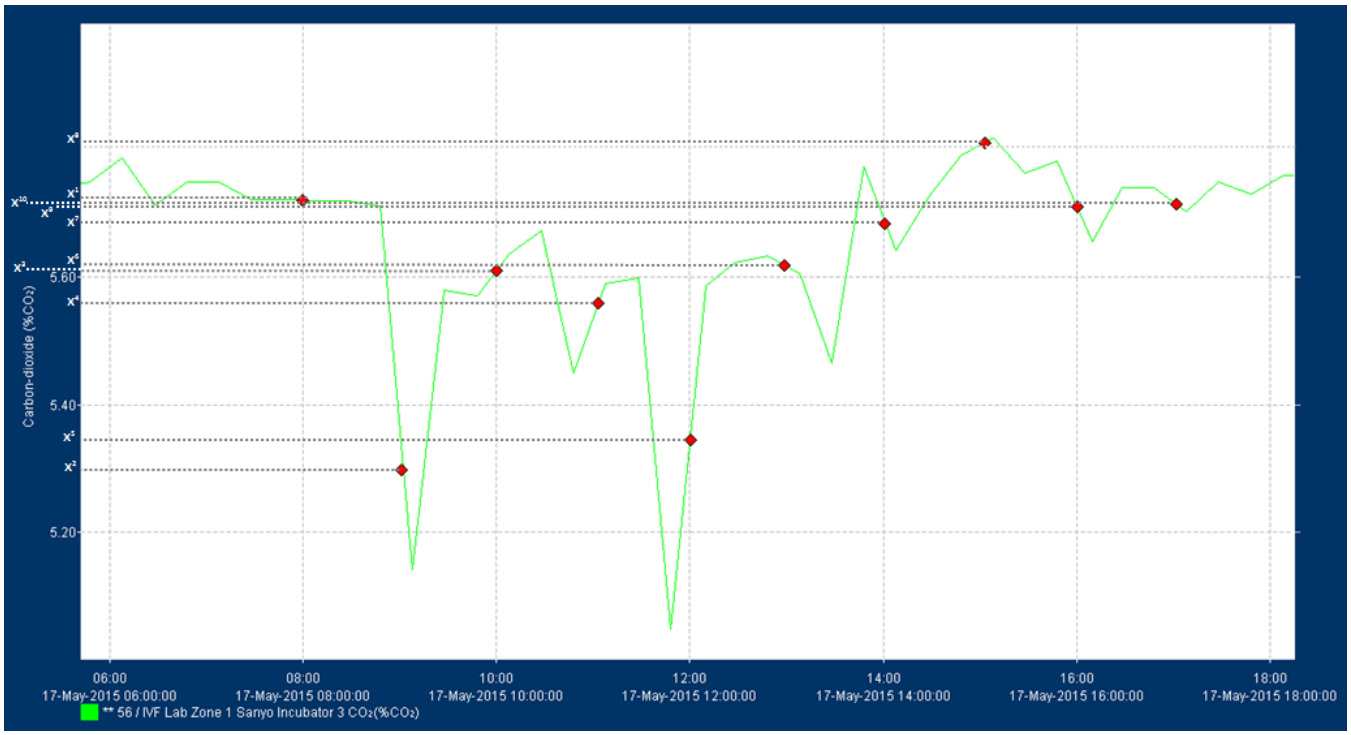


Figure 3. Standard vs. EmbryoScope® incubator traces for normal working day.

Figure 3A. Carbon dioxide trace for a standard incubator on a normal working day. Red diamonds indicate the measurement taken at each hour with a dashed line along to the x-axis indicating the CO_2 level (X_1, X_2, X_3 – X_{10})

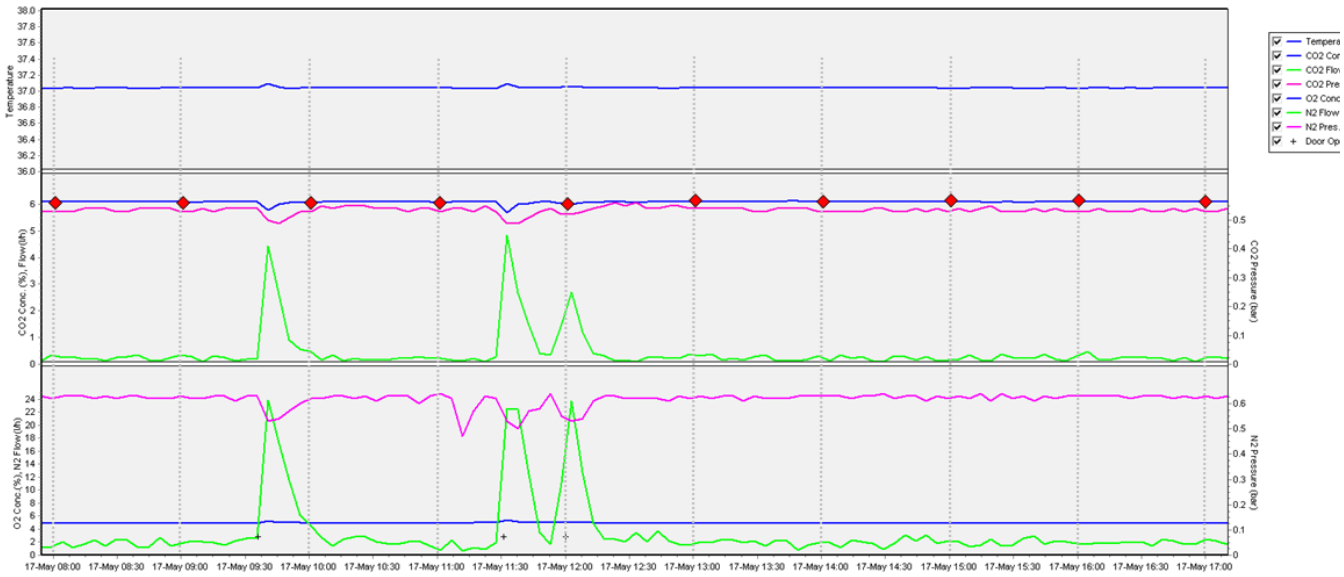


Figure 3B. A CO_2 trace for an EmbryoScope® incubator on a normal working day. Red diamonds indicate the measurement taken at each hour. As the measurements are constant dashed lines along to the x-axis were unnecessary.

Table 7. Standard vs. EmbryoScope® CO_2 readings		
	Standard % CO_2	**EmbryoScope® % CO_2**
1	5.70	6.10
2	5.30	6.10
3	5.62	6.10
4	5.55	6.10
5	5.35	6.00
6	5.63	6.10
7	5.68	6.10
8	5.81	6.10
9	5.71	6.10
10	5.72	6.10
Comparison of CO_2 readings from standard and EmbryoScope® incubation systems on a normal working day (n=10) $p < 0.0001$ (Mann-Whitney U test).		

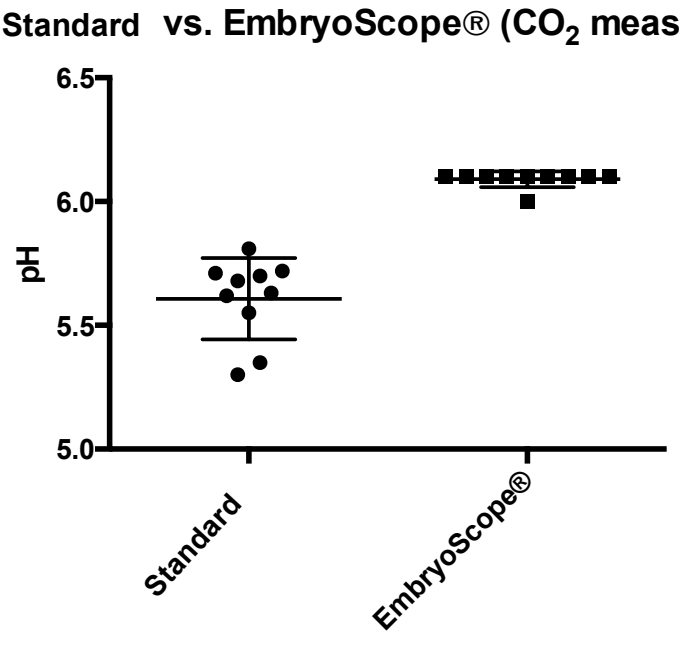


Figure 4. Standard vs. EmbryoScope® CO_2 results. Carbon dioxide measurements taken every hour over ten hours in standard and EmbryoScope® incubation systems using incubator independent monitoring traces (n=10) $p<0.0001$ (Mann-Whitney U test).

3.6 Discussion

The results presented indicate that the EmbryoScope® as an incubator is as stable as a standard incubator when considering osmolality and substantially more stable when considering pH. Specifically, when the osmolality is addressed, the mean osmolality achieved in each incubation system did not differ significantly. In addition, the standard deviations were comparable indicating a similar variation in the measurements between the two systems. This provides reassurance to users that the culture environment of this technology (the EmbryoScope®) is as stable as standard counterparts. It is not surprising that both systems maintain a relatively stable osmolality as this would be reflected and, inevitably, reported, in embryo development leading to changes in the methods of culture. Importantly, the aims of these experiments were to confirm the stability of the EmbryoScope® environment, in relation to osmolality, which has been achieved.

When considering the results obtained for the series of experiments to measure pH, converse to osmolality, a variation was seen. For those results obtained using the pH meter the variation in the results was similar (as reflected in the standard deviations) but the EmbryoScope® values were statistically significantly higher. The results obtained amount to an increase in pH of approximately 0.11. There may be a number of reasons for this increased result, discussed later, however in terms of stability, the results were comparable. Conversely, when using the CO_2 measurements as a proxy to determine a variation in pH the difference between the two systems was obvious. The standard deviation in the standard incubator versus the EmbryoScope® were 0.16 and 0.03, respectively, highlighting the variability in the readings taken from the standard incubator trace. The EmbryoScope® CO_2 readings were also significantly higher than those in the standard incubator, but not outwith the accepted limit. Due to the difficulties associated with measuring pH using a pH meter (discussed later), the CO_2 readings are likely to represent the variation in culture conditions more appropriately and thus indicate that the EmbryoScope® offers a considerably more stable culture environment in terms of pH compared to a standard incubator. This

provides further reassurance to users that the EmbryoScope® is appropriate for human embryo culture.

A significant variation in the functional components of culture media (i.e. pH and osmolality) can have detrimental effects on embryo development but is more likely to cause unseen effects. These suboptimal conditions can inflict stress on the embryo meaning it has to access coping or repair mechanisms to counteract suboptimal culture conditions. This notion is well documented and relates to the Barker hypothesis or the developmental origins of health and disease (DOHaD) (Barker and Osmond, 1986). This hypothesis was put forward when nutritional stress *in utero* was manifested as low birth weights and heightened risk of adult cardiovascular disease. The first, and arguably most, evident examples of this hypothesis were the use of thalidomide to relieve morning sickness in 1950-1960 resulting in widespread birth defects (McBride, 1961; Brent and Holmes, 1988) and the Dutch Hunger Winter study where rations were decreased to 400-800 calories per day for five months resulting in glucose intolerance, obesity and cardiac dysfunction in adult life of children born during this famine (de Rooij *et al,* 2006a; 2006b; 2006c; 2006d; 2010). The cause of these manifestations is likely, at a molecular level, to relate to changes in how genes are transcribed and expressed. The phenomenon of genomic imprinting, more specifically, it's defection, is one such example of how suboptimal conditions can affect an embryos health.

Although there is conflicting evidence for the argument that exposure of embryos to *in vitro* culture conditions can cause unseen effects such as methylation aberrations (discussed previously), the basic principle, that the *in vitro* environment is artificial and the early stages of embryo development are crucial, is clear. An embryos response to stress highlights its ability to evolve to it's surrounding environment but could affect growth and metabolism and force the embryo to divert resources away from coordination of 'normal' processes towards those that have been introduced by suboptimal culture; the basis for the quiet embryo hypothesis (Leese, 2002). It is therefore logical to assume that any method, technology or equipment that could reduce these *in vitro* insults would be beneficial. Further research is required regarding

methylation patterns in relation to *in vitro* embryo culture and the effect on the preimplantation embryo to draw firmer conclusions. However, it appears that the use of a TLS may, certainly when considering pH, reduce the amount of 'stress' an embryo is exposed to during its *in vitro* culture.

3.7 Conclusion

Osmolality and pH are pivotal in the culture of human preimplantation embryos and must be appropriately maintained for effective development and subsequent pregnancy in ART. An embryo can be affected by fluctuations in these factors, manifested in their quality, but the exposure of embryos to these stressors is also apparent later in life. With the advent of new technologies, there is the possibility that the maintenance of these environmental factors at stable levels can be easily achieved. The aim of this series of experiments was to demonstrate that the EmbryoScope® offers a stable environment in terms of pH and osmolality which is evident from the results presented here. This represents a significant step towards the reduction of *in vitro* stress inflicted on preimplantation embryos.

CHAPTER 4: The EmbryoScope® as an incubator; comparison of success rates to a standard incubator

4.1 Prelude

The previous chapter details that of environmental differences between two incubation systems; a standard incubator and a TLS (EmbryoScope®). The purpose for performing this research study was to determine if a TLS (EmbryoScope®) could provide comparable culture conditions when compared to a standard incubator employed at the HFC. The following chapter supports this aim by outlining a strict-matched pair analysis to compare treatment success rates between standard incubation and the EmbryoScope®.

4.2 Embryos cultured in a time-lapse system result in superior treatment outcomes; a strict matched pair analysis (Paper 1)

4.2.1 Abstract

A retrospective, strict matched-pair analysis on 728 treatment cycles between January 2011 and September 2014 was performed. 364 treatment cycles, where all embryos were cultured and examined in the EmbryoScope®, were matched to treatment cycles where all embryos were cultured in a standard incubator with conventional morphological examination. Matching was performed for patient age, number of oocytes collected, treatment type and date of oocyte collection (± six months). The clinical (CPR), implantation (IR), live birth (LBR) and miscarriage rates (MR) were calculated and considered significant at $p<0.05$ (Chi-square test). Clinical pregnancy rate, IR and LBR were found to be statistically significantly higher in the time-lapse system (TLS) group compared to the standard incubation group (CPR; 44.8% vs. 36.5%, p=0.023. IR; 39.3% vs. 32.2%, p=0.029. LBR; 43.1% vs. 33.8%, p=0.009). Although there was a 5.5% decrease in the MR for the TLS group when compared to the standard incubation group, this result was not statistically significant (18.9% vs. 24.4%, p=0.192). There is a paucity of well-designed studies to confirm that embryos cultured and examined in TLS can

result in superior treatment outcomes, and this strict-matched pair analysis with a large cohort of treatment cycles indicates the advantage of using TLS.

4.2.2 Introduction

The first application of TLS in embryology was recorded in 1968 where chick embryos exposed to teratogenic doses of hypoxia were analysed (Grabowski and Schroeder, 1968). Following this, studies relating to preimplantation embryonic development were published (Colly-d'Hooghe *et al*, 1977; Milligan *et al*, 1978; Lueck and Aladjem, 1980; Massip and Mulnard, 1980; Milligan *et al*, 1980; Schatten and Schatten, 1980; Alexandre and Mulnard, 1988). One of the earliest clinical applications of TLS was reported in 1997 regarding polar body extrusion and pronuclear formation in human oocytes (Payne *et al*, 1997). Subsequently, the internalisation of fragments observed in human embryos was published (Hardarson *et al,* 2002) followed by a report of mouse embryo collapse analysed using time-lapse photography (Niimura, 2003). Focus turned to the use of TLS in a clinical setting in 2008 with a number of publications exclusively studying preimplantation embryonic development using TLS and how the information these systems provided could be used to determine embryo viability (Arav *et al,* 2008; Lemmen *et al*, 2008; Mio and Maeda, 2008). The first commercially available TLS began installations in Europe in 2011. TLS for clinical application have now been readily adopted worldwide with instruments installed in numerous countries. Although the body of evidence remains weak, it suggests that TLS can increase the chances of a pregnancy for many undergoing assisted reproduction.

The use of TLS in clinical laboratories allows for a detailed analysis of embryos contained within it giving over 700 images per embryo. This is compared to the conventional snap-shot observations acquired when using an incubator without time-lapse capabilities that require translation into a written series of numbers and letters open to interpretation by other members of the scientific team. The wealth of information that a TLS provides inevitably creates the need to modify how embryos are selected for use and as such there are many reports linking time-lapse parameters (termed morphokinetics) to an embryos ability to create a pregnancy.

A recent Cochrane review retrieved 33 articles relating to the use of TLS and their relative effect in treatment success with only ten studies being potentially eligible for inclusion (Armstrong *et al,* 2015). After further evaluation, three studies were included as true randomised controlled trials (RCT). These trials totalled 994 couples with the majority contributed by one study (Rubio *et al,* 2012). Following analysis it was concluded that for all types of TLS, with or without cell-tracking, embryo selection algorithms, versus standard embryo incubation there was no conclusive evidence of a difference in clinical, live birth, miscarriage and stillbirth rates per couple randomised. The aim of the following investigation was to examine whether TLS can be considered superior to standard incubation systems when considering CPR, IR, LBR and MR by performing a strict matched-pair analysis with a large cohort of patients.

4.2.3 Material and methods

A retrospective, observational, strict matched pair investigation was designed. Data for this research were obtained from 728 treatment cycles between January 2011 and September 2014. This data comprised 364 patients having embryos cultured in a standard incubator (Sanyo Multigas MCO-18M, 37°C, 6% CO_2) (group 1) and 364 having their embryos cultured in a TLS, the EmbryoScope® (Vitrolife, Gothenburg, 37°C, 6% CO_2, 5% O_2) (group 2). Although in group 1 214 were cultured in 20% O_2 and 150 in 5% O_2, a statistical examination of the LBR showed no significant differences between these groups (20% O_2 vs. 5% O_2; 34.1% vs. 34.0%, p=0.92) which were subsequently pooled. All treatments included in this analysis were from known implantation embryos i.e. a single or a double embryo transfer where transfer of two embryos resulted in either a negative pregnancy test or two fetal heartbeats.

4.2.3.1 Patient criteria

All patients with embryos cultured in the EmbryoScope® with known outcome were matched to patients having embryos cultured in a standard incubator for patient age (exact), number of oocytes collected (exact), treatment type and date of treatment (± six months). Patients were not included twice. Patients

were allocated to either standard or TLS culture randomly, based on availability. In February 2014 the laboratory became 100% time-lapse enabled meaning all patients had all embryos cultured in a TLS.

4.2.3.2 Oocyte retrieval and embryology

Injected oocytes and embryos were cultured in G-1™ followed by G-2™. For those destined for standard incubation, injected oocytes/embryos were cultured in 4-well dishes. For those destined for EmbryoScope® culture, injected oocytes/embryos were cultured in EmbryoSlides®.

4.2.3.3 Outcome measures and statistical analyses

Clinical pregnancy rate, IR, LBR and MR were calculated. Clinical pregnancy rate was calculated as the number of patients having a fhb at 6-7 weeks gestation confirmed by ultrasound scan (regardless of number of fhb) out of the number of embryo transfers performed. Implantation rate was calculated as the total number of fhb (i.e. inclusive of higher order pregnancies) out of the number of embryos transferred. LBR was calculated as the number of all live births out of the number of embryo transfers. Finally, MR was calculated as the number of positive hCG tests (urinary sample taken 14 days following a day three transfer or 11 days following a day five transfer) that did not result in a fhb at ultrasound scan at 6-7 weeks gestation. Results were analysed using the Chi-square test (Prism® 5 (GraphPad Software©, USA)).

4.2.4 Results

A total of 728 treatment cycles were analysed and the CPR, IR, LBR and MR calculated (see Table 8 for baseline demographic information). Clinical pregnancy rate, IR and LBR were statistically significantly different between the two groups (Table 9). The CPR for group 1 (control, standard incubation) when compared to group 2 (TLS) was 36.5% vs. 44.8%, respectively, $p = 0.023$. The IR for group 1 when compared to group 2 was 32.2% vs. 39.3%, respectively, $p = 0.029$. The LBR for group 1 when compared to group 2 was 33.8% vs. 43.1%, respectively, $p = 0.009$. However, although there was a

5.5% increase in the MR for group 1 when compared to group 2, this result was not significantly different (24.4% vs. 18.9%, respectively, p = 0.192).

Table 8. Baseline demographic data for standard vs. EmbryoScope® incubation.

	Group 1 (Standard)	Group 2 (EmbryoScope®)	p-value
SET (n)	300	283	0.11
DET (n)	64	81	
Cleavage stage transfers (n)	116	101	0.20
Blastocyst stage transfers (n)	248	263	
Previous attempts (mean ± S.D)	1.35 (±0.92)	1.45 (±0.95)	-
Average patient age (mean ± S.D)	34.04 ± 4.00		
Average oocytes collected (mean ± S.D)	10.13 ± 4.72		
Embryos transferred (n)	428	445	0.64

Data includes the number of SET and DET, proportion of cleavage and blastocyst transfers, number of previous attempts, number of embryos transferred, average patient age and average oocytes collected in group 1 and group 2. Data were analysed using the Chi-square test. SET; single embryo transfer. DET; double embryo transfer. S.D; standard deviation.

Table 9. Data end point results for standard vs. EmbryoScope® incubation.

	Group 1 (Standard)	Group 2 (EmbryoScope®)	p-value
CPR (%)	133/364 (36.5)	163/364 (44.8)	<0.03*
IR (%)	138/428 (32.2)	175/445 (39.3)	<0.03*
LBR (%)	123/364 (33.8)	157/364 (43.1)	<0.01*
MR (%)	43/176 (24.4)	38/201 (18.9)	>0.1

Clinical pregnancy rate (CPR), implantation rate (IR), live birth rate (LBR) and miscarriage rate (MR) for both standard and EmbryoScope® incubation. All results were considered to be statistically significantly different between the two groups where $p<0.05$ (Chi-square test).

4.2.5 Discussion

The results of this strict matched pair analysis reveal that embryos cultured and examined in the EmbryoScope® incubator result in superior treatment outcomes in this laboratory. These results are in concordance with others (Rubio *et al,* 2014; Yang *et al,* 2014; Adamson *et al,* 2016; Basile *et al,* 2015) but have been contradicted elsewhere (Nakahara *et al,* 2010; Cruz *et al,* 2011; Kirkegaard *et al,* 2012; Kahraman *et al,* 2013; Kovacs *et al,* 2013; Armstrong *et al,* 2015; Park *et al,* 2015). A recent Cochrane review (Armstrong *et al,* 2015) suggested that there was insufficient evidence to conclude that TLS with or without cell tracking technology would be beneficial to patients undergoing ART. Included in this were three eligible RCTs the first of which contributed most of the data for the review. This study was a multi-centre RCT of patients undergoing ICSI, using donated or autologous oocytes. In total, 843 couples were randomised; 438 to TLS and 405 to standard incubation. The CPR and MR were calculated as end-points. Although this analysis revealed a significant increase in treatment outcomes, considerable reasons for bias were identified. Firstly, patients could request the intervention (TLS) therefore allocation was, in fact, non-random. Secondly, the study was classed as 'double-blinded' due to the gynaecologist and statistician being unaware of the arm to which the patients had been randomised. However, the patients and embryologists were given this information. Although unlikely to create a significant bias, this detail could invalidate the results. Finally, the heterogeneity of the sample was considerable including the use of donated, and thus both fresh and frozen oocytes (Rubio *et al,* 2014). The remaining two RCTs included in the review were conducted on a small number of couples, one being interim results only, leaving a combined total of 61 to 65 in each arm (Kahraman *et al,* 2013; Kovacs *et al,* 2013). The reviewers reported a high risk of attrition bias in one of these studies due to the principal investigator undertaking the randomisation and also because there was no blinding. Overall, the reviewers stated that there was no conclusive evidence of a difference between standard incubation and TLS when considering CPR, MR, LBR and stillbirth rates. Further analyses, not included in this Cochrane review, have also

shown no significant differences in treatment outcomes between embryos cultured in standard incubation versus TLS (Nakahara *et al,* 2010; Cruz *et al,* 2011; Kirkegaard *et al,* 2012; Park *et al,* 2015).

Differences in results found thus far in the matter of TLS could be attributed to a number of factors. Firstly, a benefit of TLS that one laboratory might enjoy may not be so with another due to the conditions of the laboratory in the first instance. In brief, a well-designed, stable culture environment (TLS) introduced into what was a relatively unstable culture condition may elicit an immediate uplift in treatment outcomes. Whereas, to place this technology into an already optimal culture environment, may not reveal such results. There are many factors that vary between laboratories that could impact this; the type of culture media (single or sequential), culture dish type, volume of media used for culturing embryos, volume of oil overlay, the type of incubator and the embryo grading and embryo transfer policies. It is therefore reasonable to conclude that some laboratories may benefit from TLS more than others.

Secondly, during the culture of embryos in the EmbryoScope® in this analysis an in-house derived embryo selection algorithm was used. This indicates that the analysis presented here does not distinguish between the two, commonly stated, major benefits of TLS; the undisturbed nature of the systems or the use of embryo selection algorithms. Whilst the authors acknowledge that this could create ambiguity, it can also be defended. This detail means that this analysis addresses TLS as a whole in the manner in which it should be utilised; using the information provided by the images. It also gives further explanation for the heterogeneity of success of TLS. Some laboratories utilising TLS have access to large amounts of data meaning in-house derivation and validation of predictive models can be performed; a method much preferred to utilising an externally derived embryo selection algorithm. In these laboratories, where internally derived models are used, although not proven, a greater benefit to using TLS would be expected. Naturally, in those laboratories that do not have access to a data-set allowing in-house derivation of predictive models, externally developed versions may be adopted, a

decision which has been cautioned (Kirkegaard *et al,* 2013a; Yalçınkaya *et al,* 2014). The earliest publications regarding TLS aimed to assess the safety of the systems (Nakahara *et al,* 2010; Cruz *et al,* 2011; Freour *et al,* 2012; Kirkegaard *et al,* 2012). Many of these studies randomised oocytes or embryos between two culture systems (standard and TLS) and found no differences in treatment outcomes of embryo quality parameters. The use of an embryo selection algorithm in these studies is not mentioned, thus these analyses assessed the effectiveness of the incubator itself, not the information it provided. Once satisfaction with the safety of the system had been reached attention was then turned to how the information from the TLS could be utilised. Further reports were then published that revealed an uplift in outcome parameters (Rubio *et al,* 2014; VerMilyea *et al,* 2014; Yang *et al,* 2014; Basile *et al,* 2015; Milewski *et al,* 2015; Siristatidis *et al,* 2015) with one obvious difference; these analyses included the use of an embryo selection algorithm and could provide evidence of the benefits of using TLS alongside an embryo selection algorithm. The study previously described (Rubio *et al,* 2014) supports the above notion whereby those embryos cultured in a standard incubator were assessed for selection based on morphology alone and those in TLS were selected using an internally derived, multivariable model. A significantly higher ongoing pregnancy rate was found in TLS compared to standard incubation (51.4% vs. 41.7% per cycle and 54.5% vs. 45.3% per embryo transfer, respectively) as well as a significantly decreased early pregnancy loss in TLS (16.6% vs. 25.8%). In addition, the implantation rate was significantly increased in the TLS group (44.9% vs. 37.1%) (Rubio *et al,* 2014). A further investigation sought to select the most competent blastocysts for transfer by combining TLS and aCGH for patients undergoing PGS designed as a prospective study with sibling oocytes. 1163 metaphase II oocytes from 138 PGS patients were included and oocytes were randomised to two groups after ICSI; group A were cultured in TLS and group B in standard incubation. Array CGH using trophectodoerm biopsy on both groups was carried out and one or two euploid blastocysts either within the morphokinetic ranges (group A) or morphological grades (group B) were transferred. The CPR and IR were found to be significantly higher in group A when compared to group B (CPR; 71.1% vs. 45.9%, IR; 66.2% vs. 42.4%,

respectively) demonstrating that when embryo selection algorithms are used as an adjunct to select embryos for transfer, superior treatment outcomes can be achieved (Yang *et al,* 2014). These investigations address TLS as an incubator whilst also using the data it provides, synonymous with the current analyses, indicating that embryo selection algorithms derived using TLS are able to select embryos more effectively than standard morphology assessments. It is not surprising that an increase in treatment outcomes is seen in these cases owing to the wealth of information that is available to the user of TLS to do basic, but powerful, embryo selection.

Literature regarding TLS now predominantly concerns development of embryo selection algorithms or reviews concluding that further evidence for its (TLS) superiority is required. The authors believe that predictive models can be very useful, in the first instance for de-selection (rather than selection) of embryos undergoing abnormal cleavage events such as DC and RC shown to have a significantly reduced chance of creating a pregnancy (Rubio *et al,* 2012; Liu *et al,* 2014) but also, if developed effectively, to select the best embryo from a cohort for a specific patient demographic. Patient characteristics including infertility diagnosis (Sundvall *et al,* 2015) and maternal age (Hampl and Stepan, 2013; Chawla *et al,* 2015) as well as treatment characteristics including treatment type and culture conditions (Lemmen *et al,* 2008; Wale and Gardner, 2010; Ciray *et al,* 2012; Cruz *et al,* 2013; Kirkegaard *et al,* 2013b) have been shown to affect an embryo's morphokinetic profile and the resulting subtle differences may be used to identify which embryo has the highest implantation potential. Herein lies a further reason for possible variation in success of TLS between laboratories; patient and treatment characteristics.

4.2.6 Conclusion

This matched pair analysis indicates that treatment cycles where embryos are cultured and examined in TLS result in superior outcomes including CPR, IR, LBR and MR. Although the notion is novel, the authors believe that the real benefit of TLS lies in the development of patient specific embryo selection algorithms. Literature thus far indicates that there is likely to be no difference

in treatment outcomes when an embryo selection algorithm is not used and future research should be geared towards developing effective embryo selection algorithms to aid in embryo selection.

4.2.7 Dissemination

The above research was prepared for publication in Human Fertility. The research was accepted and published on 24th November 2016 (appendices section 9.6).

CHAPTER 5: The validation of existing embryo selection algorithms

5.1 Prelude

Preceding chapters of this thesis have been concerned with the confirmation that a TLS (EmbryoScope®) could provide comparable culture conditions and treatment success rates to a standard incubator employed at the test site. Since this aim of the research study has been supported, subsequent chapters will be concerned with harnessing the information that the EmbryoScope® incubator provides. This will commence with an examination of existing methods for the use of TLS for effective embryo selection in the form of the validation of six, existing embryo selection algorithms (ESAs).

5.2 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 (Paper 2)

5.2.1 Abstract

The objective of this research study was to determine the efficacy of six embryo selection algorithms (ESAs) when applied to a large, exclusive set of known implantation embryos. A total of 884 IVF or ICSI treatment cycles (977 embryos) performed between September 2014 and September 2015 were included in this single-site retrospective, observational analysis. Embryos were cultured using G-TL™ (Vitrolife) at 5% O_2, 89% N_2, 6% CO_2, 37°C in EmbryoScope® instruments. The efficacy of each ESA to predict implantation was defined using specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV), area under the receiver operating characteristic curve (AUC) and likelihood ratio (LR). The differences in implantation rates (IR) in the categories outlined by each ESA were statistically analysed (Fisher's exact and Kruskall Wallis test). When applied to an exclusive cohort of known implantation embryos, the PPVs of each ESA were 42.57%, 41.52%, 44.28%, 38.91%, 38.29% and 40.45%. The NPVs

were 62.12%, 68.26%, 71.35%, 76.19%, 61.10% and 64.14%. The sensitivity was 16.70%, 75.33%, 72.94%, 98.67%, 51.19% and 62.33% and the specificity was 85.83%, 33.33%, 42.33%, 2.67%, 48.17% and 42.33%, The AUC were 0.584, 0.558, 0.573, 0.612, 0.543 and 0.629. Two of the ESAs resulted in significant differences in the embryo classifications in terms of IR ($p<0.05$). These results highlight the need for the development of in house, patient, treatment and environment specific ESAs. These data suggest that currently available ESAs may not be clinically applicable and lose their diagnostic value when externally applied.

5.2.2 Introduction

Traditional methods for embryo selection have been utilised for over twenty years. There are numerous morphological parameters that are thought to be useful for correct embryo selection; pronuclear morphology (z scoring) (Tesarik and Greco, 1999; Scott, 2003), polar body alignment and appearance (Payne *et al*, 1997; De Placido *et al*, 2002), appearance of cytoplasm and zona pellucida (Plamstierna *et al*, 1998), early cleavage (Lundin *et al*, 2001; Isiklar *et al*, 2002), multinucleation (Pickering *et al*, 1995; Jackson *et al*, 1998; Yakin *et al*, 2005), and blastomere morphology (Shapiro *et al*, 2000; Hardarson *et al*, 2001; Johansson *et al*, 2003). Basic embryo grading, including the number of blastomeres, evenness in the size of the blastomeres and the level of fragmentation remains the gold standard for embryo selection. However, using this method in a traditional sense (with a standard bench top incubator) has two limitations; a restricted overview of an embryo's development and the exposure of the embryo to suboptimal temperatures and gas concentrations. With the introduction of TLS, where an image of each embryo is taken every 10 to 20 minutes, more intricate embryo parameters can be viewed whilst leaving the embryos in an undisturbed environment. As the availability of TLS increased, attention was first focused on assessing their clinical safety. Once this had been established and the available technologies validated for clinical use (Freour *et al,* 2012; Nakahara *et al*, 2010; Basile *et al*, 2011; Cruz *et al*, 2011; Kirkegaard *et al*, 2012), research then turned to determining how the TLS could be utilised to increase pregnancy rates through in depth embryo analysis and an undisturbed culture

system.

Through both the research that followed and that performed previously, many morphokinetic parameters were identified that correlated with the embryo's ability to create a pregnancy both in humans and animals; the appearance and disappearance of pronuclei (PN) and nuclei at each cell stage (Payne *et al*, 1997; Lemmen *et al*, 2008; Scott, 2010; Azzarello *et al*, 2012), the length of time between early cytokinesis (Gonzales *et al*, 1995; Ramsing and Cellesen, 2006; Ramsing *et al*, 2007; Lechniak *et al*, 2008; Herrero *et al,* 2011; Meseguer *et al*, 2011; Cruz *et al*, 2012; Hlinka *et al*, 2012) and initiation of blastulation (Campbell *et al*, 2013a). Further embryological phenomena have been observed using time-lapse imaging including the reabsorption of fragments (Hardarson *et al*, 2002), direct cleavage (DC) of embryos from one to three cells (Rubio *et al*, 2012) and reverse cleavage (RC) (Liu *et al*, 2014). These phenomena have been shown to affect an embryos implantation potential to varying degrees however, their discovery could lead to more effective embryo selection within a laboratory utilising TLS.

Single embryo parameters, such as those named above, have been linked to embryo viability (see reference Kirkegaard *et al*, 2012 for review) and now these parameters have been used to develop ESAs. These ESAs seek to combine a number of morphokinetic parameters that have been linked to an embryo’s viability expressed either as formation of a blastocyst, implantation or a live birth. This study aims to examine the efficacy of six published ESAs (Azzarello *et al*, 2012; Basile *et al*, 2015; Cruz *et al*, 2012; Campbell *et al*, 2013a; Chamayou *et al*, 2013; Dal Canto *et al*, 2012) for predicting an embryo’s viability, expressed as IR, in a clinically applicable setting aiming to demonstrate the need to develop specific, in-house ESAs. Examined ESAs were selected based on their clinical applicability to the test site, assessed superficially prior to analysis.

5.2.3 Materials and methods

This investigation was a single site, retrospective observational design. Data were obtained from 884 treatment cycles between September 2014 and

September 2015. Clinical pregnancy was confirmed by the presence of a fhb at ultrasound scan at six weeks gestation. All treatments included in this analysis were from known implantation embryos i.e. a single (SET) or a double embryo transfer (DET) where the transfer of two embryos resulted in either a negative test or two fetal heartbeats (fhbs).

5.2.3.1 Patient criteria

No specific patient criteria were applied to the following investigation. This investigation sought to maximise the number of embryos available for inclusion as well as to include a heterogeneous cohort of embryos that would be representative of those found in an IVF laboratory.

5.2.3.2 Oocyte retrieval and embryology

All injected oocytes, fertilised oocytes and unfertilised metaphase II oocytes were placed in individual culture drops of G-TL™ and cultured in the EmbryoScope®.

5.2.3.3 Analysis of time-lapse information

A single embryologist assessed images for the required morphokinetic parameters. The parameters annotated included time to pronuclear fading (tPNf), time to two-cell (t2), three-cell (t3), four-cell (t4), five-cell (t5), eight-cell (t8), time to start of blastoculation (tSB), time to blastocyst (tB, defined when the blastocoele has filled over half of the embryo and there is a <10% increase in the embryo diameter (i.e. the beginning of expansion) quantified using the line tools on the EmbryoScope® instrument). From these annotations, two further annotations were calculated (s2; time between three and four-cell; and cc2; time to complete the second cell cycle). Each of the ESAs were then retrospectively applied to the same cohort of known implantation embryos.

5.2.3.4 Outcome measures and statistical analyses

Positive predictive value, NPV, specificity and sensitivity, likelihood ratio (LR) and AUC were used to determine the efficacy of each of the ESAs. These

methods of measurement were chosen for analysis due to their relationship to validity and predictive power. Positive predictive value (PPV) was defined as the percentage of embryos creating a fhb as well as a favourable ESA outcome. Negative predictive value was defined as the percentage of embryos not creating a fhb as well as an unfavourable ESA outcome. Sensitivity was defined as the ability of the ESA to correctly classify an embryo as viable. Specificity was defined as the ability of the ESA to correctly classify an embryo as non-viable.

Each of the test measures were determined using the following calculations:
PPV = true positives / (true positives + false positives)
NPV = true negative / (true negatives + false negatives)
Sensitivity = true positives / (true positives + false negatives)
Specificity = true negatives / (true negatives + false positives)

The likelihood ratio (LR) was determined using the following calculation:
LR = sensitivity / (1 – specificity)

The AUC was calculated for each ESA. The IR in each category of the ESA was compared using Fisher's exact test (for ESAs with two outcome categories i.e. true, false) and Kruskall Wallis test (for ESAs with more than two outcome categories i.e. A, B, C and D). Results were considered statistically significant at $p<0.05$. Statistical analysis was performed using the statistical package Prism® 5 (GraphPad Software©, USA)

5.2.4 Results

A total of 977 known implantation embryos from 884 treatment cycles were subject to retrospective analysis to determine the efficacy of six published ESAs (Table 10). Of these, 529 of these embryos were created using conventional IVF while 448 were created using ICSI. The mean patient age was 33.44 ± 4.53 with an average treatment attempt number of 1.37. The primary aetiologies for infertility were male factor (32.2%), maternal age (4.1%), ovulatory disorders (9.9%), tubal disorders (6.6%), uterine disorders (4.1%), other (including genetic disorder) (0.2%), hormonal deficiency (1%)

and unexplained (41.9%). All embryo transfers were performed on day five (blastocyst) with 93 DETs and 791 single embryo transfers performed. Agonist protocols comprised 50.36% of treatment cycles with the remainder, an antagonist protocol. An overall IR of 39.7% was achieved with 388 of the 977 embryos implanting and 589 not implanting.

The PPV for each of the ESAs did not reach above 45% in any case. The NPV was between 60-70% for all ESAs analysed (Table 10). The sensitivity and specificity were considerably more variable (Table 10), as would be expected, identifying that two ESAs had a high sensitivity (Campbell *et al*, 2013a; Chamayou *et al*, 2013) and another, a high specificity (Azzarello *et al*, 2012). Finally, the AUC analysis revealed values from 0.512 to 0.629 (Table 10).

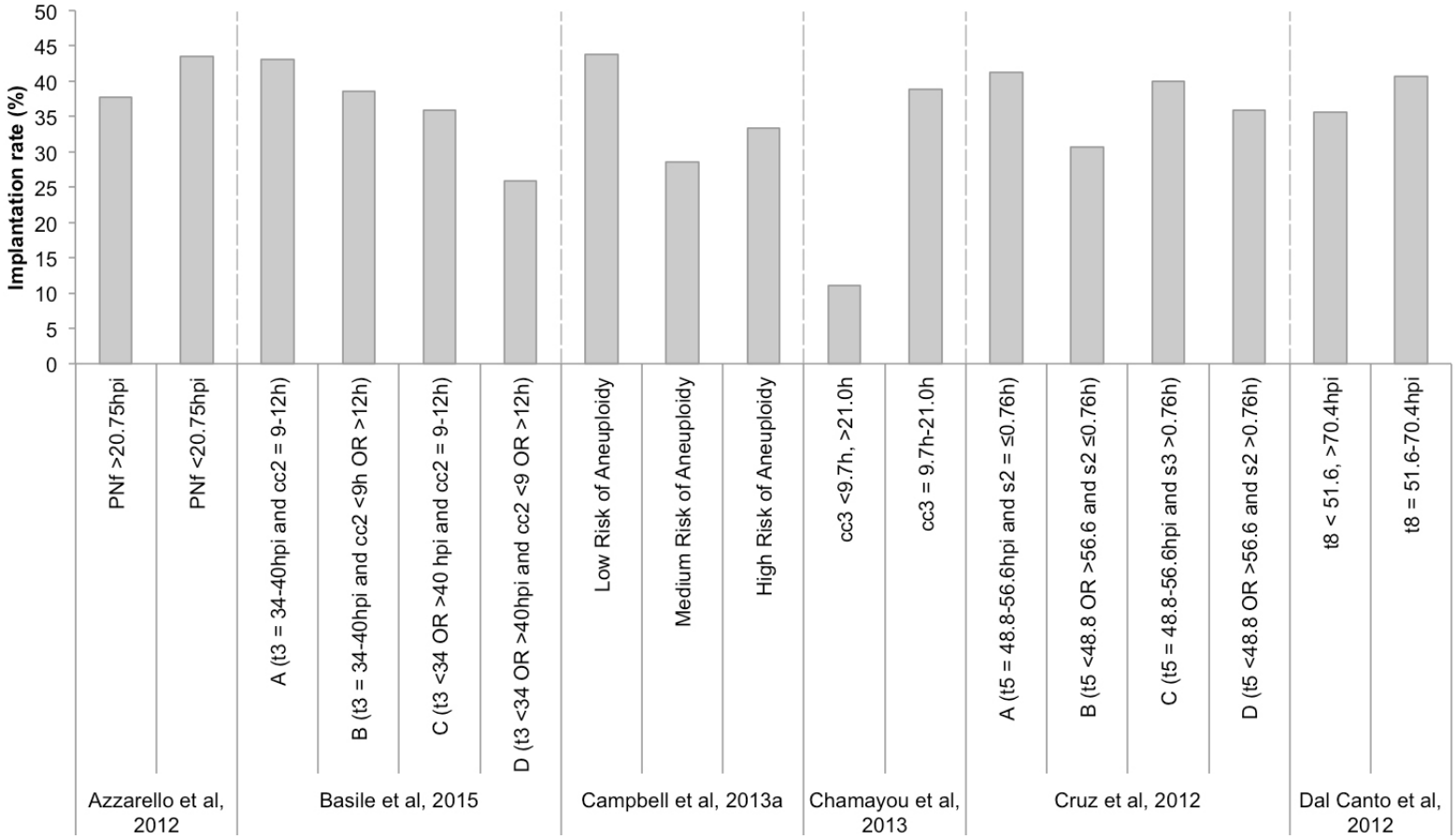
The IR for each category of four of the analysed ESAs did not vary significantly ($p>0.05$) (Figure 5). However, the IR for the three categories of the aneuploidy risk classification ESA (Campbell *et al*, 2013a) varied significantly ($p<0.0001$) as did category A with category D in the ESA developed by Basile *et al*, (2015). The aneuploidy risk classification ESA also had the strongest LR (1.26) and PPV (44.28%). Incidentally, the number of embryos classified as high risk using this ESA was just three, of which one implanted giving this category an IR of 33.33%; a potentially misleading result. The absolute difference between the IR of low and medium risk embryos was 15.19% (Figure 5).

Table 10. Summary of embryo selection criterion and main results.

	Model type	Parameter	Time frame	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC	Likelihood ratio	P-value
Azarello *et al*, 2012	Selection/ deselection	PNf	>20h 45m	16.71	85.83	42.57	62.12	0.584	1.18	0.2724 (Fisher's exact)
Basile et al, 2015	Hierarchical	t3 cc2	34-40hpi 9-12h	75.33	33.33	41.52	68.26	0.558	1.13	0.006 (Kruskall-Wallis)
Campbell *et al*, 2013a	Risk classification model	tSB tB	*Low risk*: tSB <96.2hpi tB <122.9hpi *Medium risk*: tSB ≥96.2hpi tB <122.9hpi *High risk*: tB ≥122.9hpi	72.94	42.33	44.28	71.35	0.579	1.26	<0.0001 (Kruskall-Wallis)
Chamayou *et al*, 2013	Selection/ deselection	cc3	9.7-21h	98.67	2.67	38.91	76.19	0.552	1.01	0.1817 (Fisher's exact)
Cruz *et al*, 2012	Hierarchical	t5 s2	48.8-56.6h ≤0.76h	51.19	48.17	38.29	61.10	0.517	0.99	0.1402 (Kruskal-Wallis)
Dal Canto *et al*, 2012	Selection/ deselection	t8	54.9 ± 5.2h	62.33	42.33	40.45	64.14	0.583	1.08	0.1415 (Fisher's exact)

PNf; pronuclear fading. t3; time to 3-cell. cc2; time between 2-cell and 3-cell. t5; time to 5-cell. s2; time between 3-cell and 4-cell. tSB; time to start of blastulation. tB; time to full blastocyst. cc3; time between 3-cell and 5-cell. t8; time to 8-cell. PPV; positive predictive value. AUC; area under the receiver operating characteristic curve. P-value stated when comparing the implantation rates between categories in the applicable ESA.

Figure 5. Implantation rate of each category of six published embryo selection algorithms



Azzarello et al, 2012; IR of those embryos where pronuclear fading (PNf) occurred after 20.75hpi (n=832, 37.74%) and those that faded before 20.75hpi (n=145, 43.45%) (p>0.05, Fisher's exact test). Basile et al, 2015; IR of embryos classified as A (t3 = 34-40hpi and cc2 9-12h, n=453), B (t3 = 34-40hpi, cc2 >9 OR <12h, n=231), C (t3 <34 OR >40hpi and cc2 = 9-12h, n=173) and D (t3 <34 OR >40hpi and cc2 <9 OR >12h, n=120) with respective IR of 43.05%, 38.53%, 35.84% and 25.83% (p>0.006, Kruskal-Wallis test). Campbell et al, 2013; IR for embryos classified as low risk (tSB <92.2hpi and tB <122.9hpi, n=621), medium risk (tSB ≥96.2 and tB ≤122.9hpi, n=353) and high risk (tB ≥122.9hpi, n=3) of aneuploidy with respective IR of 43.80%, 28.61% and 33.33% (p<0.05, Kruskall-Wallis test). Chamayou et al, 2013; IR of those embryos where cc3 (t5-t3) occurred between 9.7-21h (n=959, 11.11%) and those that did not (n=18, 38.79%) (p>0.05, Fisher's exact test). Cruz et al, 2012; IR of embryos classified as A (t5 = 48.8-56.6hpi and s2 ≤0.76h, n=364), B (t5 = 48.8-56.6hpi and s2 >0.76h, n=140), C (t5 = <48.8 OR >56.6hpi and s2 ≤0.76h, n=353) and D (t5 <48.8 OR >56.6 and s2 >0.76h, n=120) with respective IR of 41.21%, 30.71%, 39.94% and 35.83% (p>0.05, Kruskal-Wallis test). Dal Canto et al, 2012; IR of embryos where t8 occurred between 51.6-70.4hpi (n=578, 40.66%) and those that did not (n=399, 35.59%) (p>0.05, Fisher's exact test).

5.2.5 Discussion

All six of the examined ESAs (Azzarello *et al,* 2012; Cruz *et al*, 2012; Basile *et al*, 2015; Campbell *et al*, 2013a; Chamayou *et al*, 2013; Dal Canto *et al*, 2012) achieved an AUC less than 0.65 (0.584, 0.558, 0.573, 0.612, 0.543, 0.629, respectively), indicating reduced predictive capability. None of the ESAs achieved a PPV above 45% (42.57, 41.52, 44.28, 38.91, 38.29, 40.45%, respectively) also indicative of poor diagnostic value. The NPV reached over 60% in all of the ESAs (62.12, 68.26, 71.34, 76.19, 61.10, 64.14%, respectively). The specificity of each ESA was variable indicating that some ESAs are able to identify embryos with a reduced chance of implantation better than others (85.83, 33.33, 42.33, 2.67, 48.17, 42.33%, respectively) also reflected in the NPV. This variability was, inevitably, seen in the sensitivity of the assessed ESAs (16.71, 75.33, 72.94, 98.67, 51.59, 62.33%, respectively). In all of the ESAs assessed, the LR was close to 1 (1.18, 1.13, 1.26, 1.01, 0.99, 1.08, respectively). The LRs of all ESAs revealed that there was little predictive power of implantation where a favourable ESA result is obtained (Table 10). Likelihood ratios range from 0 to infinity and a LR close or equal to 1 indicates a lack of diagnostic value; the furthest from 1 that any of the ESAs in this investigation reached was 0.26 indicating that an embryo has a 0.26 increased chance of creating a pregnancy if a favourable ESA outcome is achieved.

Worthy of note are the ESAs that were found to have statistical significance between the categories of embryo classification (Campbell *et al,* 2013a; Basile *et al,* 2015). However, the number of embryos classified as high risk of aneuploidy in the aneuploidy risk classification ESA was just three of 977. Further validation, performed by the developers of this ESA (Campbell *et al,* 2013b) using 88 embryos, classified four as high risk of aneuploidy. Clearly, using this ESA, the chance of an embryo being classified as high risk is low which raises issues about the specificity of the ESA especially when evidence suggests that over 50% embryos are aneuploidy (Fragouli and Wells*,* 2011). With an AUC of 0.575 and a 0.26-increased chance that an embryo would create a pregnancy if classified as low risk of aneuploidy, this ESA may not

represent a robust, clinically applicable embryo selection method. Nonetheless, this ESA is the most effective out of the six assessed when a combination of specificity, sensitivity, PPV, NPV, AUC, LR and differences in implantations between each embryo classification category is considered. The other ESA to gain statistical significance between the categories when considering IR was that of Basile *et al* (2015). Statistical significance was found between the IRs of category A and D indicating that this ESA may perform well in terms of identification of poor quality embryos. This is also reflected in a high sensitivity and NPV. However, the LR remains low at 1.13 and the other measures of the effectiveness of the ESA (specificity, PPV and AUC) indicate this ESA may not be as effective at determining higher implantation potential embryos.

The analyses performed indicate that ESAs available in the literature may not provide substantial, additional aid for embryo selection in a clinically relevant setting. The current investigation highlights that externally derived ESAs are developed, inevitably, under conditions different to that of the adoptive centre (Table 11) encouraging the development of in-house, specific ESAs. It has been shown that the method by which embryos are created (IVF or ICSI) can affect their temporal behaviour (Cruz *et al,* 2013; Bodri *et al,* 2015; Liu *et al,* 2015). In addition to varying treatment types, a number of the analysed ESAs excluded certain patient groups to avoid confounding factors. This includes those with endometriosis, polycystic ovary syndrome (PCOS), severe male factor infertility and maternal age over 39 years. This exclusion constitutes a proportion of patients that make up a significant fraction of patients treated in an IVF laboratory and onto which these ESAs could be critically useful.

There is evidence to suggest that the reason for infertility could affect an embryo's morphokinetic profile in particular those with PCOS (Wissing *et al,* 2014) thus their exclusion in the ESA development is understandable but reduces its clinical applicability unless a specific ESA is developed for this specific patient group. Furthermore, one group's ESA was developed using oocyte donors only, a clear confounder for the application of this ESA in other centres.

Table 11. Summary of publications used for examination of efficacy of selection criteria.

	n (embryos)	n (cycles)	Fertilisation method	End point	Exclusion criteria	Inclusion criteria	Image capture interval (mins)	Protocol	Culture	Media change	Transfer day
Azarello *et al*, 2012	159	130	ICSI	LBR	-	Embryos transferred at 4-cell stage with equal blastomeres and <25% fragmentation, autologous gametes, female age ≤39, male factor infertility ($1\text{-}5x10^5$ motile sperm/ ejaculate)	20	Agonist	Cook® 5.5% CO_2, 5% O_2, 89.5% N_2	No	2 (44hpi)
Basile et al, 2015	754	1664	ICSI	IR	Severe male factor, severe endometriosis, BMI 30 kg/ m^2, low response (for standard patients, less than five metaphase II oocytes) and no preimplantation genetic screening (PGS) or PGD	For donors: 18–30 years old (mean: 26.9, SD = 4.7), normal menstrual cycles (26–34 days duration), a BMI of 18–28 kg/m^2 and normal ovaries and uterus as observed by transvaginal ultrasound	10-20	Antagonist (agonist trigger for analogous oocytes, hCG trigger for oocyte donors)	Site 1: Cook®, 5.5% CO_2, 20% O_2, 74.5% N_2 Site 2: Global IVF Medium (LifeGlobal), 6.5% CO_2, 20% O_2, 73.5% N_2 Site 3: Cook®, 6% CO_2, 20% O_2, 74% N_2 Site 4: Global IVF Medium (LifeGlobal), 6% CO_2, 20% O_2, 74% N_2	No	3
Campbell *et al*, 2013	88	25	ICSI	CPR and LBR	-	Patients undergoing a cycle inclusive of PGS	20	Agonist (75%) Antagonist (25%)	Global IVF medium (LifeGlobal) 5.5% CO_2, 5% O_2, 89.5% N_2	Yes	
Chamayou *et al*, 2013	178	78	ICSI	BFR	Severe endometriosis, premature ovarian failure, severe asthenoteratozoospermia	Fresh gametes	20	Agonist	Quinn's Advantage (SAGE) 5% CO_2, 5% O_2	Yes	5
Cruz *et al*, 2012	834	165	ICSI	BFR	-	Oocyte donor meeting all required criteria for donation programme	20	Agonist	Global IVF medium (LifeGlobal) 6% CO_2, 21% O_2, 37.4°C	Yes	5
Dal Canto *et al*, 2012	134	71	IVF (22) and ICSI (49)	IR	-	Indication for standard IVF or ICSI due to male factor, tubal factor, stage I or II endometriosis or PCOS, maternal age 27-42.	20	Agonist	ISM1 (day 1-3) BlastAssist (day 3-5) 6% CO_2, 5% O_2, 89% N_2	Yes	3 and 5

ICSI; intracytoplasmic sperm injection. IVF; in vitro fertilisation. LBR; live birth rate. BFR; blastocyst formation rate. CPR; clinical pregnancy rate. IR; implantation rate. PGS; preimplantation genetic screening. PCOS; polycystic ovary syndrome. CO_2; carbon dioxide. O_2; oxygen. N_2; nitrogen. hpi; hours post insemination.

In addition, the majority of the ESAs were developed on embryos created under an agonist protocol. However, one group's ESA development cohort contained a proportion of embryos created under an antagonist protocol (Campbell *et al,* 2013a). The use of agonist and antagonist protocols has yet to be shown to affect an embryo's morphokinetic profile however, they have been linked to embryo quality (Murber *et al,* 2009; Vengetesh *et al,* 2015) which could indicate that there is a potential for them to also have a temporal effect.

Finally, and perhaps most significantly, varying culture conditions were used in the development of these ESAs. It has been shown that an embryos morphokinetic profile is significantly altered in different culture media specifically between sequential and single-step media (Ciray *et al,* 2012; Barrie *et al,* 2015). This means that those developed using sequential media may not be effective in selecting embryos cultured in single-step media, and vice versa. In addition, varying CO_2 and O_2 gas concentrations were used in the development of a number of these published ESAs. Oxygen tension has been specifically linked to an embryo's morphokinetic profile in both humans (Kirkegaard *et al,* 2013c) and mice (Wale and Gardner, 2010) where those embryos cultured at 20% O_2 have reduced developmental rates and the completion of the third cell cycle is significantly delayed. Of the six ESAs analysed, one comprised multiple centres (Basile *et al,* 2015). The culture conditions varied slightly between centres therefore it could be argued that this ESA has a broader clinical use whilst maintaining similar predictive power measurements (i.e. sensitivity, specificity, PPV, NPV, LR, AUC) to the other ESAs investigated. It should be highlighted however, that the algorithm developed in this original article used oocyte donors, a natural bias for outcomes focusing on embryological features and implantation potential. These fundamental differences in the development of each ESA need to be seriously considered before their external adoption. It is highly unlikely that an external centre will have the same patient, treatment and environmental parameters as that of the developing centre.

A further consideration for the use of externally derived ESAs is the subjective

nature of annotating morphokinetic parameters, the differences in image capture analysis, such as the number of focal planes, and the varying definition of time-zero. The subjective nature of annotations creates unreliability in the external application of ESAs. There has been some development with this due to the publication of annotation guidelines in 2014 (Ciray *et al,* 2014) however, this will not eliminate the subjectivity completely. Interestingly, there are now two commercially available 'one size fits all' ESAs that, based on the results presented here, should not perform as well as expected. Variations in image acquisition are unlikely to create significant disparity however, coupled with the variability between 'annotators', an increasing level of inaccuracy could be created. Although undefined in some of the publications, the definition of t0 varies between groups with some using t0 as the time of insemination or injection, the inaugural and arguably the most common method, and others the mid-point of ICSI. It has now been largely accepted that the use of insemination/ injection is arbitrary and the exact moment that the sperm enters the oocyte is indeterminate for IVF cases and, where possible, time of PN fading should be used as t0 (Liu *et al*, 2015).

It could be argued that a limitation of the current analyses is the potential for bias due to the use of an in house ESA with similar morphokinetic parameters to one of the externally derived ESAs (Cruz *et al,* 2012) to aid in embryo selection of the analysed embryos. Owing to this, a comparison of the proportion of embryos in each of the categories (A-D) in the original manuscript for the external ESA in question (Cruz *et al,* 2012) with the current analyses was performed. From this analysis, the proportion of embryos in each category did not differ between the original manuscript of the external ESA and the current analyses (A; 39.7% (106/267) vs. 37.3% (364/977), B; 13.5% (36/267) vs. 14.3% (140/977); C; 36.0% (96/267) vs. 36.1% (353/977); D; 10.8% (29/267) vs. 12.3% (120/977), respectively) This provides reassurance that any bias created from the use of similar morphokinetic parameters in the selection of the embryos used in this analysis is minimal.

Finally, it is important to consider that the use of a TLS as a method for embryo selection has yet to be appropriately evidenced (Kaser and

Racowsky, 2014). As can be seen from the results presented here, the poor performance of the investigated ESAs allows the field to question the overall clinical applicability of the use of TLS. There is considerable heterogeneity in the origin and culture of the embryos used for the development of these ESAs and it should be considered that these parameters affect the ability of a one-size-fits all approach to function effectively. Perhaps the development of patient, treatment and environment specific, optimum morphokinetic time ranges will present a means of utilising TLS achieving a higher predictive power. There are ideal conditions under which to test the efficacy of externally derived ESAs, select embryos based only on morphology then perform the analyses presented here or, preferably, prospective application. At the study site, morphokinetics have been used since the introduction of TLS into the lab to aid in embryo selection therefore a dataset large enough to perform the former of these two methodologies would not be possible. The authors do, however, recognise the strength of a prospective methodology for the aims presented here. This will be the focus of future research in this area to better delineate the benefits of using TLS in the clinical embryology laboratory.

5.2.6 Conclusion

The development of ESAs, thus far, has not involved the control of confounding factors such as media type, patient age and treatment type, except inadvertently by virtue of availability. They are often developed under the environmental parameters available in the laboratory performing the development and thus are clinically relevant in these cases alone. For external application, the ESAs lose their predictive capabilities. The primary objective of ESAs is to allow the selection of the best embryo from a cohort in a clinical setting. Those presented here, clarify that embryo morphokinetics could be used for embryo selection however, they do not offer clinically relevant means to aid in embryo selection in other laboratories unless the development criteria are also adopted. The collective contribution of confounding factors means that derived ESAs can only be applied to that on which they were developed and when applied to a heterogeneous cohort of embryos, as would be found in an IVF laboratory, the capability of the ESA to detect the most viable embryo diminishes. Further research needs to focus on

the development of ESAs that are specific to subgroups of patients, environments and treatments. At the very least, embryology laboratories should proceed with caution when implementing ESAs derived from published sources and consider thorough in house validation of such ESAs before clinical use, if at all.

5.2.7 Dissemination

The above research was prepared for publication in Fertility and Sterility. The research was accepted and published on 6th January 2017 (appendices section 9.6).

CHAPTER 6: The analysis of abnormal embryos as a method for embryo deselection

6.1 Prelude

Chapter 5, presenting a paper demonstrating that existing, published ESAs lose clinical applicability when externally utilised, informs the research that follows. It becomes clear that more effective methods of embryo selection, or, as the case may be, deselection, are required in the first instance while the development of patient, treatment and environment specific ESAs is investigated. The use of deselection criteria may be readily available, less heavily influenced by patient, environment and treatment parameters and easily adopted by others utilising TLS. Thus, the following chapter outlines the prevalence and implantation potential of five abnormal embryonic phenotypes and their conceivable merit for use as embryo deselection criteria.

6.2 A preliminary investigation into the prevalence and implantation potential of five abnormal embryonic phenotypes assessed using time-lapse imaging (Paper 3)

6.2.1 Abstract

This retrospective, single site observational study aimed to delineate five abnormal embryonic developmental phenotypes assessing their prevalence, implantation potential and suitability for inclusion in embryo selection models in an IVF laboratory. A total of 15, 819 embryos from 4559 treatment cycles cultured in EmbryoScope® incubators between January 2014 and January 2016 were included. Time-lapse images were assessed retrospectively for five abnormal embryo phenotypes; direct cleavage, reverse cleavage, absent cleavage, chaotic cleavage and cell lysis. The prevalence of each abnormal phenotype was assessed. The embryo fate, embryo quality and implantation rate were determined and compared to a control embryo cohort. The collective prevalence for the five abnormal phenotypes was 11.39% where chaotic cleavage and direct cleavage together constituted 9.63%. The implantation rate was 17.4%, 0%, 25%, 2.1% and 0% for direct, reverse,

absent, chaotic cleavage and cell lysis, respectively. The overall implantation rate for all abnormal embryos was significantly lower compared to the control population (6.9% vs. 38.66%, $p<0.0001$, Fisher's exact). The proportion of good quality embryos in each category never reached over 24%. Embryos exhibiting an abnormal phenotype may have reduced developmental capability manifested in both embryo quality and implantation potential when compared to a control embryo cohort.

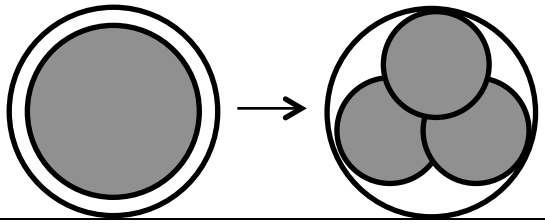
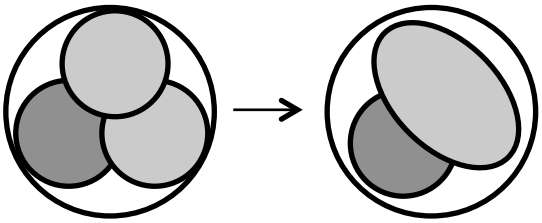
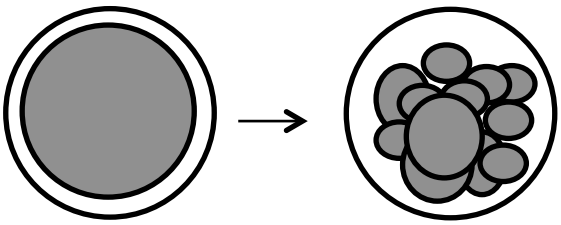
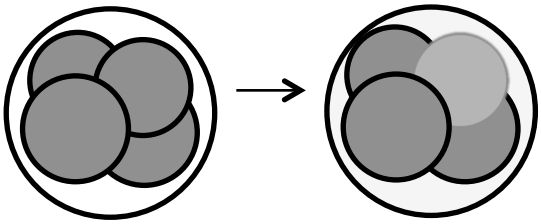
6.2.2 Introduction

Abnormal cleavage patterns exhibited by some embryos include, but are not limited to; abnormal syngamy, direct cleavage, reverse cleavage, absent cleavage, chaotic cleavage and cell lysis.

The first of five abnormal cleavage patterns investigated here is direct cleavage (DC). This is the cleavage of one blastomere into three, instead of the expected two, daughter cells (Figure 6). The ability of these embryos to create a pregnancy has been shown to be significantly reduced (Rubio *et al,* 2012) where 13.7% of all examined embryos and 6.6% of transferred embryos underwent DC, with 1.2% resulting in a clinical pregnancy. These embryos have been shown to have a markedly decreased blastocyst formation rate when compared to their normal counterparts (Athayde Wirka *et al,* 2014).

The second abnormal phenotype to be considered is reverse cleavage (RC); the phenomenon of blastomere fusion (Figure 6). Of 789 embryos assessed for RC, defined as blastomere fusion or failed cleavage, 27.4% of embryos were found to exhibit this abnormal cleavage pattern and were shown to have a reduced implantation potential (Liu *et al,* 2014). An examination of 1698 embryos detected a prevalence of RC of 6.8% however embryos appeared to have similar fragmentation, cell evenness and morphokinetic profiles compared to their non-reverse cleaved counterparts (Hickman *et al,* 2012). This research concluded that RC does not seem to impair embryo development to the blastocyst stage supported by the findings of others (Desai *et al,* 2014).

Figure 6. Schematic representation of the five abnormal embryo phenotypes.

Abnormal Phenotype	Definition	Schematic
Direct cleavage (DC)	Cleavage of one blastomere into three distinct blastomeres	
Reverse cleavage (RC)	The fusion of two blastomeres into a single blastomere	
Absent cleavage (AC)	Pronuclear/ nuclear fading followed by a cytoplasmic 'roll', no division, but an additional, or multiple, nuclei	
Chaotic cleavage (CC)	Cleavage of one cell into multiple fragments with no discernable blastomeres	
Cell lysis (CL)	The lysing of one blastomere within an embryo at any stage of development	

Schematic representation of the five abnormal embryo phenotypes. Schematic includes direct cleavage, reverse cleavage, absent cleavage, chaotic cleavage and cell lysis.

Absent cleavage (AC) is defined as the process by which a blastomere undergoes a pseudo division (seen as a 'roll') that does not produce two discernable blastomeres but a single, or multiple, extra nuclei within the single blastomere (Figure 6). Absent cleavage has previously been categorised under RC, termed type II RC (Liu *et al,* 2014). Of those embryos that underwent RC (27.4%), 82% were classed as type II; absent cleavage rather than blastomere fusion. Further evidence of this specific developmental pattern has not yet been published. This is perhaps due to the likelihood that these embryos will not be used for treatment thus circumventing a clinical need to further define this phenomenon.

Chaotic cleavage (CC) results when an embryo undergoes apparent cleavage but does not create distinctive blastomeres (Figure 6). A single investigation studying this cleavage pattern in 639 embryos found an overall prevalence of 15%, a blastocyst formation rate of 14% and an IR of 0% (Athayde Wirka *et al,* 2014). Interestingly, this investigation also found that 35.2% of those exhibiting CC had good cleavage stage quality. This was however, markedly lower than the other abnormal phenotypes observed (DC and abnormal syngamy). Again, as with AC, this phenomenon may be under investigated due to the reduced likelihood that embryos exhibiting this phenotype will be used in treatment.

Finally, an abnormal embryo developmental phenomenon that has yet to be discussed in the literature, in terms of time-lapse imaging of embryos from fresh treatment cycles, is cell lysis (CL) (Figure 6); a process often visualised in frozen thawed embryos (Rienzi *et al,* 2005; Tang *et al,* 2006; Yeung *et al,* 2009; Bottin *et al,* 2015). In an analysis of 891 frozen embryo transfer (FET) cycles, no pregnancies resulted if CL occurred in over 50% of the embryo. However, if CL accounted for 25 to 50% of the embryo the pregnancy rate was 3.2%; significantly lower than if less than 25% CL had occurred (16.6%) (Tang *et al,* 2006) supported by others (Yeung *et al,* 2009; Bottin *et al,* 2015).

Although these investigations are not entirely synonymous with the current analysis, they provide evidence that embryos with lysed cells have a reduced implantation potential.

As discussed above, there is disparity in the literature with regards to the prevalence and implication of the presence of certain abnormal phenotypes. Further investigation into these phenomena is required to determine if their presence is severe enough to exclude these embryos from selection for use in treatment. Five abnormal cleavage patterns exhibited by embryos (DC, RC, AC, CC and CL) are explored in 15,819 embryos detailing their prevalence, implantation potential, and the suitability for inclusion of these potential deselection criteria in embryo selection models.

6.2.3 Materials and methods

This investigation was a single site, retrospective observational design. Data were obtained from 4559 treatment cycles including 15,819 embryos cultured in the EmbryoScope® incubators between January 2014 and January 2016.

6.2.3.1 Patient criteria

There were no specific patient criteria applied to this investigation.

6.2.3.2 Oocyte retrieval and embryology

All injected oocytes, fertilised oocytes and unfertilised metaphase II oocytes were placed in individual culture drops of G1™ (for all cycles pre September 2014) or G-TL™ (all cycles post September 2014) and cultured in the EmbryoScope®.

6.2.3.3 Analysis of time-lapse information

For DC, embryos were classified into one of three categories; true DC (T-DC, defined as all three resultant cells cleaving on the subsequent cell cycle, each having a nucleus and each included in the morula), false DC (F-DC, one or more of the above criteria not fulfilled) and unconfirmed DC (U-DC, unable to classify as true or false). Unconfirmed DC embryos were defined as such due to either obscurity preventing categorisation or the cessation of culture before

the morula stage was reached. A justification for the choice of this classification, not reported elsewhere, lies in unit specific data where two obviously distinct DC event patterns were visualised using a TLS. This, as well as previous reports of DC patterns (Kola *et al,* 1987; Kalatova *et al,* 2015), led to the development of the three-tiered classification of DC events. With regards to the final criterion for T-DC classification (inclusion of all cells in the morula), this stage of development was used as an indicator that all cells, abnormal or not, would contribute to the eventual blastocyst and would not be excluded. Further to this, DC could be proposed as a correction mechanism whereby the DC event is a means to remove surplus genetic material thus excluding the cells from the eventual blastocyst, described here as F-DC and a more favourable type of DC event. Direct cleavage from both one to three cells and from two to five cells were included in the analysis. Reverse cleavage is defined simply as blastomere fusion. Absent cleavage is defined as the process by which a blastomere undergoes a pseudo division (seen as a 'roll') that does not produce two discernable blastomeres but a single, or multiple, extra nuclei within the single blastomere. Chaotic cleavage is observed when an embryo undergoes apparent cleavage but does not create distinctive blastomeres. Cell lysis is defined as the loss of a blastomere through cell lysis (Figure 6). Although not exclusively a phenomena visualised through time-lapse technology and one that can be visualised using standard embryo morphology assessments, CL is predominantly seen in embryos following cryopreservation whereas here CL is described in fresh embryos. Thus, this was included in the current investigation to determine the effect of CL on the viability of a fresh embryo.

6.2.3.4 Outcome measures and statistical analyses

The overall prevalence of the five abnormal embryo phenotypes was defined per embryo and per treatment cycle. The average patient age, oocytes collected and previous attempts were calculated for each of the five categories. The fate (transfer, freeze, discard) of each abnormal embryo was determined as well as their quality on the day of utilisation defined as good, average or poor (Table 12). The IR for each abnormal phenotype was

determined where the origin of the fetal heartbeat could be confirmed i.e. using known implantation data from an abnormal embryo or not. The number of single and double abnormal embryo transfers and the stage at which the abnormal embryo(s) was transferred was also determined (Table 13). Statistical analyses included the student t-test for the comparison of the abnormal phenotype baseline information (patient age, oocytes collected and previous attempts) to the control embryo baseline data. The Fisher's exact test was used to compare the IR of the abnormal embryos with normal counterparts. Results were considered significant at $p<0.05$. Statistical analysis was performed using the statistical package Prism® 5 (GraphPad Software©, USA).

Table 12. Definitions of embryo quality.	
Good quality embryo/blastocyst (GQE/B)	Cleavage stage embryos with even blastomeres (<20% difference in diameter) and <20% fragmentation Blastocyst embryos with prominent and compact inner cell mass and many cells forming a cohesive epithelium
Average quality embryo/blastocyst (AQE/B)	Cleavage stage embryos with 20-50% difference in cell diameter and/or 20-50% fragmentation Blastocyst stage embryos with easily discernable inner cell mass with many cells that are loosely grouped together
Poor quality embryo/blastocyst (PQE/B)	Cleavage stage embryo with >50% difference in blastomere diameter and/or >50% fragmentation Blastocyst stage embryos with few cells forming the inner cell mass and very few cells making up the trophectoderm
Definitions of embryo quality used to classify embryos as good, average and poor quality based on ACE/BFS embryo grading guidelines (Cutting *et al,* 2008).	

Table 13. Baseline data for each abnormal embryo phenotype.

	Abnormal embryos transferred (n)	Total transfers (n)	SET (n)	DET (n)	Cleavage stage transfers (n)	Blastocyst stage transfers (n)
T-DC	1	1	1	0	0	1
F-DC	6	6	6	0	0	6
U-DC	16	15	14	1	5	10
DC	23	22	21	1	5	17 (1xDET)
RC	9	8	7	1	2 (1xDET)	6
AC	4	3	2	1	1	2 (1xDET)
CC	48	37	26	11	20 (5xDET)	17 (6xDET)
CL	2	2	2	0	0	2
Overall	86	72	58	14	28	44

Embryo transfer baseline information for each abnormal embryo phenotype including the total number of transfers, the number of single embryo transfers (SET), double embryo transfers (DET), cleavage stage transfers and blastocyst stage transfers. T-DC; true direct cleavage. F-DC; false direct cleavage. U-DC; unconfirmed direct cleavage. DC; direct cleavage. RC; reverse cleavage. AC; absent cleavage. CC; chaotic cleavage. CL; cell lysis.

6.2.4 Results

Data were obtained from 15,819 embryos from 4559 treatment cycles cultured in the EmbryoScope® between January 2014 and January 2016. Of the 15,819 embryos, 14,008 were derived from 3273 treatment cycles where no abnormal divisions of interest (DC, CC, RC, AC and CL) were observed and thus constituted the control group. These embryos resulted in 3456 embryos transferred and 1336 fhbs (IR = 38.66%) (Table 14). The remaining embryos (1811) were found to pertain to a treatment cycle (n=1286) exhibiting an embryo with one of the abnormal division patterns of interest.

Abnormal phenotypes with the highest prevalence per embryo observed were DC and CC at 4.38% (T-DC, F-DC, U-DC, collectively) and 5.25%, respectively. The remaining phenotypes had considerably lower prevalence ranging from 0.41 to 0.84% (Table 15). The overall prevalence of abnormal division patterns per embryo observed was 11.39% (Table 15). The IR of abnormal embryos ranged from 0 to 33.3% (Table 15). Of the five abnormal division patterns the IR of U-DC, CC and RC were significantly lower than normal counterparts; 12.5% (2/16), 2.1% (1/48) and 0% (0/9), respectively (Table 15). Furthermore, the overall IR of all abnormal embryos was significantly lower than normal counterparts (6.9% (6/86) vs. 38.66%) (Table 14 and 15) and of the six implanted embryos, five resulted in a live birth, with no birth defects, and one remains ongoing. In all cases the percent of good quality embryo (GQE/B) resulting from those exhibiting abnormal division patterns never reached above 24% and the majority of embryos were classified as poor quality (Table 15). This is also reflected in the utilisation of these embryos where the highest proportion of each group was discarded (Figure 7). The proportion of embryos undergoing either DC from one-to-three or two-to-five cells in each of the DC categories was as follows, respectively; T-DC, 16 and 32; F-DC, 26 and 43; U-DC, 176 and 404.

Table 14. Baseline information for embryos not exhibiting an abnormal division pattern.	
Total embryos (n)	14008
Embryo transfers(n)	3273
Embryos transferred (n)	3456
Sum fhb (n)	1336
Count fhb (n)	1269
IR (%)	38.66
CPR (%)	38.77

Baseline information for embryos not exhibiting an abnormal division pattern Including total number of embryos, number of embryos transferred, number of embryo transfers, total (sum) fetal heartbeats (fhb), count of fhb (regardless of number), implantation rate (IR), clinical pregnancy rate (CPR). Implantation rate was calculated as sum fhb/embryos transferred. Clinical pregnancy rate was calculated as count fhb/embryo transfers.

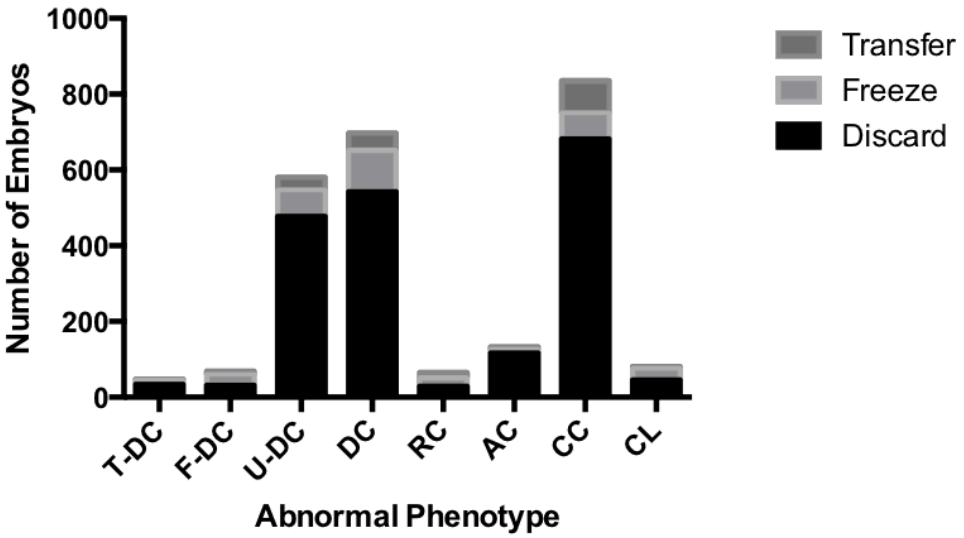
Table 15. Descriptive data regarding embryos that underwent an abnormal division pattern.

	Embryos (n)	Cycles (n)	Affected embryos/cycle	Prevalence/ embryo (%)	Prevalence/cycle (%)	Transfer	Freeze	Discard	GQE (n (%))	AQE (n (%))	PQE (n (%))	Abnormal embryos transferred (n)	FHS (n)	IR (%)	p-value
TDC	48	45	1.07	0.3	0.9	3	11	34	10 (20.8)	8 (16.7)	30 (62.5)	1	0	0	>0.05
FDC	69	64	1.08	0.43	1.2	9	29	31	11 (16.0)	21 (30.4)	37 (53.6)	6	2	33.3	>0.05
UDC	580	463	1.25	3.65	9.0	33	70	477	69 (11.9)	101 (17.4)	410 (70.7)	16	2	12.5	0.0378
DC	697	572	1.22	4.38	11.1	45	110	542	90 (12.9)	130 (18.7)	477 (68.4)	23	4	17.4	0.05
RC	65	61	1.07	0.41	1.2	14	22	29	15 (23.1)	10 (15.4)	40 (61.5)	9	0	0	0.0153
AC	133	123	1.08	0.84	2.4	7	10	116	6 (4.5)	5 (3.8)	122 (91.7)	4	1	25	>0.05
CC*	835	459	1.82	5.25	8.9	85	69	681	4 (4.7)	19 (22.4)	62 (72.9)	48	1	2.1	<0.0001
CL	81	71	1.14	0.51	1.4	5	31	45	11 (13.6)	22 (27.2)	48 (59.2)	2	0	0	0.5257
Overall	1811	1286	1.41	11.39	25.0	156	242	1413	-	-	-	86	6	6.9	<0.0001

The total number of affected embryos, number of affected treatment cycles, the number of affected embryos per treatment cycle, prevalence per embryo (defined as number of affected embryos/total number of embryos), prevalence per cycle (defined as number of affected treatment cycles/total number of treatment cycles), their fate, their quality and the IR of transferred embryos that were abnormal is shown. The IR of these embryos was then compared to that of the normal embryo cohort for statistical significance (Fisher's exact, significant at $p<0.05$).

*only transferred embryos assessed for quality for this category due to significant missing data.

Figure 7. Fate of embryos from each abnormal embryo phenotype.



Proportion of embryos transferred, frozen or discarded that underwent an abnormal division pattern where direct cleavage (DC) includes true direct cleavage (T-DC), false direct cleavage (F-DC) and unconfirmed direct cleavage (U-DC) combined. RC; reverse cleavage, AC; absent cleavage, CC; chaotic cleavage, CL; cell lysis.

Patient age was significantly lower for those undergoing DC, RC and CC to those not exhibiting an abnormal division pattern. The number of oocytes collected was found to be significantly higher in treatment cycles containing abnormal embryos than those not containing embryos exhibiting an abnormal division pattern. Finally, the number of previous attempts was not found to be significantly different between any of the abnormal division categories and the control embryo cohort (Table 16). Baseline information from treatment cycles containing an abnormal embryo did not contribute to baseline information for the control cohort.

Table 16. Baseline information for embryos undergoing an abnormal division pattern.								
	Affected embryos (n)	**Treatment cycles (n)**	**Patient age (mean ± S.D)**	**p-value**	**Oocytes collected (mean ± S.D)**	**p-value**	**Previous attempts (mean ± S.D)**	**p-value**
TDC	48	45	32.82 ± 4.7	<0.0001	12.95 ± 7.78	<0.0001	1.37 ± 0.93	>0.05
FDC	69	64						
UDC	580	463						
RC	65	61	32.5 ± 4.5	0.0097	15.7 ± 9.7	<0.0001	1.23 ± 0.6	0.2663
AC	133	95	33.16 ± 5.41	0.0629	15.09 ± 8.57	<0.0001	1.35 ± 0.8	0.8438
CC	835	459	32.93 ± 4.87	<0.0001	13.44 ± 8.5	<0.0001	1.39 ± 0.82	0.6765
CL	81	71	33.24 ± 4.27	0.1381	13.86 ± 7.79	<0.0001	1.28 ± 0.78	0.4422
Normal	14008	3273	34.08 ± 4.73	-	10.5 ± 5.99	-	1.37 ± 0.98	-
Including the total number of affected embryos, the number of treatment cycles these pertain to, the mean patient age, oocytes collected and previous attempts. The mean patient age, oocytes collected and previous attempts were statistically analysed against the normal embryo cohort for significant differences (student t-test, significant at $p<0.05$).								

6.2.5 Discussion

The prevalence of DC in the literature has been stated as 13.7% (Rubio *et al,* 2012) and 18% (Hickman *et al,* 2012). In the current analysis the overall prevalence of DC was 4.38% (U-DC, F-DC and T-DC combined) occurring in 1.22 embryos per treatment cycle. The implantation potential of embryos undergoing DC has been stated as just 1.2% (Rubio *et al,* 2012) however, in the current analysis the IR was found to be 17.4% (4/23) (T-DC, F-DC and U-DC combined); not significantly lower than that of the control embryo cohort although this could be attributed to the reduced numbers. A classification system of DC was not adopted by other publications therefore if F-DC were not considered, the IR would be significantly lower than those not exhibiting a DC. Of the three categories, those that were classed as F-DC had the highest IR, as one might expect from the definition. There is a paucity of literature regarding the exact mechanisms underlying the phenomenon of DC however a recent comprehensive review discusses both molecular and cellular mechanisms that could be related (Kalatova *et al,* 2015). In particular, centrosome defects are suggested as possible causes for DC facilitated through the lack of certain regulatory proteins such as p53. The presence of surplus centrosomes leading to DC, as suggested by Kalatova *et al* (2015), is reflected in an early investigation of tripolar oocytes. Genetic assessment of tripolar DC oocytes revealed three division patterns; DC to three cells (62%); cleavage to a morphologically normal two-cell ‘embryo’ (24%) and cleavage to a two cell ‘embryo’ plus an extrusion (14%) (Kola *et al,* 1987). All triploid oocytes that had undergone DC to three cells were chromosomally abnormal with each containing a varied number of chromosomes (here considered a T-DC). Those that cleaved to morphologically normal two cell ‘embryos’ were found to be true triploid with each blastomere containing a 69XXX/XXY chromosome complement. However, of those oocytes that cleaved to a two cell ‘embryo’ plus an extrusion, 75% were found to have two diploid blastomeres and a haploid extrusion. In the analysis presented here, the IR of F-DC, those embryos analogous to the two cell embryo plus an extrusion, was 33.3% (2/6). Caution should be taken as the numbers are considerably reduced in this group due to the need to use known implantation embryos,

however, this represents a result just over 5% lower than that of a phenotypically normal embryo. Although speculative, the findings by Kola *et al* (1987) not only corroborate the aforementioned theory by Kalatova *et al* (2015) of amplified centrosome material, but could also indicate that embryos have the potential to correct genetic abnormalities. There are many studies detailing self correction between the cleavage stage and the blastocyst stage of embryo development (Voullaire *et al,* 2000; Li *et al,* 2005b; Munne *et al,* 2005; Barbash-Hazan *et al,* 2008; Northrop *et al,* 2010). It has been noted that trisomy embryos correct more often than other aneuploidies (Barbash-Hazan *et al,* 2008) possibly occurring through the loss of a chromosome in trisomy cells (Munne *et al,* 2005). In addition, in previous reports, CC could be misinterpreted as a DC thus causing the prevalence of DC to appear falsely increased. The increased IR of DC seen in the present investigation compared to previous reports may also be due to observers having experience with the different categorisations of DC, making them proficient at recognising patterns of F-DC, such as blastomere behaviour, allowing preferential selection of a potential F-DC in U-DC cases. The reduced patient age and increased number of oocytes collected may reflect a simple association between maternal age and number of oocytes collected. However, it may also indicate that stimulation can lead to reduced oocyte quality (Aboulghar *et al,* 1997) and high oocyte numbers (>15) can reduce the chance of a live birth (Ji *et al,* 2013), which could manifest as an abnormality such as DC.

Reverse cleavage occurred in 65 embryos (1.07 embryos per treatment cycle) of which 36 were either transferred or frozen where 26 were classed as good or average quality. It is likely that embryos classed as poor quality were utilised due to unavailability of others. The IR of embryos undergoing RC in the current investigation was 0% (0/9). The prevalence of RC has been reported as 6.8, 7 and as high as 27.4% in previous reports (Hickman *et al,* 2012; Desai *et al,* 2014; Liu *et al,* 2014). However, the rate of formation of usable embryos is in conjunction with others at approximately 40% (Desai *et al,* 2014). There have been reports that RC is affected by other variables such as ICSI and gonadotrophin releasing hormone (GnRH) antagonists. Therefore

a possible explanation for the disagreement presented here could be due to the difference in baseline patient and treatment variables, a consideration for further investigation. The phenomenon of RC has been recognised previously with regards to frozen thawed embryos (Trounson, 1984; Balakier *et al,* 2000). Balakier *et al* (2000) sought to determine the chromosomal changes in blastomeres that undergo fusion following thawing. This analysis included 1141 embryos frozen on day two and 873 frozen on day three. Reverse cleavage was found in 51 embryos of which 70% were classed as good quality. The overall frequency of RC was 4.6% in day two embryos and 1.5% in day three embryos. A slightly higher incidence of blastomere fusion was found in embryos created using IVF when compared to ICSI. When a control group was observed (embryos not subject to freezing and thawing) the prevalence of RC was 0.3%, a result not far from that recorded in the present study (0.41%). The IR of embryos that underwent blastomere fusion following thawing in the above investigation was very poor with 15 embryo transfers containing one abnormal and one normal embryo resulting in a single live birth only. Again, a result similar to that seen in the present investigation where no pregnancies resulted from nine embryos transferred that had undergone RC. The chromosomal status of blastomeres resulting from fusion was also examined where embryos affected by RC were transformed into either polyploidy or mosaic embryos. The authors suggested that the occurrence of blastomere fusion could be associated with existing membrane abnormalities that could promote fusion affected by factors such as pH, temperature and osmolality differences. Interestingly, in some fields of research the production of tetraploid embryos is advantageous and it has been concluded that tetraploidy does not prohibit preimplantation development (Eglitis, 1980); corroboration for the development of approximately 40% good or average quality embryos in the present investigation. This investigation could conclude similarly to others where the presence of RC did not seem to affect an embryos ability to create a good quality embryo but does impair an embryos ability to implant.

Absent cleavage has been characterised as a type of RC in a previous report (Liu *et al,* 2014) however, in the current report it is classed as a distinct

phenotype. The prevalence per embryo of this abnormality compared to RC is more than double (0.84 vs. 0.41%) and of the four embryos that were transferred with this phenotype, one implanted. However, in a previous report, of 22 embryos, none implanted that underwent type I or type II RC (defined here as AC) (Liu *et al,* 2014). In another investigation using disaggregated human embryos, blastomeres were scored for the number of nuclei present after 16 to 20h culture and a small proportion of mononucleated blastomeres exhibited two nuclei after culture. It was hypothesised that approximately 30% of these occurred through AC (Pickering *et al,* 1995). Here, AC was shown to occur in 1.08 embryos per treatment cycle and of the 133 embryos exhibiting AC, 122 were classed as poor quality and 116 were discarded. Unlike DC, RC and CC however, the patient age was not shown to be significantly different when compared to the control embryo cohort.

Chaotic cleavage has an overall prevalence per embryo of 5.25%; by far the highest of the five abnormal phenotypes. Occurring in 1.82 embryos per treatment cycle suggestive of a patient, treatment or environmental effect rather than a spontaneous event. One comprehensive analysis identified the prevalence of CC to be 15%, with a blastocyst formation rate of 14% and an IR of 0% (Athayde Wirka *et al,* 2014). In the current analysis, the IR of these embryos was 2.1% (1/48); significantly lower than the IR of the control embryo cohort. Of the utilised embryos, just 18.2% were classed as good quality, 27.3% as average and 54.5% as poor. Interestingly, it has previously been found that 35.2% of those exhibiting CC were classed as good quality, a result not synonymous with the current analysis. A possible explanation for this disagreement is the TLS used. In the current analysis, EmbryoScope® was the TLS of choice however, in the analysis by Athayde Wirka *et al* (2014) the Eeva™ system was used. The Eeva™ system uses dark field illumination to enable the software within it to track blastomeres. The EmbryoScope® does not use dark field illumination which could make distinction of blastomeres from fragments more straightforward. An investigation conducted on patients carrying a Robertsonian translocation (the fusion of two acrocentric chromosomes), revealed that a high proportion of embryos resulting from these patients underwent numerous CC divisions and, rather than the

aneuploid segregation of the Robertsonian translocation being the only reason for the infertility, there may be a post zygotic manifestation leading to uncontrolled chromosome segregation (Conn *et al,* 1998). The presence of chaotically dividing embryos has been noted elsewhere (Harper and Delhanty, 1996; Delhanty *et al,* 1997; Laverge *et al,* 1997) and has also been identified as a patient related phenomenon (Delhanty *et al,* 1997) a statement synonymous with CC occurring in up to 1.82 embryos per treatment cycle.

Cell lysis is largely discussed in the literature when considering frozen thawed embryos and, as discussed previously, there is an associatively low IR (Tang *et al,* 2006). 59.2% of the embryos were classed as poor quality with 55.6% of the total discarded. Just 13.6% were considered good quality and 27.2% average quality, a result similar to other abnormal phenotypes. As very few embryos were shown to exhibit this phenotype, and fewer still were transferred, it is difficult to draw conclusions about the implications of this abnormal phenotype. It would be reasonable to use previous evidence regarding frozen thawed embryos to attribute their potential for success. However, CL in frozen embryos is likely as a result of cryo-damage during the freeze thaw process whereas, in fresh embryos, the CL could be as a result of exposure to another stressor such as suboptimal pH, temperature or osmolality. Cells that lyse may have a heightened sensitivity to changes in the environment, or lack a cytoplasmic constituent that regulates cell volume, for example, leading to its lysis.

6.2.5.1 Abnormal phenotypes as deselection criteria

Where possible, U-DC and T-DC embryos should not be selected for transfer if other embryos are available, even when embryo quality is considered. It is important to note at this point that embryos transferred at the cleavage stage undergoing DC (of which there were five in the current analysis) will inevitably be classed as U-DC. These embryos may have resulted in F-DC thus caution is advised due to a potential bias in the current results of U-DC cleavage stage embryos. For this reason, extended culture of DC embryos may be valuable to allow the classification into either F-DC or T-DC and thus aid

further in embryo selection and management of patient expectation. Chaotic cleavage, the most common abnormal phenotype in the current analysis, has been linked to severe chromosomal abnormalities in the literature which could be patient specific therefore it's possible that the phenomenon could occur more than once in a patient cohort indicating an underlying genetic condition. Where CC embryos are transferred the expected IR is 2.1% regardless of embryo quality. For this reason, identification of CC as a deselection tool should be considered for laboratories utilising TLS. Just fewer than 92% of embryos that exhibit AC create poor quality embryos thus they would likely be automatically discounted from clinical use. Reverse cleavage and CL each have an IR of 0%, albeit from low numbers of transferred embryos. However, the relative prevalence is low, the majority of embryos exhibiting these phenomena are poor quality and they are not able to implant therefore these embryos should not be selected for transfer where possible. These recommendations have been implemented at the study site to aid in embryo selection. In addition to the above, the need for accurate and consistent annotation of embryos is imperative for any centre utilising TLS. This issue was raised a number of years ago resulting in the publication of suggested terminology in order to create consensus among users (Ciray *et al,* 2014). Consensus is paramount and caution is advised when implementing or analysing time-lapse parameters discussed by others.

This preliminary investigation sought to determine the prevalence, implantation potential and suitability for inclusion in ESAs of five abnormal cleavage events. To determine IR, only known implantation embryos were used leading to a significant reduction in the number of embryos available for analysis. Nevertheless, this number would be difficult to achieve at another single site based on the study site using TLS for all patients and performing over 2000 treatment cycles per year. In addition, the ability to track the implantation of these embryos is made more difficult with the increased likelihood of transferring two embryos in these cases, potentially due to reduced embryo quality in the available embryo cohort. Based on the results presented here, future analyses should focus on embryos undergoing more than one abnormal division event, the cell stage at which the abnormal

cleavage event occurs, the effect of treatment parameters such as ICSI and day of transfer as well as the assessment of a relationship between the abnormal phenotypes and multinucleated blastomeres (MNB). In addition, the authors plan to perform an extension of this analysis to include embryo quality and outcome information regarding DC one-to-three versus two-to-five cells in the DC classifications presented here. Finally, scrutiny should be paid to CL where the specific timings of the CL event should be assessed and linked to the relative impact on embryo viability.

In conclusion, embryos exhibiting an abnormal phenotype appear to have reduced developmental capability expressed as both embryo quality and implantation potential. Time-lapse systems are bringing to light many unusual and, most likely, fundamentally complicated embryological phenomena requiring in depth analysis that could ultimately improve the outcome of treatment cycles.

6.2.6 Dissemination

The above research was prepared for publication in Reproductive BioMedicine Online. The research was accepted for publication on 17th February 2017 (appendices section 9.6).

CHAPTER 7: The effect of environmental factors on embryo development

7.1 Prelude

Preceding chapters, with regards to annotation, have been concerned with interrogation of the information that a TLS can provide in the form of validation of externally derived ESAs as well as the identification of five abnormal cleavage events that have been shown to reduce an embryos chance of implantation. The following chapter aims to assess the effect of patient, treatment and environmental factors on morphokinetic parameters. This chapter includes an interim analysis of a sibling oocyte study which aims to determine differences, if any, in embryo development, both morphokinetic and morphologic, between three commercially available culture media. Further to this, a regression analysis was performed to identify any patient and treatment parameters significantly affecting nineteen morphokinetic features of embryo development. This chapter aims to support the hypothesis that specific ESAs are required for the true potential of TLS to be realised.

7.2 Embryo quality and morphokinetics are affected by culture media type: an interim analysis of a sibling oocyte study.

7.2.1 Abstract

Literature suggests that treatment, environment and patient parameters can affect an embryo's early morphokinetic profile indicating that the use of standardised morphokinetic ESAs may not be clinically effective. An ongoing sibling oocyte study was commenced in August 2016 where embryos from patients having ICSI or IVF treatment, were randomised, following injection or fertilisation check, to three commercially available culture media, namely G-TL™, SAGE-1-Step™ and Continuous Single Culture® (CSC). Nineteen absolute and interval morphokinetic parameters were assessed for differences using the related-samples Friedman's two-way analysis of variance by ranks or a one-way ANOVA, dependent on qualification of

normality using D-Agostino and Pearson normality test. Blastocyst formation rate (BFR), utilisation rate (UR) and incidence of abnormal cleavage events were analysed using Fisher's exact test. All results were considered significant at $p<0.05$. Patients (n=32) contributed 432 oocytes resulting in 293 embryos. The BFR was not different between any culture media at 64.15%, 77.89% and 73.91%, for G-TL™, Sage-1-Step™ and CSC, respectively ($p=0.08$). The UR (embryos transferred and cryopreserved) were, however, significantly different; 39.62%, 65.26% and 57.61%, respectively ($p=0.0009$). A total of 80 embryos underwent an abnormal division event (cell lysis, direct cleavage, chaotic cleavage, absent cleavage and reverse cleavage) although there was no significant difference in the incidence of abnormal division between the three media at 43.75%, 22.50% and 33.75%, respectively ($p=0.07$). Absolute (t2, t3, t4, t5, t6, t7, t8, t9, tM, tSB, tB) and interval morphokinetic parameters (s2, s3, cc2, cc3, cc4, t9-tM, tM-tSB, tSB-tB) were assessed. Of 293 embryos, 36.18% were cultured in G-TL™, 32.42% in SAGE-1-Step™ and 31.40% in CSC. Of the nineteen morphokinetic parameters assessed, tM ($p=0.03$) and t9-tM ($p=0.005$) were significantly reduced in embryos cultured in CSC. This investigation allows for the control of confounding factors of patient or treatment origin therefore, it is surmised, any observed effect is a true reflection of the culture media. This implies that the development and validation of ESAs must be specific, robust and prospective before being introduced for clinical use.

7.2.2 Introduction

Since the introduction of TLS into the IVF laboratory, many have sought to utilise its capability to visualise an embryo's morphokinetic timeline as a proxy for embryo viability. This aim led to the development of ESAs. Such ESAs have, thus far, been developed on the premise that preimplantation embryos are classed as independent observations therefore the interference of confounding factors has not been accounted for (Kirkegaard *et al*, 2016) creating a one-size-fits-all approach. The external validation of certain developed ESAs has highlighted that they lose clinical effectiveness and are not easily transferred as shown in Chapter 5 and by others (Freour *et al*, 2015; Kirkegaard *et al*, 2014; Yalcinkaya *et al*, 2014). In addition to the failure

of external application of developed ESAs, evidence also emerged suggesting that embryo morphokinetics could be affected by a number of patient and treatment parameters such as BMI (Bellver *et al*, 2013), lifestyle choices such as smoking (Freour *et al*, 2013), the use of IVF or ICSI for fertilisation (Cruz *et al*, 2013) and the drugs used for controlled ovarian stimulation (Munoz *et al*, 2012; 2013). Following this, regression analyses were performed to determine the effect of patient, treatment and environmental parameters on an embryos morphokinetic timeline as outlined later in Chapter 7 as well as by others (Kirkegaard *et al*, 2016). That carried out by Kirkegaard *et al* (2016) observed that embryos had delayed development when maternal age, FSH dose and attempt number were increased. In addition to the effect of patient and treatment parameters, the environment has also been assessed; specifically the culture media.

Human embryo culture has been, and remains, extensively investigated. However, human embryo metabolism is far from being fully understood. This is primarily because of its complex nature but also because of the lack of human material to perform investigations as well as the absence of an appropriate *in vivo* animal model (Menezo *et al*, 2013). The effect of culture media on embryo development has focused on the two culture systems available to the laboratory; single-step and sequential (Biggers and Racowsky, 2002; Sepulveda *et al*, 2009; Biggers *et al*, 2005; Perin *et al*, 2008; Hentemann and Bertheussen, 2009). The principles of each system relate to two hypotheses regarding embryo metabolism; ‘let the embryo choose’ and ‘back to nature’ (Summers and Biggers, 2003). The former is one that is currently favoured and is the principle underpinning the use of a single-step culture medium. This system provides the embryo with all the nutrients required for development to the blastocyst stage and relies upon the embryo utilising that which it requires. The latter denotes the use of a sequential culture system where a change in culture medium is required on day three of embryo development and each formulation of culture media (pre and post day three of development) provides only those substrates that the embryo requires at each particular stage. Analyses seeking to determine whether culture media affects embryo morphokinetics have, primarily, been interested

in comparing fundamentally different culture systems; single-step versus sequential (Ciray *et al*, 2012; Basile *et al*, 2013). This may reflect the increased availability of commercial versions of single-step culture media in recent years as well as the rapid interest in TLS and the notion of an undisturbed environment. Although single-step culture media are now common place in IVF laboratories, there remains of paucity of data relating to the morphokinetic effects of such culture media, particularly beyond the five-cell stage.

The current investigation aimed to observe the effect of three different types of commercially available single-step culture media (G-TL™ (Vitrolife), SAGE 1-Step™ (Origio), Continuous Single Culture® (CSC) (Irvine Scientific)) on nineteen morphokinetic parameters and embryo quality using a sibling oocyte study design. The detrimental effects of ammonium on embryo development on *in vitro* embryo development have been previously described (Lane *et al*, 2001; Zander *et al*, 2006; Lane and Gardner, 2003; Gardner and Lane, 1993; Lane and Gardner, 1994; Lane and Gardner, 1996). As such, it was considered prudent to also investigate ammonium accumulation in the three culture media over the course of a standard incubation period in both the EmbryoScope® incubator in EmbryoSlides® and in a standard incubator in tubes. It was deemed appropriate that an interim analysis be performed to assess the results thus far in terms of fulfilling the original power calculation (appendices section 9.4.2).

7.2.3 Materials and methods

Patient recruitment commenced in August 2016. Patients were approached at their initial consultation or initial clinical appointment where they were given the patient information sheet and consent form (appendices section 9.4.3) by a research nurse. Patients were given at least 14 days to consider the information and sign the consent form. The patients were then re-approached at their baseline scan to confirm participation. For those that had consented to participation, this was re-confirmed on the day of oocyte collection prior to the procedure.

7.2.3.1 Patient criteria

Inclusion criteria for participation in the trial included; both partners less than 38 years old; first treatment cycle; fresh, autologous gametes used in treatment; conventional IVF or ICSI used to create embryos; six oocytes injected for ICSI or three embryos created following IVF. A total of 32 patients fulfilled the inclusion criteria, consented to participate and had oocytes/embryos randomised.

7.2.3.2 Oocyte retrieval and embryology

Following ICSI or fertilisation check (for IVF cases only), injected oocytes (ICSI) or embryos (IVF) were randomly assigned, equally, to one of the three culture media; G-TL™, SAGE 1-Step™ and CSC. For this analysis, G-TL™ was classed as the control media by virtue of its established use at the test site. As such, where one surplus injected oocyte or embryo was available, it was assigned to G-TL™. Where two surplus injected oocytes or embryos were available, one was assigned to G-TL™ and one to SAGE 1-Step™. Those oocytes/embryos destined for culture in G-TL™ received a thorough wash in one well of a 4-well dish containing 0.65ml of equilibrated G-TL™ before being cultured in drops 1-4 of an EmbryoSlide®. Those oocytes/embryos destined for culture in SAGE 1-Step™ received a thorough wash in one well of a 4-well dish containing 0.65ml of equilibrated SAGE 1-Step™ before being cultured in drops 5-8 of an EmbryoSlide®. Those oocytes/embryos destined for culture in CSC received a thorough wash in one well of a 4-well dish containing 0.65ml of equilibrated CSC before being cultured in drops 9-12 of an EmbryoSlide®. Where more than 12 oocytes/embryos were to be randomised, a second EmbryoSlide® was used in the same manner as the first. Culture conditions for the three culture media were consistent and no media-specific optimisation was performed prior to the study.

7.2.3.3 Ammonium assessment

Ammonium assessment was carried out in two vessels; EmbryoSlide® dishes and 5ml tubes. EmbryoSlides® were set-up to allow repeated measurements

of 100µl of media every 24h for a total of 168h (day -1 to day 6) from the three media types (n (G-TL™) = 24, n (SAGE 1-Step™) = 24, n (CSC) = 24). EmbryoSlides® were prepared including 12 micro wells of 25µl of either G-TL™, SAGE 1-Step™ or CSC with 1.4ml oil overlay. Dishes were placed in EmbryoScope® instruments and the time entered recorded as time zero. Three 100µl samples of each culture media was taken immediately (day -1) and every 24h thereafter, with immediate snap freezing (placed in a -50°C freezer), up to 168h. Each sample was taken by placing the pipette directly into the culture drops and removing the required volume of media whilst ensuring no oil was aspirated. The pipette tip was cleaned with a lint-free tissue to remove oil residue and the sample expelled into a pre-labeled Eppendorf® tube. This process was repeated until the Eppendorf® tube contained 100µl. The sample was then snap frozen until analysis. 5ml tubes containing 1ml of each culture media were prepared. Three 100µl samples of each culture media was taken immediately (day -1) and every 24h thereafter, with immediate snap freezing, up to 168h (n (G-TL™) = 24, n (SAGE 1-Step™) = 24, n (CSC) = 24). Analysis of ammonium accumulation was performed using a glutamate dehydrogenase methodology (MULTIGENT Ammonia Ultra).

7.2.3.4 Outcome measures and statistical analyses

Nineteen absolute and interval morphokinetic parameters were assessed between three culture media using the related-samples Friedman's two-way analysis of variance by ranks or a one-way ANOVA based on a test for normality (D'Agostino and Pearson normality test); t2-t9, tM, tSB, tB, s2, s3, cc2, cc3, cc4, t9-tM, tM-tSB, tSB-tB. Blastocyst formation rate (BFR), utilisation rate (UR), proportion of top quality blastocysts and incidence of abnormal cleavage events were analysed using Fisher's exact test. A two-tailed sample size calculation with 95% power value and 0.05 alpha value was performed (appendices section 9.4.2) however as this is an interim analysis these requirements were not fulfilled. Differences in ammonium build-up were determined between the three culture media in the two vessel types also using related-samples Friedman's two-way analysis of variance by ranks or a

one-way ANOVA based on a test for normality (D'Agostino and Pearson normality test). All results were considered significant at $p<0.05$.

7.2.4 Results

7.2.4.1 Media trial results

A total of 293 embryos resulted from the participation of 32 patients in the media trial (data summary in Table 20). Of the 32 participants, 20 underwent IVF and 12 underwent ICSI. For clinical reasons, in seven cycles, all embryos were electively frozen, and the remaining 25 resulted in a blastocyst transfer (Table 20). The fates of embryos varied significantly between G-TL™, SAGE 1-Step™ and CSC with a lower proportion of embryos cultured in G-TL™ resulting in utilisation ($p=0.0009$, Table 20). Of the 293 embryos, 210 created blastocysts (Table 21). The proportion of good, average and poor quality blastocysts (GQB, AQB, PQB) varied significantly between culture media with fewer top quality embryos being created from embryos cultured in G-TL™ ($p=0.04$, Table 21). A total of 80 of the 293 embryos underwent an abnormal division event; CL, DC, CC, AC or RC (Table 22). The proportion of abnormal embryos in each culture media did not differ significantly ($p=0.07$) (Table 22).

Full annotation from tPNf through to tB was undertaken in 210 blastocysts, however, 72 of these embryos did not have a full match with all culture media. This is a result of an uneven number of embryos cultured in the media either following ICSI (unable to predict fertilisation therefore some media contained no fertilised oocytes) or from the fertilisation of a number of oocytes not divisible by three following IVF fertilisation check. A further 51 of the 210 embryos reaching the blastocyst stage underwent an abnormal division event, therefore, one or more annotations were not performed. The remaining embryos (87) had full annotation from tPNf to tB and there was appropriate matching to perform the statistical analysis. Statistical differences were found between the three culture media when considering tM ($p=0.03$) and the time between t9 and tM ($p=0.005$). All other morphokinetic parameters did not vary significantly between the three culture media (Table 23).

Table 20. Data summary of participants of the three culture media trial.				
Patients (n)	32			
IVF cycles (n)	20			
ICSI cycles (n)	12			
Blastocyst transfer (n)	25			
Freeze all cycles (n)	7			
	G-TL™	**SAGE 1-Step™**	**CSC**	**Total**
Oocytes (n)	130	114	104	348
Embryos (n)	106	95	92	293
Embryos transferred (n)	6	13	7	26
Embryos frozen (n)	36	49	46	131
Embryos discarded (n)	64	33	39	136
Utilisation rate (%)	39.62*	65.26	57.61	53.58

Number of participants, IVF and ICSI cycles, blastocyst transfers and freeze all cycles, number of oocytes, embryos and the utilisation of embryos in each of the culture media. The utilisation rate is significantly reduced when embryos are cultured in G-TL™ (p=0.0009, Fisher's exact test). No media-specific optimisation was performed prior to the study.

Table 21. Proportion of embryos reaching the blastocyst stage and their quality.						
	G-TL™		**SAGE 1-Step™**		**CSC**	
	n	**%**	**n**	**%**	**n**	**%**
Cleavage stage embryos	12	11.32	6	6.32	14	15.22
M/CM stage embryos	13	12.26	7	7.37	6	6.52
Necrotic embryos	13	12.26	8	8.42	4	4.35
Blastocyst stage embryos	68	64.15	74	77.89	68	73.91
GQB	33	48.53*	50	67.57	44	64.71
AQB	10	14.71	12	16.22	11	16.18
PQB	25	36.76	12	16.22	13	19.12

Number of embryos reaching the cleavage stage, morula (M) or cavitating morula (M), those that became necrotic and those reaching the blastocyst stage. There is a significantly lower proportion of good quality blastocysts (GQB) obtained when embryos are cultured in G-TL™ (p=0.04, Fishers exact test). No media-specific optimisation was performed prior to the study. AQB; average quality blastocyst. PQB; poor quality blastocyst.

Table 22. Number of embryos undergoing an abnormal division event in each of the three culture media.

	G-TL™	SAGE 1-Step™	CSC	Total
Abnormal division events (n)	35	18	27	80
CL (n)	2	0	0	2
DC (n)	9	4	11	24
CC (n)	20	11	10	41
AC (n)	3	3	3	9
RC (n)	1	0	3	4

No significant difference in the proportion of embryos undergoing an abnormal division event was found (p=0.07, Fishers exact test). CL; cell lysis. DC; direct cleavage. CC; chaotic cleavage. AC; absent cleavage. RC; reverse cleavage.

Table 23. The effect of culture media type on nineteen morphokinetic parameters of 87 embryos.

	G-TL™ (mean ± S.D)	SAGE 1-Step™ (mean ± S.D)	CSC (mean ± S.D)	Statistical test	P value
tPNf (hpi)	23.00±2.97	23.44±2.42	23.67±2.55	Non-parametric	0.08
t2 (h)	2.57±0.42	2.63±0.54	2.56±0.45	Non-parametric	0.79
t3 (h)	13.39±1.43	13.62±1.20	13.44±1.19	Non-parametric	0.42
t4 (h)	14.45±3.12	14.35±1.76	14.08±1.36	Non-parametric	0.08
t5 (h)	26.50±2.68	26.85±2.39	26.83±2.46	Parametric	0.33
t6 (h)	27.89±3.60	28.39±4.67	27.74±2.60	Non-parametric	0.97
t7 (h)	30.14±6.66	30.09±5.55	30.54±5.28	Non-parametric	0.52
t8 (h)	33.53±8.58	32.53±6.28	32.95±6.72	Non-parametric	0.87
t9 (h)	47.14±5.32	46.77±4.86	47.11±6.12	Non-parametric	0.79
tM (h)	56.24±6.35	57.39±8.82	53.41±7.15	Parametric	0.03*
tSB (h)	70.08±6.39	71.13±7.82	69.25±5.37	Parametric	0.34
tB (h)	81.86±9.53	82.30±9.82	79.88±7.52	Non-parametric	0.73
s2 (h)	1.06±2.65	0.74±1.27	0.64±0.63	Non-parametric	0.94
s3 (h)	7.03±7.47	5.67±5.52	6.12±6.40	Non-parametric	0.97
cc2 (h)	10.82±1.20	10.99±1.24	10.88±0.96	Non-parametric	0.49
cc3 (h)	12.05±2.48	12.50±2.08	12.75±1.66	Non-parametric	0.52
cc4 (h)	13.60±6.24	14.24±5.36	14.16±6.35	Parametric	0.9
t9tM (h)	9.11±4.26	10.62±7.63	6.31±3.78	Parametric	0.005*
tMtSB (h)	13.84±4.26	13.73±4.97	15.84±6.06	Non-parametric	0.26
tSBtB (h)	11.78±5.00	11.18±4.55	10.63±5.63	Non-parametric	0.79

Absolute morphokinetic parameters assessed include tPNf (time to pronuclear fading), time to two-cell (t2) through to time to nine-cell (t9), time to start of morula (tM), blastulation (tSB) and blastocyst (tB). Interval morphokinetic parameters include s2 (t3-t4), s3 (t5-t8), cc2 (t2-t3), cc3 (t4-t5), cc4 (t8-t9), t9-tM, tM-tSB and tSB-tB. Statistically significant differences were found between embryos in the three culture media when considering tM and t9-tM (p=0.03, 0.005, respectively, one-way ANOVA). S.D.; standard deviation. hpi; hours post insemination. h; hours.

7.2.4.2 Ammonium results

Ammonium levels increased over the course of 168h starting at a minimum level of 15.92µmol/l (CSC in EmbryoSlides®) up to a maximum of 56.64µmol/l (CSC in EmbryoSlides®) (Table 24, Figure 8). Of the total 144 measurements taken, seven were returned as 'null'; CSC in a 5ml tube at 120h, G-TL™ in an EmbryoSlide® at 144h, CSC in an EmbryoSlide® at 0h, 48h, 72h, 96h and 168h. 'Null' indicated that the assay was unable to be performed for a number of possible reasons including insufficient sample volume, which is the most likely, or reagent or equipment malfunction. Considerable intra-sample variability was observed between the triplicate measurements as shown in the standard deviations (0.0 to 17.52). The levels of ammonium in CSC increased significantly more than SAGE 1-Step™ when cultured in tubes ($p=0.0009$, Friedman test) (Table 25, Figure 9). However, the 'rate' of accumulation of ammonium did not appear to differ in any other culture media in either tubes or EmbryoSlides® ($p<0.05$, Friedman test).

Table 24. Ammonium levels (µmol/l) in three commercially available culture media from EmbryoSlides®

	G-TL™ (µmol/l) (mean ± S.D)	SAGE 1-Step™ (µmol/l) (mean ± S.D)	CSC (µmol/l) (mean ± S.D)
0h	28.54 ± 9.87	20.95 ± 2.56	15.92 ± 0.49*
24h	37.51 ± 1.77	25.07 ± 0.64	23.38 ± 1.44
48h	46.38 ± 2.08	24.42 ± 1.42	27.08 ± 1.27*
72h	40.15 ± 4.47	31.38 ± 7.16	33.48 ± 0.00*
96h	42.26 ± 1.2	26.26 ± 1.38	39.34 ± 2.43*
120h	35.41 ± 1.53	31.47 ± 5.96	42.58 ± 2.03
144h	34.86 ± 0.59*	30.98 ± 3.16	51.63 ± 1.47
168h	39.95 ± 1.99	42.36 ± 7.67	56.64 ± 3.7*

Ammonium levels in G-TL™, SAGE 1-Step™ and CSC sampled from EmbryoSlides® cultured in the EmbryoScope® over 168h. No significant differences were detected between groups (p=0.0789, Friedman test). S.D; standard deviation.

* - one of three results returned as ‘null.’

Table 25. Ammonium levels (μmol/l) in three commercially available culture media from 5ml tubes

	G-TL™ (μmol/l) (mean ± S.D)	SAGE 1-Step™ [a] (μmol/l) (mean ± S.D)	CSC[a] (μmol/l) (mean ± S.D)
0h	25.23 ± 4.47	18.71 ± 1.0	29.18 ± 8.19
24h	27.62 ± 2.15	19.52 ± 2.62	27.27 ± 2.52
48h	28.73 ± 3.08	21.01 ± 4.45	32.99 ± 0.56
72h	30.94 ± 3.08	20.64 ± 2.25	36.07 ± 2.68
96h	32.41 ± 1.76	24.49 ± 3.1	40.18 ± 2.91
120h	29.55 ± 0.96	27.25 ± 2.25	56.14 ± 12.84*
144h	34.61 ± 1.61	28.53 ± 2.35	59.35 ± 17.52
168h	32.71 ± 0.68	42.95 ± 0.39	51.76 ± 0.83

Ammonium levels in G-TL™, SAGE 1-Step™ and CSC sampled from 5ml tubes cultured in a standard bench-top incubator over 168h. S.D; standard deviation.

* - one of three results returned as 'null.'

[a] – significance between groups (p=0.0009, Friedman test)

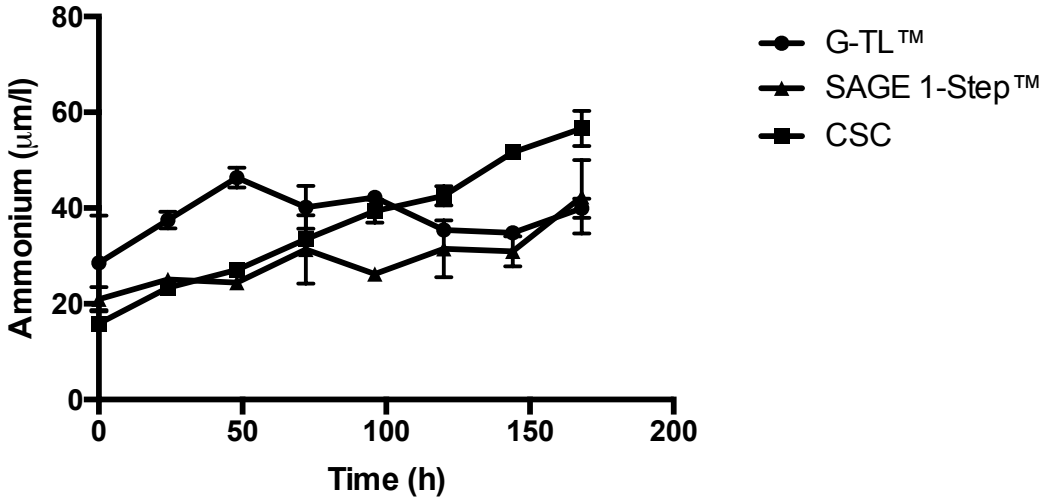


Figure 8. **Mean (and standard error bars) ammonium levels (μmol/l) in three commercially available culture media**. G-TL™, SAGE 1-Step™ and CSC sampled from EmbryoSlides® cultured in the EmbryoScope® over 168h. No significant differences were detected between groups (p=0.0789, Friedman test).

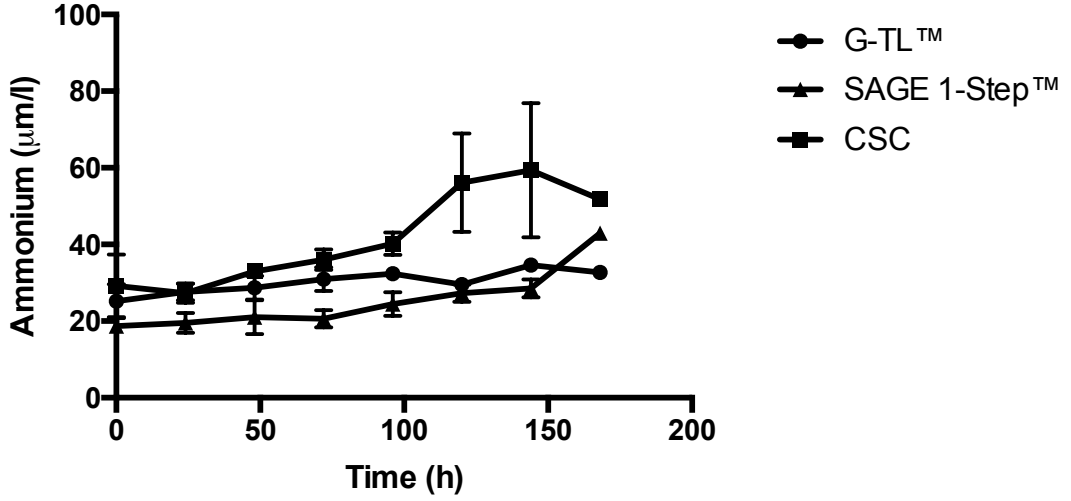


Figure 9. Mean (and standard error bars) ammonium levels (μmol/l) in three commercially available culture media. G-TL™, SAGE 1-Step™ and CSC sampled from 5ml tubes cultured in a standard incubator over 168h. CSC ammonium levels were statistically significantly higher than SAGE 1-Step™ (p=0.0009, Friedman test). No other significant differences between groups (p>0.05, Friedman test).

7.2.5 Discussion

The morphokinetic differences of human embryos cultured in three commercially available single-step culture media were assessed using a sibling oocyte study design. The ammonium concentration in each was determined over the course of 168h to establish any differences in this unavoidable feature of human embryo culture media. Differences in ammonium concentrations may have confounded any true morphokinetic differences arising from the culture media *per se*. Embryos cultured in CSC underwent compaction significantly earlier in their development than those cultured in G-TL™ or SAGE 1-Step™. In addition, although not statistically significant, those stages that follow compaction (tSB, tB) also occurred sooner in the morphokinetic timeline for those embryos cultured in CSC. When considering embryo development broadly, the proportion of GQB was significantly reduced in embryos cultured in G-TL™ and consequently, embryos in this culture media were not utilised as often as those in SAGE 1-Step™ or CSC. There was a noticeable increase in the concentration of ammonium over the course of the 168h in all three culture media and there was a statistically higher concentration of ammonium in CSC over the course of incubation when sampled from a 5ml tube in a standard incubator compared to SAGE 1-Step™.

These data suggest that embryos are affected, both morphologically and morphokinetically, by the environment in which they are cultured. There have been numerous accounts of the effect of culture media on static observations of embryos (Quinn, 2004; Balaban and Urman, 2005; Lane and Gardner, 2007; Sepulveda *et al*, 2009). Unfortunately, these are not particularly relevant to this discussion as most lack the information regarding the specific time points or cell numbers meaning the variation could simply be as a result of the time of observation. Where morphokinetics are concerned, there is a lack of evidence for the comparison of different single-step culture media for parameters beyond the cleavage stage of development. Nevertheless, they provide evidence for the embryonic effect of the culture environment. The first published study to assess the effect of culture media on embryo

morphokinetics (Ciray *et al*, 2012) aimed to compare that of early embryo development in sequential and single-step culture media from the same manufacturer. This study involved a total of 446 oocytes from 51 couples that were randomly assigned to either the single-step or sequential culture media after injection. The authors concluded that embryos cultured in single-step culture media exhibited shorter cleavage times when compared to sequential media for all cell divisions up to the five-cell stage. The authors observed a significantly higher proportion of utilised embryos in the sequential culture media than the single-step media. Although the first publication to highlight these morphokinetic differences, the article did not demonstrate a sample size calculation therefore it is difficult to determine if it was powered appropriately. These results are similar to a service evaluation study performed at the HFC where a comparison was made between sequential and single-step culture media from the same manufacturer. In this retrospective comparison, a total of 6392 embryos over six months were analysed. A statistically significant difference for all cleavage events up to the five-cell stage was observed. In contrast to the aforementioned study, these data suggested that a higher proportion of embryos were utilised that were cultured in the single-step medium when compared to the sequential medium (Barrie *et al*, 2015). A further investigation (Basile *et al*, 2013) assessed the morphokinetic parameters of 723 embryos from 75 couples in a single-step culture media and a sequential culture media from different manufacturers. This experiment found no statistically significant differences in any cleavage event up to the five-cell stage, contradictory to those previously published. The authors highlighted some pitfalls of their experimentation; primarily, the sole use of donor oocytes. Although this methodological choice eliminates some confounding factors regarding oocyte quality from the infertile female, it also restricts the value of the results, as they are less transferrable to the population in question. A more recent prospective analysis compared the early embryo cleavage kinetics between two sequential culture systems using 620 sibling oocytes (Zhang *et al*, 2016). This study reported no significant differences in any morphokinetic timing, morphology, UR or IR. However, this research used lax inclusion criteria (patients less than 45 with a BMI less than 35), it also used time of insemination as time-zero, which has been advised

against (Liu et al, 2015), along with no mention of the statistical test used thus making it difficult to determine if the statistical analysis was appropriate.

There have been two analyses, using mouse embryos, investigating the effects of culture media on morphokinetics beyond the five-cell stage showing that certain morphokinetic parameters are affected by constituents in the culture media; in the absence of protein t2, cc2, t5-t8 were delayed however, in the presence of protein t8-tSB were delayed (Morbeck *et al*, 2014). More recently, a similar analysis has been performed using single-step culture media only (Morbeck *et al*, 2017). This analysis revealed that mouse embryos had similar morphokinetic timelines between four commercially available single-step culture media however, they were differentially affected by oxygen concentration. These analyses, assessing morphokinetic differences between culture media, provide evidence that the environment in which embryos are cultured can affect morphokinetic parameters.

For the analysis presented here, it is important to consider the constituent differences in each of these culture media in order to determine possible reasons for the variations seen however, this information is not readily available to the end-user which is a long-standing grievance of scientists undertaking embryo culture in an IVF laboratory. Morbeck and colleagues sought to determine the constituent differences of commercially available culture media to elucidate the disparity in their composition; a feat not achieved before. Firstly, the composition of seven culture media was assessed for glucose, organic acids, amino acids, electrolytes and other compounds found in human embryo culture media (Morbeck *et al,* 2014). Stark differences were discovered, notably glucose, one of the main energy substrates for embryo development, where concentrations ranged from 0.1 to 3.2mM. The lactate to pyruvate ratio, also key substrates for embryo development, varied considerably ranging from 5-126 and 1.2-105 for cleavage, and blastocyst stages, respectively. Other profound differences included the discovery of three metals in one media (aluminium, iron and manganese) and a 30-times higher concentration of amino acids in one compared to the other six that were analysed. Owing to the increased

popularity of single-step culture media, the same group then analysed four commercially available culture media, three of which are those analysed in the present research, namely, G-TL™, SAGE 1-Step™, and CSC. This examination revealed marked differences in these culture media. G-TL™ had five-times higher concentration of glucose than SAGE 1-step™ and two-times higher than CSC. Also, G-TL™ also had a two-fold increase in pyruvate compared to both CSC and SAGE 1-Step™ (Morbeck *et al*, 2017).

Using this information it is possible to theorise reasons for the variations in both embryo quality and embryo morphokinetics in the current research study. Firstly, consideration should be given to the significant differences in glucose concentrations as documented by Morbeck *et al* (2017). It has been suggested that glucose has inhibitory effects on embryo development at certain stages (Schini and Banister, 1988; Chatot *et al,* 1989). A significant increase in the number of blastocyst cells has also been observed when glucose is removed from the culture media at the preimplantation stages (Conaghan *et al*, 1993). It was hypothesised that this could have been a result of enhanced cleavage rate at the earlier stages of embryo development. This evidence could provide a theory for the reduced number of good quality blastocysts in G-TL™ compared to other culture media as G-TL™ had a substantially higher concentration of glucose present when analysed in a previous report (Morbeck *et al*, 2017).

In terms of the differences in morphokinetics seen in embryos cultured in CSC (i.e. reaching tM faster) these constituent differences may have an influence although it is difficult to ascertain the complex interactions that are inevitably at work in these circumstances. It is known that pyruvate is the primary energy source for pre-compaction embryos and glucose for post-compaction embryos (Gardner, 1998). In addition there is a complex relationship between metabolites, specifically glucose and amino acids, where negative effects of the presence of glucose in the pre-compaction stages in single-step media can be counterbalanced by the presence of amino acids (Menezo *et al*, 2013; Guyader-Joly *et al*, 1997). In the analysis conducted by Morbeck *et al* (2017), CSC was found to have the lowest amino acid concentration of the three

culture media assessed here as well as half as much glucose and pyruvate compared to G-TL™ and SAGE 1-Step™.

Another obvious difference in the constituents of these three culture media is the calcium to magnesium ratio. Calcium is essential for compaction to occur *in vitro* and, of all of the culture media, CSC had the highest calcium to magnesium ratio (2.4) when compared to G-TL™ and SAGE 1-Step™ (0.6, 1.2, respectively) (Morbeck *et al*, 2017). Interestingly, the use of compaction as a marker for embryo viability has been addressed where those embryos that compacted earlier have an increased chance of implantation (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011; Le Cruguel *et al*, 2013). In addition, delayed compaction has been observed as a result of a developmental perturbation such as embryo biopsy (Bar-El *et al*, 2016). Clearly, these culture media have vast differences in terms of the metabolites that they contain and it may be the case that these differences have an effect on embryo morphokinetics.

Where ammonium concentration is concerned, the data presented demonstrate an increase over the course of 168h in all three culture media in both vessels (EmbryoSlide® and 5ml tube). However, when sampled from 5ml tubes, there was a significantly increased level of ammonium in CSC compared to SAGE 1-Step™. Amino acids spontaneously breakdown in culture to produce ammonium and the embryo also metabolises amino acids to produce more ammonium (Gardner and Lane, 1993; Lane *et al*, 2001). Amino acids act as pHi regulators, osmolytes and energy substrates and have the capacity to either stimulate or inhibit embryo development (Bavister and Arlotto, 1990; Bavister and McKiernan, 1992). Increased ammonium concentrations in culture media have been significantly linked to embryo and fetal development, predominantly in mice (Lane *et al*, 2001; Zander *et al*, 2006; Lane and Gardner, 2003; Gardner and Lane, 1993; Lane and Gardner, 1994; Gardner and Lane, 1996). To determine these effects on human embryos is difficult, as this would require supplementation of the culture media with a compound known to be toxic to other species' embryonic development. However, using animal models, ammonium has been

suggested to effect embryo development through three possible pathways; decreasing the concentration of alpha-ketoglutarate by its conversion to glutamate impairing the flux through the Krebs cycle leading to serious depletion of ATP in the cell. Second, ammonium can activate the enzyme phosphofructokinase, which creates an increase in glycolytic activity in turn shown to be detrimental to embryo development. Finally, ammonium as a weak base can also increase the internal pH of cells *in vitro* (Gardner and Lane, 1993; Lane and Gardner, 2003).

The results of the ammonium analysis here mirror those found elsewhere where those media based on a KSOM formulation (CSC) have a build-up of ammonium over the course of 120h up to 55µmol/l (Lane and Gardner, 1993). Although the level of ammonium in each of the culture media in the present analysis did not reach the critical level of 75µmol/l described in the literature (Gardner *et al*, 2013), this analysis only involved unused culture medium. Pertinent to the results in the current analysis are discoveries relating to amino acids and pH of the culture media analysed. The analysis conducted by Morbeck *et al* (2017) found that G-TL™, SAGE 1-Step™ and CSC had similar pH (7.32, 7.35 and 7.25, respectively) but substantial differences in the total amino acid concentrations; 2250µmol/l, 2075µmol/l, 1863µmol/l, respectively, with nine essential and five non-essential amino acids higher in G-TL™ and SAGE 1-Step™. In addition, this group revealed that G-TL™ lacked glutamate, was low in aspartate and additionally contained taurine. G-TL™ and SAGE 1-Step™ also had low levels of glutamine. The expected results based on this information are not what were observed in the current analysis; the media having the highest concentration of amino acids (G-TL™) did not have the highest overall ammonium concentrations. It could be however, together with the knowledge that there is approximately a three-fold increase in ammonium concentration when embryos are present (Gardner and Lane, 1993) that if there are more amino acids available to the embryo, they may metabolise them and generate higher levels of ammonium thus leading to an overall reduction in embryo quality, and as such utilisation, as seen in those embryos cultured in G-TL™. In addition, various non-essential amino acids have an inhibitory effect on blastocyst development (Lane and Gardner, 1994)

therefore if these are present in higher concentrations in G-TL™ this offers further explanation for the reduction in embryo quality and utilisation seen. It is however, important to note, that no media-specific optimisation was performed prior to this study and, owing to the study design (i.e. all three culture media in one dish), it would not have been possible to introduce differing CO_2 concentrations for each culture media. This may be a consideration for the reduction in performance of G-TL™ in the present study where the pH of the culture media may have been operating at a sub-optimal level causing a reduction in the embryo quality and subsequent utilisation rate.

7.2.6 Conclusion

There is a lack of literature comparing morphokinetics, firstly, between different single-step culture media and secondly, those parameters beyond the five-cell stage. The current analysis presents evidence that the culture environment may affect embryo morphokinetics however this is not as obvious as might be expected based on the substantial differences in constituents found in the three culture media. Differences in embryo development in the three single-step culture media examined were observed where the number of good quality blastocysts and the utilisation rate was significantly reduced in one type. This assessment represents an interim analysis that has not yet fulfilled the sample size required for appropriate statistical power. It would be prudent to include additional outcome analyses such as pregnancy outcomes however considerably more data would be required to determine differences in IR, CPR, and LBR, if any. The results of this analysis should be considered when utilising externally derived ESAs and the development of patient, treatment and environment specific ESAs should be encouraged to account for morphokinetic differences observed in variations of these influential parameters.

7.3 Morphokinetic parameters of embryo development are affected by its origin

7.3.1 Abstract

Since the introduction of time-lapse imaging, more effective methods for embryo selection are being sought. As a result, basic investigations linking an embryo's viability to it's morphokinetic profile have been produced with many developing embryo selection algorithms (ESAs). However, there is a lack of consideration for the effect of confounding factors such as patient and treatment parameters on morphokinetic parameters. This research study aimed to determine the effect of patient and treatment parameters on nineteen embryo morphokinetic parameters. A total of 2376 embryos from 639 treatment cycles were analysed in this single-site, retrospective cohort analysis using a multiple regression to determine the effect of maternal patient age, maternal BMI, suppression protocol, infertility diagnosis and treatment type on nineteen morphokinetic parameters using pronuclear fading as time-zero (t0); time to each cellular division (tn) including t2, t3, t4, t5, t6, t7, t8, t9, time to start of compaction (tM), start of blastulation (tSB), full blastocyst (tB) and the intervals between each stage including s2, s3, cc2, cc3, cc4, t9-tM, tM-tSB and tSB-tB. Data were collated between September 2014 and January 2016. Patients were included once and embryos were cultured for six days in a time-lapse enabled incubator (EmbryoScope®). Results were considered significant at $p<0.05$ and beta coefficients were analysed to quantify any significant effects of patient and treatment factors on morphokinetic parameters. Complex relationships between various morphokinetic parameters and specific patient and treatment factors exist rather than any systemic effect. Maternal age was shown to significantly affect t2, t4, tB and tM-tSB. An increase in one year of age results in a decrease in t2 by 0.006 hours (h), t4 by 0.029h, an increase in tB by 0.78h and an increase in tM-tSB by 0.92h. Maternal BMI was shown to affect t2 alone where a one-unit increase BMI resulted in a decrease in t2 by 0.009h. Those embryos created using ICSI (excluding those utilising donor sperm) had significantly different t2, tSB, tB, cc2 and tM-tSB measurements compared to those created using IVF with ICSI derived embryos undergoing t2 0.098h earlier, tSB 1.157h later

and tB 1.510h later. Embryos derived from ICSI also have significantly longer cc2 (by 0.185h) and tM-tSB (by 0.637h). Suppression protocol had no significant effect on any morphokinetic parameter. Morphokinetic analyses are, by their nature, subjective. The investigated confounders were not exhaustive, as paternal factors, such as age, and the dose of gonadotrophins for example, were not considered. In addition, the cohort of embryos available to examine the more rare infertility diagnoses and treatment types were limited thus conclusions must be made tentatively. The findings outline the need for the consideration of confounding factors when assessing an embryo's ability to achieve implantation. Although morphokinetic parameters have been related to embryo viability, it is likely that this will vary dependent on the embryo's origin. These data highlight the need for the development of patient and treatment specific ESAs that have been prospectively validated in appropriate randomised, controlled trials.

7.3.2 Introduction

Time-lapse systems (TLS) are no longer a novel technique for the culturing of human embryos. It is employed by many internationally and has gained a high degree of attention based on little scientific evidence (Armstrong *et al,* 2015). In theory, TLS offer two potential benefits; a highly controlled, undisturbed culture environment and an increased level of detail when analysing the embryos contained within the system. However, a recent Cochrane review concluded that 'there is insufficient evidence of differences in CPR, LBR, MR still birth rate to choose between TLS [time-lapse systems] and standard incubation' (Armstrong *et al,* 2015). It is notoriously difficult for clinics to perform the much-needed randomised controlled trials for a multitude of reasons; funding availability, lack of patient interest and difficulty in the approval process. As a result, many turn to retrospective, observational investigations to determine the relevance and significance of the environment and the information that TLS can provide, of which the pitfalls of such experimental designs have been highlighted (Kirkegaard *et al,* 2016).

What does remain novel about TLS is not their use *per se*, in the simplest form, but how the information gleaned from them is used. Time-lapse systems

can capture images of embryos every five to ten minutes over a period of six days, generating over 700 images per embryo. The wealth of information available to the user regarding one embryo is, undeniably, astronomical but exactly how to use this information is a problem posed and the reason this feature of TLS remains novel.

Morphokinetic data (the timings at which an embryo reaches a developmental milestone) provided by TLS have been identified that correlate with the embryo's ability to create a pregnancy both in humans and animals; the appearance and disappearance of PN and nuclei at each cell stage (Payne *et al,* 1997; Lemmen *et al,* 2008; Scott, 2010; Azzarello *et al,* 2012) the length of time between early cytokineses (Gonzales *et al,* 1995; Ramsing and Cellesen, 2006; Ramsing *et al,* 2007; Lechniak *et al,* 2008; Herrero *et al,* 2011; Meseguer *et al,* 2011; Cruz *et al,* 2012; Hlinka *et al,* 2012) direct one to three cell divisions (Rubio *et al,* 2012), and start times of blastulation (Campbell *et al,* 2013a).

With this information in tow, many pursued the development of models that generate an embryo score known as ESAs. As is well known, ESAs incorporate a set of instructions for the user where, depending on the answers to the questions asked, a score is given that will aid in the selection, or deselection, of embryos in any given cohort. Many ESAs have now been developed and published each using differing outcome parameters, exclusion and inclusion criteria and morphokinetic parameters to define the selection of an embryo (Wong *et al,* 2010; Meseguer *et al,* 2011; Azzarello *et al,* 2012; Cruz *et al,* 2012; Campbell *et al,* 2013a; Chamayou *et al,* 2013; Dal Canto *et al,* 2012; Basile *et al,* 2015). Crucially, a number of these ESAs have been validated externally with varying degrees of success (Kirkegaard *et al*, 2014; Yalcinkaya *et al*, 2014; Freour *et al*, 2015; Barrie *et al,* 2017).

Unfortunately, the lack of control for confounding variables in time-lapse investigations, especially those involving the derivation of ESAs, reduces their transferability and means they are likely to only be applicable to the patients on which, and environment in which, they were derived. This effect of

potential confounders on embryo morphokinetics has been addressed recently where seven timing events were analysed to determine the effects of various potential confounders including maternal age, treatment type, BMI, cumulative gonadotrophin dose and the number of previous attempts (Kirkegaard *et al,* 2016). From this investigation, the authors concluded that a high degree of embryo timing variability can be explained by the patient demographic rather than an embryos' viability when considered as part of a large cohort of embryos.

The investigation presented here sought to determine the effects of a number of patient and treatment parameters on nineteen morphokinetic parameters using a multiple regression analysis methodology. The purpose of this investigation was to echo that previously found, to inform future directions of research, specifically the consideration of embryo origin during the derivation of ESAs, and to highlight that the power of TLS in embryo selection is yet to be exposed.

7.3.3 Materials and methods

Data were obtained from 639 treatment cycles including 2376 embryos cultured in the EmbryoScope® incubators between September 2014 to January 2016.

7.3.3.1 Oocyte retrieval and embryology

All injected oocytes (following ICSI) and fertilised oocytes (following IVF fertilisation check) were placed in individual culture drops of G-TL™ (Vitrolife) and cultured in the EmbryoScope® (Vitrolife).

7.3.3.2 Analysis of time-lapse information

The absolute morphokinetic parameters assessed included time to two-cell (t2), three-cell (t3), four-cell (t4), five-cell (t5), six-cell (t6), seven-cell (t7), eight-cell (t8), nine-cell (t9), time to start of compaction (tM), start of blastulation (tSB) and full blastocyst (tB). The interval morphokinetic parameters assessed included the time between t2 and t3 (cc2), t4 and t5

(cc3), t8 and t9 (cc4), t3 and t4 (s2), t5 to t8 (s3), t9 and tM, tM and tSB, tSB and tB.

7.3.3.3 Outcome measures and statistical analysis

A multiple regression was performed on 2376 embryos to determine the effect on t2, t3, t4, t5, t6, t7, t8, t9, tM, tSB, tB, cc2, cc3, cc4, s2, s3, t9-tM, tM-tSB and tSB-tB of maternal age, maternal BMI, suppression protocol and primary infertility diagnosis. All morphokinetic parameters were classed as continuous, dependent variables. Maternal age and BMI were classed as continuous, independent variables. Treatment type, primary diagnosis and suppression protocol were categorical independent variables. However, because treatment type and infertility diagnosis were polytomous they required the use of a reference category for statistical analysis. The reference category for treatment type was IVF and the reference category for infertility diagnosis was male origin. As was the case for all morphokinetic parameters, linearity was assessed by partial regression plots and a plot of studentised residuals against the predicted values. There was independence of residuals, as assessed by a Durbin-Watson statistic (1.00-2.00). There was homoscedasticity, as assessed by visual inspection of a plot of studentised residuals versus unstandardised predicted values. There was no evidence of multicollinearity, as assessed by tolerance values greater than 0.1. No studentised deleted residuals were excluded from the analysis as they did not have leverage values greater than 0.2 and values for Cook's distance above 1. The assumption of normality was met, as assessed by Q-Q Plot (appendices section 9.5). Results were considered significant at $p<0.05$. Statistical analysis was performed using the statistical package for the social sciences (SPSS) (IBM corporation, 2015) (statistical analysis excerpt in appendices section 9.5).

7.3.4 Results

In total, 2376 embryos from 639 patients were included in this analysis. None of the patient or treatment parameters affected the morphokinetics of embryo development as a whole (Table 17 and 18).

Table 17. Multiple regression analysis results for the effect of maternal age, maternal BMI, suppression protocol, infertility diagnosis and treatment type on absolute morphokinetic parameters.

	t2		t3		t4		t5		t6		t7		t8		t9		tM		tSB		tB	
	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B
Maternal Age	.007*	-.006	.050	-.013	.007*	-.029	.791	-.004	.809	.004	.464	-.020	.152	-.052	.964	.001	.404	-.029	.058	.063	.043*	.078
Maternal BMI	.001*	-.009	.295	-.008	.362	-.012	.622	-.010	.093	-.037	.302	-.033	.267	-0.49	.330	-.036	.305	-.043	.133	-.060	.272	-.052
Suppression	.573	-.012	.613	-.030	.251	-.113	.754	0.47	.971	-.006	.558	-.144	.552	-.199	.625	.136	.577	.179	.843	.060	.229	.429
Infertility Diagnosis																						
Ovarian	.913	-.004	.261	-.111	.866	-.028	.877	-.038	.326	-.269	.352	-.378	.928	.050	.472	.331	.863	-.091	.437	.390	.977	-.017
Uterine	.223	.045	.262	-.119	.958	-.009	.958	.014	.716	-.108	.662	.192	.173	.809	.593	.266	.494	.391	.156	.768	.204	.806
Donor	.027*	-.310	.019*	-.945	.044*	-1.340	.168	-1.388	.161	-1.572	.036*	-3.478	.230	-2.698	.021*	-4.343	.327	-2.121	.238	-2.419	.014*	-5.894
Unexplained	.571	-.019	.968	.004	.432	.123	.230	.285	.558	.155	.705	.148	.564	.306	.485	.310	.375	.454	.157	.685	.254	.647
Endocrine	.103	-.178	.802	.078	.403	.432	.385	.678	.216	1.077	.315	1.293	.220	2.140	.713	.536	.568	.960	.108	2.559	.404	1.557
Secondary	.002*	-.329	.418	-.250	.263	-.572	.746	-.250	.184	-1.143	.156	-1.806	.313	-1.741	.668	-.619	.013*	-4.137	.051	-3.069	.021*	-4.256
Treatment Type																						
ICSI	.001*	-.098	.281	.087	.114	.211	.539	.124	.245	.262	.516	.216	.618	.255	.990	.005	.232	.520	.005*	1.157	.002*	1.510
IMSI	.306	-.074	.377	.184	.421	.277	.830	-.112	.512	-.381	.427	-.682	.501	-.783	.683	.397	.009*	2.938	.073	1.905	.210	1.560
TESE ICSI	.435	-.076	.462	.203	.811	.110	.337	.664	.275	.841	.455	.851	.373	1.378	.726	.453	.576	.831	.050	2.769	.272	1.817
D-IVF	.084	.183	.164	.422	.245	.583	.107	1.222	.514	.552	.090	2.121	.152	2.424	.024*	3.199	.407	1.353	.101	2.535	.007*	4.882
D-ICSI	.014*	.341	.001*	1.304	.084	1.137	.008*	2.650	.033*	2.367	.107	2.642	.248	2.571	.030*	4.036	.941	.160	.206	2.568	.099	3.930

Time to two-cell (t2), three-cell (t3), four-cell (t4), five-cell (t5), six-cell (t6), seven-cell (t7), eight-cell (t8), nine-cell (t9), start of compaction (tM), blastulation (tSB) and time to full blastocyst (tB) are included. P-values (P) and beta coefficients (B) are shown for each parameter. Statistically significant results are indicated by *. A negative beta coefficient indicates a decrease in the parameter for every unit increase in the independent variable. BMI; body mass index. ICSI; intra-cytoplasmic sperm injection. IMSI; intra-cytoplasmic morphologically selected sperm injection. TESE; testicular sperm extraction. D-IVF; donor-IVF. D-ICSI; donor-ICSI.

Table 18. Multiple regression analysis results for the effect of maternal age, maternal BMI, suppression protocol, infertility diagnosis and treatment type on interval morphokinetic parameters

	cc2		cc3		cc4		s2		s3		t9-tM		tM-tSB		tSB-tB	
	P	B	P	B	P	B	P	B	P	B	P	B	P	B	P	B
Patient Age	.285	-.007	.094	.025	.082	.053	.081	-.016	.141	-.048	.348	-.031	<.001*	.092	.454	.016
BMI	.940	.001	.904	.002	.726	.013	.759	-.003	.319	-.039	.847	-.008	.584	-.017	.736	.009
Suppression	.749	-.018	.240	.160	.236	.335	.331	-.083	.410	-.245	.886	.043	.610	-.119	.055	.369
Infertility Diagnosis																
Ovarian	.260	-.107	.962	-.011	.546	.381	.556	.083	.858	.088	.392	-.422	.211	.481	.200	-.407
Uterine	.108	-.164	.924	.023	.279	-.543	.470	.110	.134	.795	.814	.125	.363	.377	.912	.038
Donor	.102	-.635	.958	-.048	.387	-1.645	.494	-.394	.514	-1.309	.271	2.222	.850	-.298	.007*	-3.475
Unexplained	.805	.023	.456	.162	.993	.004	.380	.120	.964	.021	.763	.143	.534	.231	.902	-.038
Endocrine	.394	.256	.730	.246	.277	-1.604	.430	.354	.348	1.462	.787	.424	.190	1.599	.319	-1.002
Secondary	.052	-.579	.648	.322	.441	1.122	.467	-.322	.333	-1.491	.023*	-3.518	.375	1.068	.232	-1.188
Treatment Type																
ICSI	.018*	.185	.636	-.087	.564	-.220	.283	.124	.802	.101	.203	.515	.044*	.637	.175	.353
IMSI	.198	.258	.413	-.389	.230	1.180	.755	.093	.518	-.671	.015*	2.541	.204	-1.033	.606	-.346
TESE ICSI	.296	.279	.380	.555	.479	-.924	.813	-.094	.605	.714	.785	.377	.073	1.938	.285	-.952
D-IVF	.414	.239	.355	.639	.588	.776	.711	.161	.427	1.201	.224	-1.846	.318	1.182	.016*	2.348
D-ICSI	.012*	.963	.096	1.513	.436	1.465	.770	-.167	.968	-.079	.052	-3.876	.121	2.408	.288	1.362

Duration of second cell cycle (cc2; t3-t2), third cell cycle (cc3; t5-t4), fourth cell cycle (cc4; t9-t8), synchrony of the second cell cycle (s2; t3-t4), synchrony of the third cell cycle (s3; t8-t5), time between t9 and tM, time between tM and tSB, time between tSB and tB are included. P-values (P) and beta coefficients (B) are shown for each parameter. Statistically significant results are indicated by *. A negative beta coefficient indicates a decrease in the parameter for every unit increase in the independent variable. BMI; body mass index. ICSI; intra-cytoplasmic sperm injection. IMSI; intra-cytoplasmic morphologically selected sperm injection. TESE; testicular sperm extraction. D-IVF; donor-IVF. D-ICSI; donor-ICSI.

Instead, complex relationships appeared to exist between specific morphokinetic parameters and patient and treatment parameters. Of all assessed parameters, suppression protocol (buserelin or cetrotide) had no significant effect on any morphokinetic parameter. Maternal age was shown to significantly affect t2, t4, tB and tM-tSB. Female patient BMI was shown to affect t2 alone. The assessment on infertility diagnosis revealed an affect on t2, t3, t7, t9, tB and tSB-tB when donor sperm was used (e.g. in a same sex relationship or in severe male factor cases). In addition, and as a reflection of this, when assessing the morphokinetic parameters based on treatment type, t2, t3, t5, t6, t9 and cc2 were affected when ICSI using donor sperm was performed and t9, tB and tSB-tB were affected when IVF using donor sperm was performed. In addition, those embryos created using ICSI (excluding those utilising donor sperm) had significantly different t2, tSB, tB, cc2 and tM-tSB measurements compared to those created using IVF.

Beta coefficients, indicating the amount of change elicited by the dependent variable (morphokinetic parameter) when a one-unit change in the independent variable (patient or treatment characteristics) is made give additional information about the extent to which morphokinetic parameters are affected by patient and treatment characteristics. With regards to maternal age, an increase of one year results in a decrease in t2 by 0.006h, t4 by 0.029h, an increase in tB by 0.78h and an increase in tM-tSB by 0.92h. This result indicates that embryos from younger patients undergo t2 slower than those from older patients however are overall faster than older counterparts. Furthermore, where ICSI treatment has been performed (excluding donor sperm) embryos undergo t2 0.098h earlier, tSB 1.157h later and tB 1.510h later than those undergoing IVF. Embryos derived from ICSI also have significantly longer cc2 (by 0.185h) and tM-tSB (by 0.637h). This result indicates that embryos derived from ICSI undergo the first cleavage of preimplantation embryo development earlier than those undergoing IVF however by the blastocyst stage of development ICSI embryos are overall slower than those derived from IVF.

Baseline characteristics of the patient cohort are shown in Table 19.

Table 19. Baseline patient information for the analysed embryo cohort in the multiple regression analysis.	
Number of embryos	2376
Number of patients	639
Number of cycles	639
Maternal age (mean +/- SD)	32.9 +/- 4.4
Maternal BMI (mean +/- SD)	24.3 +/- 3.7
Suppression protocol (n / %)	
Buserelin	275 / 41%
Cetrotide	364 / 59%
Cause of infertility (n / %)	
Male factor	225 / 35.2%
Ovarian	114 / 17.8%
Uterine	88 / 13.8%
Donor	4 / 0.6%
Unexplained	193 / 30.2%
Endocrine	8 / 1.3%
Secondary	7 / 1.1%
Treatment Type (n / %)	
IVF	343 / 53.7%
ICSI	266 / 41.6%
IMSI	17 / 2.7%
TESE-ICSI	7 / 1.1%
D-IVF	4 / 0.6%
D-ICSI	2 / 0.3%
Number of eggs collected (mean +/- S.D)	14.7 +/- 7.3
Number of embryo transfers	503
Number of embryos transferred	550
Number of positive pregnancy tests (n / BPR)	213 / 42.3%
Number of fhbs (n / IR)	219 / 39.8%
S.D; standard deviation. BMI; body mass index. IVF; *in vitro* fertilisation. ICSI; intra-cytoplasmic sperm injection. IMSI; intra-cytoplasmic morphologically selected sperm injection. TESE; testicular sperm extraction. D-IVF; donor-IVF. D-ICSI; donor-ICSI. BPR; biochemical pregnancy rate (number of positive hCG tests/number of embryo transfers). fhbs; fetal heartbeats. IR; implantation rate (number of fetal hearts/ number of embryos transferred).	

7.3.5 Discussion

The effect of patient and treatment parameters on nineteen morphokinetic parameters was tested using a large group of embryos revealing a number of complex relationships between specific patient and treatment parameters and certain morphokinetic parameters. The analysis presented adds to the results seen by others (Kirkegaard *et al,* 2016) and together with these highlight the presence of confounders when considering morphokinetics.

Firstly, maternal age significantly affected four morphokinetic parameters; t2, t4, tB and tM-tSB demonstrating that embryos from younger patients undergo t2 slower than those from older patients however are, overall, faster. Although the evidence is lacking in the literature regarding the specific relationship between morphokinetic parameters and maternal age, aneuploidy could be used as a proxy. It is well accepted that the rate of aneuploidy increases with maternal age (Franasiak *et al,* 2014) and a particular investigation observed significant differences in blastulation morphokinetic parameters and risk of aneuploidy as determined through trophectoderm biopsy (Campbell *et al,* 2013a). From this investigation, a risk classification model was developed and, although when externally applied lost efficacy (Kirkegaard *et al*, 2014; Yalcinkaya *et al*, 2014; Freour *et al*, 2015; Barrie *et al,* 2017), supports the notion that patient age affects morphokinetic parameters. Differences of up to a 0.1h increase were observed in embryos from older patients; information that could be useful when selecting embryos using ESAs that may not have taken patient age into consideration.

The relationship between maternal BMI and embryo quality is yet to be determined however, there are interesting investigations emerging assessing the composition of follicular fluid from patients with varying BMIs. The effect of BMI seen in this analysis could be due to the requirement for a higher dose of gonadotrophins (Zander-Fox *et al,* 2012), which has been demonstrated to affect an embryo's morphokinetic profile (Kirkegaard *et al,* 2016). The influence of maternal BMI was evident only on t2; a gold standard for embryo viability dating back 20 years (Shoukir *et al,* 1997). Although this effect is not sustained throughout embryo development the association could be a reflection of embryo viability. For every one-unit increase in BMI t2 occurs 0.009h earlier. This effect is not likely to be clinically applicable at this stage and future

research should be directed to determining the effect of extreme BMIs specifically. It has been demonstrated that patients with increased BMI have reduced pregnancy rates compared to normal BMI patients (Zander-Fox *et al,* 2012) thus a clinically relevant effect on a morphokinetic parameter so heavily related to viability such as t2 is not outwith the realms of possibility.

To consider differences in categories in infertility diagnosis the number of treatment cycles in these categories must first be highlighted. For those undergoing treatment using donor sperm the number of treatment cycles accounted for 0.6% of the total number of treatment cycles. Where infertility was secondary to another disorder, seven treatment cycles were included. Nonetheless, significant results were obtained that should be included in the discussion as they may indicate a need for further investigation. At the test site, donor sperm is cryopreserved before use. The freezing of spermatozoa has been shown to affect the integrity of the cells in a variety of ways (reviewed by Di Santo *et al,* 2012). More pertinent to this discussion is the effect cryopreservation of sperm has on the genes required for fertilisation and embryo development (Valcarce *et al,* 2013). It stands to reason that this effect could be exhibited in embryo morphokinetics as well as embryo quality presenting a possible reason differences are seen in various morphokinetic parameters. As well as the use of donor sperm as an infertility diagnosis, infertility as a result of another disorder (secondary) was the only other infertility diagnosis that affected any of the nineteen morphokinetic parameters, specifically t2, tM, tB and t9-tM. This group included those with, or having previously suffered from cervical or breast cancer, patients with diabetes, a genetic condition, and Hodgkin's disease or Rokatinsky syndrome. This group included just 20 embryos from seven patients therefore conclusions are speculative and the information is not necessarily immediately clinically relevant. However, these data support the overall aim of the investigation; that embryo development could be affected by subtle demographical differences and in order to support this finding, larger numbers of embryos need to be assessed before this is considered when developing ESAs.

The effect of method of insemination on an embryos morphokinetic profile has been demonstrated previously (Bodri *et al,* 2015; Lemmen *et al,* 2008) however, many used an arbitrary time for t0, the most popular of which is time of insemination or injection.

The use of these time points as t0 is obviously confounding as they are ambiguous and could vary by hours from oocyte to oocyte. In support of this, differences observed in embryo morphokinetics have been shown to disappear when an observable time point is used for t0 (Cruz *et al,* 2013). In the current analysis, time of pronuclear fading was used as t0 therefore any observed differences in treatment type are more reliable than those using time of insemination or injection. In particular those embryos created using ICSI had significantly different t2, tSB, tB, cc2 and tM-tSB measurements when compared to embryos created through IVF. This result is particularly useful as the majority of embryos are created using standard IVF or standard ICSI in an IVF laboratory. These significant differences of up to 1.5h indicate that, at the very least, ESAs should be developed to accommodate differing treatment types even when a definable t0 is used. In agreement with others (Lammers *et al,* 2015), there were no significant differences in morphokinetic parameters when ICSI using testicular retrieved sperm was used. However, a few cautionary notes must be made regarding this result. Firstly, due to the polytomous nature of this group in the analysis this is in comparison to IVF as a fertilisation method whereas other analyses have compared ejaculated sperm with surgically retrieved sperm for use in ICSI (Lammers *et al,* 2015). Secondly, this group only accounted for seven patients and 27 embryos. There must be further investigations into the more rare treatment types to better examine the need for alternative optimum ranges for various morphokinetic parameters to be used in ESAs but this result does highlight that a one-size-fits-all approach is unlikely to be appropriate.

The current analysis does not include other suspected confounders such as dose of gonadotrophins, paternal age or endogenous maternal hormone levels therefore it is by no means exhaustive. The analysis serves to demonstrate the effect of certain patient and treatment parameters in order to inform future areas of research and highlight that variability seen in embryo development is not necessarily an effect of embryo viability, as is suggested by those using morphokinetic parameters to predict an embryos ability to implant. This is also an indication regarding the use of ESAs and their inability to be externally applied with the same efficacy as is observed at the development site. Embryo selection algorithms should be developed with variations in patient and treatment parameters in mind. It is important that any developed ESAs be prospectively applied in RCTs to eliminate known and unknown confounders. It is very possible that

embryologists select the most viable embryo in a cohort in terms of morphology, morphokinetics and chromosomal complement and yet implantation still does not occur. This highlights an obvious confounder that is often overlooked and is likely to only be able to be controlled through RCTs; endometrial receptivity. Embryo development is seemingly affected in subtle ways by a multitude of factors. The formulation of ESAs is not likely to be able to account for the effect of confounders entirely and until such a time that appropriate trials have been completed it may be beneficial to use macro-morphokinetic markers that are less variable and potentially less heavily influenced by confounding factors. In the first instance, these parameters can be used to perform effective deselection of those embryos undergoing abnormal division events such as DC and RC, both shown to significantly reduce implantation potential of embryos (Rubio *et al,* 2012; Athayde Wirka *et al,* 2014; Liu *et al,* 2014; Barrie *et al*, 2017).

7.3.6 Conclusion

This analysis provides a comprehensive account of the effect of confounding factors on an embryos morphokinetic profile. It highlights the subtle nature of embryo development and the need to perform appropriate and robust production and validation of ESAs if they are to be employed to perform embryo selection in an IVF laboratory. Where some of the more rare infertility diagnoses or treatment types are concerned, conclusions should be considered tentative but this analysis provides evidence that further investigations should be carried out to clarify the complex relationships between confounders and morphokinetic parameters. Until the development of ESAs that consider the effect of confounders and that have been prospectively applied in RCTs, other macro-morphokinetic markers should be considered to perform simple but effective deselection using TLS.

7.3.7 Dissemination

The above research was prepared for publication in Human Reproduction. The research was submitted for consideration on 30th January 2017.

CHAPTER 8: General discussion, limitations and conclusions

8.1 Chapter 3 general discussion

Chapter 3 was concerned with analysing fundamental parameters in a culture system; osmolality and pH. These parameters were measured in a series of experiments comparing standard incubation and the EmbryoScope® to determine if the EmbryoScope® could provide a culture environment comparable to standard incubation. These experiments also sought to represent that the use of this TLS could represent a positive step towards the reduction of *in vitro* stress that a preimplantation embryo experiences. From these experiments it was observed that osmolality did not vary significantly between standard and EmbryoScope® incubation when culture media, over the course of 144h, was examined. However, there were significant differences where pH was concerned. As will be delineated, considerable experimental issues were experienced in terms of pH measurement however, when a pH meter was used as well as the CO_2 measurements in the incubators as a proxy for pH, significant differences were found between the two systems indicating that the pH is likely to be much more variable in standard incubation than in an EmbryoScope®. This result provides an answer for the aim of this investigation; to compare the EmbryoScope® to the standard incubation technique employed at the HFC in order to determine if the EmbryoScope® provides a comparably stable culture environment for the embryos contained within it.

There is minimal evidence in the literature regarding any previous experimentation with a similar aim but there are a number of studies assessing a TLS in terms of the undisturbed environment. An assessment using day two embryo morphology as the outcome parameter concluded that embryo quality was not superior in the TLS (Park *et al,* 2015) with others concluding the same (Nakahara *et al,* 2010; Cruz *et al,* 2011; Kirkegaard *et al,* 2012). The effect of the microenvironment on human embryo development has been documented where the ‘good quality’ embryo rate and blastocyst formation rate (BFR) were compared between a standard, front-loading incubator and a mini, top-loading incubator after a five second door opening/closing (n=348 zygotes). The good embryo and BFR were significantly higher in the mini, top-

loading incubator (39.5% vs. 28.4% and 15.1% vs. 7.8%). In addition, the recovery time for the front-load incubator was significantly longer than the top-loading incubator (31.5 ± 2.9 minutes vs. 4.9 ± 0.5 minutes) and the authors attribute this to the large gas-exchange volume when the door was opened as well as the considerable cold gas infusion required to replace that which was lost (Fujiwara *et al,* 2007). Further to this, in an assessment of 285 IVF/ICSI cycles it was found that the total BFR, proportion of good blastocysts and number of cryopreserved blastocysts per patient was significantly lower in cycles where embryos were assessed everyday for six days as opposed to four times over six days. Although there was no difference in implantation rate (IR), the authors concluded that a reduction in the observation frequency of embryos outside of the incubator could enhance embryo quality and BFR (Zhang *et al,* 2010).

It would be reasonable to suggest that one of the most likely contributing factors to any increase in embryo quality or pregnancy rates in an undisturbed environment is a consistent culture temperature. The human body is homeostatic, operating at 37°C and it can be assumed that, *in vivo*, the developing embryo is regulated under the homeostasis of the host as with protection against pathogens, for example. *In vitro*, the culture environment is responsible for maintaining a temperature that would be experienced *in vivo* as consistently as possible, thus mimicking *in vivo* homeostasis. Based on this, and that provided from previously successful cell culture, human embryos were cultured at 37°C. There is an abundance of evidence regarding the detrimental effects a reduction in temperature has on human oocytes (Zenzes *et al,* 2001; Sathananthan *et al,* 1988; Pickering *et al,* 1990) however, little available information regarding human embryos, for obvious reasons. Nonetheless, logically, such a drastic effect on oocyte ultrastructure is likely to persist into embryonic development therefore, it is crucial to maintain a 37°C environment *in vitro*. This is often achieved through the use of heated equipment in the laboratory such as tube warmers and, importantly, heated observation stages. However, when embryos are removed from their culture environment the temperature is likely to fall immediately, due to the ambient temperature upon removal.

Shortly after the first live birth following *in vitro* fertilisation, it was suggested that the female reproductive tract had a lower basal temperature than the rest of the body and culture at 37°C may not be appropriate (Grinstead *et al,* 1980; Hunter and Nichol,

1986; Grinstead *et al,* 1985). Two investigations of particular interest assessed the pregnancy rates in humans following embryo culture at reduced temperatures. A retrospective analysis using 209 patients allocated embryos to one of two incubators, A or B. This allocation was performed based on workload and simply reflected an operational need within the laboratory. Temperature measurements were taken over the course of culture and A was found to be below 37°C for 47% of the measurements compared to 20% for B. The actual mean temperature difference between A and B was 0.07°C while media pH and CO_2 levels were similar. The CPR for incubator A was 61% compared to 42% for incubator B (p=0.009). However the proportion of 'excellent' transfers (no blood or mucus on the catheter) was higher in those cultured in incubator A. All other distributions including patient age, male and female risk factors, per cent ICSI, embryo quality and number of embryos transferred did not differ between the two groups (Higdon *et al,* 2006). This experiment assessed the chance of pregnancy in operationally different temperatures rather than setting the temperatures deliberately lower. In a further investigation, the culture temperature was investigated by randomising 52 couples' oocytes (805 in total) into either an incubator set to 37°C or an incubator set to 36°C. This investigation resulted in a higher proportion of blastocysts in the 37°C group (48.4% vs. 41.2%) however all other outcome parameters were comparable; fertilisation rate, aneuploidy rate and IR (Hong *et al,* 2014). These results indicate that the cumulative pregnancy rate is augmented if embryo culture is carried out at 37°C which is a contradiction to that originally thought in the 1980's. It is clear that further evidence is required to determine if embryo culture at less than 37°C could be beneficial however the experiments thus far highlight that embryo development can be affected by the *in vitro* culture temperature.

A caveat of experiments regarding temperature is firstly the difficulties in measuring temperature accurately as most measuring devices have a tolerance of ±1°C and often drift out of calibration over time (Higdon *et al,* 2008). Secondly, by virtue of incubator use, it is likely that many may already be culturing embryos lower than 37°C. When a TLS is considered, there is substantial difficulty in accurate measurement of the temperature of culture media in the culture dishes. This is primarily due to the dishes themselves (requirement of a lid and placing the probe through the lid and fixing in an appropriate position) and the electronic mechanisms within the incubator creating difficulties for the wires used in temperature measurement. Although pH and

osmolality represent fundamental embryo culture measurements, temperature measurement is also imperative. It is likely that the temperature in a TLS is measurably more stable than that of a standard incubator and the determination of this through feasible scientific experimentation should be considered for future areas of research.

The measurements made in this research also presented technical difficulties. Due to the number of EmbryoSlides® required to perform the osmolality analyses, an entire EmbryoScope® instrument was dedicated to this experiment. However, due to the reduced space within the working IVF laboratory and the demand on standard incubators at particular time points in a treatment pathway, the experimental dishes contained within the standard incubator were cultured alongside treatment dishes not for use within the experiment protocol. This therefore meant that the standard incubator door was opened considerably more times when compared to the EmbryoScope®. It appears that this did not affect the osmolality readings for the standard incubator, as there was no difference in the osmolality over the culture period in either replicate. Conversely, it was unknown whether the osmolality of culture media within the EmbryoScope® would have varied more if the incubator had been subject to the same number of door openings during normal working conditions. To try and resolve this issue, a trace was taken from the same EmbryoScope® but from a normal working day to quantify the number of door openings and compare it to those during the experimental period. From the trace taken, the EmbryoScope® door was opened ten times which was comparable to the twelve times it was opened during the experimental period. It is reasonable to assume that, as the EmbryoScope® was subject to a similar number of door openings during the experimental period as it would have been under normal working conditions, the osmolality measurements are reflective of those that would be obtained from culture media contained in dishes for clinical use. This same consideration must be made when assessing the pH results.

A further difficulty that the authors encountered was the measurement of pH itself. It has been concluded by some that there is ‘no point’ in measuring pH routinely (Mortimer and Mortimer, 2004) due to its direct dependence on CO_2 and the ability to measure CO_2 easily. pH measurements are often confirmed by two or three methods to ensure accurate sampling owing to the difficulty in its measurement. Once a dish is removed from the incubator the pH drifts immediately and within two minutes has

changed significantly. This introduces difficulty for accurate sampling and measurement. Initially, the measurement of pH using a blood-gas analyser was attempted however, the instrument did not respond to the volumes (25µl) and type of sample (culture media) that could be provided. Failing this, the authors attempted measurement of the pH using Clinitek Status™ device (Bayer HealthCare) routinely used to perform urine panels but with the capacity to detect pH. This instrument was able to take a measurement and provide a pH value but it was not sensitive enough to detect minor changes in pH needed for the proposed experiment.

For these reasons (lack of an appropriate measuring device for sampling of culture media and the inaccuracy that sampling culture media brings) *in situ* measurements of pH (i.e. culture media in a culture dish) were not made and an *in situ* pH meter was used instead. This overcame the difficulty of obtaining results, let alone accurate ones, but also brought limitations to the extrapolation of results to the culture conditions an EmbryoScope® or standard incubator provides. The *in situ* pH meter constitutes a small well that can be filled with culture media that is exposed to the pH probe. The media can be covered with oil to prevent evaporation, and thus fluctuations in pH, and the probe can be placed anywhere within the incubator allowing the data logger to sit outside the incubator. In the standard incubator, the probe was placed on a shelf where a culture dish would normally sit and so measurements were reflective of embryos cultured in this environment. However, within the EmbryoScope®, due to the size of the probe and the movement of the dish tray within the incubator, the probe had to be placed below the dish tray just within the door of the incubator. The position reflected where dishes would be when the door was to be opened but was not entirely accurate in terms of dish placement and movement within the incubator. This is unlikely, however, to have caused a significant variation, as the incubator should maintain consistent gas levels throughout the chamber. The sensitivity of the CO_2 probes inside the EmbryoScope® and the standard systems should be taken into account. The traces taken were compared to one another yet different probes are likely to be used to detect the gas levels in each system. Although this may not pose a substantial issue it is still an obvious difference between the systems and therefore using the traces to determine the pH should be considered as an adjunct to the measurements using the pH meter; although not flawless themselves.

There is also a single limitation of this research that was unavoidable but would be relatively easily rectified. One replicate result was performed in the osmolality experiments. To perform any more than this would have incurred further consumable costs that were not available to the researchers. The lack of replicates is reflected in the standard deviations of the results; 11.81 and 11.93. Although they are similar, and therefore indicate a similar breadth of variation in the two groups, they are also large which represents an increased range of values. If triplicates were to be performed, should the results mirror those already attained, a higher number of results are likely to cluster around the mean and thus reduce the standard deviation.

This research serves to demonstrate the stability of the EmbryoScope® in terms of pH and osmolality and provides a platform for future developments, which should focus on other fundamental *in vitro* culture parameters such as temperature.

8.2 Chapter 4 general discussion

Having established that the EmbryoScope® incubator provides a comparable, if not superior, culture environment in terms of fundamental culture conditions, pH and osmolality, focus was then brought to the treatment outcomes. This chapter was concerned with comparing the success rates in the form of CPR, IR, LBR and MR between standard incubation employed at the HFC and the EmbryoScope® using a strict matched-pair analysis design. A total of 728 treatment cycles were assessed and the CPR, IR and LBR were found to be significantly higher in those embryos cultured in the EmbryoScope®. The MR, although reduced in those embryos cultured in the EmbryoScope®, was not significantly different between the two systems.

This research represents a robust, retrospective design and the results indicate a clear benefit of culturing embryos in a TLS. Nonetheless, a limitation is it's retrospective nature and the need to perform the gold standard of research methodologies in the form of an RCT. A Cochrane review retrieved 33 potential articles that assessed TLS for improvements in success rates (Armstrong *et al*, 2015) however of the 33, only three were eligible for scrutiny (Kahraman *et al*, 2013; Kovacs *et al*, 2013; Rubio *et al*, 2014). The review highlighted that further RCTs were required to draw conclusions regarding the improved success rates some enjoy when utilising a TLS. The results

attained here may indeed be observed if an RCT were to be performed at the study site however, a restriction for a centres ability to perform RCTs on this technology is it's widespread, successful use. It would have been ideal to control for other confounding factors in this analysis such as primary infertility diagnosis and stimulation regimen. However, due to the limited amount of data relating to patients cultured in standard and TLS within a sensible time period, due to the switch over at the study site to 100% TLS culture, if other suspected confounders had been controlled for the data set would have significantly reduced and it may have been necessary to loosen the matching on other parameters such as the number of oocytes collected or maternal age. It is with an RCT that only the true control of confounders can be achieved.

The future of this area of research would be to perform an RCT using three arms; standard incubation, time-lapse incubation without annotation capabilities and time-lapse incubation with annotation capabilities. This would build on that presented here and test the hypothesis that time-lapse annotations contribute to the benefits of utilising an incubator that is time-lapse enabled. This is, in part, addressed in the research presented. The selection of embryos cultured in a standard incubator is performed using a national grading scheme however those in TLS receive the added benefit of an ESA. This means that the group cultured in TLS received an enhanced method of embryo selection potentially leading to a higher chance of choosing the most viable embryo when compared to standard incubation. Some could argue this as a limitation to the research design however, it represents an analysis of TLS as it was intended to be used clinically; as an incubator as well as an embryo selection method.

The authors believe that the study design used is a valid one that provides further reassurance of, at the very least, the lack of harmful effects of the use of TLS for human embryo culture demonstrated through enhanced CPR, IR and LBR.

8.3 Chapter 5 general discussion

The following chapters were concerned with a deeper interrogation of the annotation aspect of the EmbryoScope® in confidence that the EmbryoScope® as a basic incubator provided comparable if not superior environmental stability. To set the premise for the basis of a large part of the research it was first necessary to investigate

the efficacy of available ESAs to determine if the further proposed research was justifiable. Although the lack of transferability of pre-constructed ESAs had already been demonstrated (Kirkegaard *et al*, 2014; Yalcinkaya *et al*, 2014) the concomitant assessment of multiple ESAs had not yet been performed. In this chapter, the efficacy of six published ESAs to predict implantation was examined using 977 known implantation embryos. The efficacy was demonstrated through the sensitivity, specificity, PPV, NPV, AUC and LR. The aim of this chapter was to demonstrate that these ESAs lost their clinical applicability when applied in an external setting i.e. another fertility centre. This was achieved through the results gained where none of the tested ESAs reached an AUC higher than 0.6; an indication of a lack of predictive capabilities.

The rationale for the validation of the selected six ESAs was to demonstrate the lack of transferability of these ESAs to an external setting. Their selection was based on their potential popularity in the field as well as being from established research groups. The unit-specific generation of ESAs appears to have slowed to be replaced with a focus on commercial generation of ESAs presumably due to their [commercial entities] accessibility to extremely large multicentre datasets. The development of such ESAs has a clear advantage; they will be developed on a large group of heterogeneous embryos indicating that any resulting ESA is likely to be applicable to a heterogeneous group of patients. However, the research presented in this chapter provides evidence that externally derived ESAs cannot be applied with the same efficacy outside of the development and validation site; a limitation that may persist even with larger datasets.

This research is retrospective however, the ideal study design for the aim of this research (to demonstrate externally derived ESAs' dimished transferability) is the randomisation of patients to one of six groups where embryo selection would be performed using one of six ESAs only. This methodology has not been employed thus far, most likely due to the belief that externally derived ESAs are relatively transferrable. However, following this initial retrospective analysis, it is likely that this is not the case. Wherever a new method for embryo selection is to be introduced clinically, a prospective trial should be considered. Certainly, any developed ESAs as a result of the research presented here will be prospectively applied in RCTs before their full clinical use.

A further limitation of the research presented in Chapter 5 is that all embryos that had the six ESAs applied to them had been selected using an internal ESA. This internal ESA used similar morphokinetic parameters to one analysed (Cruz et al, 2012). It was highlighted to the authors that this may cause bias in the results obtained and so a brief analysis was performed to determine if the proportion of embryos obtained in each category in the original manuscript for the ESA in question differed from that obtained in the research presented here. From this, it was observed that the proportions did not differ providing reassurance that any bias created from the use of similar morphokinetic parameters in the selection of embryos used in this analysis was minimal. However, this does reiterate the need for externally derived ESAs to be applied in a prospective, randomised manner to eliminate all bias where possible.

8.4 Chapter 6 general discussion

After gathering sufficient data to support the notion that externally derived ESAs lose their clinical applicability when employed at the HFC, the next research aim was to develop improved ways of selecting embryos. The development of patient, treatment and environment specific ESAs is a complex and imperfect process therefore, to utilise the information that the EmbryoScope® provided without creating complicated ESAs, macro-morphokinetic parameters were assessed in the form of abnormal embryonic phenotypes (including CC, RC, AC, DC and CL). These macro-morphokinetic parameters are easily identified, annotated and analysed and appear to be less influenced by patient and treatment parameters than absolute or interval morphokinetic parameters. This research analysed 15,819 embryos to determine the incidence and IR of five of the most commonly visualised abnormal division events; CC, DC, AC, CL and RC. A number of these phenomena have been documented previously (Athayde-Wirka *et al*, 2014; Rubio *et al*, 2012; Liu *et al*, 2014; Hickman *et al*, 2012) and reports demonstrate a reduction in an embryo's IR if they were to exhibit these abnormal division events. The same was observed in this research where the IR of certain abnormal division events was 0%. Owing to the sensitive nature of this field and with the knowledge that there is evidence to suggest that the chance of success is reduced with embryos undergoing an abnormal division event, it is difficult to design a more robust methodology to test the hypothesis posed here. As such, the results of this

chapter will inform methods for embryo deselection at the test site where embryos seen to undergo one or more of the abnormal division events should be avoided for embryo transfer where possible. Importantly, this research also allows appropriate management of patient expectations in cases where only embryos that have undergone an abnormal division event are available to transfer. Finally, a novel classification system of one of the abnormal division events (DC) was developed where a scale of severity is suggested that again, can be used clinically to aid in embryo selection and manage patient expectations. It may be the case that, with time, more embryonic phenomena will be revealed that need to be considered in the same manner. It may be prudent, for example, to concentrate future research efforts towards the examination of multinucleation at the cell stages and an analysis of those embryos that undergo more than one abnormal division event.

The main limitation of the research regarding deselection criteria and the incidence and implantation potential of embryos undergoing abnormal division events is the limited number of embryos on which conclusions can be drawn. In some of the categories, the number of embryos transferred was as low as two. There is an obvious, unavoidable, reason for this; they are abnormally developing embryos and if the patient has others then they are likely to be chosen over any undergoing an abnormal cleavage event. However, the limitation remains. It would be impossible to eliminate this limitation by way of a change of study design as there is no available alternative. A prospective study design would not be possible in this case for obvious ethical reasons. The study site has, most likely, the largest dataset of embryos cultured in TLS in the UK therefore, as far as single site analyses are concerned, it would be difficult for others to achieve numbers to draw more definitive conclusions than that of the research presented in this chapter.

8.5 Chapter 7 general discussion

Chapter 7 was concerned with determining if patient, treatment and environmental factors affect an embryos morphokinetic profile. Where the assessment of the three culture media is concerned there are a number of limitations that should be highlighted. Firstly, the culture system for each media was not used from the point of oocyte collection. All oocytes for this analysis were cultured in G-IVF Plus™ or G-TL™ up until

the point of time-lapse culture (i.e. following ICSI or IVF fertilisation check). This could introduce a bias towards the control culture media (G-TL™) and should be noted when assessing the results of the analysis. In addition, if a patient achieved an unequal amount of oocytes/embryos (i.e. not a multiple of three) then additional oocytes/embryos would be placed in G-TL™ (if a one surplus) and then SAGE 1-Step™ (if two surplus). The justification for this methodology was that, in order to perform robust statistical analysis, each embryo would have to have a match in each culture media. In addition, SAGE 1-Step™ was the closest of the two to the control media in terms of constituents as CSC is based on KSOM formula optimised for mouse embryos and G-TL™ and SAGE 1-Step™ were developed on the basis of concentrations of nutrients in the human fallopian tube (Gardner *et al*, 1996; Morbeck *et al*, 2017). This meant that CSC was naturally at a disadvantage in terms of the number of oocytes/embryos cultured within it. However, due to the statistical test used (Friedman's two-way analysis of variance by ranks) and the need to analyse like-for-like embryos, this bias was removed when considering the effects on morphokinetic parameters.

This chapter also provides an account of the susceptible nature of embryo morphokinetics and, after determining that the TLS (the EmbryoScope®) is likely to be superior in terms of incubation, success rates and existing methods for embryo selection are lacking clinical applicability as well as highlighting certain, appropriate deselection criteria, this chapter concludes the whole research study suitably. This chapter constituted two parts; firstly a sibling oocyte study where a patients oocytes/embryos were randomised between three commercially available culture media; second, a regression analysis was performed assessing how maternal age, maternal BMI, suppression protocol, infertility diagnosis and treatment type influence an embryos morphokinetic profile. The results of these investigations revealed that embryos are indeed affected, to differing levels, by patient, treatment and environmental factors thus supporting the hypothesis that specific ESAs should be developed to accommodate these confounders. Once developed, specific ESAs should be tested clinically, following retrospective validation, in the form of RCTs; it is in this where the power of time-lapse imaging can be discovered.

The first obvious limitation of this analysis was the reduced numbers of embryos available from patients with the more rare treatment types and infertility diagnoses assessed in the regression analysis. This research highlights that some treatment types (such as the use of donor sperm) could considerably alter the morphokinetic profile of embryos therefore any developed ESAs should take this into consideration. However, because the number of treatment cycles that are performed that use donor gametes is reduced at the study site, it is difficult to draw robust conclusions. It does however, emphasise the need to continue the research into these less common treatment types and infertility diagnoses to better understand their effects on an embryos morphokinetic profile. It would take a substantial amount of time to collate data of a suitable sample size to be able to investigate this with any reliability, certainly at the study site, nonetheless, this research should provide a springboard for others to investigate a similar relationship; perhaps most suitable to centres that perform a high number of treatment cycles utilising donor gametes. There are numerous infertility diagnoses that are unlikely to vary in volume between centres, for example. those that are secondary to other disorders/diseases such as Rokatinsky syndrome, breast/cervical cancer or diabetes. Although significant effects on morphokinetic parameters were found when considering this infertility diagnosis, the results must be taken, universally, tentatively. When considering these results in the development of ESAs it may be possible to apply ESAs to these patient groups as well as the deselection criteria described in Chapter 6 to aid in embryo selection and, as always, as an adjunct to standard morphology assessments.

8.6 Overall limitations

Perhaps the most prominent limitation of the presented research is the subjective nature of annotations. This project required the manual ‘annotation’ of all embryos used for data analysis. Upon commencement, the researchers had to decide whether one person should perform these subjective measurements providing a reduced amount of data with a high level of validity or should many people perform these annotations to provide a high volume of data with a reduced validity? In consideration of this question, although the validity of the measurements may be compromised, this would be minimal with the correct training as well as continual quality control and assurance. An internal scheme was introduced and maintained throughout the project to monitor the embryo

annotations and rectify any unusual responses. In short, this scheme involved intense, supervised training given by the researcher to all members of the scientific team at the study site. Following the training, each 'annotator' was required to annotate one embryo per week with the researcher confirming the annotations made. Each 'annotator' was then enrolled onto the monthly quality assurance program involving the full annotation of three embryos of the researchers choosing.

There are, of course, inherent biases from this process. Firstly, as the researcher provided all training, the aim of this training (all 'annotators' being consistent) creates a reduction in the transferability of any developed algorithms. It would not be possible for the researcher to provide training to whomever utilises the produced ESAs but as the annotation consensus definitions (Ciray *et al*, 2014) were used throughout the research, as long as adopting centres also adhered to these consensus definitions, the results should be largely transferable. The overriding consideration that the researcher faced is the means by which any developed ESAs from this investigation would be used; they are intended for use in multiple, international clinics by many users. It stands to reason that the *use* of these ESAs should inform their development.

A further limitation of the presented research is that it considers the assessment of one form of TLS only; the EmbryoScope®. Although, arguably, the most popular of the TLS, others have merits. Currently, to our knowledge, there are four other variations that are commercially available; Eeva™ (Merck Serono, Germany), Primo Vision™ (Vitrolife, Sweden), Miri® Time-Lapse Incubator (Esco Medical ApS, Denmark), and Geri® (Merck KGaA, Germany). The two former variations are scopes that are fitted within a standard incubation system. This would therefore indicate that potentially the initial stage of this research would not be applicable, however success rates may still vary. The latter two are stand-alone incubators similar to the EmbryoScope® and so may offer more stable culture conditions as in Chapter 3 of the presented research. However, where annotations are concerned, some of these systems have crucial, potentially beneficial, differences. Firstly, the Eeva™ system uses dark-field illumination to allow intelligent software to track individual cells and record electronically when a division event has occurred. This function is to facilitate the production of a score from a programmed ESA that the user does not have access to. Essentially, the Eeva™ system performs embryo selection without embryologist involvement. This is the only

system to have this facility, which has been shown to have a level of efficacy in predicting the formation of a blastocyst rather than IR (Conaghan *et al*, 2013; Diamond *et al*, 2015). Other TLS do not differ significantly from the EmbryoScope® except that both Miri® and Geri® boast individual culture compartments to further stabilise the culture conditions for dishes contained within the system other than the one that is being removed or entered.

A final general limitation to the research involving time-lapse imaging is the definition of time-zero. Originally, time-zero was assigned to the time of injection or insemination of the oocytes. However, it was quickly realised that the time of insemination/injection represented an arbitrary point in preimplantation development owing to the fact that it is impossible to know the exact time that a sperm enters the oocyte in the those embryos created using IVF. The sperm could enter the oocyte at any point in approximately 16h (time of insemination to time of fertilisation check) thus indicating that embryo development (cleavage) would begin sooner in those where the sperm entered earlier. Although much more likely to happen sooner than 16hpi, even a difference of two hours between oocytes would cause disparity in results and a reduction in the efficacy of any applied ESAs. Regarding embryos that were created using ICSI, it is known when the sperm enters each oocyte however, none of the commercially available TLS have a function to input a different time of injection for each oocyte. Although this would be a more specific time point, it still introduces a level of ambiguity, as it is likely that the full integration of the sperm would take different amounts of time for each oocyte and sperm combination. Absolute timings that were visible during time-lapse incubation, the most popular of which is pronuclear fading (Liu et al, 2015) then became favourable. When using this parameter, relationships between certain morphokinetic parameters and embryo viability as well as differences in these between treatment types were no longer apparent (Cruz *et al*, 2013; Liu *et al*, 2015). Especially when some of the observed differences in the described research here are subtle, the use of a consistent time zero, such as pronuclear fading, is imperative. In addition to the use of an arbitrary time-zero, another difference in the literature is the time between image acquisitions. As shown in Table 11 where the differences in the publications regarding the external application of ESAs are listed, many used different image acquisition lengths. Again, when the differences detailed in the research here are subtle, they could be exacerbated by differences in image acquisition timings.

8.7 Conclusions of thesis

The aims of the research presented were firstly to compare the EmbryoScope® (a time-lapse enabled incubator) to standard incubation employed at the HFC in terms of the culture environment measured in the form of pH, osmolality and treatment success rates; CPR, IR, LBR and MR. Secondly, the research aimed to determine if the information that a TLS provides can contribute to effective embryo selection in an IVF laboratory in the form of patient, treatment and environment specific ESAs. This aim was achieved by first, assessing if existing published ESAs could be reliably applied to an external cohort of embryos. Second, by determining deselection criteria from a maximised dataset and third, by examining the effects of patient, treatment and environment specific parameters on an embryos morphokinetic profile.

In the first instance, it can be concluded that the EmbryoScope® provides a stable culture environment comparable, and in some instances (pH, CPR, LBR) superior to that of a standard incubator employed at the study site. Regarding embryo annotation using TLS, it can be concluded that externally derived ESAs lose their clinical effectiveness when applied to an independent cohort of known implantation embryos. It can also be concluded that effective deselection criteria can be identified that indicate an embryos reduced ability to implant. Finally, patient, treatment and environmental factors have been identified using robust methodology that affect an embryo's morphokinetic profile thus indicating that future research should focus on the development of specific ESAs after which prospective application should be considered.

The research study conducted represents novel work in this area in a number of ways. The pH and osmolality had, to the researcher's knowledge, not yet been compared between a TLS and a standard incubation system. Furthermore, the external validation of more than one ESA on a large embryo cohort had not been performed. In addition, the assessment of abnormal division events on a sample size of over 15,000 had not yet been achieved. Finally, a sibling oocyte investigation on three commercially available culture media assessing the effects on morphokinetics past the five-cell stage using human embryos had also not been attempted.

Overall, the current research holds clinical relevance and it is the opinion of the researcher that the true benefits of TLS are yet to be unlocked. It is in the form of specific embryo selection methods that the real benefits of a TLS lie. These complex methods of embryo selection should account for variations in patient-demographic as well as treatment type and environmental factors such as culture media. Once developed, these specific ESAs would, ideally, be prospectively applied in a clinical setting to evaluate their effectiveness and impact on the outcome of an ART treatment cycle. It is with this that the efficacy of embryo selection will be increased which will in turn lead to a higher proportion of successful treatment cycles where a TLS is employed.

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Appendices

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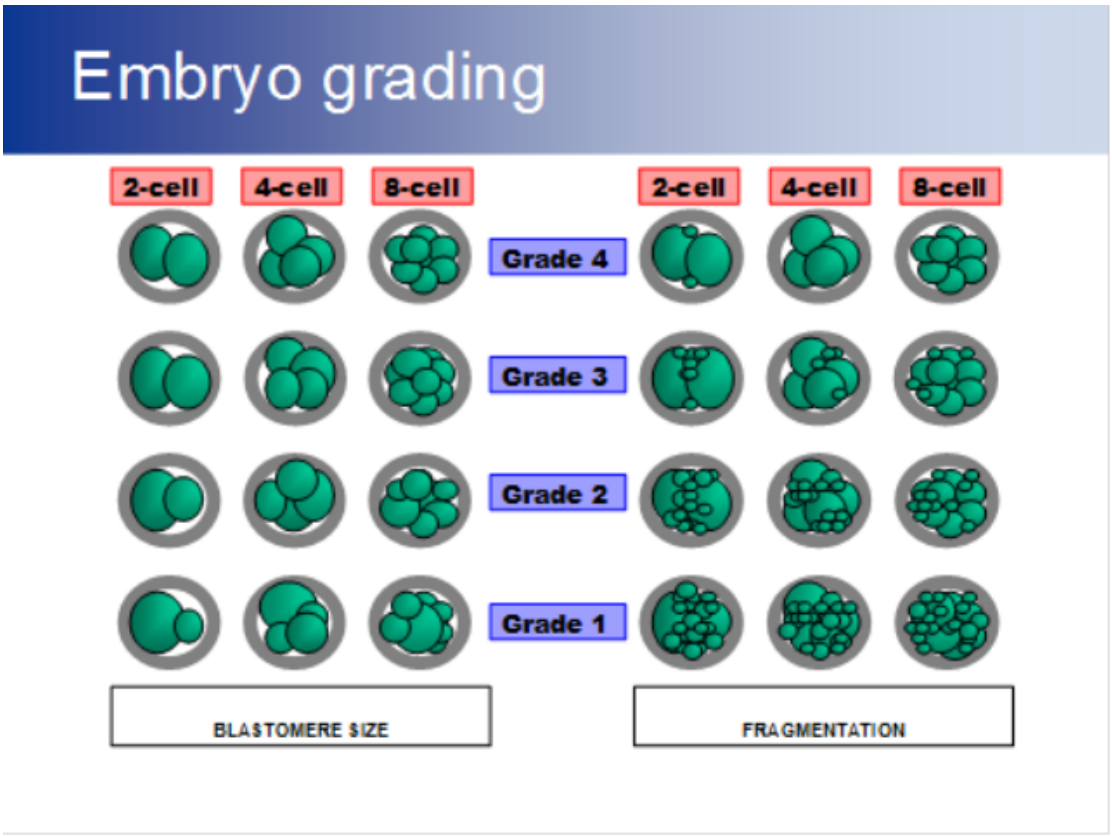
9.1 Appendix 1 - The EmbryoScope® and its use at the Hewitt Fertility Centre

Figure 1. The EmbryoScope® Incubator

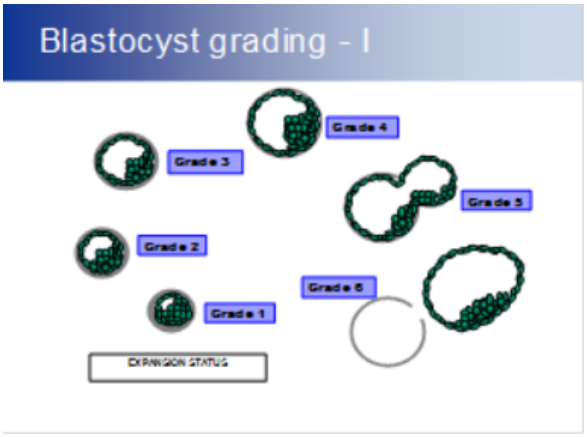
Incubator (right) and the viewer where embryo annotations are made (left).

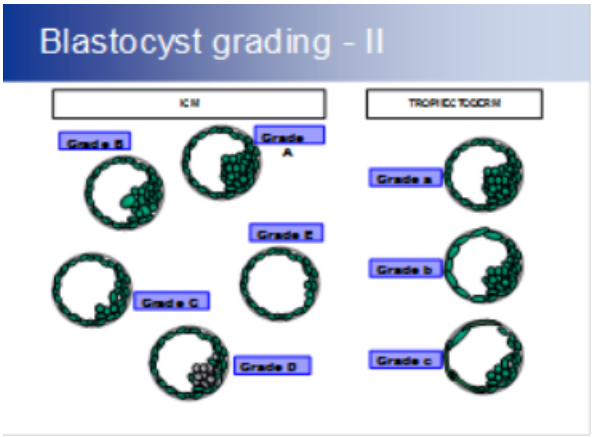
Figure 2. Cleavage stage embryo grading scheme (Cutting *et al*, 2008)



Blastomere number		
Blastomere size	4 = regular, even division 3 = <20% difference (blast diam) 2 = 20-50% difference 1 = >50% difference	*after Hardarson et al 2001*[33]
Fragmentation	4 = <10% frags by volume 3 = 10-20% 2 = 20-50% 1 = >50%	*after van Royen et al 2003*[35]

Figure 3. Blastocyst stage embryo grading scheme (Cutting *et al*, 2008)





Expansion Status	1 = Early blastocyst; blastocoel less than half the volume of the embryo, little or no expansion in overall size, zona pellucida (ZP) still thick
	2 = Blastocyst; blastocoel more than half the volume of the embryo, some expansion in overall size, ZP beginning to thin
	3 = Full blastocyst; blastcoel completely fills the embryo.
	4 = Expanded blastocyst: blastocoel volume now larger than that of the early embryo. ZP very thin
	5= Hatching blastocyst; trophectoderm has started to herniated through the ZP
	6 = Hatched blastocyst; the blastocyst has evacuated the ZP
ICM grading	A = ICM prominent, easily discernible and consisting of many cells, cells compacted and tightly adhered together
	B= Cells less compacted so larger in size, cells loosely adhered together, some individual cells may be visible
	C = Very few cells visible, either compacted or loose, may be difficult to completely distinguish from trophectoderm
	D = Cells of the ICM appear degenerate or necrotic
	E = No ICM cells discernible in any focal plane
Trophectoderm	a = Many small identical cells forming a continuous trophectoderm layer
	b = Fewer, larger cells, may not form a completely continuous layer
	c= Sparse cells, may be very large, very flat or appear degenerate

Figure 4. The published embryo selection algorithm employed at the HFC

The first tier of this algorithm is t5 which must fall between 48.8hpi – 56.6hpi. The second tier is s2 (time between t3 and t4) which must fall between 0h – 0.76h. The final tier is cc2 (time between t2 and t3) and must be less than 11.9h (Meseguer *et al*, 2011).

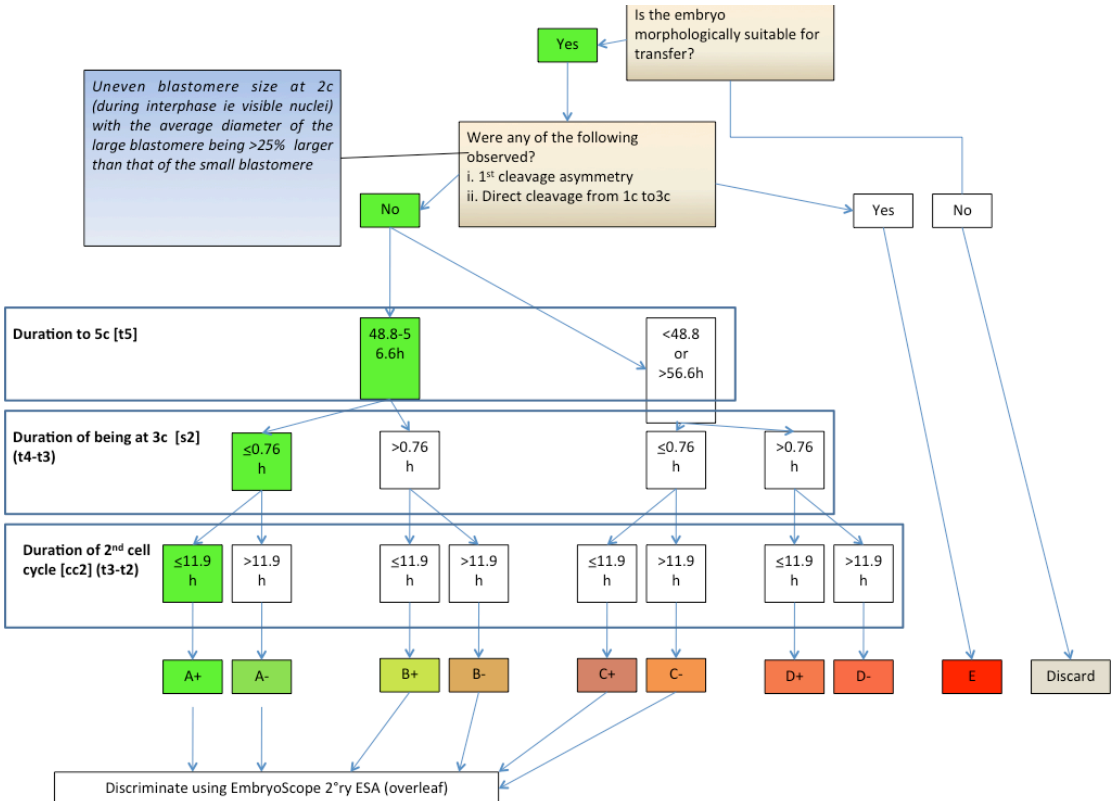


Figure 5. Results of the preliminary validation of the published ESA

Validation performed at the HFC on 173 known implantation embryos. The x-axis represents the score assigned by the algorithm (A+-D-). The y-axis represents the implantation rate as a percentage.

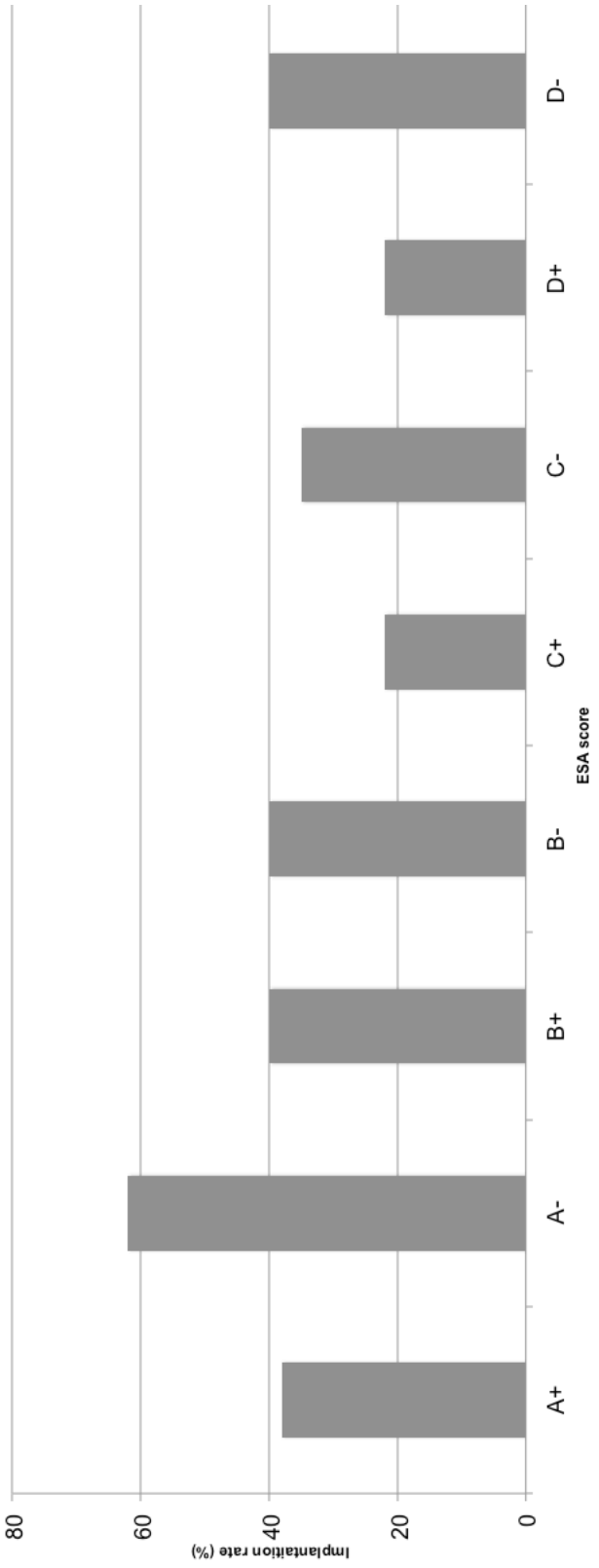


Figure 6. The HFC v1.0 ESA

The primary tier of the HFC v1.0 algorithm is s2 (the time between t3 and t4), which must be between 0-1.7h. The second tier is cc3 (time between t4 and t5) which must be between 9.5h – 11.4h. The final tier is t5 which must be between 43.46h – 51.04hpi.

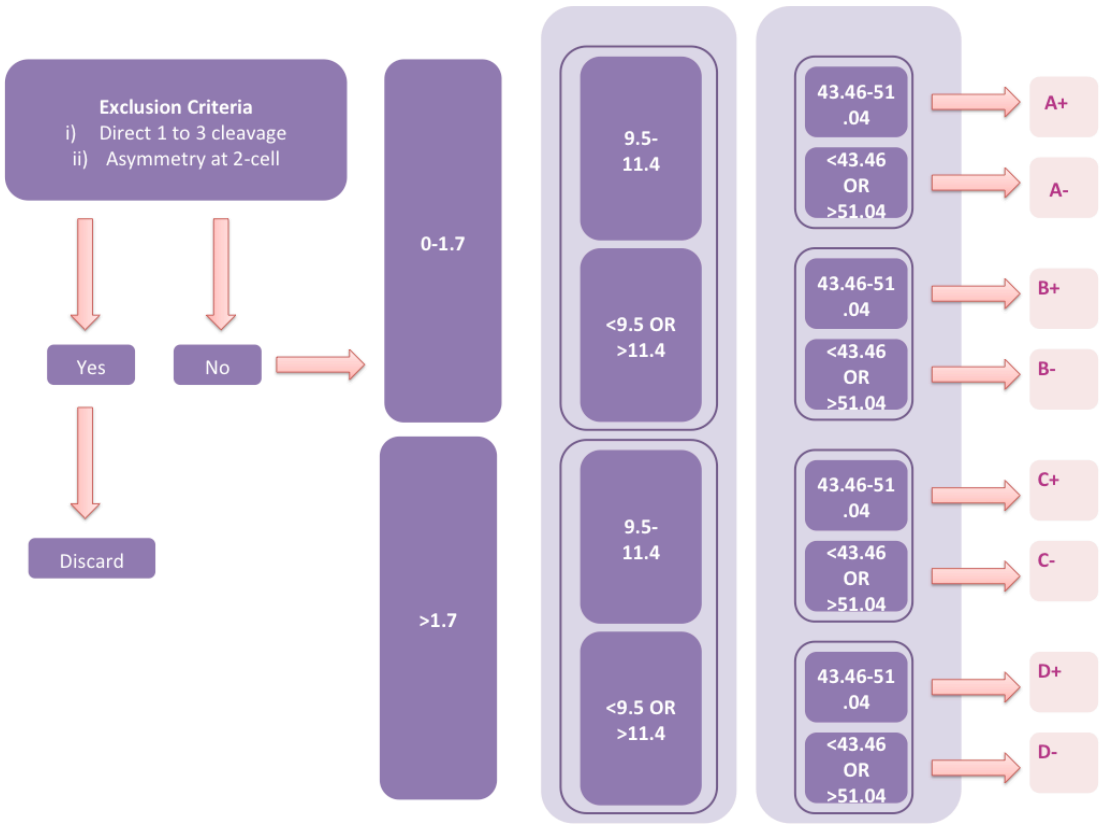


Figure 7. Results of the HFC v1.0 development on 173 known implantation embryos

The x-axis represents the embryo score given by the ESA described in Figure 6. The y-axis represents implantation rate as a percentage. Difference in implantation rates between A+ and D- was statistically significant (p=0.001, chi-square test).

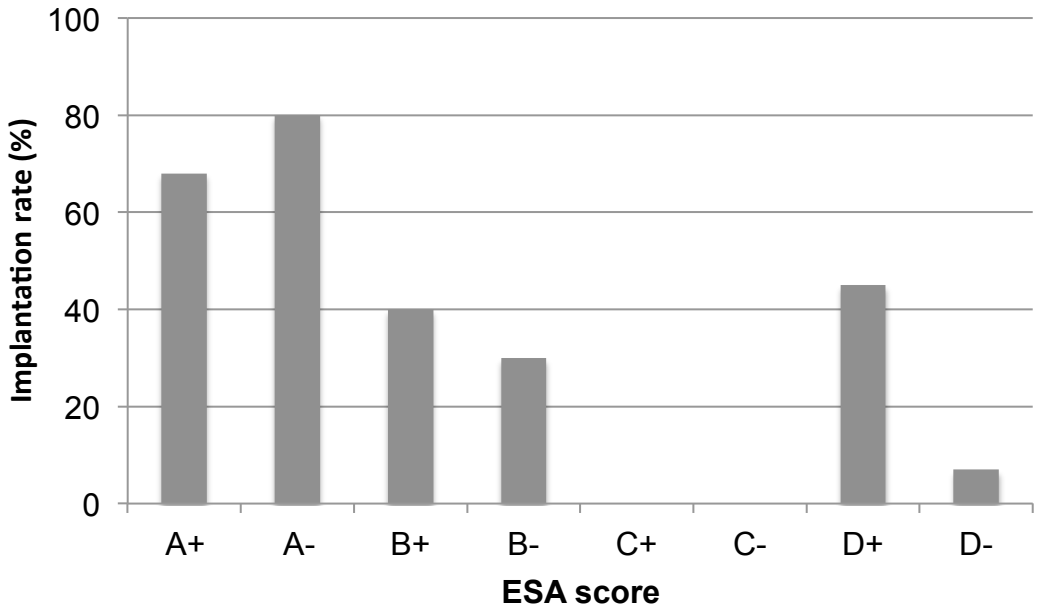


Figure 8. Results of the validation of HFCv1.0 and published ESA

The x-axis represents the score assigned by each ESA and the y-axis represents implantation rate as a percentage.

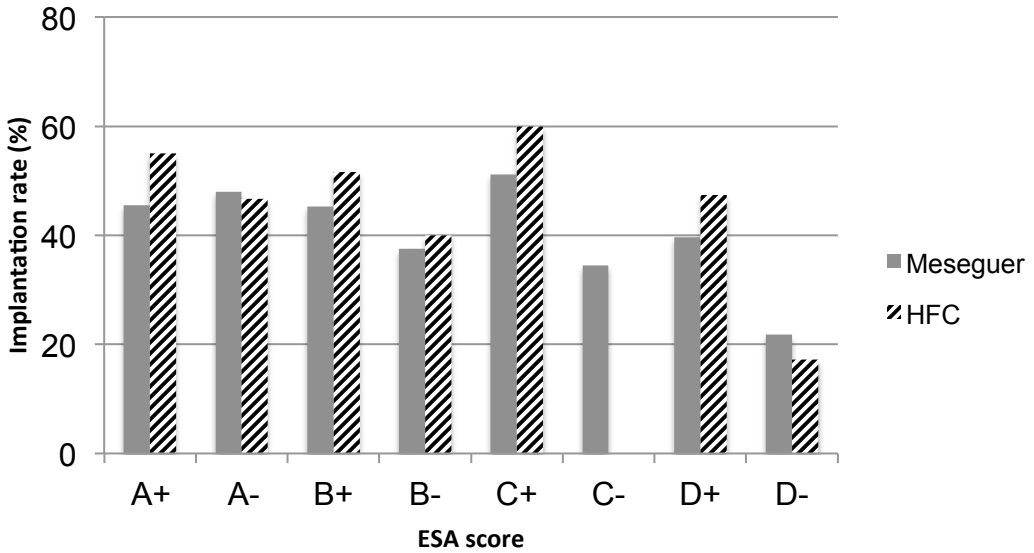


Table 1. The proposed guidelines on the nomenclature and annotation of human embryos using time-lapse imaging (Ciray *et al*, 2014).

Annotation	Definition
tPNa	Time of first visualization of both pronuclei
tPNf/tPNB	Time immediately following last visualization of both pronuclei
t2PB	Time of second polar body extrusion
t1	tPNf to the time immediately before cleavage furrow is visualized for first cell cycle tPNB - (t2-DurFirCyt)
DurFirCyt	Appearance of cleavage furrow to two distinct cells
t2	Time that the first cleavage has occurred (two distinct cells)
cc1	Total time of first cell cycle (tPNf – t2)
t3	Time at which the 3-cell stage is reached
t4	Time at which the 4-cell stage is reached
cc2	Total time of second cell cycle
t5	Time at which the 5-cell stage is reached
t6	Time at which the 6-cell stage is reached
t7	Time at which the 7-cell stage is reached
t8	Time at which the 8-cell stage is reached
cc3	Total time of third cell cycle
t9	Time at which the 9-cell stage is reached
tMx	Time that full compaction has occurred
tMy	Time that embryo is partially compacted
tSB	Time to start of blastulation – first sign of blastocele cavity forming
tByz	Time before zona pellucida thinning
tEyz	Time of initiation of zona pellucida thinning
tB	Time of formation of full blastocyst –blastocele cavity fills embryo, ICM/TE distinguishable, no more than 10% increase in outer diameter of zona pellucida
tHyz	Time of initiation of herniation
tBCi (n)	Blastocyst expansion
tBCend (n)	Blastocyst collapse
tSER(i)	Time of first visualization of translucent vacuole (smooth endoplasmic reticulum)
tSER(e)	Time of last visualization of translucent vacuole (smooth endoplasmic reticulum)
tRoll(i)	Time of initiation of blastomere movement
tRoll(e)	Time to end of blastomere movement
tCW(i)	Time of initiation of cytoplasmic waves
tCW(e)	Time to end of cytoplasmic waves
tCS	Time of first visualization of cytoplasmic strings
MNB	Multinucleation of blastomeres
%ftn	Percentage of fragmentation (to be annotated after each cell division)

Table 2. Results of logistic regression analysis

Logistic regression analysis performed in order to determine statistical significance of different embryological parameters in order to develop the HFC v1.0 embryo-scoring algorithm.

Parameter	Unit Specific Range	Unit Specific	P Value
t2	23.36-28.88	Low	0.803
t3	34.16-39.6	Medium	0.7923
t4	35.67-38.86	Medium	0.6689
t5	43.46-51.04	Medium	0.2387
t2-t3	9-13.5	Medium	0.7308
t3-t4	0-1.7	High	0.0197*
t4-t5	9.5-11.4	High	0.0429*

9.2 Appendix 2 – Research governance

NHS
Health Research Authority
National Research Ethics Service

NRES Committee North West - Haydock
3rd Floor - Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7827
Fax: 0161 625 7299

12 January 2015

Dr Stephen Troup
Liverpool Women's NHS Foundation Trust
The Hewitt Fertility Centre
Crown Street
Liverpool
L8 7SS

Dear Dr Troup

Study title:	**The evaluation of human embryo incubation and annotation using time-lapse imaging**
REC reference:	**14/NW/1310**
Protocol number:	**N/A**
IRAS project ID:	**155032**

Thank you for your submission of 30 December 2014, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Lead Reviewers.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager, Rachel Katzenellenbogen, nrescommittee.northwest-haydock@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from NRES. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see

"Conditions of the favourable opinion" below).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering letter on headed paper		28 May 2014
Letter from sponsor		15 May 2014
Letter Of Intention To Appeal		28 August 2014
Other [Consent Process]	1	01 November 2014
Participant consent form	1	02 May 2014
Participant information sheet (PIS)	2	01 November 2014
REC Application Form [REC_Form_09062014]		09 June 2014
Referee's report or other scientific critique report		28 April 2014
Research protocol or project proposal	1.0	02 May 2014
Summary CV for Chief Investigator (CI) [Troup]		
Summary CV for student [Barrie]		
Summary CV for supervisor (student research) [McDowell]		
Summary CV for supervisor (student research) [Brown]		

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and

the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

14/NW/1310	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

On behalf of
Dr Tim S Sprosen
Chair

Email: nrescommittee.northwest-haydock@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: Ms Gillian Vernon, Liverpool Women's NHS Foundation Trust

Edge Hill University

Research & Development Department
Liverpool Women's NHS Foundation Trust
Crown Street
Liverpool
L8 7SS

13th May 2014

Dear Sir/Madam

Co-sponsor Letter
Title of Project:
Time-lapse imaging: incubation and annotation.

Applicant:
Amy Barrie, Clinical Embryologist, The Hewitt Fertility Centre
Liverpool Women's NHS Foundation Trust

Supervisors:
Dr Jeremy Brown
Reader in Health Service Research, Edge Hill University

Dr Garry McDowell
Reader in Health and Biomedical Science, Manchester Metropolitan University

Research to be conducted at:
Liverpool Women's NHS Foundation Trust

I can confirm that the University is acting as academic co-sponsor for this project and has suitable indemnity in place. The application conforms to all appropriate Edge Hill University policies, and has been scrutinised by appropriate quality assurance mechanisms.

Yours faithfully,

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21st January 2015

Dr Stephen Troup
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Dear Steve

LWH1018 - The evaluation of human embryo incubation and annotation using time-lapse imaging

Following submission of project documents, associated paperwork and approvals to the Trust's R&D Department, I am pleased to inform you that your research project has been approved by the R&D Director. This approval relates to the documentation listed below:

- Ethics approval letter [14/NW/1310] dated 12th January 2015
- Protocol [Version 1.0] dated 2nd May 2014

The research is registered on the Trust's R&D database under the reference LWH1018, which I would be grateful if you could quote in all future correspondence regarding the project.

The Sponsor(s) of this research project under the Research Governance Framework for Health and Social Care (RGF) is the Trust.

Having gained approval to conduct this research under the auspices of Liverpool Women's NHS Foundation Trust, you will be expected to comply with the principles of Good Clinical Practice and the Department of Health RGF. Please refer to your delegated duties outlined overleaf. Our Trust R&D Department must be kept informed of regulatory amendments, updates and approvals – this is your responsibility as site investigator.

Where your research involves the use of equipment otherwise not readily used in clinical care as part of LWH clinical guidelines, it is your responsibility to ensure such equipment is removed from use in clinical areas at the end of your research project.

It is also your responsibility to assure the confidentiality and protection of patient identifiable information. To gain a thorough understanding of your information governance responsibilities, the Trust R&D Department recommends that you refer to the NHS IG Toolkit, accessing the online training materials where necessary (www.connectingforhealth.nhs.uk/igtrainingtool).

I would like to take this opportunity to wish you the best of luck with this research and to request a copy of the final report and any subsequent publications.

Yours sincerely

Louise Hardman
Research & Development Manager

Liverpool Women's

9.3 Appendix 3 – Standard operating procedures

9.3.1 Setting up for treatment

9.3.1.1 Required equipment

Safety cabinet with heated surface
Electronic BibbyJet
Electronic pipette
Printed label/permanent marker pen

9.3.1.2 Reagents and consumables

10ml graduated pipettes (Falcon)
1ml graduated pipettes (Falcon)
Extra long pipette tips (Scientific Lab Supplies)
G-MOPS™ Plus
G-MOPS™
G-TL™
EmbryoGlue®
20% protein supplement (Irvine Scientific)
OVOIL™
60mm round petri dishes (Nunc™)
14ml round bottom tubes (ReproMed)
5ml round bottom tube (ReprodMed)
50ml flasks (Falcon)
4-well dishes
EmbryoSlides®
Coloured incubator tape

9.3.1.3 General information

- All culture dishes should be set up using aseptic techniques and allowed to equilibrate overnight as described below.

- Prior to the setup of any dishes or tubes staff MUST have the media checked to ensure it is suitable for use and the relevant section of the To Do list signed.
- Culture dishes are numbered as 1-4 working in a clockwise manner starting from the top left well.

9.3.1.4 Temperature, pH and osmolality

Crucial considerations for embryo culture are temperature, pH and osmolality. Temperature and pH changes occur rapidly and are related to the amount of time the dish stays out of the incubator. It is important to ensure culture dishes are out of the incubator for the shortest time possible.

- The heated stage in a safety cabinet can be switched off when setting up dishes. Alternatively, the unheated surface within the workstation can be used.
- No more than two dishes are to be prepared at one time.
- The OVOIL™ must be added immediately to avoid evaporation and pH changes.
- Dishes must equilibrate in the incubator for a minimum of six hours.
- Wherever possible media should be warmed/equilibrated for no longer than 72h (including duration of embryo culture). This may be exceeded for patients being cultured in G-TL™.

NB. Six hours is the minimum recommended time by the manufacturer for the medium to reach the correct pH under OVOIL™ (18h is the maximum).

9.3.1.5 Spare media

- Spare tubes of G-TL™ and G-IVF™ Plus should be put in an incubator to equilibrate at 5% O_2 and 6% CO_2 for use the following day. These can be used for any new or converted treatments or surplus oocytes/embryos/sperm samples.

- Spare OVOIL™ should also be placed in a warmer tight-capped (pre-warmed) and incubator loose-capped (gas-equilibrated) overnight for use the next day.
- The volume of spare media to be pre-equilibrated should be decided on a day-to-day basis. The following factors should be taken into consideration when calculating volumes of spare media:
  - Number of day 3 patients the following day (G-TL™).
  - Number of patients for oocyte collection with >12 follicles (spare G-IVF™ Plus).
  - Spare 0.5ml tube of G-TL™ per embryo transfer list.

NB. Media can only be kept warm in an incubator/warmer for a maximum of three days (G-IVF™, G-MOPS™ and EmbryoGlue®) with the exception of G-TL™ which can be kept for up to seven days.

If insufficient spare media has been pre-equilibrated, it can be incubated immediately and allowed to equilibrate for a minimum of six hours before use.

9.3.1.5 Incubator labels

- Prior to setting up, incubator labels for each patient must be created.
- Two pieces of coloured tape; each piece of tape should have an RI label (which contains the patients details) attached. One of the labels should detail the treatment type, and one label should detail the date of the oocyte collection or warm/thaw. The labels are loosely stuck to the laminated sheet on the outer door of the set-up incubator.

9.3.1.6 Setting up prior to oocyte collection

The following must be made up for each oocyte collection and left to equilibrate overnight in the incubator:

- 1 x G-MOPS™ Plus dish (to hold oocytes during collection).
- 1 x O/C wash dish (to wash oocytes through after oocyte collection for both IVF and ICSI).

- IVF Insemination dish/es (for IVF only) (number to be made decided based on follicle number).
- ICSI holding dish/es (for ICSI only) (number to be made decided based on follicle number).
- EmbryoSlide® (for ICSI only) (number to be made decided based on follicle number).
- 1-2 x 50ml G-MOPS™ (Follicle Flush) for patients with 5 or less follicles. Number is decided based on follicle number.
- 1 x 8ml G-IVF™ Plus into sperm prep incubator (2 x 8ml needed for viral positive sperm samples).

G-MOPS™ Plus dish

This dish is for holding the oocytes during the oocyte collection procedure.

1. Add a patient label (in absence of label use a permanent marker pen and write the female patient's full name and date of birth) to a 4-well dish.
2. Write the dish type at the top in permanent marker (i.e. 'G-MOPS™ Plus').
3. Add an RFID tag to the underside of the dish.
4. Add 0.65ml of G-MOPS™ Plus to each well and then carefully add 0.35ml of OVOIL™ (see schematic below) and replace lid.
5. Place the dishes in the warmer overnight.

O/C wash dish (for all oocyte collections)

The O/C wash dish is used to wash the oocyte cumulus complexes (OOCs) through after the oocyte collection to ensure that there is no introduction of G-MOPS Plus and therefore possible pH changes to the IVF insemination dish or ICSI holding dish.

1. Add a patient label (in absence of label use a permanent marker pen and write the female patient's full name and date of birth) to a 4-well dish, discard lid.
2. Write the dish type on the top of the dish in permanent marker
3. Add an RFID tag to the underside of the dish.
4. Add 0.65ml of G-IVF™ Plus to each well and then carefully overlay with 0.35ml of OVOIL™ (see schematic below).

5. Place the dishes in the incubator to equilibrate overnight.

ICSI holding dish (for ICSI and IVF/ICSI back-up only)

1. Add a patient label (in absence of label use a permanent marker pen and write the female patient's full name and date of birth) to a 4-well dish, discard lid.
2. Write the dish type on the top of the dish in permanent marker
3. Add an RFID tag to the underside of the dish.
4. Add 0.65ml of G-IVF™ Plus to wells 1 & 2 and then carefully overlay with 0.35ml of OVOIL™ (see schematic below). These wells are for holding the oocytes in prior to stripping (max. 10 oocytes per well).
5. Add 0.7ml of G-TL™ to well 3. This well is to wash the denuded oocytes through.
6. Add 3 x 10µl drops of G-TL™ to well 4 (taken from well 3) and immediately cover with 0.7ml OVOIL™ (to avoid evaporation).
7. Increase the drop sizes in well 4 to 20µl by adding another 10µl G-TL™ (taken from well 3). These drops are for holding the denuded oocytes post stripping.
8. Immediately cover well 3 with 0.35ml OVOIL™
9. Place the dish in the incubator to equilibrate overnight.

IVF insemination dishes (for IVF only)

1. Add a patient label (in absence of label use a permanent marker pen and write the female patient's full name and date of birth) to a 4-well dish, discard lid.
2. Write the dish type on the top of the dish in permanent marker
3. Add RFID tag to the underside of the dish.
4. Add 0.65ml G-IVF™ Plus to each well and then cover with 0.35ml OVOIL™.
5. Place the dish in the incubator to equilibrate overnight

Embryo transfer dish

1. Add a patient label (in absence of label use a permanent marker pen and write the female patient's full name and date of birth) to a 4-well dish, retain lid.
2. Write the dish type on the top of the dish in permanent marker
3. Add 0.5ml G-TL™ to well 4
4. Add 0.5ml EmbryoGlue® to well 3
5. Replace lid.
6. Place the dishes in the incubator to equilibrate overnight.

EmbryoSlides®

For a guide to making EmbryoSlides® refer to section 9.3.9.4.

Embryo transfer tubes

- A loose capped 0.5ml aliquot of G-TL™ should be placed in the set up incubator for each patient having an embryo transfer, along with 1 x spare aliquot for each list of transfers.

Follicle flush media

- For all patients with ≤5 follicles place 1-2 x 50ml flasks of G-MOPS™ in the warmer. Professional judgment should be used to decide on the number..

OVOIL ™

Non-gassed OVOIL™ is required for patients undergoing embryo thaws, ICSI treatment and oocyte vitrification. Bottles of OVOIL™ should be placed in the warmer (tight-capped) the day before the treatment is scheduled. The following amount of OVOIL™ is required for each procedure:

- 10ml of OVOIL™ (tight-capped) is required for every embryo thaw
- 15ml of OVOIL™ (tight-capped) is required for every ICSI procedure.
- 10ml of OVOIL™ (tight-capped) is required for every patient booked in for oocyte vitrification.

Gassed OVOIL™ is required for additional dishes required on the same day. A minimum of 25ml should be available in the set up incubator.

Dishes required for a patient having IVF using EmbryoScope®

Day prior to oocyte collection:

- G-MOPS™ Plus dish
- O/C wash dish
- IVF insemination dish/es

Day of oocyte collection:

- EmbryoSlides® depending on number of oocytes collected

Dishes required for a patient having ICSI using EmbryoScope®

Day prior to oocyte collection:

- G-MOPS™ Plus dish
- O/C wash dish
- ICSI holding dish
- EmbryoSlides® dependent on follicle number

9.3.2 Oocyte collection

9.3.2.1 Required equipment

Safety Cabinet with heated surface
Permanent marker pen/printed label
Bright field stereo-microscope
Hot block
Pipette rest

9.3.2.2 Reagents and consumables

Extra long pipette tips
G-MOPS™ Plus

G-IVF™ Plus
G-TL™
OVOIL™
60mm round petri dishes
4-well dishes
14ml round bottom tube
50ml flasks
Sterile Pasteur pipettes (Origio)
Rubber bung (Fisher Scientific)
Disposable gloves (non-powdered)
Disposal container with gel sachet inside
Paper towel (to sit under above container in case of spillages)
Oosafe® (Parallabs)

9.3.2.3 General considerations

One Pasteur pipette should be used for the oocyte collection procedure from picking up the oocyte cumulus complexes (OCC's) from the follicular fluid and placing them in the G-MOPS™ Plus holding dish. Once all oocytes are in the G-MOPS™ Plus dish use a clean Pasteur pipette to transfer the OCC's from the G-MOPS™ Plus dish through the O/C wash dish and into the IVF insemination dish/ICSI holding dish. This will reduce the risk of carrying over any suboptimal components from the follicular fluid into the culture system. This is the same for both IVF and ICSI oocytes but ICSI oocytes could potentially be at a higher risk due to the fact that they lack the protection from the cumulus cells.

9.3.2.4 Prior to oocyte recovery

1. The embryologist must use alcohol gel on her/his hands upon entering the lab.
2. An RI Witness™ ID card should be assigned prior to the oocyte collection procedure. The patient's full name, date of birth and unit number, along with the partners name (if ID card is already assigned) or partners name and date of birth (if a new card has been made) should be witnessed with a second embryologist using the ID card, embryology pack and RI Witness™. In addition, a check needs to be

made to ensure the presence or absence of ‘F’ following the hospital number is the same on the ID card, embryology pack and RI Witness™.

3. All information on the embryology pack should be checked and all discrepancies amended before the treatment begins.
4. Set out pre-warmed petri dishes (bases and lids) on heated surface.
5. Place a disposal container with a gel sachet inside inside in the safety cabinet, with a piece of paper towel underneath to soak up any spillages.
6. A sterile Pasteur pipettes should be removed from the packaging, a rubber bung attached and placed on the pipette rest.
7. Using the case notes, the embryology pack and the RI Witness™ ID card, the embryologist, the oocyte collecting doctor/fertility nurse specialist (FNS) and the nurse should ask the patients to state their names and dates of births and the photo ID checked. The appropriate section of the witness form must then be signed and ticked, respectively, and the RI Witness™ ID card is placed in the card holder.
8. The embryologist should take receipt of a completed pre oocyte collection lab check list form from the nurse and ensure that it is completed fully to include verbal confirmation of the patients treatment type, consent to cryopreservation of suitable embryos and any telephone numbers. Any consent issues which have been documented at the bottom of the form should be indicated as resolved by the nurse initialing next to each. The form should be signed as complete by the nurse.

9.3.2.5 The oocyte retrieval procedure

1. The embryologist must wear a pair of powder free gloves.
2. As the aspirate is collected it is placed in the hot block by the nurse prior to examination by the embryologist. The first follicle is placed in the first position in the front row of the hot block with any flushes from this follicle going directly behind. The second follicle will then go in the second position again with any flush going behind. This is to assist the clinical and nursing team in identifying which tube the oocyte came from and also prioritise looking through follicle aspirate over flush once an oocyte has been identified.
3. Approximately 5mls of follicular fluid should be carefully tipped out into a petri dish (use both the base and the lid) for examination and identification of the oocyte OCCs using the microscope only (no ‘eye-balling’).

This procedure should be carried out as quickly as is safely possible, and without delay to minimise any drop in temperature.

4. When the first oocyte is located the G-MOPS™ Plus dish should be removed from the warmer. Assign the dish to the patient using RI Witness™.
5. As the oocytes are located they should be immediately transferred to the G-MOPS™ Plus dish. Oocytes are washed in well 4 then transferred to separate wells to minimise changes in temperature. (The first oocyte is added to well 1, the 2nd to well 2, the 3rd to well 3, the 4th to well 1, the 5th to well 2, the 6th to well 3 etc). The oocytes remain in this dish for the duration of the collection.
6. After being checked, the follicular fluid is poured carefully into a disposal container and the petri dish discarded in the sharps bin
7. The oocyte number is circled on the embryology record form to help keep a track of the number of oocytes collected so far.
8. The oocyte collection is officially finished when the nurse informs the embryologist that the last tube has been placed into the hot block.
9. Once all the tubes have been checked through, the lid of the waste tub is secured and the tub is discarded in the sharps bin, gloves removed and discarded and the used Pasteur pipette replaced with a clean one.
10. The procedure room door must then be left open until a 2nd member of the nursing team comes in to the procedure room. This is in case there is an emergency and the nurse needs someone to call for help.
11. The oocytes are distributed into new dishes depending on their planned treatment type as per below.
12. At the end of the procedure the embryologist must wipe down the surface with Oosafe® and wash his/her hands.
13. When the workstation is clear of all tubes and dishes the embryologist must get a second person to check their workstation ensuring there are no tubes in the hot blocks or dishes in the workstation area. Both members of staff must then sign the witness form.

<u>Distribution of oocytes for ICSI patients</u>

1. The O/C wash dish is removed from the incubator.

2. Assign the O/C wash dish using RI Witness™.
3. Using a clean Pasteur pipette, the OCC's are transferred from the G-MOPS™ Plus dish and washed through drops 1-4 (clockwise) of the O/C wash dish
4. The ICSI holding dish is removed from the incubator and the OCC's are transferred and left in wells 1 & 2 of the ICSI holding dish until the time of cumulus removal.
5. If possible the embryologist should try to avoid placing more than 10 oocytes in a drop; drop 2 should be used as a backup if a high number of oocytes are collected.
6. The oocytes are graded at this stage and then placed back into the incubator for four hours prior to injection. Oocytes can be rolled using a Pasteur pipette to aid visualisation of the OCC, however the duration of time the dish is outside the incubator should be considered.
7. All remaining dishes must be checked before placing them in the sharps bin..
8. The number of oocytes collected should be written on the white board with the insemination time next to the patient name and the oocyte collection time removed.
9. The oocyte collection details must then be added onto the Infertility Database for Embryology and Andrology System (IDEAS V.5.3™).
10. The embryology record form is then placed in day 0 tray in the embryology laboratory in order of insemination time.

Distribution of oocytes for IVF patients

1. The O/C wash dish is removed from the incubator.
2. Assign the O/C wash dish using RI Witness™.
3. The oocytes are transferred from the G-MOPS™ Plus dish and washed through drops 1 - 4 of the O/C wash dish.
4. The IVF insemination dish is then removed from the incubator and assigned
5. The oocytes are then transferred to the IVF insemination dish (es).
6. The oocytes should be split evenly between the IVF insemination dishes with a maximum of 4 oocytes per well, 1-3 (clockwise) with the fourth well of each dish remaining clean as a wash well for fertilisation check.

7. The oocytes are graded at this stage and then placed back into the incubator for four hours prior to insemination. Oocytes can be rolled using a Pasteur pipette to aid visualisation of the OCC, however the duration of time the dish is outside the incubator should be considered.
8. All remaining dishes must be checked before placing them in the sharps bin.
9. The number of oocytes collected should be written on the white board with the insemination time next to the patient name and the oocyte collection time removed.
10. The oocyte collection details must then be entered onto the IDEAS™ V.6 system.
11. The embryology record form is then placed in day 0 tray in the embryology laboratory in order of insemination time.
12. Any spare IVF insemination dishes for the patient should be checked, patient label removed, and the dish transferred to the 'spare dishes' shelf of an incubator (recording the date 'made') on the side.

<u>IVF cases with large numbers of oocytes (>12) collected</u>

1. A second insemination dish must be used, ensuring that the oocytes are distributed as described above.
2. If a second dish has to be made following an oocyte collection (i.e. not set up on previous day) then half of the oocytes are transferred into the first IVF insemination dish and the remaining oocytes must remain in the O/C wash dish. The number of oocytes remaining in the O/C wash dish must be recorded on the white board directly next to the number of oocytes in total and the insemination time.
3. Both the IVF insemination dish and the O/C wash dish containing the 'extra' oocytes are transferred to the patient's allocated position in the incubator. The embryologist must then record on the lab white board that a dish is required (including patient name and dish type).
4. The extra dish must be made using pre-equilibrated G-IVF™ Plus and OVOIL™ as soon as a member of staff is free to do so. Once made up, the dish request is removed from the lab white board.

5. The first well of the second dish is labelled ‘5’ etc.

Distribution of oocytes for IVF/ ICSI back up cases

1. When the OCC’s have been collected they must be left in the O/C wash dish until a decision has been made with regards to treatment type
2. This must be recorded on the white board by writing ‘n oocytes in O/C wash dish’ directly next to the insemination time

9.3.2.6 OCC grading

Grade 1- (immature) OCC:	-tightly packed, unexpanded cumulus cells, -occasionally compact clumps of parietal granulosa cells, and dense layer of coronal cells -the ooplasm is barely visible through the cumulus.
Grade 1 (mature) OCC:	-fully radiating corona surrounded by a loose mass of cumulus cells. -the oocyte can be clearly seen.
Grade 1+ (post-mature) OCC:	-cumulus with clusters of darkened cells -coronal cells are dark and tight. -the oocyte is clearly visible.

9.3.3 Intracytoplasmic sperm injection (ICSI)

9.3.3.1 Required equipment

Integra Ti Micromanipulator, Research Instruments.
Safety cabinet with heated stage
Pipette handle and rest

9.3.3.2 Reagents and consumables:

5ml round bottom tube
ICSI dishes (Nunc™)

60mm round petri dishes
10ml graduated pipette
Sterile ICSI holding pipette (Smiths Medical)
Sterile ICSI injection pipette (Smiths Medical)
G-IVF™ Plus
G-MOPS™ Plus
HYASE-10X™
OVOIL™
ICSI -100™ (PVP) (Vitrolife)

9.3.3.3 Reagent preparation

The day before ICSI:

Put OVOIL™ tight capped in the 37^{o} C warmer overnight.

The day of ICSI (am):

Place one vial of HYASE-10X™ for every four ICSI cases in the warmer.
Place one 1.0ml G-MOPS™ Plus aliquot (tight-capped) per two ICSI cases & per vial of HYASE-10X™ in the warmer.
Remove PVP from fridge and place in safety cabinet (do not place on heated stage).

Setting up a stripping dish

Enzymatic digestion (*hyaluronidase)* of cumulus and corona (stripping) is performed up to a max. of one hour prior to injection.

1. Remove one 1.0ml G-MOPS™ Plus aliquot from the warmer. Remove a second aliquot of G-MOPS™ Plus if a new vial of HYASE-10X™ is required.
2. Add a patient label and an RFID tag to a 60mm round petri dish.
3. Place the dish on a cold surface when making up the stripping dish and ensure that the process is performed quickly
4. Add 0.9mls of G-MOPS™ Plus directly into a vial of HYASE-10X™ using a graduated pipette- ensure complete mixing.

5. Prepare a dish for stripping by adding 5-6 x ~50ml drops of G-MOPS™ Plus from the tube to the stripping dish
6. Then transfer ~250ml of the diluted HYASE-10X™ to the dish making two large droplets (only one droplet is necessary if the oocyte number is less than five). If using a Pasteur to make drops ensure that the G-MOPS™ Plus drops are made first, this is to prevent any HYASE-10X™ being transferred into the G-MPOS™ Plus.
7. **C**over with 10 ml warmed OVOIL™.
8. Place the stripping dish in the warmer to equilibrate.

9.3.3.4 Stripping of cumulus cells

1. Set up a flamed Pasteur pipette and the following denudation pipettes: 1x 200µm, 1 x 155µm, 1x 135µm. Alternatively finely drawn pipettes of appropriate diameter may be used.
2. Remove the stripping dish from the warmer followed by the ICSI holding dish from the incubator.
3. Assign the stripping dish accordingly using RI Witness™
4. In the absence of RI Witness™ a second embryologist must cross check the patient details on the ICSI holding dish, the stripping dish and the embryology forms, and sign the appropriate section of the witnessing form.
5. Using a Pasteur pipette transfer no more than half of the OOCs to the droplet containing pre-equilibrated HYASE-10X™ solution.
6. Aspirate a maximum of five OOCs in and out of the pipette. The cumulus cells will begin to disperse.

NB. Maximum exposure time to HYASE-10X™ is 30 seconds.

7. Transfer the partly denuded oocytes into the first of the wash droplets containing G-MOPS™ Plus, taking care to carry over a minimum amount of HYASE-10X™ solution.
8. Aspirate each oocyte up and down, using the denudation pipettes to remove any corona. Transfer to next clean wash drop and replace the ICSI holding dish in the incubator.
9. Repeat step 8 until cells have been removed.

10. Although all cells do not need to be removed, it is important to clean oocytes sufficiently enough to avoid problems with polar body visualisation, or holding and rotating the oocyte on the holding pipette during the ICSI procedure
11. Assess oocyte maturity as follows:
    - Metaphase II oocyte (MII) - one complete or fragmented polar body
    - Metaphase I oocyte (MI) - no polar body
    - Germinal vesicle stage oocyte (GV) - one nucleus with single nucleolus
12. Wash all mature (MII) oocytes thoroughly in the wash well (well number 3) in the ICSI holding dish before being placed in a fresh culture micro-drop.
13. Immature oocytes (MI) should be placed into a separate micro-drop. Incubate until needed for injection.
14. GV oocytes are discarded following the stripping procedure. MII and MI are kept until the ICSI has been performed.

9.3.3.5 Preparation of ICSI dish

- ICSI dishes must be prepared approximately one hour prior to the ICSI on a unheated surface, the lid replaced and then the dish placed in the warming oven until required.
- For ICSI's with >12 oocytes make up a second injection dish and leave in the warmer until required.

1. Add a patient sticker and RFID tag to the ICSI dish.
2. Add 10µl of warmed G-MOPS™ Plus to the centre of the dish and then surrounded by eight 10µl droplets (one per oocyte) of G-MOPS™ Plus medium
3. Remove the 10µl of warmed G-MOPS™ Plus and replace with 5µl PVP. This creates a wide flat drop of PVP which helps with observing sperm.
4. Cover immediately with 4ml OVOIL™

ICSI dishes should be made up quickly and not more than one at a time to avoid evaporation of the droplets.

9.3.5.6 ICSI procedure

General information

- Injection is carried out at approximately 40h post hCG.
- An ICSI dish should remain on the rig for a maximum of 10 minutes. Please use professional judgement regarding how many oocytes are placed in the dish such that you can comfortably inject that number within the 10 minute time period.
- When extremely poor sperm samples (e.g. TESE) are encountered consideration should be given to 'harvesting' sperm before oocytes are placed in the dish such that the above time restriction can be adhered to.

The injection procedure

1. The holding and injection pipettes are attached to the manipulators: holding to the left and injection to the right.
2. Needles are aligned with the `high' objective. Correct alignment in this position means that when the needle is lowered with the coarse movement lever to its lowest point it should be positioned correctly in focus with the periphery of the drop, requiring the minimum of fine alteration with the joystick.
3. Immediately before the ICSI procedure collect the final sperm preparation tube from the sperm preparation lab.
4. Remove the ICSI dish from the warmer and add an appropriate volume of sperm suspension to the central PVP drop.
5. Ensure a witness is available and then remove the ICSI holding dish from the incubator.
6. Add one MII oocyte to each surrounding droplet.

NB. Do not pick all the oocytes up at the same time, split them into two or more 'batches' (i.e. 5 oocytes moved as 3 and then 2, 6 as 3 & 3 etc)

7. For samples with low sperm count, motility or progression (e.g. TESE) the sample may be concentrated (100ml) prior to use and separate droplets may be used.
8. Replace the ICSI holding dish in the incubator
9. Align the holding pipette first and equilibrate this in a drop of G-MOPS™ Plus medium, allowing medium to enter the pipette by capillary action.

10. Using the high objective, align the injection pipette with the holding pipette. Using the low objective, lower the injection pipette into the PVP drop and allow PVP to enter the pipette by capillary action.
11. At high magnification a motile sperm is selected.
12. Immobilise the selected sperm by drawing the injection pipette in a downward swipe across its tail. Do not damage the midpiece as this contains the centriole, but do ensure that the membrane is broken, this is indicated by ‘kinks’ in the tail. Permanent immobilisation is important.
13. Aspirate the sperm tail first into the injection pipette. Ensure that the sperm moves freely up and down in the needle. If it does not, select another sperm. Raise the pipette and move to a drop containing an oocyte.
14. Using the holding pipette apply gentle suction to the oocyte so that it is immobilised on the pipette with the polar body at six o'clock. This is important to avoid injecting the site of the metaphase spindle.
15. Position the sperm close to the tip of the injection pipette and push the injection pipette through the zona pellucida and oolemma at three o'clock. Do not push more than half way. Do not push to the opposing membrane, as this will damage the oocyte. Move straight in and out, not up and down.
16. The inner membrane may invaginate and, in order to break the oolemma, gentle suction is applied through the injection pipette until a rush of cytoplasm is observed, signalling that the membrane has been broken.
17. Gently release the suction and replace all the cytoplasm back into the oocyte together with the sperm. Insert the sperm slowly with the smallest volume of PVP possible.
18. Slowly withdraw the injection needle, which can then be used to help dislodge the oocyte from the holding pipette.
19. Return to the sperm drop to select further sperm.
20. The above process is repeated for each oocyte.
21. At the end of the ICSI procedure the needles are lifted out of the way of the dish. The lid of the dish is replaced and then the dish is transferred to the designated safety cabinet and placed on the heated stage.
22. The pre-equilibrated culture dish for that patient is removed from the incubator.
23. The injected oocytes are transferred to the embryo culture dish.
24. The embryo culture dish is then returned to the incubator and incubated overnight.

25. The ICSI dish and pipettes are then disposed of in the appropriate manner.
26. In cases of poor sperm samples (i.e. TESE, concentrate before use) the ICSI practitioner must make a note on the sperm preparation form of the quality of the sperm used for the ICSI procedure. This is then added to the IDEAS V.5.3™ sperm preparation data entry to ensure the information is available for future treatments.

9.3.4 IVF insemination

9.3.4.1 Required equipment

Safety cabinet with heated stage
Pipette handle and rest
Automatic pipette

9.3.4.2 Reagents and consumables

Extra long tips

9.3.4.3 Sperm concentration

The final concentration of sperm for the insemination should be 100,000 normal motile sperm /ml. In order to calculate the insemination volume:

Normal Motile Sperm (NMS) = Concentration x % motility (A+B) x % normal forms

Insemination Volume = 65 / NMS (for a 650μl drop)

9.3.4.4 Signing out the sperm for insemination

1. In the absence of RI Witness™ a second embryologist must be present for witnessing this procedure.
2. The rack containing all the preparation tubes is removed from the incubator in the sperm preparation lab. The rack is placed into the RI Witness™ tube reader.

3. All the labels on all the tubes in the allocated rack are checked (female name) against the details on the sperm preparation form and the RI Witness™ screen by the embryologist. On the 'final prep' tube both partners' names and dates of birth are checked against the sperm preparation form (as this is the tube used for insemination). The number and type of tubes in the rack are checked against the RI Witness™ screen to ensure all tubes are assigned correctly.
4. The 'final prep' tube is removed from the rack, and the 'Discard' option on RI Witness™ is used to discard all of the tubes. The label on the front of the rack is removed and placed on top of one of the tubes and rack is then placed on the bench until the insemination has been performed.
5. The final preparation tube is transferred to the embryology lab to perform the insemination.

9.3.4.5 Insemination

1. Set up the automatic pipette according to the insemination volume required and number of wells containing oocytes to be inseminated, attach a pipette tip and place the pipette on the pipette rest ready for use.

NB. It is the responsibility of the embryologist performing the insemination to check the number of oocytes to be inseminated in each well and to ensure that the pipettor is set up correctly prior to the insemination.

2. Remove the insemination dish from the incubator.
3. Cross check the patient details on the embryology forms, the insemination dish and the final sperm tube with the 2nd witness who must then enter their unique PIN into RI Witness™ to authorise the insemination and sign the appropriate section on the witness form.

NB. Each well should be briefly checked to ensure the number of oocytes in each well corresponds with the embryology paperwork. This must be a brief check and must not put the oocytes at an unnecessary risk of cooling.

4. Gently shake the final sperm tube to mix the sample evenly.
5. Pick up the pipette from the rest and place the tip in the sperm preparation tube and draw up the calculated volume of sperm suspension.
6. If using the automatic pipette, pre-pipette some of the sample out onto the side of the preparation tube.

7. Position the first well to be inseminated under the microscope so that the well can be visualised, place the tip into the well and press the yellow button on the pipette.
8. Move the dish so that each well can be inseminated as described above.
9. Once all the oocytes have been inseminated check all inseminated wells to visualise motile sperm
10. Place the dish back in the incubator
11. Discard the sperm 'final prep' tube on RI Witness™ and place in a sharps bin
12. Wipe surface with Oosafe®.
13. The embryologist must then sterilize their hands using alcohol gel.
14. The insemination procedure should be entered onto IDEAS V.5.3™.
15. The patients name should be wiped off the laboratory whiteboard.

9.3.5 Fertilisation check

9.3.5.1 Required equipment

Safety cabinet with heated stage
Pipette handle and rest

9.3.5.2 Reagents and consumables

135µm denudation pipette (Research Instruments)
155µm denudation pipette (Research Instruments)

9.3.5.3 Timescale for checking fertilisation

Oocytes should be checked in the morning following oocyte collection:

IVF - 16-22hpi

ICSI - 12-18h after injection as PN may appear earlier. Oocytes cultured in the EmbryoScope® can be checked at any point in the morning but the patient call must be before 12pm.

9.3.5.4 General information for checking fertilisation

- 'Cleaning' of oocytes is carried out using a 135µm denudation pipette (or a Pasteur pipette - drawn so its internal diameter is just greater than the diameter of a human oocyte. The tip of the drawn pipette should be free of jagged edges and at right-angles to the length).
- Denuded oocytes should be handled using a pipette no smaller than a 146-155µm pipette
- Ensure only the minimum volumes of medium are transferred.
- Care must be taken not to catch the end of the denuding pipette when transferring oocytes from well to well.
- Any oocyte in which normal fertilisation cannot be confirmed should not be considered suitable for replacement or cryopreservation without confirmation from a Consultant Embryologist.
- Care must be taken not to use a drawn pipette with an internal diameter that is too small.
- A new pipette must be used for every patient.
- Pipettes should be discarded immediately at the end of the procedure.
- If RI Witness™ is not operational, the patient details on the embryology notes and on the bottom of the dishes must be witnessed by a second member of staff prior to transferring embryos from one dish to another.

9.3.5.5 IVF fertilisation checks

1. To remove the cumulus and corona cells the oocytes should be gently aspirated in and out of a 135µm pipette until the majority of the coronal cells have become detached to the extent that PN and polar bodies can be clearly observed.
2. If the cumulus cells are not dispersed, needles should be used to dissect the oocyte from the OCC.
3. The oocytes must then be washed in the 4th 'clean' well of the insemination dish.
4. The denuded oocytes are then inspected under high power on the dissecting microscope for the presence of PNs, polar bodies and other inclusions. The inverted microscope is used to obtain a higher magnification. It is also important

to observe the general appearance of the oocyte. Factors to note are micro-PNs, cytoplasmic texture and shape, zona integrity and shape, clarity etc.

NB. Micro-PN's are classified as nuclear envelopes which are less than 20% in size of the two larger PNs and contain no more than one nucleoli. If normally fertilised embryos contain micro-PN then this should be clearly documented on the embryology pack.

5. Once all the oocytes have been denuded, check for signs of fertilisation and record the results on the embryology form, and then remove the EmbryoSlide® from the incubator.
6. All normally fertilised oocytes (2PNs) are washed through the 4 wash wells of the EmbryoSlide® (labelled A-D) before placing them in their designated drops.
7. Remaining vacant wells in the EmbryoSlide® are filled with unfertilised oocytes after they have been washed through the wash wells. These will be rechecked for signs of fertilisation at the early cleavage check.
8. In the event the EmbrosSlide® is full, additional 2PN's are kept temporarily in wash well D, 1PN's in wash well C and 0PN's in wash well B. A 2nd EmbryoSlide® should be prepared and the embryos/unfertilised oocytes should be moved as soon as possible.
9. Load the EmbryoSlide® containing the embryo(s) into an EmbryScope® incubator.
10. Any immature GV and MI oocytes, along with any abnormally fertilised embryos are discarded.
11. The location of all oocytes must be clearly noted.
12. In cases of OHSS all embryos are cryopreserved at the blastocyst stage, following discussion with a Senior Embryologist.
13. In cases of low or failed fertilisation a record should be made regarding sperm motility and binding.
14. In cases of complete fertilisation failure, all 1PN and 0PN oocytes should be placed in the EmbryoScope® to be rechecked for signs of late fertilisation. Where late fertilisation is not observed but the unfertilised oocytes show signs of embryo development, a Senior Embryologist should be informed.

9.3.5.6 ICSI fertilisation checks

1. Oocytes are inspected on the viewer for the relevant EmbryoScope®.
2. Degenerate oocytes must be discarded by colouring the panel red, annotating the panel on the right hand side of the viewer to indicate “0PN” as the fertilisation status and selecting ‘Dead’ from the dropdown box on the right hand-side of the viewer.
3. 3PN and >3PN oocytes must be discarded by colouring the panel red and also annotating the panel on the right hand-side of the viewer to indicate a “3PN” or “4PN” (where 4PN indicates anything over 3PN).
4. **All** oocytes should be assigned a PN status on the viewer i.e. 0PN, 1PN, 2PN, 3PN, >4PN
5. The presence of micro-PN should be noted in the ‘If applicable’ dropdown box on the right hand-side of the viewer.
6. The fertilisation status of each oocyte must be clearly noted on the embryology paperwork along with the location and fate if any are discarded.

9.3.5.7 Data entry

All clinical information should be entered electronically into the IDEAS V.5.3™ system immediately after the procedure has taken place.

9.3.5.8 Embryo transfer scheduling

Patients who require an embryo transfer are then scheduled accordingly dependent on age, number of embryos, number of embryos for transfer and treatment history. The patient is booked in to the IDEAS V.5.3™ diary and the scheduled time is noted on the front of the embryology pack.

9.3.5.9 Post fertilisation calls

1. Following fertilisation checks, the patients are phoned by an embryologist.
2. This normally takes place around mid-late morning, but always before 12pm.

3. The patients are informed about their fertilisation results, a discussion is had regarding the number of embryos to be transferred and the patient is asked whether they have any question or concerns.
4. The day and time of embryo transfer is given to the patient and they are asked to remain contactable via telephone in case the embryo transfer date and time changes. This decision is dictated by the quality of the embryos.
5. In cases of complete fertilisation failure, the patient is informed and any questions answered. The patient is also informed that the unfertilised oocytes will be rechecked for signs of fertilisation until day two and to expect another phone call the following day.

9.3.6 Embryo grading

9.3.6.1 Cleavage Stages (day 2 and day 3 embryos)

Embryo quality is assessed by light microscopy and is based on observing cleavage rate and morphology. Embryo morphology is primarily assessed based on the number of blastomeres, evenness of cell division, and degree of fragmentation (Figure 2, appendices section 9.1).

Embryos are given a grade comprised of three numbers, in the following order:

- Blastomere number
- Blastomere evenness
- Fragmentation

Example: The grade is recorded as [blastomere number] (size/fragmentation); therefore a four-cell embryo with slightly uneven cell division (~10% difference in cell size) and around 30% fragmentation by volume will be scored as 4(3/2).

9.3.6.2 Blastocyst Stage (day 5 and day 6 embryos)

Blastocyst quality is assessed by light microscopy and is based on the degree of blastocoel expansion and the presence and morphology of both the inner cell mass (ICM) and trophectoderm (Figure 3, appendices section 9.1).

Embryos are given a grade comprised of a number and two letters, in the following order:

- Expansion status
- ICM
- Trophectoderm

Example: The grade is recorded as [expansion status] (ICM/trophectoderm); therefore, a full blastocyst, where the blastocoel completely fills the embryo, with no ICM cells and a trophectoderm made up of sparse cells which may be very large, flat or degenerate will be scored as 3 (E/c)

Embryos are not routinely graded on day 4 of their development, however, when an embryo is somewhere between the cleavage stage and the blastocyst stage, they are graded as follows:

M = Morula. This term describes an embryo in which the cells are starting to or have compacted, i.e. the individual cell membranes are becoming or have already become indistinguishable.

CM = Cavitating Morula. This term describes an embryo at the morula stage which is also showing sign of a developing blastocoel (fluid filled cavity).

9.3.7 EmbryoScope® general use

9.3.7.1 Required equipment

Safety cabinet with heated stage
Pipette handle and rest
Electronic BibbyJet
Electronic pipette
Printed label/permanent marker pen EmbryoSlide®

9.3.7.2 Reagents and consumables

155µm denudation pipette

1ml graduated pipettes

Extra long tips

G-TL™

OVOIL™

60mm round petri dishes

5ml round bottom tube

9.3.7.3 General information

The Incubator

- Please refer to pages seven, 10-12 in the manufacturer's user manual
- The EmbryoScope® provides an environment with controlled temperature, CO_2 (and other gases) for the development of embryos.
- It has an integrated inverted microscope and imaging system for embryo viewing.
- The device includes time-lapse microscopy at multiple focal planes and logging of incubation conditions
- Air is purified by HEPA and active carbon filters
- The EmbryoScope® provides incubation of up to 72 individual embryos in six sterile disposable polymer slides each with 12 embryos (e.g. up to six patients with 12 embryos each).
- Built-in tri-gas incubator, which controls temperature, CO_2 and O_2 levels. The device uses N_2 and CO_2 to maintain desired oxygen partial pressure and pH in a bicarbonate buffer system.
- The device is suitable for continuous operation
- The EmbryoScope® MUST be operated by trained personnel according to instructions contained in this SOP.
- In the event of system failure or fault the EmbryoSlides® must be immediately removed from the EmbryoScope® using the emergency procedures (instructions located in pouch under the service lid).

Manufacturers safety & maintenance instructions

- Please refer to page five of the manufacturers user manual
- The device includes moving parts with safety stops.
- Do not try to block safety sensors to insert a finger or a hand into the device while it is turned on. This is dangerous and may cause injury.
- Do not touch any moving parts when power is ON or during operation.
- Mishandling or misuse of the EmbryoScope® may result in serious injury to the user
- The EmbryoScope® must remain at the location where it was installed
- Maintenance/service visits of the device should be arranged every six months
-

Activation of the incubator alarm

An audible alarm will activate if:

- CO_2/O_2 deviates by more than 0.5% of the set value or if CO_2/N_2 pressure is too low

- Temperature deviates by more than 0.5ºC of the set value

Re-setting the incubator alarm

Press the re-set button (triangle icon) on the control panel. The red LED light will remain flashing until the set values have been reached again.

Activation of the computer alarm

The following malfunctions of the built-in PC and failure to close the load door correctly will activate another audible alarm. The computer alarm cannot be reset, the alarm condition must be resolved i.e. closing the door properly or re-booting the computer system (see Emergency procedures in EmbryoScope® User Manual).

The computer alarm will sound in case of:

- EmbryoScope® software failure or failure of the operating system of the built-in PC
- Load door open for extended period of time (> 30 seconds)
- EmbryoScope® software is not running properly (e.g. in case of problems with the PC operating system or if the software has accidentally been turned off)
- Errors in data communication between EmbryoScope® software and the separate unit controlling the incubation environment (Temperature and Gas).

Should the instrument 'freeze', it can be prompted to restart by pressing 'cmd' + 'L' and entering the appropriate username and password. This is the following for all instruments including the viewers:

Username: embryo
Password: scope

Following a generator test the EmbryoScopes® will lose contact with the databases and need approximately 15 minutes to reconnect. The computers must not be restarted more than once in this time frame whilst the viewers are trying to connect with the database.

In the event that 'Fertilitech support' is activated on the viewer (all slide entries will appear with red writing indicating that remote access is activated) the window that is running the support program must be closed.

Ensure that the support has been completed before closing the program.

NB. A computer failure may result in a loss of time-lapse images, but will not pose an immediate threat to the embryos incubated in the EmbryoScope®, as the temperature and gas concentration is controlled separately.

9.3.7.4 The EmbryoSlide®

Please refer to pages 26-29 of the manufacturer's user manual.

- Only the EmbryoSlide® must be used with the EmbryoScope® device. The lid must be replaced before placing the EmbryoScope® into the device.
- An EmbryoSlide® contains a large reservoir for an OVOIL™ overlay with 12 wells for single incubation of 12 individual embryos and 4 wash wells (A-D)
- Each well holds a volume of 25 µl.
- Inside each culture well there is a central micro-well where the embryo resides, i.e. the *micro-well* has a diameter of approximately 250 µm.
- Individual well numbers (1-12) and wash wells (A-D) are indicated beside the bottom of each well, which are legible using a stereomicroscope during embryo handling.
- EmbryoSlides® and lids are individually wrapped in a sterile pouch. The pouches must only be opened in a safety cabinet.

<u>Setting up an EmbryoSlide®</u>

It is essential that the preparation of the EmbryoSlide® is performed exactly as described below to minimise evaporation.

Please refer to pages 27-28 of the manufacturer's user manual.

EmbryoSlides® must be set up according to the treatment type that is being performed which involves the incubation of media prior to EmbryoSlide® set-up in some cases.

For IVF patients:

- 0.5ml of G-TL™ media must be placed in the set-up incubator on the afternoon of day of oocyte collection for every EmbryoSlide® that has to be made for the following day's IVF fertilisation checks
- After at least two hours of equilibration, the EmbryoSlides® can be set up for the appropriate cases
- Once the EmbryoSlides® have been set up they must be placed in the set-up incubator for re-equilibration overnight until use the following day
- The embryos are then placed in the prepared EmbryoSlides® after the fertilisation check has been performed on day one

For ICSI patients:

- 0.5ml of G-TL™ media must be placed in the set-up incubator on the afternoon of the day before the oocyte collection for every ICSI case scheduled
- On the day of oocyte collection an Embryoslide® must be set up as soon as possible after oocytes have been collected
- Depending on the number of oocytes collected a maximum of two Embryoslides® can be set up for each patient
- If a low number of oocytes are collected then an Embryoslide® can be made following the stripping procedure

If Embryoslides® are prepared with pre-warmed medium that is un-gassed then it must be allowed to equilibrate for 16h before being used.

To set up an EmbryoSlide® do the following;

1. Place an EmbryoSlide® on an ambi-plate on a heated stage in an appropriate cabinet.
2. Place a patient sticker along the front of the dish, with the ‘fin’ at the left, and a RFID tag under the ‘fin’.
3. Fill a 155µm pipette with pre-warmed G-TL™ and place the tip of the plastic pitpette at the bottom of the well and fadd directly to the micro-well until it domes
4. Repeat this for all micro-wells
5. Using the electronic pipette immediately add 25µl of G-TL™ media to each well including the wash wells.
6. Carefully layer up to 1.4ml of warm pre-equilibrated OVOIL™ into the EmbryoSlide® while observing down a microscope to ensure ample coverage of the wash wells.
7. Ensure all air bubbles are removed using a 155µm pipette by pushing the pipette tip down to the bottom of the micro-well and then moving the tip in a ‘stirring’ motion while sucking the air bubble/s up into the pipette ensuring that minimal media is removed.
8. All bubbles floating within the well **must** then be removed as they could distort images if they move over where the camera is taking an image.
9. Replace the EmbryoSlide® lid and place the dish in the designated incubator for a minimum of 16h if the dish has been set up using uneqilibrated media and OVOIL™ or a minimum of one hour if set up with pre-equilibrated media and OVOIL™.

NB. Bubbles must be removed relatively fast to avoid evaporation of medium from the well, and take care not to remove medium during removal of bubbles. Bubbles should be removed before adding the OVOIL™ so OVOIL™ droplets are not dragged down to the well.

9.3.7.5 Loading the EmbryoSlide® with embryos

1. Please refer to pages 28-29 of the manufacturer’s user manual
2. Remove the culture dish and the equilibrated EmbryoSlide® from

the incubator and place on a heated surface within an appropriate safety cabinet. Care must be taken when handling the EmbryoSlide® as grease from hands can distort the image. Avoid contact with the bottom of the dish at all times.

3. Select the first embryo to be transferred to the EmbryoSlide® using an appropriate handling pipette.
4. Draw up the embryo into the pipette from the culture dish. Ensure there is enough medium in the pipette to be able to 'waft' the embryo into position. The embryo must sit in the middle of the micro-well (see diagram below).
5. Wash the embryo in the designated wash wells (A-D)
6. Deposit the embryo at the top of the micro-well and allow it to float down into the micro-well.
7. Once all the embryos have been transferred into the EmbryoSlide®, and have had sufficient time to settle into the micro-well, use the handling pipette to 'waft' them into the centre of the micro-well.

9.3.7.6 Adding a patient to the EmbryoScope®

Please refer to pages 35-36 in the manufacturer's user manual

1. Press start on the Welcome screen to start using the EmbryoScope®
2. On the "Home" screen press the "Add slide" button
3. The warning light at the EmbryoSlide® load door lock will change from red to green indicating that the door is unlocked and may be opened
4. Open the EmbryoSlide® load door and place the EmbryoSlide® containing the embryos in the empty and only accessible position of the EmbryoSlide® holder

NB. The first EmbryoSlide® is placed in position 1; subsequent EmbryoSlides® swill be placed in the next available slots. The EmbryoScope® software keeps track of unoccupied positions and will automatically move the EmbryoSlide® holder to the next

available position. The EmbryoSlide® should be inserted with the handling tail fin towards the front of the EmbryoScope®.

5. Press “OK”
6. Enter the patient ID, patient name and the date and time of insemination of the treatment along with the treatment ID i.e. TX12345

NB. for patients who have had more than 12 oocytes injected and therefore have two EmbryoSlides® the insemination time for the second dish must reflect the witness/start of injection time of that batch of oocytes, not the time the ICSI was first started

7. Select on the right hand side which wells in the EmbryoSlide® contain embryos by clicking the numbers appropriately
8. Select “Done”
9. A dialogue box will appear asking if you would like to “Add more slides”
10. If more slides need to be added that are ready to do so then click “Yes” and follow steps 3-7
11. If there are no more EmbryoSlides® to add then click “No”
12. The EmbryoScope® software will automatically find the wells and will acquire the optimal focal planes for all wells
13. If auto-focus did not find the best focused image then it can be manually corrected by selecting “Live View” then following section 7.5 of the manufacturers user manual
14. If an instrument is constantly not finding the best focus then the re-calibration of the camera should be undertaken (please refer to the user manual for further instruction)

9.3.7.7 Changing medium in EmbryoSlide® wells

- Please refer to pages 29, 46-48 of the manufacturers user manual
- The culture medium is only changed in unusual circumstances and not part of a normal culture period from day 0 to day 6 (where a single-step medium is used)
- New medium must be pre-equilibrated at the appropriate temperature and gas concentrations

1. Place a 155µm pipette, a manual pipette set to 25µl and a 60mm round

petri dish in an appropriate safety cabinet

2. Select the patient requiring a media change on the home screen of the EmbryoScope®.
3. Press “Pause”. The slide holder will move to bring the selected EmbryoSlide® to the door which can take up to 20 seconds. During this time a tube of pre-equilibrated media that the wells are to be replenished with must be removed from the set-up incubator, tight capped and placed in the safety cabinet.
4. When the correct EmbryoSlide® is in the removal position the EmbryoScope® door will unlock and a green light will appear below the door latch
5. A box will appear with ‘Ready to proceed’ – select OK.
6. Remove dish.
7. Place the dish on the heated stage
8. Using the manual pipette set at 25µl, attach a tip and place it in first well of the EmbryoSlide®. **DO NOT PLACE THE TIP NEAR THE EMBRYO IN THE MICROWELL AS THE EMBRYO COULD BE ACCIDENTALLY ASPIRATED INTO THE PIPETTE.**
9. Remove 25µl of media out of every well in the EmbryoSlide®, dispelling the used media between each well into the petri dish
10. Change the pipette tip and remove the cap of the media tube
11. Depress the pipette to the furthest point and aspirate media from the tube
12. Place the pipette tip in the first well of the EmbryoSlide® and depress the pipette to the first stop allowing some media to remain in the pipette acting as a buffer preventing the formation of bubbles
13. Repeat this for each well in the EmbryoSlide®
14. Check the dish for bubbles and remove these as described above using the 155µm pipette
15. Ensure that the time the dish is out of the EmbryoScope® is as short as possible
16. Select ‘Reinsert’ on the touchscreen of the EmbryoScope® and, when prompted, replace the EmbryoSlide® in the EmbryoScope®
17. Ensure that the focus is re-checked after the media change has

completed to ensure that all the wells are correctly aligned and the embryos are in focus

NB. While an EmbryoSlide® is paused, no images are acquired from the remaining EmbryoSlides®. If an EmbryoSlide® has been paused for more then one hour the EmbryoScope® will alarm. This alarm can be reset by pressing 'Reset' on the incubator control panel on the upper left side of the EmbryoScope®

9.3.7.8 Additional information

Please refer to the manufacturer's user manual for additional information regarding the following:

- Incubator temperature
- CO_2 and O_2 setting of the incubator
- Camera reset and calibration
- Live inspection and refocusing of embryos
- Home screen information
- Data storage on the EmbryoScope®
- Routine validation of EmbryoScope®
- Emergency procedures
- Technical specifications
- Symbols
- Disposal of waste

9.3.8 EmbryoScope® annotation and selection

9.3.8.1 Annotation pathway

Full annotation is only required for those embryos that are utilised. It is recommended that annotations take place on the day of use to minimise additional, unnecessary annotations.

DAY 1 (16-20hpi) – Fertilisation Check

1. At fertilisation check for ICSI patients annotate PN(n) for ALL oocytes

DAY 1 (26-28hpi) – Late Fertilisation/Early Cleavage Check

1. Annotate PN(n) (for IVF only), t2PB, tPNa and tPNf using the annotation panel on the EmbryoScope® shown below
2. Perform a 'late fertilisation check' on any unfertilised oocytes by annotating their PN(n) again and if they remain unfertilised "discard" them by colouring the embryo red using the discard tool
3. A witness is not required for this as a physical discard is not taking. If however, the oocyte being "discarded" was once a normally fertilised embryo and has converted to a 3PN then "discard" must NOT be written next to the oocyte, instead a line must be drawn to indicate that no further grading or annotations are required. This oocyte will then be counted in the final discard witness check at the end of the treatment as it was once an embryo.

NB. Where the PNs fade unequally (i.e. more than one frame apart) select 'tPNf unequal' from the If Applicable menu when the first PN fades then 'PN faded' when the second disappears

DAY 2 (40-44hpi)

1. Annotate t2, t3, t4, using the cell number drop down menu on the annotation panel
2. Annotate ONLY the % fragmentation and blastomere evenness at the two and four-cell stages using the relevant boxes on the annotation panel (shown below). The percentage fragmentation relates to the current grading scheme used i.e. 0-10% = 4, 10-20% = 3, 20-50% = 2 and over 50% = 1. Blastomere evenness options are simply "even" and "uneven"; if the embryo has been graded a 4 or 3 for evenness using the grading system then it is to be classed as even, if it is a 1 or a 2 then it is to be classed as uneven

3. Assess the embryo for MNB at two and four-cell stage. If both cells of the two-cell embryo show MNB then click ‘2’ – this will then indicate that 100% of cells have MNB.
4. Assess for irregular division. Where an irregular division occurs the irregular division tick box must be selected, the irregular division selected from the drop-down menu and the embryo coloured yellow using the ‘?’ tool
5. Where DC occurs select ‘DC1-3 TBC’ from the If applicable drop-down menu. At the end of the culture period, the day 5/6 annotater will confirm if it is a ‘True DC1-3’ (i.e. all cells divide in next cell cycle, have a nucleus or are incorporated into blastocyst) or a ‘False DC1-3’ (i.e. cells do not divide in next cell cycle, they don’t have nuclei or they are not incorporated into resulting blastocyst)

NB

- **Do not annotate MNB if the fragmentation is 20-50%, if the fragmentation obscures the view or if the embryo undergoes DC. In this case annotate as ‘N/A’.**
- **Do not annotate further if the embryo has undergone DC (this will need to be confirmed by the day 5/6 annotater when the nature of DC has been established)**
- **If the embryo is uneven at the two-cell stage the annotations should continue**

DAY 3 (64-68hpi)

1. Annotate t5, t6, t7 and t8 using the same tool as used for annotating t2 as shown above
2. Annotate the % fragmentation and blastomere evenness as performed for the day 2 check shown above for eight-cell stage only
3. Assess for irregular divisions as above
4. Perform the % fragmentation and evenness for any two and four-cell stages that were unable to be performed on day 2 following the instructions provided above

DAY 4 (88-92hpi)

1. Annotate t9+ and tM using the same tool as used for annotating all other cell stages (shown above)
2. If compaction begins before t9+ select ‘tM (early)’ from the if applicable menu
3. If blastulation begins before tM select ‘tSB (early)’ from the if applicable menu
4. Perform the % fragmentation and evenness for any eight-cell stages that were unable to be performed on day 3 following the instructions provided above
5. Assess for irregular division as in point 4 above.

DAY 5 (112-116hpi) – AM Check

1. Annotate tSB and tB using the same tool as used for annotating all other cell stages
2. Perform any other annotations that have been unable to be performed on previous days and confirm any DC (if it cannot be confirmed select ‘DC1-3 unconfirmed’ from the irregular division menu) and annotate evenness and fragmentation at two-cell where applicable

Additional information

- The final morphology grade should be assigned at the last frame of imaging for each embryo
- If the embryo cannot be annotated due to irregular division or quality then the irregular division tick box must be checked and the reason for the irregularity selected from the irregular division drop down menu
- Every irregularity MUST have ‘irregular division’ ticked and the reason for the irregular division selected from the irregular division drop down menu i.e. DC, RC, CL, CC etc

- If any embryo cannot be annotated due to quality, click irregular division and select ‘Fragmented Embryo’ from the irregular division drop down menu
- Where a slide has to be removed from one instrument to another, they must be linked by writing the following statement in the slide description box; ‘Split run – slide Dxxx.xx.xx_Sxxxx_Ixxx is same slide’
- Do not write any free text in any of the comments boxes
- If a treatment cycle is an FET, enter ‘FET’ in the fertilisation comment box
- If annotations are unable to be made due to instrument errors, select the appropriate error from the ‘Instrumental Failures’ drop down menu at the point at which the error occurs

9.3.8.2 Selection

The following process should be followed in order to select embryos for transfer, cryopreservation and discard.

1. Grade all embryos on the last image using the ‘View Slide’ option
2. Identify, based on morphology, those embryos that will be utilised
3. Every embryo that is selected for utilisation should have its time-lapse images reviewed in detail to ensure there are no abnormalities in development. For example, a late occurring 3PN.
4. Highlight three of these and click ‘Annotate’
5. Watch the videos from start to finish for all three embryos alongside one another on normal speed
6. Use this to identify any irregular divisions
7. Rank the embryos based on the footage and leave the top ranked embryo selected
8. Return to the ‘View Slide’ pane and select further embryos to be utilised
9. Watch the videos for these embryos from start to finish and continue in this way until a top ranked embryo is identified
10. Use the ‘Compare and Select’ tool to determine each embryos ESA score

11. Select “HFC v1.1” from the drop-down menu at the top of the screen
12. The timings for the appropriate division events will be displayed along with the dynamic grade
13. Where an embryo has not been annotated for one of the reasons above a “?” or “(Late)” will be displayed.
14. The HFC v1.1 grade should then be written in the designated column on the embryology pack
15. Ensure the embryo selected for use (transfer or cryopreservation) does not have an unusually low score from the HFCv1.1 ESA
16. If there are two embryos that are morphologically the same, the ESA should be used to choose between them

9.3.9 Performing an embryo transfer

9.3.9.1 Required equipment

Safety cabinet with heated stage
Pipette handle and rest

9.3.9.2 Reagents and consumables

60mm round petri dishes
4-well dish
Equilibrated aliquot of G-TL™
1ml syringe (Hunter Scientific)
Transfer catheter and stylet (Smiths Medical)
Disposable scissors (Rocket Medical)
Gloves
Needle

9.3.9.3 Embryo transfer

Embryo transfers are performed on day 3 of day 5, depending on embryo development and what the embryologists deem as the best day for transfer based on patient age, number of embryos, number of GQE on day 3 and the number of embryos to be replaced.

Setting up for embryo transfer

1. The day before embryo transfer ensure there are enough catheters and stylets and syringes in the warming oven for all transfers the following day.
2. The day before embryo transfer an embryo transfer dish is prepared and placed in the appropriate incubator overnight for equilibration

Prior to ET:

3. Remove one petri dish from the warmer and place on the heated stage to warm.
4. Place a pack of disposable scissors in the safety cabinet with the plastic removed.
5. Have a needle for 'popping' bubbles in the safety cabinet.

Selection of embryos for transfer

1. All day 3 embryos are checked on the morning of embryo transfer and their grades and cleavage stage are recorded on the embryology record form and the IDEAS V.5.3™ system
2. Day 5 embryos are 'eye-balled' on the morning of embryo transfer and EmbryoScope® annotations are performed on all blastocysts
3. Wherever possible the embryologist responsible for performing a list of embryo transfers should check all embryos for all patients scheduled for embryo transfer before the list starts. The grade and developmental stage is recorded on the embryology record form and on the IDEAS V.5.3™ system for each patient.
4. The embryo(s) for transfer are then selected with respect to their stage and grade.

   NB. Normally fertilised embryos which displayed micro-PN's may be transferred in cases where no other normally fertilised embryos are available.
5. In cases where an IVF/ICSI spilt has been performed and an embryo from each type of treatment is to be transferred, the circumstances

justifying such a transfer should be clearly documented in the patient's notes.

6. A transfer of both an ICSI and IVF embryo during the same treatment cycle should only be carried out in exceptional circumstances, with an upper limit of 2% of all ICSI embryo transfers.

Embryo transfer procedure

Pre-embryo transfer chat with embryologist

1. The nurse should let the embryologist know when the first patient is in the procedure room by telephoning the laboratory.
2. The embryologist should enter the procedure room from the embryology lab.
3. The embryologist must confirm the patient identity by asking both partners to confirm their full names and dates of birth.
4. The embryologist must then summarise the treatment cycle, confirming the following:

- Treatment type
- Number of oocytes collected
- Number of oocytes injected (if applicable)
- Number fertilised
- Number of embryos to be replaced
- Number to be frozen (if applicable)
- Embryo stage and quality
- Inform the patients that they will receive a letter confirming the outcome of any supernumerary embryos within 10 days.
- Ask the patients if they have any questions
- Ask the patients if they would like to see their embryos on the monitor prior to transfer.

eSET strategy non-compliance

The Hewitt Fertility Centre Multiple Birth Minimisation Strategy (MBMS) – Patient Management Algorithm should be used as guidance to the number of embryos to be transferred.

If more embryos are transferred than set out in the MBMS, the MBMS - non-compliance log must be completed and the reasons for more embryos being replaced should be included. A pre-printed sticker should be placed in the clinical case notes at the time of transfer. The patient must sign the sticker to acknowledge that they are going against clinical advice and are happy with the risks of potential multiple pregnancy.

Three embryos are only transferred in exceptional circumstances – this should be following review of the patients with a Consultant or the Scientific Director and the appropriate form completed (Consent to transfer three embryos form) and details of decision written in the casenotes**.**

Information to be discussed with patient

Risks to mother

- Early and late miscarriage
- Induced hypertension
- Pre-eclampsia
- Gestational diabetes
- Caesarian section
- Haemorrhage
- Stress and depression

Risks to child

- Prematurity
- Early death (twice as high as a singleton pregnancy)
- Cerebral palsy

- Reduced IQ and ADHD
- Language problems

EmbryoGlue®

1. Once embryos have been selected and the patient informed of the selection, the embryo(s) should be transferred to a pre-labelled, pre-equilibrated embryo transfer dish.
2. The embryo(s) for transfer are washed through G-TL™ (well 4) and moved to EmbryoGlue® (well 3) ready for embryo transfer.
3. The move to EmbryoGlue® **MUST** be recorded on IDEAS V.5.3™ for each patient – indicating the time of the move and which embryos were moved.
4. Embryos can be left in EmbryoGlue® for 10-15 minutes and **NO MORE THAN** 30 minutes for day 3 and **NO MORE THAN** 45 minutes for day 5 embryos.
5. The embryologist should then ring the nurse recovery station to inform the nurse that the embryologist is ready to proceed with the embryo transfer.

ID check

1. Patient identity and date of birth must be verbally confirmed for both patients, prior to transfer, in the presence of the embryologist, the doctor/FNS and the assisting nurse. All parties must check the names and dates of birth on the patient identification sheet in the patient notes and on RI Witness™ ID card along with the photo ID then sign the appropriate section on the embryology pack to record that this has taken place.
2. The embryologist should then insert the ID card into the card reader.
3. The doctor/FNS informs the embryologist of the size of catheter required
4. Remove the catheter and a 1ml syringe should be removed from the warmer.

5. The syringe is loosened by moving the plunger up and down several times. The syringe is then loaded with the 0.5ml G-TL™ from the embryo transfer tube. The media should be drawn up and down the syringe to remove any air bubbles.
6. Only after the doctor/FNS, informs the lab that he/she is ready is the embryo transfer dish to be removed from the incubator.
7. Switch the camera on and ask the patients to observe the monitor and confirm that their names and dates of birth on the dish are correct.

NB. If the patient is having transfer under sedation then the partner must confirm that they are satisfied with the identifying details.

8. If the patients wish to view their embryos prior to replacement the embryologist should place the embryos that have been selected for transfer under the camera and 'zoom' in. The embryologist should very briefly describe the salient features of the embryos to the patients bearing in mind that the embryos should be out of the incubator for the shortest possible time.
9. In the absence of a camera the embryologist performing the transfer requires a second witness prior to loading the catheter.

Loading the catheter

1. The packaging for the catheter should be cut so that its packet is used for handling by the embryologist at all times.
2. After attaching the G-TL™-loaded 1ml syringe, the catheter should be charged with the G-TL™ and the remainder emptied into the petri dish.
3. The embryo(s) are loaded into the centre of approximately 0.5cm of medium with approximately 0.5cm of air on either side.
4. The embryo(s) location within the catheter must then be checked to ensure they have been loaded correctly. This can be done by visualising the embryos within the column just as it is loaded, or can be done by placing the lower part of the catheter in the petri dish containing the expelled G-TL™ media and visualising the location of the embryo(s). If the embryo(s) are not located within the media

column, they should be carefully expelled into the well containing EmbryoGlue® and reloaded. The loading procedure is then repeated and the embryo location rechecked.

5. The catheter should be carefully handed (the appropriate way round i.e. tip of the catheter should be facing towards the patient) to the doctor/FNS, clearly stating the patient's name and the number of embryos to be replaced. As the doctor/FNS takes hold of the syringe and catheter, the packaging is removed.

Use of a stylet

1. If the embryologist is asked for a stylet once the catheter has been handed over to the doctor/FNS, the embryologist must remove the appropriate stylet from the warmer and with one hand take the catheter from the doctor/FNS and hand over the stylet with the other.
2. The embryologist must return the catheter containing the embryos back into the embryology laboratory and immediately place on the heated stage until the doctor/FNS is ready.
3. The outer packaging can be used to carefully place the tip of the catheter containing the embryos onto the heated stage.
4. When the doctor/FNS is ready to proceed, the embryologist slowly removes the outer sheath from the inner catheter, stopping short of the end so that the doctor/FNS can ‘feed’ the inner catheter into the outer sheath positioned within the cervix of the patient.
5. If at any stage the embryologist feels the embryos have been out too long i.e. during a difficult transfer, they must return the embryos to the embryo transfer dish and re-incubate.

ET catheter check

1. Following the embryo transfer the catheter is passed back to the embryologist in order for the catheter to be carefully checked to ensure that the embryo(s) have been transferred
2. The catheter is emptied into the empty dish lid on the heated stage.
3. The dish of expelled G-TL™ is carefully checked for the presence of the embryo(s).

4. Any bubbles should be removed using a sterile needle.
5. The embryologist informs the doctor/FNS if the catheter is clear.
6. If the embryo(s) are present then the process for re-loading should be undertaken

Completing the transfer

1. The empty embryo transfer dish is checked and discarded.
2. All used petri dishes, scissors, syringes and catheters are discarded in the nearest sharps bin.
3. The embryologist must remove the ID card from the reader. The ID card is filed in the embryology pack if there are supernumerary embryos to freeze.
4. Supernumerary embryos from fresh cycles are either cryopreserved or incubated for further assessment for possible cryopreservation, or discarded.
5. All records relating to the transfer are completed including the IDEAS V.5.3™ systems.
6. If the cycle is complete (i.e. no embryos remaining post-transfer or any remaining embryos are discarded):
    - an electronic HFEA treatment form must be completed, validated and sent to the HFEA using the IDEAS V.5.3™ system.
    - If there are no supernumerary embryos (i.e. all embryos transferred), the embryology pack must be filed in the ‘Cycles to close’ tray ready for the cycle to be closed and the pack to be filed. If the embryos were cultured using the EmbryoScope®, the embryology pack should be placed in the ‘ES Update’ tray.
    - If supernumerary embryos were discarded and not cryopreserved, a cryopreservation letter must be generated from IDEAS V.5.3™ and printed. The embryology pack must then be filed in the ‘Cycles to close’ tray ready for the printed letter to be filed in the pack, the cycle closed and the pack to be filed. If the embryos were cultured using the EmbryoScope®, the embryology pack should be placed in the ‘ES Update’ tray.

9.4 Appendix 4 – Triple media trial documentation

9.4.1 Standard operating procedure

1. The day before the oocyte collection set-up the appropriate dishes according to section 9.3.3.
2. Prepare one EmbryoSlide® per 18 follicles on the day prior to oocyte collection as per Figure 9 referring to section 9.3.9.3 on instructions on how to prepare an EmbryoSlide®.
3. On the day of oocyte collection and just prior to the procedure starts, the member of staff performing the pre oocyte collection checks should reconfirm the patient's participation in the trial
4. Perform the oocyte collection, oocyte culture and oocyte stripping (if applicable) according to section 9.3.4 and 9.3.5.
5. If more than 12 oocytes are available for injection, ensure there are two EmbryoSlides® available. If additional EmbryoSlides® are required, prepare using spare culture media placed in the set-up incubator the previous day
6. Perform the ICSI procedure according section 9.3.5 (if applicable)
7. Divide the injected oocytes into three equal groups. Wash the injected oocytes in the relevant wash wells based on which media the oocytes are destined for as per Figure 9
8. If an odd number of oocytes have been injected, assign the additional oocyte to the control group. If two surplus oocytes are injected then one should be assigned to the control group (G-TL™) and the other to SAGE 1-Step™.
9. Perform the IVF insemination according to 9.3.6 (if applicable)
10. Carry out fertilisation check at 16hpi according to 9.3.7
11. Divide the embryos into three equal groups. Wash the embryos in the relevant wash wells based on which media the embryos are destined for as per Figure 9. Any unfertilised oocytes should be equally divided between the three culture media and washed in the relevant wash wells as per Figure 9. If an odd number of oocytes/embryos are available then the instructions in step 8 should be adhered to.

12. Annotate all embryo characteristics according to section 9.3.10
13. Complete embryo selection and embryo transfer according to sections 9.3.10 and 9.3.11.
14. Take a final grade for all embryos immediately before their utilisation/fate i.e. discard/ freezing/ transfer

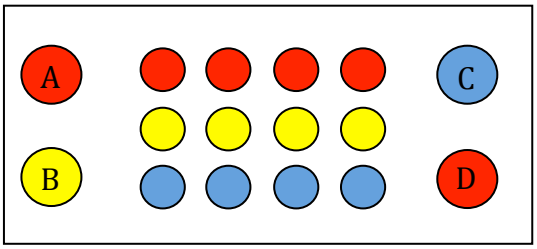


Figure 9. A schematic to represent an EmbryoSlide® to be used in TMT. Red fill indicates control media, yellow fill indicates culture media B and blue fill represents culture media C. Wash well D should be filled with control media in case of surplus oocytes and no second EmbryoSlide®.

9.4.2 Sample size calculations

Using data from the HFC, a sample mean and standard deviation were calculated. From this, the change in minutes was calculated for a 2.5 and 5% change for each morphokinetic parameter. Sample sizes were calculated for both a head-to-head investigation (A vs. B) and a mutli-variant investigation (A vs. B vs. C). The sample size calculation was two-tailed, used a 95% power value and alpha as 0.05 (Table 4).

The principle of such calculations is to determine a predicted difference that the investigation should yield as significant if this change is present. For example, where a 5% change from the mean is chosen, this indicates that if there is statistical significance in the investigation equal to, or greater than, a 5% change either way, it will be detected based on the sample sizes provided. Thus, for t2 in the head to head investigation, in order to detect at least a 5% change in the actual mean as statistically significant the sample size would need to be 198. However, if the sensitivity needs to be higher, i.e. be able to

detect a smaller change as statistically significant then the sample size will also need to be higher. Again, consider t2, for a 2.5% change to be detected, the sample size would need to be 792. This is related to the wide variation seen in the measurements. If a measurement has a large standard deviation compared to the mean, there is already a level of sampling error and so it is more difficult to determine if a difference is as a result of a true difference or simply sampling error. This is likely to be a common problem in the parameters being measured in this investigation due to the human involvement in the observations.

Table 3. Head-to-head (A vs. B) Sample Size Calculation; power = 95%, alpha =0.05, two-tailed

	Mean (min)	Standard deviation	n	2.5% change (min)	Sample size (2.5% change)	5% change (min)	Sample size (5% change)
t2PB	232.20	145.20	295	5.81	8117	11.61	2033
tPNa	493.80	189.00	312	12.35	3044	24.69	762
tPNf	1479.00	246.00	769	36.97	576	73.95	144
t2	1635.60	319.20	681	40.89	792	81.78	198
t3	2300.40	148.20	586	57.51	87	115.02	22
t4	2412.60	378.60	620	60.32	512	120.63	129
t5	3129.00	466.80	568	78.22	463	156.45	116
t6	3265.80	521.40	549	81.65	530	163.29	133
t7	3417.60	591.00	544	85.44	622	170.88	156
t8	3621.00	666.00	548	90.53	704	181.05	176
t9+	4360.80	585.60	500	109.02	375	218.04	94
tM	5121.00	602.40	478	128.03	288	256.05	72
tSB	5913.00	575.40	454	147.83	197	295.65	50
tB	6663.00	636.60	383	166.58	190	333.15	48

Table 4. Multivariate (A vs. B vs. C) Sample Size Calculation; power = 95%, alpha =0.05, two-tailed

	Mean (min)	Standard deviation	n	2.5% change (min)	Sample size (2.5% change)	5% change (min)	Sample size (5% change)
t2PB	232.20	145.20	295	5.81	20307	11.61	5266
tPNa	493.80	189.00	312	12.35	7615	24.69	1906
tPNf	1479.00	246.00	769	36.97	1440	73.95	360
t2	1635.60	319.20	681	40.89	1982	81.78	496
t3	2300.40	148.20	586	57.51	216	115.02	54
t4	2412.60	378.60	620	60.32	1281	120.63	321
t5	3129.00	466.80	568	78.22	1158	156.45	290
t6	3265.80	521.40	549	81.65	1326	163.29	332
t7	3417.60	591.00	544	85.44	1556	170.88	389
t8	3621.00	666.00	548	90.53	1760	181.05	440
t9+	4360.80	585.60	500	109.02	939	218.04	233
tM	5121.00	602.40	478	128.03	720	256.05	180
tSB	5913.00	575.40	454	147.83	493	295.65	124
tB	6663.00	636.60	383	166.58	475	333.15	119

Table 5. A summary for the required sample sizes				
	A vs. B 2.5%	A vs. B 5%	A vs. B vs. C 2.5%	A vs. B vs. C 5%
t2PB	8117	2033	20307	5266
tPNa	3044	762	7615	1906
tPNf	576	144	1440	360
t2	792	198	1982	496
t3	87	22	216	54
t4	512	129	1281	321
t5	463	116	1158	290
t6	530	133	1326	332
t7	622	156	1556	389
t8	704	176	1760	440
t9+	375	94	939	233
tM	288	72	720	180
tSB	197	50	493	124
tB	190	48	475	119

As can be seen from Table 5, the sample sizes required to perform the multi-variant analyses are higher. In addition, in order to detect the smaller percent change the sample size is greater. Based on the scientific background and the clinical relevance of performing a multi-variant analysis, this is the study methodology that will be chosen with sensitivity for 5% change detection. The rationale for selecting a 5% change detection rate is two-fold. Firstly, if a 2.5% change is required to be detected the sample sizes for some parameters are too great. For example, to detect a 2.5% change in t2PB using a multi-variant analysis the sample size would need to be more than 20,000 observations; an unattainable amount due to resource limitations. Secondly, a 2.5% change in some parameters equates to less than fifteen minutes and the clinical relevance of this change is likely to be small. In the literature, most embryo selection algorithms have optimum time ranges of a couple of hours therefore detecting a change that is small could lead to over-fitting of the models. If the difference equates to over 60 minutes then it is likely that this will be enough to effect a change in an algorithm score; the eventual aim of the research.

It has been concluded that a maximum of 500 observations (embryos) will be made for each morphokinetic parameter in each arm of the investigation totaling 1500 embryos. This means that approximately 150-200 patients will need to be recruited to the study based on the average number of oocytes collected from each patient at the HFC (ten) and the average fertilization rate. Where 500 observations are made, the sample size required for t2PB and

tPNa are not attained thus any the investigation will not be powered enough to detect true significant differences in these parameters. This therefore means, that as these parameters are only relevant for patients undergoing ICSI, patients that have both IVF and ICSI will be included in this investigation.

9.4.3 Patient information sheet

TIME-LAPSE IMAGING: INCUBATION AND ANNOTATION

PATIENT INFORMATION SHEET

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

The Hewitt Fertility Centre is the only centre in the UK that offers time-lapse imaging incubation as a standard for all patients at no extra cost. Time-lapse imaging is a new technology that allows an image of each of your embryos to be taken every ten minutes while it is inside the incubator. Using these machines, that have time-lapse capabilities, we are able to see a lot more about an embryos development than we ever could before. Because of this, we are developing different ways to select embryos based on very specific embryo development timings (called morphokinetics). It is well known that an embryos development and quality is linked to the success of a treatment cycle therefore we are carrying out a series of studies that will help us develop the service offered to all patients at the Hewitt Fertility Centre and get the most out of time-lapse imaging.

Why have I been invited?
You have been invited to take part in this study because you are attending the Hewitt Fertility Centre for fertility treatment.

Do I have to take part?
No. It is up to you to decide. We will describe the study and go through this information sheet, which we will then give to you. We will then ask you to sign a consent form to show you have agreed to take part. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive. You can obtain independent information about being involved in a research study from the local NHS Patients Advisory Liaison Service (PALS) on telephone number 01517024160.

What will happen to me if I take part?
You will be asked to sign a consent form for taking part in the study. The consent form covers three different studies, which will be outlined for you. You can consent to be considered for participation in all, none or some of the studies.

<u>Study 1</u>
The purpose of this study is to validate embryo selection algorithms (ESA's) developed on over 1000 embryos using time-lapse imaging facilitated by the EmbryoScope® incubator at the Hewitt Fertility Centre. An ESA is a list of

questions that are asked, the answers to which help to identify the best embryo for transfer. During this study you will be chosen (at random) to either have your embryos selected using an ESA or to have them selected using standard procedures (the way the embryos look); the way embryos have been chosen for the last 30 years.

Study 2
The purpose of this study is to determine if the environment in which embryos are cultured has an effect on their morphokinetics. This study will involve culturing your embryos in three different types of culture media. You will have two-thirds of your embryos cultured in two 'experimental' media and one-third in the 'control' media (the one used routinely at the Hewitt Fertility Centre). The 'experimental' culture medias used in this study are all commercially available and are used routinely around the world for the culture of embryos in fertility units. The information from this study will be used to improve developed ESA's.

Study 3
The purpose of this study is to see if different patient, treatment and embryo characteristics have an effect on their environment. This study involves the sampling of the culture media used in your treatment cycle once it has been completed i.e. after embryo transfer and embryo freezing. We will take a sample of the 'used' culture media once the embryos have been removed from it and run tests to see if there are variations between patient groups and embryo qualities.

What will I have to do?
You will only have to complete the consent form provided. You will not be required to attend the unit any more often or take part in any questionnaires or surveys.

What are the possible disadvantages and risks of taking part?
There are no known risks or disadvantages from taking part in any of the studies. The ESA's developed in study 1 have been done so robustly and many centres around the world do not prospectively apply them before using them clinically, the retrospective validation is seen as sufficient. We are conducting this part of the research to fulfil a requirement of a postgraduate research qualification. The culture media used in study 2 is commercially available and although it is not routinely used at the Hewitt Fertility Centre, they are used in many centres around the world. They have all been CE marked. A product that is CE marked indicates that it has complied with all EU laws for sale within the EU. We require your consent for this study because it involves something that isn't 'routine' in a treatment cycle. In study 3, we are simply sampling the 'used' culture media at the end of your treatment therefore there will be no effect on the care/service you receive.

What are the possible benefits of taking part?
As the ESA's used in study 1 have been developed to be particularly robust, it is hoped that this method of embryo selection will supersede that of standard embryo selection methods. Therefore, a higher chance of success may be

seen when using ESA's to select embryos for transfer as compared to standard embryo selection methods, however this cannot be guaranteed. If you are randomly assigned to the control group (no use of ESA's) you will not have any benefit. A possible benefit to participating in study 2 is that the 'experimental' culture media performs better than the 'standard' culture media resulting in higher quality embryos and an increased chance of success but we simply do not know if this is the case which is why we are conducting the research. There are no benefits to participating in study 3.

What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

Will my taking part in the study be kept confidential?
Yes. We will follow ethical and legal practice and all information about you will be kept strictly confidential. Information gathered will only be used for the purpose of the research and the results presented such that the information from a single individual cannot be identified.

Who is organising and funding the research?
The study is being organised and conducted by the Hewitt Fertility Centre team at Liverpool Women's Hospital. Liverpool Women's Hospital are also funding the research. This research is being conducted as part of a postgraduate research degree registered at Edge Hill University.

Who has reviewed the study?
All research within the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by the Ethics Committee.

What do I do now?
Please sign the consent form and hand it to us or return them in the envelope provided.

If you want to know the results of the study, a summary can be sent to you by post. Please contact Ms Amy Barrie (details below).

Thank you very much for considering taking part in our research.

Ms Amy Barrie
Clinical Embryologist
The Hewitt Fertility Centre
Crown Street
Liverpool Women's Hospital
Liverpool L8 7SS

9.4.4 Patient consent form

CONSENT FORM

Title of Study: Time-lapse imaging: incubation and annotation.

Name of Researcher: Ms Amy Barrie/ Dr Stephen Troup

Please initial box

1. I confirm that I have read and understand the information sheet **dated September 2015 Version 2.0** for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the research and care team, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in:

   i) all studies described ☐

   ii) study 1 ☐

   iii) study 2 ☐

   iv) study 3 ☐

_____	_____	_____
Name of female patient	Date	Signature
_____	_____	_____
Name of male patient	Date	Signature
_____	_____	_____
Name of person taking consent	Date	Signature

When completed, 1 copy for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

9.5 Appendix 5 – Statistical analysis excerpt

A multiple regression was run to predict the time of certain morphokinetic parameters using pronuclear fading (tPNf) as time-zero (t0) from female patient age (patage), female BMI (patbmi), infertility diagnosis (primidiag) and suppression protocol (suppressionprotocol). The assumptions of independence of errors, linearity, homoscedasticity, unusual points and normality of residuals were met. Detailed below is that of t2 as an example of the statistical output generated for this analysis.

There are 8 assumptions that must not be violated in order to obtain valid results from a multiple regression:

1. The dependent variable must be measured on a continuous scale (i.e. the morphokinetic parameter)
2. There are two or more independent variables that are either categorical or continuous (i.e. patient age, patient BMI, infertility diagnosis and suppression protocol)
3. There should be independence of observations
4. A linear relationship should exist between the dependent variable and each of the independent variables
5. Homoscedasticity must be present; variances along the line of best fit remain similar as you move along the line
6. Multicollinearity must not be present; when two or more independent variables are highly correlated with each other
7. No significant outliers, high leverage points or highly influential points
8. Errors should be approximately normally distributed

Independence of observations

As patient parameters are often related in an IVF setting, for example, the short suppression protocol (cetrotide) is often suited to those patients prone to hyperstimulation thus having an infertility diagnosis of polycystic ovary syndrome, it was imperative to test for independence of observation, the lack

of which could lead to invalid regression results. To test the independence of observations the Durbin-Watson test was used. To indicate that there is no correlation between the independent variables a value of approximately 2 is desirable from the Durbin-Watson statistic.

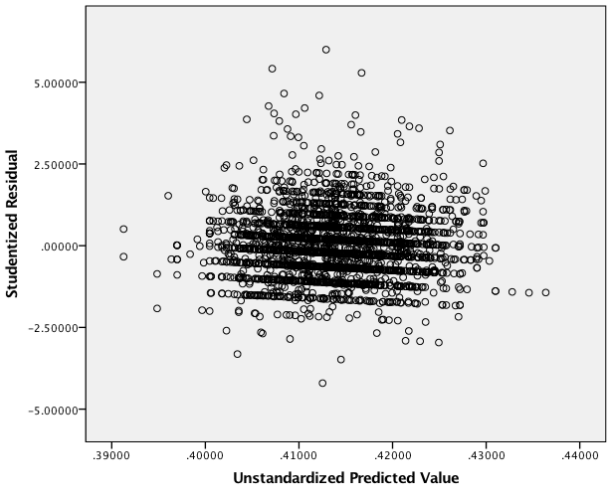
Model Summary[b]

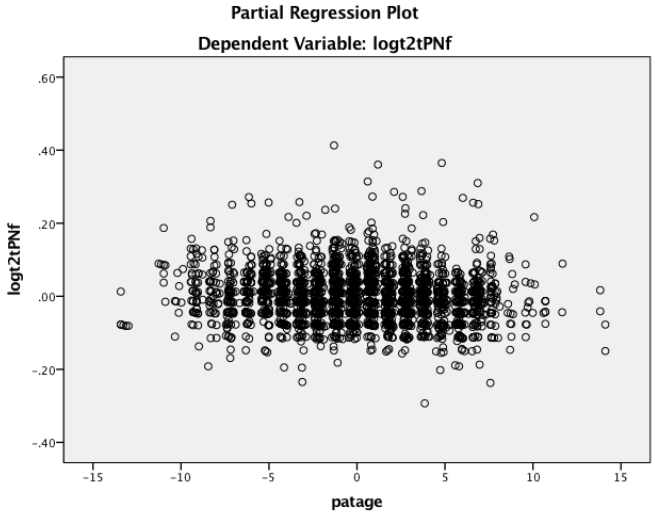
Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.089[a]	.008	.007	.06860	1.955

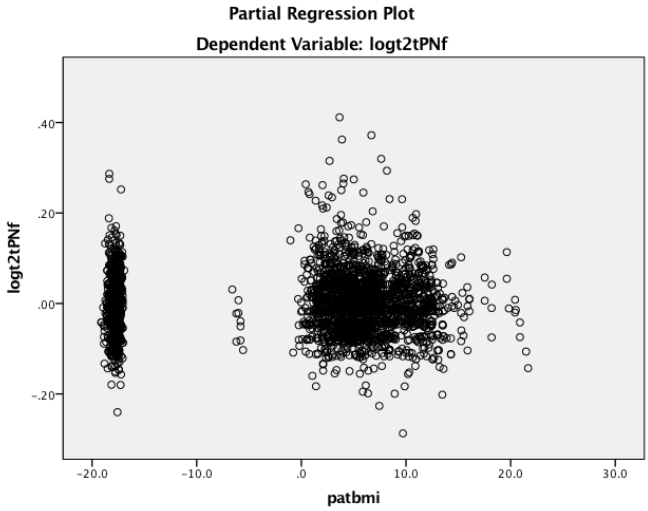
a. Predictors: (Constant), patbmi, suppressantprotocol, patage, primidiag
b. Dependent Variable: logt2tPNf

Linear Relationship

The overall linear relationship was determined using the stundentised residuals and the unstandardised predicted values of the dataset. The relationship is shown to be linear if a horizontal band is formed in the scatterplot. Individual linear relationships were then determined for each of the continuous independent variables (patbmi and patage) against the dependent variable and linearity was confirmed for all continuous independent variables as well as overall.

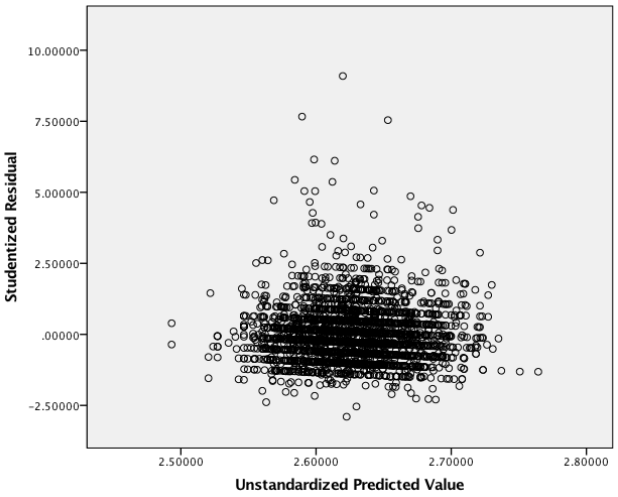


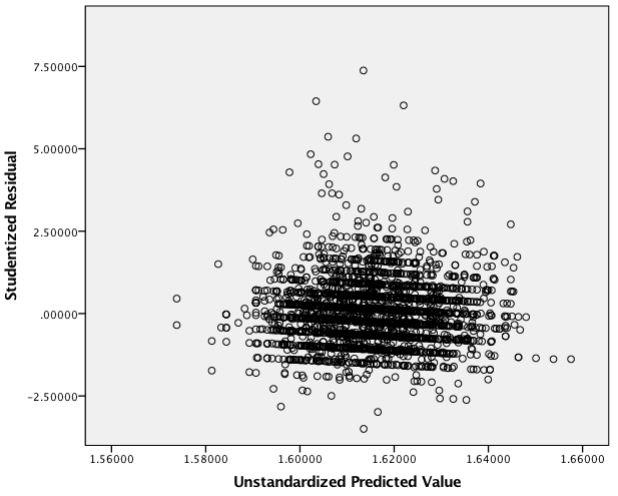




Homoscedasticity

This assumption tests whether the residuals are equally spread over the predicted values of the dependent variable. In the first instance, without transformation of the data, heterscedasticity was seen (see graph below). It was clear that the spread of values increased as the predicted values of the dependent variable increased. Therefore a root transformation as performed based on the premise that the data was moderately skewed (see graph below). However, this transformation was not appropriate as heterscedasticity remained. Therefore, a log transformation was performed (for severely skewed data) and homoscedasticity resulted.


Studentized Residual
10.00000
7.50000
5.00000
2.50000
.00000
-2.50000
2.50000
2.60000
2.70000
2.80000
Unstandardized Predicted Value


Studentized Residual
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5.00000
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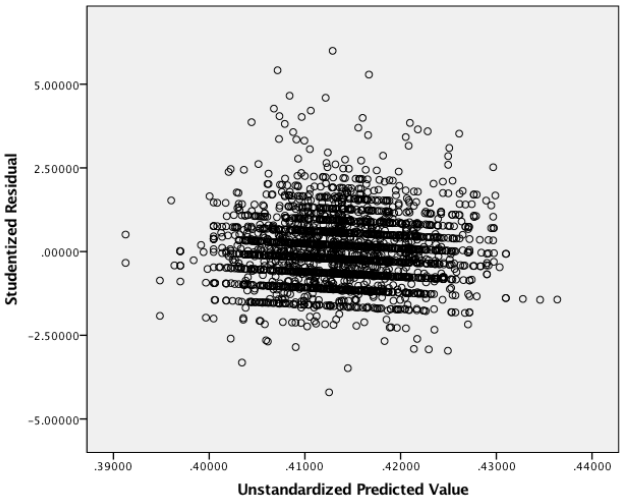
1.60000
1.62000
1.64000
1.66000
Unstandardized Predicted Value


Studentized Residual
5.00000
2.50000
.00000
-2.50000
-5.00000
.39000
.40000
.41000
.42000
.43000
.44000
Unstandardized Predicted Value

Multicollinearity

Multicollinearity results when two or more independent variables are highly correlated with each other. In these data there could be a correlation between patient BMI and infertility diagnosis as there is evidence to suggest that adipose tissue can lead to hormone deficiencies.

Collineratity was determined using two methods; inspection of correlation coefficients and Tolerance/ VIF values. The first method requires that none of the independent variables are larger than 0.7. Those of interest are highlighted and none are higher than 0.7. The second method requires the tolerance value to be greater than 0.1 or its recirpical (VIF) to be less than 10. As can be seen from the table below, the tolerance value is greater than 0.1 in all cases.

Correlations

		logt2tPNf	patage	suppressant protocol	primidiag	patbmi
Pearson Correlation	logt2tPNf	1.000	-.084	.013	.027	.014
	patage	-.084	1.000	.011	-.031	.014
	suppressantprotocol	.013	.011	1.000	-.015	-.007
	primidiag	.027	-.031	-.015	1.000	.033
	patbmi	.014	.014	-.007	.033	1.000
Sig. (1-tailed)	logt2tPNf	.	.000	.248	.075	.229
	patage	.000	.	.282	.052	.233
	suppressantprotocol	.248	.282	.	.211	.358
	primidiag	.075	.052	.211	.	.040
	patbmi	.229	.233	.358	.040	.
N	logt2tPNf	2763	2763	2763	2763	2763
	patage	2763	2763	2763	2763	2763
	suppressantprotocol	2763	2763	2763	2763	2763
	primidiag	2763	2763	2763	2763	2763
	patbmi	2763	2763	2763	2763	2763

	Coliniarity Statistics	
	Tolerance	VIF
Patage	.999	1.001
Suppressantprotocol	1.000	1.000
primidiag	.998	1.002
patbmi	.999	1.001

Detecting outliers

An outlier is defined as an observation that does not follow the usual pattern of points. Using the Casewise Diagnostics tool in SPSS, 29 outliers were identified based on the standardized residuals being greater than ±3 standard deviations. Having assessed the data, these outliers were not removed from the dataset until their leverage and influential points had been assessed. The basis for this being that a prediction model for embryo selection needs to be clinically applicable and to be so must be able to rank those that have unusually short or delayed division patterns. However, if they have a high leverage or influential points then they should be removed.

Leverage points

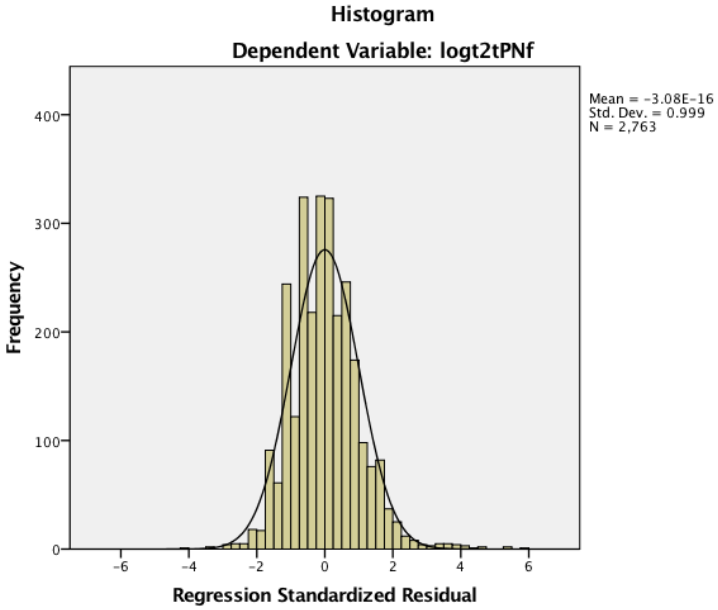
Using the leverage values for each observation those with leverage values less than 0.2 are considered safe, 0.2-0.5 as risky and over 0.5 as dangerous therefore any with a leverage point above 0.2 should be removed from the analysis. On this dataset there were no observations with leverage points above 0.2 with the lowest as 0.00571.

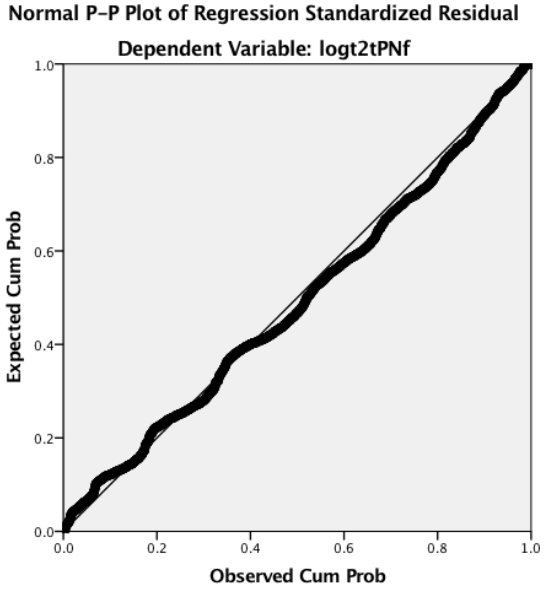
Influential points

Using the Cook's distance measure the influence of each data point was analysed. Any values above 1 should be investigated and potentially removed from the dataset. In this dataset the highest Cooks value obtained was 0.01984 therefore no observations needed to be excluded.

Normality

In order to perform a regression analysis effectively the data needs to be normally distributed when considering the errors in prediction i.e. the residuals. A histogram and a P-P plot were analysed for normality. Both methods were used as histograms can often be deceptive based on the dependence of their appearance of the correct column width. From both plots, the data was considered normally distributed.





Results

In summary, the predictive capacity of the independent variables on the dependent variable are weak with an R value of 0.089 and only 8.9% of the variance in the dependent variable being explained by the independent variables (R^2) (*Adj.* R^2 = 0.07).

Patient age, BMI, infertility diagnosis and suppression protocol statistically significantly predict t2, $F(2, 2758) = 5.565$, $p<0.0005$ however patient age was the only independent variable that added statistically significantly to the prediction, $p<0.0005$.

Model Summary[b]

Model	R	R Square	Adjusted R Square
1	.089[a]	.008	.007

a. Predictors: (Constant), patbmi, suppressantprotocol, patage, primidiag
b. Dependent Variable: logt2tPNf

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	.105	4	.026	5.565	.000[b]
	Residual	12.979	2758	.005		
	Total	13.083	2762			

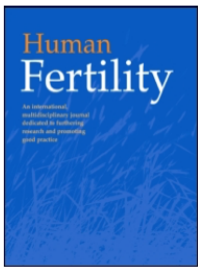
a. Dependent Variable: logt2tPNf
b. Predictors: (Constant), patbmi, suppressantprotocol, patage, primidiag

Coefficients[a]

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B		Correlations			Collinearity Statistics	
	B	Std. Error	Beta			Lower Bound	Upper Bound	Zero-order	Partial	Part	Tolerance	VIF
1 (Constant)	.449	.011		40.934	.000	.427	.470					
patage	-.001	.000	-.083	-4.380	.000	-.002	-.001	-.084	-.083	-.083	.999	1.001
suppressant protocol	.002	.003	.014	.755	.450	-.003	.007	.013	.014	.014	1.000	1.000
primidiag	.001	.001	.025	1.291	.197	.000	.002	.027	.025	.024	.998	1.002
patbmi	9.130E-5	.000	.015	.769	.442	.000	.000	.014	.015	.015	.999	1.001

a. Dependent Variable: logt2tPNf

9.6 Appendix 6 – Published articles



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Embryos cultured in a time-lapse system result in superior treatment outcomes: a strict matched pair analysis

Amy Barrie, Roy Homburg, Garry McDowell, Jeremy Brown, Charles Kingsland & Stephen Troup

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ORIGINAL ARTICLE

Embryos cultured in a time-lapse system result in superior treatment outcomes: a strict matched pair analysis

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ABSTRACT

A retrospective strict matched-pair analysis of 728 treatment cycles between January 2011 and September 2014 was performed. A total of 364 treatment cycles, where all embryos were cultured and examined in EmbryoScope®, were matched to treatment cycles where all the embryos were cultured in a standard incubator with conventional morphological examination. Matching was performed for patient age, number of oocytes collected, treatment type and date of oocyte collection (±six months). The clinical (CPR), implantation (IR), live birth (LBR) and miscarriage rates (MR) were calculated and considered significant when $p < 0.05$ (Chi-square test). CPR, IR and LBR were found to be significantly higher in the time-lapse system (TLS) group compared to the standard incubation group (CPR = 44.8% versus 36.5%, $p = 0.02$; IR = 39.3% versus 32.2%, $p = 0.03$; and LBR = 43.1% versus 33.8%, $p = 0.01$). Although there was a 5.5% decrease in the MR for the TLS group when compared to the standard incubation group, this result was not statistically significant (18.9% versus 24.4%, $p = 0.19$). There is a paucity of well-designed studies to confirm that embryos cultured and examined in TLS can result in superior treatment outcomes, and this strict-matched pair analysis with a large cohort of treatment cycles indicates the advantage of using TLS.

ARTICLE HISTORY
Received 26 January 2016
Accepted 10 June 2016

KEYWORDS
IVF; time-lapse; EmbryoScope; matched-pair

Introduction

The first application of time-lapse systems (TLS) in embryology was published in 1968 when chick embryos exposed to teratogenic doses of hypoxia were observed and analyzed (Grabowski & Schroeder, 1968). Further studies relating to preimplantation embryonic development were published over the next two decades (Alexandre & Mulnard, 1988; Colly-d'Hooghe, Valleron, & Malaise, 1977; Lueck & Aladjem, 1980; Massip & Mulnard, 1980; Milligan, Harris, & Dennis, 1978; Milligan, Harris, & Dennis, 1980; Schatten & Schatten, 1980). One of the earliest clinical applications of TLS was reported in 1997, when polar body extrusion and pronuclear formation in human oocytes were observed (Payne, Flaherty, Barry, & Matthews, 1997). Subsequently, the internalization of fragments observed in human embryos was published (Hardarson et al., 2002) followed by a report of mouse embryo collapse analyzed using time-lapse photography (Niimura, 2003). Focus turned to the use of TLS in a clinical setting in 2008 with a number of publications exclusively studying preimplantation embryonic development using TLS and how the information these systems provided could be used to determine embryo viability (Arav, Aroyo, Yavin, & Roth, 2008; Lemmen, Agerholm, & Ziebe, 2008; Mio & Maeda, 2008). The first commercially available TLS began installations in Europe in 2011 and TLS for clinical application have now been readily adopted worldwide with instruments installed in numerous countries. Although the body of evidence remains weak, it suggests that TLS can increase the chances of a pregnancy for many couples undergoing assisted reproduction. However, contradictory evidence exists. The use of TLS in clinical laboratories allows for a detailed analysis of embryos contained within it giving over 700 images per embryo. This is compared to the conventional snap-shot observations acquired when using an incubator without time-lapse capabilities that require translation into a written series of numbers and letters open to interpretation by other members of the scientific team. The wealth of information that TLS provides inevitably creates the need to modify how embryos are selected for use and as such there are many reports linking time-lapse parameters

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(termed morphokinetics) to an embryos ability to create a pregnancy.

A recent Cochrane review retrieved 33 articles relating to the use of TLS with only ten studies being potentially eligible for inclusion (Armstrong, Arroll, Cree, Jordan, & Farquhar, 2015). After further evaluation, three studies were included as true randomized, controlled trials (RCTs). These trials totalled 994 couples with the majority contributed by one study (Rubio et al., 2012). Following analysis, it was concluded that for all types of TLS, with or without cell-tracking, embryo-scoring algorithms, versus standard embryo incubation there was no conclusive evidence of a difference in clinical pregnancy, miscarriage, live birth, and stillbirth rates per couple randomized.

With this in mind, the aim of this paper was to examine whether TLS can be considered superior to standard incubation systems when considering CPR, IR, LBR and MR by performing a strict matched-pair analysis with a large cohort of patients.

Materials and methods

Study design

A retrospective, observational, matched pair data analysis was designed and approved by the NHS Research Ethics Committee in the North West (ref: 14/NW/1043). Data for this research were obtained from 728 treatment cycles between January 2011 and September 2014. This data comprised 364 patients having embryos cultured in a standard incubator (Sanyo Multigas MCO-18M (Moriguchi, Japan), 37 °C, 6% carbon dioxide (CO_2)) (group 1) and 364 having their embryos cultured in a time-lapse enabled incubator, (EmbryoScope® (Vitrolife, Gothenburg, Sweden) 37 °C, 6% CO_2, 5% oxygen (O_2)) (group 2). Although in group 1 the embryos from 214 patients were cultured in 20% O_2 and 150 in 5% O_2, a statistical examination of the LBR showed no significant differences between these groups (20% O_2 vs. 5% O_2; 34.1% vs. 34.0%, $p = 0.92$); as such the data was subsequently pooled. All the treatments included in this analysis were from known implantation embryos (i.e. a single embryo transfer or a double embryo transfer where transfer of two embryos resulted in either a negative pregnancy test or two foetal heartbeats). Clinical pregnancy (CPR), implantation (IR), live birth (LBR) and miscarriage (MR) rates were calculated.

Patient criteria

All the patients with embryos cultured in the EmbryoScope® with known outcome were matched to patients having embryos cultured in a standard incubator for patient age (exact), number of oocytes collected (exact), treatment type and date of treatment (± six months). Patients were not included twice. The patients were allocated to either standard or TLS culture randomly, based on availability. In February 2014 the laboratory became 100% time-lapse enabled meaning all patients had all embryos cultured in a TLS.

Ovarian stimulation

Pituitary downregulation was achieved with either a gonadotrophin-releasing hormone agonist (buserelin, Suprecur®, Sanofi-Aventis, Guildford, UK) or antagonist (cetrorelix acetate, Cetrotide®, Merck Serono, Darmstadt, Germany). Ovarian stimulation was performed using urine derived or recombinant follicle stimulating hormone (Progynova (Bayer, Leverkusen, Germany), Fostimon, Merional (IBSA, Lugano, Switzerland), Menopur® (Ferring Fertility, Saint-Prex, Switzerland), Gonal-f® (Merck Serono). Doses were adjusted based on patient demographic and response.

Oocyte retrieval and embryology

Ultrasound-guided oocyte collection was performed transvaginally under sedation (Diprivan, Fresenius Kabi, Lake Zurich, IL). Collected oocyte-cumulus complexes were cultured in 4-well dishes (Nunc™, Thermo Scientific, Waltham, MA) containing 0.65ml G-IVF™ (Vitrolife) covered with 0.35ml OVOIL™ (Vitrolife) in a standard incubator. Sperm preparation was performed using a standard gradient separation at 0.3 relative centrifugal force (rcf) for ten minutes (ISolate®, Irvine Scientific, Santa Ana, CA) followed by two washes at 0.6 rcf for 10 min using G-IVF™. Those oocytes destined for ICSI were prepared using enzymatic (HYASE-10X™, Vitrolife) and mechanical digestion. ICSI was performed approximately 4 h following collection after which time all injected oocytes were placed in individual culture drops of G-1™ (Vitrolife) and cultured in either an EmbryoSlide® in the EmbryoScope® or a 4-well dish in a standard incubator. Those oocytes destined for standard insemination had this performed approximately 4 h after collection and were replaced into a standard incubator until fertilization check the following day. Oocytes were then checked for fertilization approximately 16–18 h post-insemination (hpi) and all fertilized oocytes along with all unfertilized metaphase II oocytes were placed in individual culture drops of G-1™ and cultured in either an EmbryoSlide® in the EmbryoScope® or a 4-well dish in a standard incubator. Where culture to day five was undertaken, a

Table 1. Baseline data indicating the number of SET and DET, proportion of cleavage and blastocyst transfers, number of previous attempts, number of embryos transferred, average patient age, average oocytes collected and fertilization rate in group 1 (standard culture) and group 2 (EmbryoScope®).

	Group 1 (Standard)	Group 2 (EmbryoScope®)	*p*-value
Single embryo transfers (SET, *n*)	300	283	0.11
Double embryo transfers (DET, *n*)	64	81	
Cleavage stage transfers (*n*)	116	101	0.20
Blastocyst stage transfers (*n*)	248	263	
Previous attempts (mean ± SD)	1.35 ± 0.92	1.45 ± 0.95	
Average patient age (mean ± SD)	34.04 ± 4.00		
Average oocytes collected (mean ± SD)	10.13 ± 4.72		
Fertilization rate (%)	62.02	63.98	0.09
Embryos transferred (*n*)	428	445	0.64

Data were analyzed using the Chi-square test.

media change was performed on day three. For those embryos cultured in the EmbryoScope®, 20μl from each well was aspirated and replaced with 20μl of G-2™ (Vitrolife). For those embryos cultured in standard incubation, all embryos were moved to a new 4-well culture dish comprising individual 20μl drops of G-2™. Embryo selection for those cultured in the EmbryoScope® was performed using the ACE/BFS national guidelines (Cutting, Morroll, Roberts, Pickering, & Rutherford, 2008) with an internally derived embryo-scoring, time-lapse algorithm as an adjunct if a decision could not be made between embryos of similar morphology. Embryo selection for those cultured in the standard incubator was performed using the national grading scheme only. The internally derived embryo-scoring, time-lapse algorithm used three morphokinetic parameters; the time between three-cell and four-cell, the time between four-cell and five-cell, the time to five-cell. This algorithm was developed using 173 known implantation embryos and validated on a further 250 (unpublished data). The national grading scheme (Cutting et al., 2008) combines three parameters for day three embryos; cell number (*n*), blastomere evenness (1–4) and fragmentation (1–4). For day five embryos, the national grading scheme includes the level of expansion (1–6), quality of the inner cell mass (A–E) and quality of the trophectoderm (A–C). Embryo transfer was performed using the highest-grade embryo(s) either three or five days post collection depending on the number of good quality embryos the patient had on day three as well as how many were to be transferred. Selected embryos were cultured in EmbryoGlue® (Vitrolife) prior to embryo transfer.

Statistical analyses

CPR was calculated as the number of patients having a foetal heart beat (fhb) at 6–7 weeks gestation confirmed by ultrasound scan (regardless of number of fhb) out of the number of embryo transfers performed. IR was calculated as the total number of fhb (i.e. inclusive of higher order pregnancies) out of the number of embryos transferred. LBR was calculated as the number of all live births out of the number of embryo transfers. Finally, MR was calculated as the number of positive human chorionic gonadotrophin (hCG) tests (urinary sample taken 14 days following a day three transfer or 11 days following a day five transfer) that did not result in a fhb at ultrasound scan at 6-7 weeks gestation. Results were analyzed using the Chi-square test (GraphPad Software Inc., La Jolla, CA).

Table 2. Data end point results.

	Group 1 (Standard)	Group 2 (EmbryoScope®)	*p*-value
CPR (%)	133/364 (36.5)	163/364 (44.8)	0.02*
IR (%)	138/428 (32.2)	175/445 (39.3)	0.03*
LBR (%)	123/364 (33.8)	157/364 (43.1)	0.01*
MR (%)	43/176 (24.4)	38/201 (18.9)	0.19

Clinical pregnancy rate (CPR), implantation rate (IR), live birth rate (LBR) and miscarriage rate (MR) for both standard (Group 1) and EmbryoScope® (Group 2) incubation. All results were considered to be statistically significantly different between the two groups where $p < 0.05$ (Chi-square test) as indicated by the asterix.

Results

A total of 728 treatment cycles were analyzed and the CPR, IR, LBR and MR calculated (see Table 1 for baseline demographic information). CPR, IR and LBR were found to be statistically significantly different between the two groups (Table 2). The CPR for group 1 (standard incubation) when compared to group 2 (TLS) was 36.5% versus 44.8%, respectively ($p = 0.02$). The IR for group 1 when compared to group 2 was 32.2% versus 39.3%, respectively ($p = 0.03$). The LBR for group 1 when compared to group 2 was 33.8% versus 43.1%, respectively ($p = 0.01$). However, although there was a 5.5% increase in the MR for group 1 when compared to group 2, this result was not statistically significant (24.4% versus 18.9%, respectively, $p = 0.19$).

Discussion

The results of this matched pair analysis reveal that embryos cultured and examined in the EmbryoScope®

incubator result in superior treatment outcomes in this laboratory. These results are in concordance with others (Adamson et al., 2015; Basile et al., 2015; Rubio et al., 2014; Yang et al., 2014) but have been contradicted elsewhere (Armstrong et al., 2015; Cruz et al., 2011; Kahraman, Cetinkaya, Pirkevi, Yelke, & Kumtepe, 2013; Kirkegaard, Hindkjaer, Grondahl, Kesmodel, & Ingerslec, 2012; Kovacs et al., 2013; Nakahara et al., 2010; Park, Bergh, Selleskog, Thurin-Kjellberg, & Lundin, 2015). A recent Cochrane review (Armstrong et al., 2015) suggested that there was insufficient evidence to conclude that TLS with or without cell-tracking technology would be beneficial to patients undergoing assisted reproductive treatment (ART). Included in this were three eligible RCTs the first of which contributed most of the data for the review. This study was a multi-centre RCT of 843 couples undergoing ICSI, using donated or autologous oocytes; 438 were randomized to TLS and 405 to standard incubation. The CPR and MR were calculated as endpoints. Although this analysis revealed a significant increase in treatment outcomes, reasons for bias were identified. Firstly, patients could request the intervention (TLS) therefore allocation was, in fact, non-random. Secondly, the study was classed as 'double-blinded' due to the gynaecologist and statistician being unaware of the arm to which the patients had been randomized. However, the patients and embryologists were given this information. Although unlikely to create a significant bias, this detail could invalidate the results. Finally, the heterogeneity of the sample was considerable including the use of donated, and thus both fresh and frozen oocytes (Rubio et al., 2014). The remaining two RCTs included in the review were conducted on a small number of couples, one being interim results only, leaving a combined total of 61 to 65 in each arm (Kahraman et al., 2013; Kovacs et al., 2013). The reviewers reported a high risk of attrition bias in one of these studies due to the principal investigator undertaking the randomization and also because there was no blinding. Overall, the reviewers stated that there was no conclusive evidence of a difference between standard incubation and TLS when considering CPR, MR, LBR and stillbirth rates. Further analyses, not included in this Cochrane review, have also shown no significant differences in treatment outcomes between embryos cultured in standard incubation versus TLS (Cruz et al., 2011; Kirkegaard et al., 2012; Nakahara et al., 2010; Park et al., 2015).

Differences in results found thus far in the matter of TLS could be attributed to a number of factors. Firstly, a benefit of TLS that one laboratory might enjoy may not be so with another due to the conditions of the laboratory in the first instance. In brief, a well-designed, stable culture environment (TLS) introduced into what was a relatively unstable culture condition may elicit an immediate uplift in treatment outcomes. Whereas, to place this technology into an already optimal culture environment, may not reveal such results. There are many factors that vary between laboratories that could impact this: the type of culture media (single or sequential), culture dish type, volume of media used for culturing embryos, volume of oil overlay, the type of incubator and the embryo grading and embryo transfer policies. It is, therefore, reasonable to conclude that some laboratories may benefit from TLS more than others.

Secondly, during the culture of embryos in the EmbryoScope® in this analysis an in-house derived embryo-scoring algorithm was used. This indicates that the analysis presented here does not distinguish between the two, commonly stated, major benefits of TLS: the undisturbed nature of the systems or the use of embryo-scoring algorithms. Whilst the authors acknowledge that this could create ambiguity, it can also be defended. This detail means that this analysis addresses TLS as a whole in the manner in which it should be utilized; using the information provided by the images. It also gives further explanation for the inconsistency of success of TLS. Some laboratories utilizing TLS have access to large amounts of data meaning in-house derivation and validation of predictive models can be performed; a method much preferred to utilizing a published embryo-scoring algorithm developed externally. In these laboratories, where internally derived models are used, although not proven, a greater benefit to using TLS would be expected. Naturally, in those laboratories that do not have access to a data-set allowing in-house derivation of predictive models, externally developed versions may be adopted, a decision which has been cautioned (Kirkegaard, 2013; Yalçınkaya et al., 2014).

The earliest publications regarding TLS aimed to assess the safety of the systems (Cruz et al., 2011; Freour, Lammers, Splingart, Jean, & Barriere, 2012; Kirkegaard et al., 2012; Nakahara et al., 2010). Many of these studies randomized oocytes or embryos between two culture systems (standard and TLS) and found no differences in treatment outcomes of embryo quality parameters. The use of an embryo-scoring algorithm in these studies is not mentioned, thus these analyses assessed the effectiveness of the incubator itself, not the information it provided. Once satisfaction with the safety of the system had been reached attention was then turned to how the information from the TLS could be utilized. Further reports

were then published that revealed an uplift in outcome parameters (Basile et al., 2015; Milewski et al., 2015; Rubio et al., 2014; Siristatidis et al., 2015; VerMilyea et al., 2014; Yang et al., 2014) with one obvious difference: these analyses included the use of an embryo-scoring algorithm and could provide evidence of the benefits of using TLS alongside an embryo-scoring algorithm. The study previously described (Rubio et al., 2014) supports the above notion whereby those embryos cultured in a standard incubator were assessed for selection based on morphology alone and those in TLS were selected using an internally derived, multivariable model. A significantly higher ongoing pregnancy rate was found in TLS compared to standard incubation (51.4% versus 41.7% per cycle and 54.5% versus 45.3% per embryo transfer, respectively) as well as a significantly decreased early pregnancy loss in TLS (16.6% versus 25.8%). In addition, the implantation rate was significantly increased in the TLS group (44.9% versus 37.1%) (Rubio et al., 2014). A further investigation sought to select the most competent blastocysts for transfer by combining TLS and array comparative genomic hybridization (aCGH) for patients undergoing preimplantation genetic screening (PGS) designed as a prospective study with sibling oocytes. A total of 1163 metaphase II oocytes from 138 PGS patients were included and oocytes were randomized to two groups after ICSI: group A were cultured in TLS and group B in standard incubation. Array CGH using trophectoderm biopsy on both groups was carried out and one or two euploid blastocysts either within the morphokinetic ranges (group A) or morphological grades (group B) were transferred. The clinical pregnancy and implantation rates were found to be significantly higher in group A when compared to group B (CPR; 71.1% versus 45.9%, IR; 66.2% versus 42.4%, respectively) demonstrating that when embryo-scoring algorithms are used as an adjunct to select embryos for transfer, superior treatment outcomes can be achieved (Yang et al., 2014). These investigations address TLS as an incubator whilst also using the data it provides, synonymous with the current analyses, indicating that embryo-scoring algorithms derived using TLS are able to select embryos more effectively than standard morphology assessments. It is not surprising that an increase in treatment outcomes is seen in these cases owing to the wealth of information that is available to the user of TLS to do basic, but powerful, embryo selection.

Literature regarding TLS now predominantly concerns development of embryo-scoring algorithms or reviews concluding that further evidence for its (TLS) superiority is required. The authors believe that predictive models can be very useful, in the first instance for de-selection (rather than selection) of embryos undergoing abnormal cleavage events such as direct cleavage and reverse cleavage shown to have a significantly reduced chance of creating a pregnancy (Liu, Chapple, Roberts, & Matson, 2014; Rubio et al., 2012) but also, if developed effectively, to select the best embryo from a cohort for a specific patient demographic. Patient characteristics including infertility diagnosis (Sundvall, Kirkegaard, Ingerslev, & Knudsen, 2015) and maternal age (Chawla et al., 2015; Hampl & Stěpán, 2013) as well as treatment characteristics including treatment type and culture conditions (Ciray, Aksoy, Goktas, Ozturk, & Bahceci, 2012; Cruz et al., 2013; Kirkegaard, Hindkjaer, & Ingerslev, 2013; Lemmen et al., 2008; Wale & Gardner, 2010) have been shown to affect an embryo's morphokinetic profile and the resulting subtle differences may be used to identify which embryo has the highest implantation potential. Herein lies a further reason for possible variation in success of TLS between laboratories; patient and treatment characteristics.

Conclusion

This matched pair analysis indicates that treatment cycles where embryos are cultured and examined in TLS result in superior outcomes including clinical pregnancy, implantation, live birth and miscarriage rates. Although the notion is novel, the authors believe that the real benefit of TLS lies in the development of patient-specific embryo-scoring algorithms. Literature thus far indicates that there is likely to be no difference in treatment outcomes when an embryo-scoring algorithm is not used and future research should be geared towards developing effective embryo-scoring algorithms to aid in embryo selection.

Disclosure statement

The authors report no declaration of interest.

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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

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Objective: To study the efficacy of six embryo-selection algorithms (ESAs) when applied to a large, exclusive set of known implantation embryos.
Design: Retrospective, observational analysis.
Setting: Fertility treatment center.
Patient(s): Women undergoing a total of 884 in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment cycles (977 embryos) between September 2014 and September 2015 with embryos cultured using G-TL (Vitrolife) at 5% O_2, 89% N_2, 6% CO_2, at 37°C in EmbryoScope instruments.
Intervention(s): None.
Main Outcome Measure(s): Efficacy of each ESA to predict implantation defined using specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV), area under the receiver operating characteristic curve (AUC), and likelihood ratio (LR), with differences in implantation rates (IR) in the categories outlined by each ESA statistically analyzed (Fisher's exact and Kruskal-Wallis tests).
Result(s): When applied to an exclusive cohort of known implantation embryos, the PPVs of each ESA were 42.57%, 41.52%, 44.28%, 38.91%, 38.29%, and 40.45%. The NPVs were 62.12%, 68.26%, 71.35%, 76.19%, 61.10%, and 64.14%. The sensitivity was 16.70%, 75.33%, 72.94%, 98.67%, 51.19%, and 62.33% and the specificity was 85.83%, 33.33%, 42.33%, 2.67%, 48.17%, and 42.33%, The AUC were 0.584, 0.558, 0.573, 0.612, 0.543, and 0.629. Two of the ESAs resulted in statistically significant differences in the embryo classifications in terms of IR.
Conclusion(s): These results highlight the need for the development of in-house ESAs that are specific to the patient, treatment, and environment. These data suggest that currently available ESAs may not be clinically applicable and lose their diagnostic value when externally applied. (Fertil Steril® 2017;107:613–21. ©2016 by American Society for Reproductive Medicine.)
Key Words: Embryo development, embryo selection algorithm, morphokinetics

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Traditional methods for embryo selection have been used for over 20 years. Numerous morphologic parameters are thought to be useful for correct embryo selection: pronuclear morphology (Z scoring) (1, 2), polar body alignment and appearance (3, 4), appearance of cytoplasm and zona pellucida (5), early cleavage (6, 7), multinucleation (8–10), and blastomere morphology (11–13). Basic embryo grading, including the number of blastomeres, evenness in the size of the blastomeres, and the level of fragmentation, remains the gold standard for embryo selection. However, using this method in a traditional sense (with a standard bench-top incubator) has two limitations: a restricted overview of an embryo's development and the exposure of the embryo to suboptimal temperatures and gas concentrations. With the introduction of time-lapse imaging, where an image of each embryo is taken every 10 to 20 minutes, more intricate embryo parameters can be viewed while leaving the embryos in an undisturbed environment. As the availability of time-lapse technologies increased, attention was first focused on assessing their clinical safety. Once the safety had been established and the available technologies were validated for clinical use (14–18), research then turned to determining how the time-lapse imaging systems could be used to increase pregnancy rates through in-depth embryo analysis and an undisturbed culture system.

Through the research performed previously and subsequently, many morphokinetic parameters were identified that correlated with the embryo's ability to create a pregnancy both in humans and animals: the appearance and disappearance of pronuclei and nuclei at each cell stage (3, 19–21), the length of time between early cytokinesis (22–29) and initiation of blastulation (30). Further embryologic phenomena have been observed using time-lapse imaging, including the reabsorption of fragments (31), direct cleavage of cells within embryos from one to three cells (32), and reverse cleavage (33). These phenomena have been shown to affect an embryo's implantation potential to varying degrees, but their discovery could lead to more effective embryo selection within a laboratory using time-lapse technology.

Single-embryo parameters such as those mentioned here have been linked to embryo viability (18), and now these parameters have been used to develop embryo-selection algorithms (ESAs). These ESAs seek to combine a number of morphokinetic parameters that have been linked to an embryo's viability expressed as the formation of a blastocyst, implantation, or a live birth. Here, the efficacy of six published ESAs for predicting an embryo's viability was examined, expressed as implantation rate (IR), in a clinically applicable setting (21, 27, 30, 34, 35) to demonstrate the need to develop specific, in-house ESAs. The ESAs examined were selected based on their clinical applicability to the test site, assessed superficially before analysis.

MATERIALS AND METHODS

This investigation was a single site, retrospective observational design approved by the North West Research Ethics Committee (ref: 14/NW/1043) and the institutional review board where necessary. All procedures and protocols complied with United Kingdom regulations (Human Fertilisation and Embryology Act, 1990, 2008). The data were obtained from 884 treatment cycles between September 2014 and December 2015. Clinical pregnancy was confirmed by the presence of a fetal heartbeat at ultrasound scan at 6 weeks' gestation. All treatments included in this analysis were from known implantation embryos; a single-embryo transfer or a double-embryo transfer where the transfer of two embryos resulted in either a negative test or two fetal heartbeats.

Ovarian Stimulation

Pituitary down-regulation was achieved using either a gonadotropin-releasing hormone agonist (buserelin, Suprecur; Sanofi Aventis) or antagonist (cetrorelix acetate, Cetrotide; Merck Serono). Ovarian stimulation was performed using urine-derived or recombinant follicle-stimulating hormone (Progynova [Bayer Germany]; Fostimon and Merional [IBSA]; Menopur [Ferring Fertility]; or Gonal f [Merck Serono]). Doses were adjusted based on the patient's demographic and response. Patients were given 5,000 IU of subcutaneous human chorionic gonadotropin (Gonasi HP; IBSA Pharmaceuticals) 36 hours before oocyte collection. Luteal support was provided via 400 mg of progesterone pessaries, twice daily (Cyclogest; Actavis), until the pregnancy test was taken.

Oocyte Retrieval and Embryology

Ultrasound-guided oocyte collection was performed transvaginally under sedation (Diprivan; Fresenius Kabi). Collected oocyte–cumulus complexes were cultured in four-well dishes (Nunc; Thermo Scientific) with each well containing 0.65 mL of G-IVF (Vitrolife) covered with 0.35 mL of OVOIL (Vitrolife) in a standard incubator (Sanyo Multigas MCO 18M). Sperm preparation was performed using a standard gradient separation at 0.3 relative centrifugal force (rcf) for 10 minutes (ISolate; Irvine Scientific) followed by two washes at 0.6 rcf for 10 minutes using G-IVF. The oocytes destined for intracytoplasmic sperm injection (ICSI) were prepared using enzymatic (Hyase 10X; Vitrolife) and mechanical digestion. Intracytoplasmic sperm injection was performed approximately 4 hours after collection, following which all injected oocytes were placed in individual culture drops of G-TL (Vitrolife) and cultured in the EmbryoScope (Vitrolife). The oocytes destined for standard insemination had this performed approximately 4 hours after collection and replaced into a standard incubator until the fertilization check the next day. Oocytes were then checked for fertilization approximately 16 to 18 hours postinsemination (hpi), and all fertilized oocytes along with all unfertilized metaphase II oocytes were placed in individual culture drops of G-TL and cultured in the EmbryoScope.

Embryo selection was performed using the national grading scheme (36) along with an internally derived ESA. This ESA was used as an additive to morphology at the test site and only used when two or three (where double-embryo transfer was to be performed) embryos of similar morphology were available for transfer. Morphology remained the gold

standard for embryo selection. This ESA included three morphokinetic parameters: s2 (the time between three-cell [t3] and four-cell [t4]), cc3 (defined at the study site as the time between t4 and five-cell [t5]), and t5 with embryos graded in one of eight categories from A+ to D−. Embryo transfer was performed using the highest grade embryo(s) 5 days after collection. Selected embryos were cultured in EmbryoGlue (Vitrolife) for 10 to 30 minutes in a standard incubator before embryo transfer. All embryos were cultured at 37°C in 6% CO_2, 5% O_2, and 89% N_2 throughout.

Analysis of Time-lapse Information

The image interval on the EmbryoScope was set to 15 minutes with seven focal planes. Images were collected for the duration of culture immediately after ICSI or fertilization check (for IVF-derived embryos) to utilization. A single embryologist assessed images for the required morphokinetic parameters with t0 defined as the time of insemination/injection. The parameters annotated included time to pronuclear fading (tPNf), time to two-cell (t2), t3, t4, t5, eight-cell (t8), time to start of blastoculation (tSB), time to blastocyst (tB, defined when the blastocele has filled over half of the embryo and there is a <10% increase in the embryo diameter (i.e. the beginning of expansion) quantified using the line tools on the EmbryoScope instrument). From these annotations, two further annotations were calculated (s2 and cc2, the time to complete the second cell cycle). Accuracy of annotation was corroborated by the participation of the embryologist in an internal quality assurance scheme for morphokinetic analysis. Each of the ESAs (Table 1) was then retrospectively applied to the same cohort of known implantation embryos.

Statistical Analysis

Positive predictive value (PPV), negative predictive value (NPV), specificity, and sensitivity were used to determine the efficacy of each of the ESAs. These methods of measurement were chosen for the analysis because of their relationship to validity and predictive power. We defined PPV as the percentage of embryos creating a fetal heartbeat as well as a favorable ESA outcome. We defined NPV as the percentage of embryos not creating a fetal heartbeat as well as an unfavorable ESA outcome. Sensitivity was defined as the ability of the ESA to correctly classify an embryo as viable. Specificity was defined as the ability of the ESA to correctly classify an embryo as nonviable.

Each of the test measures were determined using the following calculations:

PPV = True positives/(True positives + False positives)

NPV = True negatives/(True negatives + False negatives)

Sensitivity = True positives/(True positives + False negatives)

Specificity = True negatives/(True negatives + False positives)

The likelihood ratio (LR) was determined using the following calculation:

TABLE 1

Summary of embryo selection criterion and main results.

Study	Model type	Parameter	Time frame	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC	Likelihood ratio	*P* Value
Azarello et al. 2012 (21)	Selection/deselection	PNf	>20 h 45m	16.71	85.83	42.57	62.12	0.584	1.18	.2724[a]
Basile et al. 2015 (37)	Hierarchical	t3	34–40 hpi	75.33	33.33	41.52	68.26	0.558	1.13	.006[b]
		cc2	9–12 h							
Campbell et al. 2013 (30)	Risk classification model	tSB	Low risk: tSB <96.2 hpi tB <122.9 hpi	72.94	42.33	44.28	71.35	0.579	1.26	<.0001[b]
		tB	Medium risk: tSB ≥96.2 hpi tB <122.9 hpi							
			High risk: tB ≥122.9 hpi							
Chamayou et al. 2013 (34)	Selection/deselection	cc3	9.7–21.0 h	98.67	2.67	38.91	76.19	0.552	1.01	.1817[a]
Cruz et al. 2012 (27)	Hierarchical	t5	48.8–56.6 h	51.19	48.17	38.29	61.10	0.517	0.99	.1402[b]
		s2	≤0.76 h							
Dal Canto et al. 2012 (35)	Selection/deselection	t8	54.9 ± 5.2 h	62.33	42.33	40.45	64.14	0.583	1.08	.1415[a]

Note: *P* value stated when comparing the implantation rates between categories in the applicable ESA. AUC = area under the receiver operating characteristic curve; cc2 = time between two-cell and three-cell; cc3 = time between three cell and five cell; hpi = hours post-insemination; PNf = pronuclear fading; PPV = positive predictive value; NPV = negative predictive value; s2 = time between three cell and four cell; t3 = time to three cell; t5 = time to five cell; t8 = time to eight cell; tB = time to full blastocyst; tSB = time to start of blastulation.

[a] Fisher's exact test.

[b] Kruskal-Wallis test.

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LR = sensitivity/(1 − Specificity)

The area under the receiver operating characteristic curve (AUC) was calculated for each ESA. The IR in each category of the ESA was compared using Fisher's exact test (for ESAs with two outcome categories—true or false) and Kruskal-Wallis test (for ESAs with more than two outcome categories: A, B, C, and D). $P<.05$ was considered statistically significant. Statistical analyses were performed using the statistical package Prism 5 (GraphPad Software).

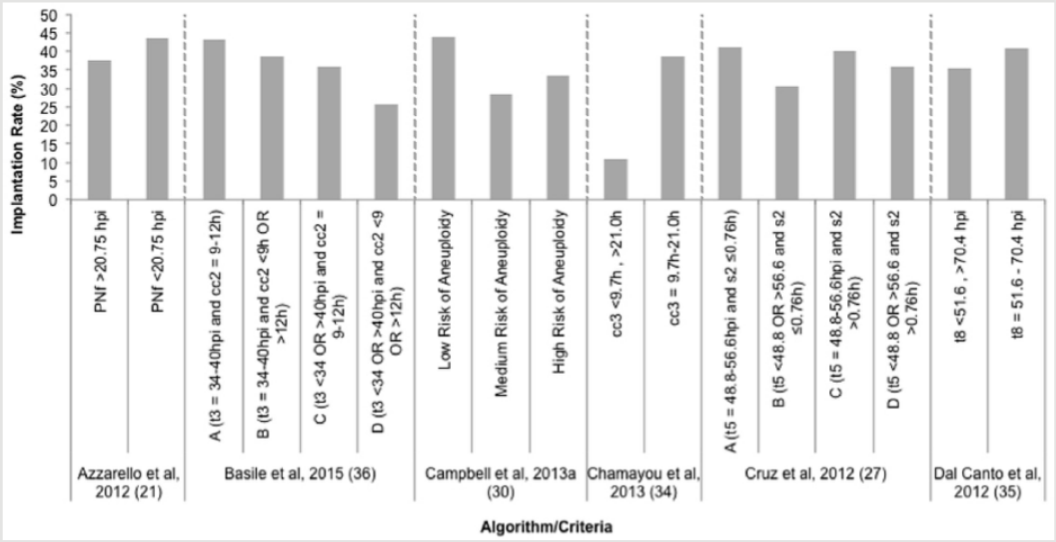
RESULTS

A total of 977 known implantation embryos from 884 treatment cycles were subject to retrospective analysis to determine the efficacy of six published ESAs (Table 1). Of these, 529 embryos were created using conventional IVF, and 448 were created using ICSI. The mean patient age was 33.44 ± 4.53 years with an average treatment attempt number of 1.37. The primary etiologies for infertility were male factor (32.2%), maternal age (4.1%), ovulatory disorders (9.9%), tubal disorders (6.6%), uterine disorders (4.1%), other (including genetic disorder) (0.2%), hormone deficiency (1%), and unexplained (41.9%). All embryo transfers were performed on day 5 (blastocyst). Ninety-three double-embryo transfers and 791 single-embryo transfers were performed. Among the treatment cycles, 50.36% were an agonist protocol; the remainder of the cycles were an antagonist protocol. An overall IR of 39.7% was achieved with 388 of the 977 embryos implanting and 589 not implanting.

The PPV for each of the ESAs did not reach above 45% in any case. The NPV was between 60% to 70% for all ESAs analyzed (Table 1). The sensitivity and specificity were considerably more variable (Table 1), as would be expected, identifying that two ESAs had a high sensitivity (30, 34), and another a high specificity (21). Finally, the AUC analysis revealed values from 0.512 to 0.629 (Table 1).

The IR for each category of four of the analyzed ESAs did not statistically significantly vary ($P>.05$) (Fig. 1). However, the IR for the three categories of the aneuploidy risk classification ESA (30) statistically significantly varied ($P<.0001$), as

FIGURE 1



Implantation rates (IRs) of the embryo classification categories in each of the analyzed embryo selection algorithms (ESAs). Azzarello et al. (21): IR of embryos where pronuclear fading (PNf) occurred after 20.75 hpi (n = 832, 37.74%) and embryos that faded before 20.75 hpi (n = 145, 43.45%) ($P>.05$, Fisher's exact test). Basile et al. (37): IR of embryos classified as A (t3 = 34–40 hpi and cc2 9–12 hours, n = 453), B (t3 = 34–40 hpi, cc2 >9 or <12 hours, n = 231), C (t3 <34 or >40 hpi and cc2 = 9–12h, n = 173) and D (t3 <34 or >40 hpi and cc2 <9 or >12 hours, n = 120) with respective IR of 43.05%, 38.53%, 35.84%,and 25.83% ($P>.006$, Kruskal-Wallis test). Campbell et al. (38): IR for embryos classified as low risk (tSB <92.2 hpi and tB <122.9 hpi, n = 621), medium risk (tSB ≥96.2 and tB ≤122.9 hpi, n = 353) and high risk (tB ≥122.9 hpi, n = 3) of aneuploidy with respective IR of 43.80%, 28.61% and 33.33% ($P<.05$, Kruskal-Wallis test). Chamayou et al. (34): IR of those embryos where cc3 (t5–t3) occurred between 9.7–21 hours (n = 959, 11.11%) and those that did not (n = 18, 38.79%) ($P>.05$, Fisher's exact test). Cruz et al. (27): IR of embryos classified as A (t5 = 48.8–56.6 hpi and s2 ≤0.76 hours, n = 364), B (t5 = 48.8–56.6 hpi and s2 >0.76 hours, n = 140), C (t5 ≤48.8 *or* >56.6 hpi and s2 ≤0.76 hours, n = 353) and D (t5 <48.8 *or* >56.6 and s2 >0.76 hours, n = 120) with respective IR of 41.21%, 30.71%, 39.94% and 35.83% ($P>.05$, Kruskal-Wallis test). Dal Canto et al. (35): IR of embryos where t8 occurred between 51.6–70.4 hpi (n = 578, 40.66%) and those that did not (n = 399, 35.59%) ($P>.05$, Fisher's exact test). cc2 = time to complete the second cell cycle; cc3 = time to complete the third cell cycle; hpi = hours postinsemination; s2 = time between three and four cell; t2 = time to two cell; t3 = time to three cell; t4 = time to four cell; t5 = time to five cell; t8 = time to eight cell; tB = time to blastocyst (when the blastocele has filled over half of the embryo and there is a <10% increase in the embryo diameter); tPNf = time to pronuclear fading; tSB = time to start of blastoculation.

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did category A with category D in the ESA developed by Basile et al. (37). The aneuploidy risk classification ESA also had the strongest LR (1.26) and PPV (44.28%). Incidentally, the number of embryos classified as high risk using this ESA was just three, of which one implanted, giving this category an IR of 33.33%—a potentially misleading result. The absolute difference between the IR of low- and medium-risk embryos was 15.19% (Fig. 1).

DISCUSSION

All six of the examined ESAs (21, 27, 30, 34, 35, 37) achieved an AUC <0.65 (0.584, 0.558, 0.573, 0.612, 0.543, and 0.629, respectively), indicating reduced predictive capability. None of the ESAs achieved a PPV above 45% (42.57%, 41.52%, 44.28%, 38.91%, 38.29%, and 40.45%, respectively), which was also indicative of poor diagnostic value. The NPV reached over 60% in all the ESAs (62.12%, 68.26%, 71.34%, 76.19%, 61.10%, and 64.14%, respectively). The specificity of each ESA was variable, indicating that some ESAs are able to identify embryos with a reduced chance of implantation better than others (85.83%, 33.33%, 42.33%, 2.67%, 48.17%, and 42.33%, respectively) also reflected in the NPV. This variability was also, inevitably, seen in the sensitivity of the assessed ESAs (16.71%, 75.33%, 72.94%, 98.67%, 51.59%, and 62.33%, respectively).

In all the ESAs assessed, the LR was close to 1 (1.18, 1.13, 1.26, 1.01, 0.99, and 1.08, respectively). The LRs of all ESAs revealed that there was little predictive power of implantation where a favorable ESA result is obtained (Table 1). Likelihood ratios range from 0 to infinity and a LR close or equal to 1 indicates a lack of diagnostic value; the farthest from 1 that any of the ESAs in this investigation reached was 0.26, indicating that an embryo has a 0.26 increased chance of creating a pregnancy if a favorable ESA outcome is achieved.

Worthy of note are the ESAs that were found to have statistical significance between the categories of embryo classification (30, 37). However, the number of embryos classified as high risk of aneuploidy in the aneuploidy risk classification ESA was just three of 977. Further validation, performed by the developers of this ESA (38) using 88 embryos, classified four as high risk of aneuploidy. Clearly, using this ESA, the chance of an embryo being classified as high risk is low, which raises issues about the specificity of the ESA especially when evidence suggests that over 50% of embryos exhibit aneuploidy (39). With an AUC of 0.575 and a 0.26 increased chance that an embryo would create a pregnancy if classified as low risk of aneuploidy, this ESA may not represent a robust, clinically applicable embryo selection. Nonetheless, this ESA is the most effective out of the six assessed when a combination of specificity, sensitivity, PPV, NPV, AUC, LR, and differences in implantations between each embryo classification category is considered.

The other ESA to gain statistical significance between the categories when considering IR was that of Basile et al. (37). Statistical significance was found between the IRs of category A and D, indicating that this ESA may perform well in terms of identification of poor quality embryos. This is also reflected in a high sensitivity and NPV. However, the LR remains low at 1.13, and the other measures of the effectiveness of the ESA (specificity, PPV and AUC) indicate this ESA may not be as effective at determining higher implantation potential embryos.

The analyses performed indicate that ESAs available in the literature may not provide substantial, additional aid for embryo selection in a clinically relevant setting. The current investigation highlights that externally derived ESAs are developed, unavoidably, under conditions different to that of the adoptive center (Table 2) encouraging the development of in-house, specific ESAs. It has been shown that the method by which embryos are created (IVF or ICSI) can affect their temporal behavior (41–43). In addition to varying treatment types, a number of the analyzed ESAs excluded certain patient groups to avoid confounding factors. This includes those with endometriosis, polycystic ovary syndrome (PCOS), severe male factor infertility, and maternal age over 39 years. This exclusion constitutes a proportion of patients that make up a significant fraction of patients treated in an IVF laboratory and for which these ESAs could be critically useful.

There is evidence to suggest that the reason for infertility could affect an embryo's morphokinetic profile, in particular those with PCOS (44), thus their exclusion in the ESA development is understandable but reduces its clinical applicability unless a specific ESA is developed for this specific patient group. Furthermore, one group's ESA was developed using oocyte donors only, a clear confounder for the application of this ESA in other centers.

In addition, the majority of the ESAs were developed on embryos created under an agonist protocol. However, one group's ESA development cohort contained a proportion of embryos created under an antagonist protocol (30) The use of agonist and antagonist protocols has yet to be shown to affect an embryo's morphokinetic profile however they have been linked to embryo quality (45, 46), which could indicate that there is a potential for them to also have a temporal effect.

Perhaps most importantly, varying culture conditions were used in the development of these ESAs. It has been shown that an embryos morphokinetic profile is greatly altered in different culture media, specifically between sequential and single-step media (47, 48). This means that ESAs developed using sequential media may not be effective in selecting embryos cultured in single-step media, and vice versa. In addition, varying CO_2 and O_2 gas concentrations were used in the development of a number of these published ESAs. Oxygen tension has been specifically linked to an embryo's morphokinetic profile in both humans (49) and mice (40) where those embryos cultured at 20% O_2 have reduced developmental rates and the completion of the third cell cycle is significantly delayed. Of the six ESAs analyzed, one comprised multiple centers (37). The culture conditions varied slightly between centers, so it could be argued that this ESA has a broader clinical use while maintaining similar predictive power measurements (i.e., sensitivity, specificity, PPV, NPV, LR, AUC) to the other ESAs investigated. It should be highlighted however, that the algorithm developed in this original article used oocyte donors, a natural bias for outcomes focusing on embryologic features and implantation potential. These fundamental differences in the development of each ESA need to be seriously considered before their external adoption. It is highly unlikely that an external center

TABLE 2

Summary of publications used for examination of efficacy of selection criteria.

Study	Embryos, n	Cycles, n	Fertilization method	End point	Exclusion criteria	Inclusion criteria	Image capture interval(min)	Protocol	Culture	Media change	Transfer day
Azarello et al. 2012 (21)	159	130	ICSI	LBR	—	Embryos transferred at four-cell stage with equal blastomeres and <25% fragmentation, autologous gametes, female age ≤39, male factor infertility ($1–5 \times 10^5$ motile sperm/ejaculate)	20	Agonist	Cook, 5.5% CO_2, 5% O_2, 89.5% N_2	No	2 (44 hpi)
Basile et al. 2015 (37)	754	1664	ICSI	IR	Severe male factor, severe endometriosis, BMI 30 kg/m^2, low response (for standard patients, less than five metaphase II oocytes) and no PGS or PGD	For donors: 18–30 y old (mean: 26.9, SD 4.7), normal menstrual cycles (26–34 d' duration), a BMI of 18–28 kg/m^2 and normal ovaries and uterus as observed by transvaginal ultrasound	10–20	Antagonist (agonist trigger for analogous oocytes, hCG trigger for oocyte donors)	Site 1: Cook, 5.5% CO_2, 20% O_2, 74.5% N_2 Site 2: Global IVF Medium, 6.5% CO_2, 20% O_2, 73.5% N_2 Site 3: Cook, 6% CO_2, 20% O_2, 74% N_2 Site 4: Global IVF Medium, 6% CO_2, 20% O_2, 74% N_2	No	3
Campbell et al. 2013 (30)	88	25	ICSI	CPR and LBR	—	Patients undergoing a cycle inclusive of PGS	20	Agonist (75%) Antagonist (25%)	Global IVF medium, 5.5% CO_2, 5% O_2, 89.5% N_2	Yes	
Chamayou et al. 2013 (34)	178	78	ICSI	BFR	Severe endometriosis, premature ovarian failure, severe asthenoteratozoospermia	Fresh gametes	20	Agonist	Quinn's Advantage, 5% CO_2, 5% O_2	Yes	5
Cruz et al. 2012 (27)	834	165	ICSI	BFR	—	Oocyte donor meeting all required criteria for donation program	20	Agonist	Global IVF medium, 6% CO_2, 21% O_2, 37.4°C	Yes	5

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TABLE 2

Continued.

Study	Embryos, n	Cycles, n	Fertilization method	End point	Exclusion criteria	Inclusion criteria	Image capture interval(min)	Protocol	Culture	Media change	Transfer day
Dal Canto et al. 2012 (35)	134	71	IVF (22 cycles) and ICSI (49 cycles)	IR	—	Indication for standard IVF or ICSI due to male factor, tubal factor, stage I or II endometriosis, or PCOS, maternal age 27–42 y.	20	Agonist	ISM1 (day 1–3), BlastAssist (day 3–5), 6% CO_2, 5% O_2, 89% N_2	Yes	3 and 5

Note: BFR = blastocyst formation rate; CO_2 = carbon dioxide; CPR = clinical pregnancy rate; hpi = hours post insemination; ICSI = intracytoplasmic sperm injection; IR = implantation rate; IVF = in vitro fertilization; LBR = live birth rate; N_2 = nitrogen; O_2 = oxygen; PCOS = polycystic ovary syndrome; PGD = preimplantation genetic diagnosis; PGS = preimplantation genetic screening.

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will have the same patient, treatment and environmental parameters as that of the developing center.

A further consideration for the use of externally derived ESAs is the subjective nature of annotating morphokinetic parameters, the differences in image-capture analysis such as the number of focal planes, and the varying definition of t0. The subjective nature of annotations creates unreliability in the external application of ESAs. There has been some development with this due to the publication of annotation guidelines in 2014 (50), but this will not eliminate the subjectivity entirely. It is interesting that there are now two commercially available one-size-fits-all ESAs that, based on the results presented here, should not perform as well as expected. Variations in image acquisition are unlikely to create significant disparity, but coupled with the variability between "annotators," an increasing level of inaccuracy could be created. Although it is undefined in some of the publications, the definition of t0 varies between groups; some use t0 as the time of insemination or injection, the inaugural and arguably the most common method, and others use it as the midpoint of ICSI. It has now been largely accepted that the use of insemination/injection is arbitrary, the exact moment that the sperm enters the oocyte is indeterminate for IVF cases, and where possible, the time of pronuclei fading should be used as t0.

It could be argued that a limitation of the current analyses is the potential for bias due to the use of an in-house ESA with similar morphokinetic parameters to one of the externally derived ESAs (27) to aid in embryo selection of the analyzed embryos. Owing to this, we compared the proportion of embryos in each of the categories (A–D) in the original manuscript for the external ESA in question (27) with the current analyses. From this analysis, the proportion of embryos in each category did not differ between the original manuscript of the external ESA and the current analyses: A, 39.7% (106 of 267) versus 37.3% (364 of 977); B, 13.5% (36 of 267) versus 14.3% (140 of 977); C, 36.0% (96/267) versus 36.1% (353 of 977); and D, 10.8% (29 of 267) versus 12.3% (120 of 977), respectively. This provides reassurance that any bias created from the use of similar morphokinetic parameters in the selection of the embryos used in this analysis is minimal.

Finally, it is important to consider that the use of time-lapse imaging as a method for embryo selection has yet to be appropriately evidenced (51). As can be seen from the results presented here, the poor performance of the investigated ESAs allows the field to question the overall clinical applicability of the use of time-lapse systems. There is considerable heterogeneity in the origin and culture of the embryos used for the development of these ESAs, and it should be considered that these parameters affect the ability of a one-size-fits all approach to function effectively. Perhaps the development of optimum morphokinetic time ranges that are patient, treatment, and environment specific will present a means of using time-lapse systems to achieve a higher predictive power.

There are ideal conditions under which to test the efficacy of externally derived ESAs; select embryos based only on morphology, then perform the analyses presented here or, preferably, prospectively apply ESAs. At the study site, morphokinetics have been used since their introduction in the laboratory to aid in embryo selection, so a data set large enough to perform

the former of these two methodologies would not be possible. We do, however, recognize the strength of a prospective methodology for the aims presented here. This will be the focus of future research in this area to better delineate the benefits of using time-lapse systems in the clinical embryology laboratory.

CONCLUSION

The development of ESAs thus far has not involved the control of confounding factors such as media type, patient age, and treatment type, except inadvertently by virtue of availability. They are often developed under the environmental parameters available in the laboratory performing the development and thus are clinically relevant in these cases alone. For external application, the ESAs lose their predictive capabilities.

The primary objective of ESAs is to allow the selection of the best embryo from a cohort in a clinical setting. Those presented here clarify that embryo morphokinetics could be used for embryo selection, but they do not offer a clinically relevant means to aid in embryo selection in other laboratories unless the development criteria are also adopted. The collective contribution of confounding factors means that derived ESAs can only be applied to the conditions under which they were developed; when applied to a heterogeneous cohort of embryos, as would be found in an IVF laboratory, the capability of the ESA to detect the most viable embryo diminishes. Further research needs to focus on the development of ESAs that are specific to subgroups of patients, environments, and treatments. At the very least, embryology laboratories should proceed with caution when implementing ESAs derived from published sources and consider thorough in-house validation of such ESAs before clinical use, if ESAs are to be used at all.

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Article

Preliminary investigation of the prevalence and implantation potential of abnormal embryonic phenotypes assessed using time-lapse imaging

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KEY MESSAGE
Embryos with abnormal division patterns as revealed by time-lapse microscopy have reduced developmental capacity and implantation potential compared with their normal counterparts. These findings emphasize the utility of time-lapse technologies in the embryology laboratory.

A B S T R A C T

This retrospective, single site observational study aimed to delineate five abnormal embryonic developmental phenotypes, assessing their prevalence, development potential and suitability for inclusion in embryo selection models for IVF. In total, 15,819 embryos from 4559 treatment cycles cultured in EmbryoScope® incubators between January 2014 and January 2016 were included. Time-lapse images were assessed retrospectively for five abnormal embryo phenotypes: direct cleavage, reverse cleavage, absent cleavage, chaotic cleavage and cell lysis. The prevalence of each abnormal phenotype was assessed. Final embryo disposition, embryo quality and implantation rate were determined and compared with a control embryo cohort. The collective prevalence for the five abnormal phenotypes was 11.4%; chaotic cleavage and direct cleavage together constituted 9.7%. Implantation rates were 17.4%, 0%, 25%, 2.1% and 0% for direct, reverse, absent, chaotic cleavage and cell lysis, respectively. The overall implantation rate for all abnormal embryos with known implantation status was significantly lower compared with the control population (6.9% versus 38.7%, $P < 0.0001$). The proportion of good quality embryos in each category of abnormal cleavage remained below 25%. Embryos exhibiting an abnormal phenotype may have reduced developmental capability, manifested in both embryo quality and implantation potential, when compared with embryos of normal phenotype.

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Introduction

Abnormal cleavage patterns exhibited by some embryos include, but are not limited to: abnormal syngamy, direct cleavage (DC), reverse cleavage (RC), absent cleavage in the presence of karyokinesis (AC), chaotic cleavage (CC) and cell lysis (CL).

The first of five abnormal cleavage patterns investigated here is DC. This is the cleavage of one blastomere into three, instead of the expected two, daughter cells (**Supplementary Figure S1**). The ability of these embryos to establish a pregnancy has been shown to be significantly reduced: 13.7% of all examined embryos and 6.6% of transferred embryos underwent DC, with 1.2% resulting in a clinical pregnancy (Rubio et al., 2012). These embryos have been shown to have a markedly decreased blastocyst formation rate when compared with their normal counterparts (Athayde Wirka et al., 2014).

The second abnormal phenotype to be considered is RC, the phenomenon of blastomere fusion (**Supplementary Figure S1**). Of 789 embryos assessed for RC, defined as blastomere fusion or failed cleavage, 27.4% of embryos were found to exhibit this abnormal cleavage pattern and were shown to have a reduced implantation potential (Liu et al., 2014). An examination of 1698 embryos detected a prevalence of RC of 6.8%; however, embryos appeared to have similar fragmentation, cell evenness and morphokinetic profiles compared with their non-RC counterparts (Hickman et al., 2012). This research concluded that RC does not seem to impair embryo development to the blastocyst stage, a finding supported by those of others (Desai et al., 2014).

AC is defined as the process by which a blastomere undergoes a pseudo division (seen as a 'roll') that does not produce two discernable blastomeres but a single, or multiple, extra nuclei within the single blastomere (**Supplementary Figure S1**). AC has previously been categorized under RC, termed type II RC (Liu et al., 2014). Of those embryos that underwent RC (27.4%), 82% were classified as type II: absent cleavage rather than blastomere fusion. Further evidence of this specific developmental pattern has not yet been published. This is perhaps due to the likelihood that these embryos will not be used for treatment, thus circumventing a clinical need to further define this phenomenon.

CC results when an embryo undergoes apparent cleavage but does not create distinctive blastomeres (**Supplementary Figure S1**). A single investigation studying this cleavage pattern in 639 embryos found an overall prevalence of 15%, a blastocyst formation rate of 14% and an implantation rate (IR) of 0% (Athayde Wirka et al., 2014). Interestingly, this investigation also found that 35.2% of those exhibiting CC had good cleavage-stage quality. This was, however, markedly lower than the other abnormal phenotypes observed (DC and abnormal syngamy). Again, as with AC, this phenomenon may be under-investigated due to the reduced likelihood that embryos exhibiting this phenotype will be used in treatment.

Finally, an abnormal embryo developmental phenomenon that has yet to be discussed in the literature, in terms of time-lapse imaging of embryos from fresh treatment cycles, is CL (**Supplementary Figure S1**), a process often visualized in frozen-thawed embryos (Bottin et al., 2015; Rienzi et al., 2005; Tang et al., 2006; Yeung et al., 2009). In an analysis of 891 frozen embryo transfer (FET) cycles, no pregnancies resulted if CL occurred in over 50% of the embryos. However, if CL accounted for 25 to 50% of the embryos, the pregnancy rate was 3.2%, significantly lower than if less than 25% CL had occurred (16.6%) (Tang et al., 2006). This finding is supported by others (Bottin et al., 2015; Yeung et al., 2009).

Although these investigations are not entirely synonymous with the current analysis, they provide evidence that embryos with lysed cells have a reduced implantation potential.

As discussed above, there is disparity in the literature regarding the prevalence and implication of the presence of certain abnormal phenotypes. Further investigation into these phenomena is required to determine if their presence is reason to exclude these embryos from selection for use in treatment. Five abnormal cleavage patterns exhibited by embryos (DC, RC, AC, CC and CL) are explored in 15,819 embryos, detailing their prevalence, implantation potential, and the suitability for inclusion of these potential deselection criteria in embryo selection models.

Materials and methods

This investigation was a single site, retrospective observational design approved by the North West Research Ethics Committee (ref: 14/NW/1043) and the Institutional Review Board of Edge Hill University. All procedures and protocols complied with UK regulation (Human Fertilization and Embryology Act, 1990, 2008). Data were obtained from 4559 treatment cycles including 15,819 embryos cultured in the EmbryoScope® incubators between January 2014 and January 2016.

Ovarian stimulation

Pituitary down-regulation was achieved using either a gonadotrophin-releasing hormone (GnRH) agonist (buserelin, Suprecur®, Sanofi Aventis, UK) or antagonist (cetrorelix acetate, Cetrotide®, Merck Serono, Germany). Ovarian stimulation was performed using urine-derived or recombinant FSH (Progynova (Bayer, Germany), Fostimon, Merional (IBSA, Switzerland), Menopur® (Ferring Fertility, Switzerland), Gonal f® (Merck Serono). Doses were adjusted based on patient demographic and response. Patients were given 5000 IU of subcutaneous human chorionic gonadotrophin (HCG) (Gonasi® HP, IBSA Pharmaceuticals, Italy) 36 h prior to oocyte collection. Luteal support was provided using 400 mg of progesterone pessaries twice daily (Cyclogest®, Actavis, UK) until the pregnancy test was performed.

Oocyte retrieval and embryology

Ultrasound-guided oocyte collection was performed transvaginally under sedation (Diprivan, Fresenius Kabi, USA). Collected oocyte cumulus complexes were cultured in four-well dishes (Nunc™, Thermo Scientific, USA) each well containing 0.65 ml GIVF™ (Vitrolife, Gothenburg, Sweden) covered with 0.35 ml OVOIL™ (Vitrolife) in a standard incubator (Sanyo Multigas MCO 18M). Sperm preparation was performed using a standard gradient separation (ISolate®, Irvine Scientific, USA) at 0.3 relative centrifugal force (rcf) for 10 min followed by two washes at 0.6 rcf for 10 min using GIVF™. Those oocytes destined for intracytoplasmic sperm injection (ICSI) were prepared using enzymatic (HYASE 10X™, Vitrolife) and mechanical digestion. ICSI was performed on all metaphase II (MII) oocytes approximately 4 h following collection, after which time all injected oocytes were placed in individual culture drops of G1™ (for all cycles before September 2014) or GTL™ (all cycles after September 2014) (Vitrolife) and cultured in the EmbryoScope® (Vitrolife). Those oocytes destined for

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standard insemination (IVF) had this performed approximately 4 h after collection and were replaced into a standard incubator until the following day. Oocytes were then checked for fertilization approximately 16 to 18 h post-insemination (hpi) and all fertilized oocytes along with all unfertilized MII oocytes were placed in individual culture drops as with ICSI derived embryos and cultured in the EmbryoScope®. Embryo selection was performed using the national grading scheme (ACE/BFS guidelines (Cutting et al., 2008, **Supplementary Table S3**)) along with an internally derived, embryo scoring algorithm (ESA). An ESA seeks to combine a number of morphokinetic parameters that have been linked to an embryo's viability. The ESA employed here was used as an additive to morphology, with the latter remaining the gold standard. This ESA included three morphokinetic parameters; s2 (time between t3 and t4), cc3 (time between t4 and t5) and t5 with embryos graded in one of eight categories from A + to D- (**Supplementary Table S4**). Embryo transfer was performed using the highest grade embryo(s) either 3 or 5 days post-collection, depending on the number of GQE the patient had on day 3 as well as how many were to be transferred. Selected embryos were cultured in EmbryoGlue® (Vitrolife) for 10 to 30 min in a standard incubator prior to embryo transfer. Embryos were cultured at 37°C, 6% CO_2, 5% O_2, 89% N_2 throughout.

Analysis of time lapse information

The image interval on the EmbryoScope® was set to 10 min with seven focal planes. Images were collected for the duration of culture immediately following ICSI or fertilization check (for IVF derived embryos) to utilization. Images were assessed by an embryologist for the abnormal embryonic phenotypes of interest. For DC, embryos were classified into one of three categories: true DC (TDC, defined as all three resultant cells cleaving on the subsequent cell cycle, each having a nucleus and each included in the morula); false DC (FDC, one or more of the above criteria not fulfilled); and unconfirmed DC (UDC, unable to classify as true or false). UDC embryos were defined as such due to either obscurity preventing categorization or the cessation of culture before the morula stage was reached. A justification for the choice of this classification, not reported elsewhere, lies in unit-specific data whereby two obviously distinct DC event patterns were visualized using time-lapse technology. This, as well as previous reports of direct cleavage patterns (Kalatova et al., 2015; Kola et al., 1987), led to the development of the three-tiered classification of DC events. Regarding the final criterion for TDC classification (inclusion of all cells in the morula), this stage of development was used as an indicator that all cells, abnormal or not, would contribute to the eventual blastocyst and would not be excluded. Further to this, DC could be proposed as a correction mechanism whereby the DC event is a means to remove surplus genetic material, thus excluding the cells from the eventual blastocyst, described here as FDC and a more favourable type of DC event. Direct cleavage from both one to three cells (DC1–3) and from two to five cells (DC2–5) were included in the analysis. RC is defined simply as blastomere fusion. AC is defined as the process by which a blastomere undergoes a pseudo division (seen as a 'roll') that does not produce two discernable blastomeres but a single, or multiple, extra nuclei within the single blastomere. CC is observed when an embryo undergoes apparent cleavage but does not create distinctive blastomeres. CL is defined as the loss of a blastomere through cell lysis (**Supplementary Figure S1**). Although not exclusively a phenomena visualized through time-lapse technology and one that can be visualized using standard embryo morphology assessment, CL is predominantly seen in embryos following cryopreservation, whereas here we describe cell lysis in fresh embryos. Thus, this was included in the current investigation to determine the effect of cell lysis on the viability of a fresh embryo.

Outcome measures and statistical analysis

The overall prevalence of the five abnormal embryo phenotypes was defined per embryo and per treatment cycle. The average patient age, oocytes collected and previous attempts were calculated for each of the five categories. The fate (transfer, freeze, discard) of each abnormal embryo was determined as well as its quality on the day of utilization, defined as good, average or poor (**Supplementary Table S1**). The IR for each abnormal phenotype was determined. For this analysis, no transfers in which an abnormal embryo was transferred with a normal embryo were included and only those for which the origin of the fetal heart was confirmed were included, i.e. known implantation data, using single embryo transfers of an abnormal embryo or double embryo transfers where two abnormal embryos were transferred resulting in a negative outcome or two fetal heartbeats. The number of single and double abnormal embryo transfers and the stage at which the abnormal embryo(s) was transferred was also determined (**Supplementary Table S2**). Statistical analyses included Student's *t*-test for the comparison of the abnormal phenotype baseline information (patient age, oocytes collected and previous attempts) to the control embryo baseline data. The Fisher's exact test was used to compare the IR of the abnormal embryos with normal counterparts. Results were considered significant at $P < 0.05$. Statistical analysis was performed using the statistical package Prism® 5 (GraphPad Software©, USA).

Results

Data were obtained from 15,819 embryos from 4559 treatment cycles cultured in the EmbryoScope® between January 2014 and January 2016. Of the 15,819 embryos, 14,008 were derived from 3273 treatment cycles where no abnormal divisions of interest (DC, CC, RC, AC and CL) were observed and thus constituted the control group. The remaining embryos (1811) were found to pertain to a treatment cycle ($n = 1286$) exhibiting an embryo with one of the abnormal division patterns of interest.

Abnormal phenotypes with the highest prevalence per embryo observed were DC and CC at 4.4% (TDC, FDC, UDC, collectively) and 5.3%, respectively. The remaining phenotypes had considerably lower prevalence ranging from 0.41 to 0.8% (**Table 1**). The overall prevalence per embryo observed of abnormal division patterns was 11.4% (**Table 1**).

The embryos from the control group (not undergoing an abnormal division event; 14,008) resulted in 3456 embryos transferred and 1336 fetal heartbeats, giving an IR of 38.7% (**Table 1**). These transfers resulted in a clinical pregnancy rate (CPR) of 38.8% (presence of fetal heart(s) in 1269 of 3273 embryo transfers). The transfer of a total of 86 abnormal embryos resulting in 6 fetal heartbeats gave an overall IR of 7.0% (**Table 1**), resulting in a CPR of 8.3% (presence of fetal heart(s) in 6 of 72 embryo transfers). Overall CPR for transfers including at least one abnormal embryo can be found in **Supplementary Table S5**. Of the five abnormal division patterns, the IR of UDC, CC and RC were significantly lower than normal counterparts: 12.5% ($P = 0.0378$), 2.1% ($P < 0.0001$) and 0% ($P = 0.0153$),

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Table 1 – Descriptive data for embryos showing an abnormal division pattern and normal embryos.

	No. of embryos *n* (%)	No. of cycles *n* (%)	Affected embryos/ cycle	No. of embryos transferred	No. of embryos frozen	No. of embryos discarded	No. of GQE (%)	No. of AQE (%)	No. of PQE (%)	No. of abnormal embryos transferred with known implantation			IR % (n fhs)	*P*-value[a]
										Total	D3	D5		
TDC	48 (0.3)	45 (1.0)	1.07	3	11	34	10 (20.8)	8 (16.7)	30 (62.5)	1	0	1	0	NS
FDC	69 (0.4)	64 (1.4)	1.08	9	29	31	11 (15.9)	21 (30.4)	37 (53.6)	6	0	6	33.3 (2)	NS
UDC	580 (3.7)	463 (10.1)	1.25	33	70	477	69 (11.9)	101 (17.4)	410 (70.7)	16	5	11 (1xDET)	12.5 (2)	0.0378
Total DC	697 (4.4)	572 (12.5)	1.22	45	110	542	90 (12.9)	130 (18.7)	477 (68.4)	23	5	18 (1xDET)	17.4 (4)	NS
RC	65 (0.4)	61 (1.3)	1.07	14	22	29	15 (23.1)	10 (15.4)	40 (61.5)	9	3 (1xDET)	6	0	0.0153
AC	133 (0.8)	123 (2.7)	1.08	7	10	116	6 (4.5)	5 (3.8)	122 (91.7)	4	1	3 (1xDET)	25 (1)	NS
CC*	835 (5.3)	459 (10.1)	1.82	85	69	681	28 (18.2)	42 (27.3)	84 (54.5)	48	25 (5xDET)	23 (6xDET)	2.1 (1)	<0.0001
CL	81 (0.5)	71 (1.6)	1.14	5	31	45	11 (13.6)	22 (27.2)	48 (59.3)	2	0	2	0	NS
Overall total	1811 (11.4)	1286 (28.2)	1.41	156	242	1413	–	–	–	86	34 (6xDET)	52 (8xDET)	7.0 (6)	<0.0001
Normal embryos	14008 (88.6)	3273 (71.8)	–	3456	4574	5978	5128 (36.6)	2468 (17.6)	6412 (45.8)	–	–	–	38.7 (1336)	–

Total DC constitutes TDC, FDC and UDC collectively.
AQE = average quality embryos; GQE = good quality embryos; PQE = poor quality embryos.
[a] For implantation rate (IR) of affected embryos compared with normal embryo cohort (Fisher's exact, significant at $P < 0.05$).
* Only transferred and frozen embryos assessed for quality for this category owing to significant missing data.

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Table 2 – Baseline characteristics for cycles with embryos undergoing an abnormal division pattern and cycles with normal embryos.

	Patient age (mean ± SD)	*P*-value	Oocytes collected (mean ± SD)	*P*-value	Previous attempts (mean ± SD)	*P*-value
TDC	32.82 ± 4.7	<0.0001	12.95 ± 7.78	<0.0001	1.37 ± 0.93	NS
FDC						
UDC						
RC	32.5 ± 4.5	0.0097	15.7 ± 9.7	<0.0001	1.23 ± 0.6	NS
AC	33.16 ± 5.41	NS	15.09 ± 8.57	<0.0001	1.35 ± 0.8	NS
CC	32.93 ± 4.87	<0.0001	13.44 ± 8.5	<0.0001	1.39 ± 0.82	NS
CL	33.24 ± 4.27	NS	13.86 ± 7.79	<0.0001	1.28 ± 0.78	NS
Total abnormal	32.93 ± 4.75	<0.0001	14.21 ± 8.47	<0.0001	1.32 ± 0.79	NS
Normal	34.08 ± 4.73	–	10.5 ± 5.99	–	1.37 ± 0.98	–

P-values for cycles with abnormal embryos versus cycles with normal embryos (Student's *t*-test, significant at $P < 0.05$).
AC = absent cleavage; CC = chaotic cleavage; CL = cell lysis; FDC = false direct cleavage; NS = not statistically significant; RC = reverse cleavage; TDC = true direct cleavage; UDC = unconfirmed direct cleavage.

respectively (**Table 1**). Furthermore, the overall IR of all abnormal embryos was significantly lower than normal counterparts (7.0% versus 38.7%, $P < 0.0001$) (**Table 1**) and of the six implanted abnormal embryos, five resulted in a live birth, with no birth defects, and one remains ongoing. In all cases the percentage of GQE resulting from those exhibiting abnormal division patterns never reached above 24% and the majority of embryos were classified as poor quality (**Table 1**). This is also reflected in the utilization of these embryos; the highest proportion of each group was discarded (**Supplementary Figure S2**). The number of embryos undergoing either DC1–3 or DC2–5, respectively, in each of the DC categories was as follows: TDC, 16 (2.3%) and 32 (4.6%); FDC, 26 (3.7%) and 43 (6.2%); UDC, 176 (25.3%) and 404 (58.0%).

Patient age was significantly lower for cycles with embryos exhibitng DC ($P < 0.0001$), RC (0.0097) and CC ($P < 0.0001$) compared with those not exhibiting an abnormal division pattern. The number of oocytes collected was found to be significantly higher in treatment cycles containing abnormal embryos than in those not containing embryos exhibiting an abnormal division pattern ($P < 0.0001$). Finally, the number of previous attempts was not found to be significantly different between any of the abnormal division categories and the control embryo cohort (**Table 2**).

Discussion

The prevalence of DC in the literature has been stated as 13.7% (Rubio et al., 2012) and 18% (Hickman et al., 2012). In the current analysis the overall prevalence of DC was 4.4% (UDC, FDC and TDC combined) occurring in 1.22 embryos per treatment cycle. The implantation potential of embryos undergoing DC has been stated as just 1.2% (Rubio et al., 2012); however, in the current analysis the IR was found to be 17.4% (4/23; TDC, FDC and UDC combined), not significantly lower than that of the control embryo cohort. A classification system of DC was not adopted by other publications; therefore, if FDC were not considered, the IR would be significantly lower than those not exhibiting a DC. Of the three categories, those that were classed as FDC had the highest IR, as one might expect from the definition. There is a paucity of literature regarding the exact mechanisms underlying the phenomenon of DC; however, it has been speculated that the presence of a multipolar spindle could be a contributing factor (reviewed in Kalatova et al., 2015). In addition, the presence of surplus centrosomes leading to DC is reflected in an early investigation of tri-pronucleate oocytes. Genetic assessment of tri-pronucleate DC oocytes revealed three division patterns: DC to three cells (62%); cleavage to a morphologically normal 2-cell 'embryo' (24%); and cleavage to a 2-cell 'embryo' plus an extrusion (14%) (Kola et al., 1987). All tri-pronucleate oocytes that had undergone DC to three cells were chromosomally abnormal with each containing a varied number of chromosomes (here considered a TDC). Those that cleaved to morphologically normal 2-cell 'embryos' were found to be true triploid with each blastomere containing a 69XXX/XXY chromosome complement. However, of those oocytes that cleaved to a 2-cell 'embryo' plus an extrusion, 75% were found to have two diploid blastomeres and a haploid extrusion. In the analysis presented here, of those embryos analogous to the 2-cell embryo plus an extrusion, two of six implanted. Caution should be taken as the numbers are small in this group due to the need to use known implantation embryos, however, this represents a result just over 5% lower than that of a phenotypically normal embryo. Although speculative, the findings by Kola et al. (1987) could also indicate that embryos have the potential to correct genetic abnormalities. There are many studies detailing self-correction between the cleavage stage and the blastocyst stage of embryo development (Barbash-Hazan et al., 2008; Li et al., 2005; Munne et al., 2005; Northrop et al., 2010; Voullaire et al., 2000). It has been noted that trisomic embryos correct more often than other aneuploidies (Barbash-Hazan et al., 2008), possibly occurring through the loss of a chromosome in trisomic cells (Munne et al., 2005). In addition, in previous reports, CC could be misinterpreted as a DC, thus causing the prevalence of DC to appear falsely increased. The increased IR of DC seen in the present investigation compared with previous reports may also be due to observers having experience with the different categorizations of DC, making them proficient at recognizing patterns of FDC, such as blastomere behaviour, allowing preferential selection of a potential FDC in UDC cases. The reduced patient age and increased number of oocytes collected may reflect a simple association between maternal age and number of oocytes collected. However, it may also indicate that stimulation can lead to reduced oocyte quality (Aboulghar et al., 1997) and high oocyte numbers (>15) can reduce the chance of a live birth (Ji et al., 2013), which could manifest as an abnormality such as DC.

RC occurred in 65 embryos (1.07 embryos per treatment cycle), of which 36 were either transferred or frozen, and 25 of those were classed as good or average quality. It is likely that embryos classed as poor quality embryos (PQE) were utilized due to the unavailability of others. None of the nine transferred embryos that underwent RC

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implanted in the current investigation. The prevalence of RC has been reported as 6.8, 7 and as high as 27.4% in previous reports (Desai et al., 2014; Hickman et al., 2012; Liu et al., 2014). However, the rate of formation of usable embryos is in conjunction with others at approximately 40% (Desai et al., 2014). There have been reports that RC is affected by other variables such as ICSI and GnRH antagonists. Therefore a possible explanation for the disagreement presented here could be due to the difference in baseline patient and treatment variables, a consideration for further investigation. The phenomenon of RC has been recognized previously with regards to frozen-thawed embryos (Balakier et al., 2000; Trounson, 1984). Balakier et al. (2000) sought to determine the chromosomal changes in blastomeres that undergo fusion following thawing. RC was found in 51 of 2014 embryos of which 70% were classed as good quality. The overall frequency of RC was 4.6% in day 2 embryos and 1.5% in day 3 embryos. A slightly higher incidence of blastomere fusion was found in embryos created using IVF when compared with ICSI. When a control group was observed (embryos not subject to freezing and thawing) the prevalence of RC was 0.3%, a result not far from that recorded in the present study (0.4%). The IR of embryos that underwent blastomere fusion following thawing in the above investigation was very poor, with 15 embryo transfers containing one abnormal and one normal embryo resulting in only one live birth –again, a result similar to that seen in the present investigation. The chromosomal status of blastomeres resulting from fusion was also examined where embryos affected by RC were transformed into either polyploidy or mosaic embryos. The authors suggested that the occurrence of blastomere fusion could be associated with existing membrane abnormalities that could promote fusion affected by factors such as pH, temperature and osmolality differences. Interestingly, in some fields of research the production of tetraploid embryos is advantageous, and it has been concluded that tetraploidy does not prohibit preimplantation development (Eglitis, 1980), corroboration for the development of approximately 40% good/average quality embros (G/AQE) in the present investigation. This investigation could conclude, similarly to others, that the presence of RC does not seem to affect an embryo's ability to create a GQE but does impair an its ability to implant.

Absent cleavage has been characterized as a type of RC in a previous report (Liu et al., 2014); however, in the current report it is classed as a distinct phenotype. The prevalence per embryo of this abnormality compared with RC is more than double (0.8% versus 0.4%) and of the four embryos that were transferred with this phenotype, one implanted. However, in a previous report of 22 embryos, none implanted that underwent type I or type II RC (defined here as AC) (Liu et al., 2014). In another investigation using disaggregated human embryos, blastomeres were scored for the number of nuclei present after 16 to 20 h culture, and a small proportion of mononucleated blastomeres exhibited two nuclei after culture. It was postulated that approximately 30% of these occurred through AC (Pickering et al., 1995). Here, AC was shown to occur in 1.08 embryos per treatment cycle and of the 133 embryos exhibiting AC, 122 were classed as PQE and 116 were discarded. Unlike DC, RC and CC, however, patient age was not shown to be significantly different when compared with the control embryo cohort.

CC has an overall prevalence per embryo of 5.3%: by far the highest of the five abnormal phenotypes, occurring in 1.82 embryos per treatment cycle, suggestive of a patient, treatment or environmental effect rather than a spontaneous event. One comprehensive analysis identified the prevalence of CC to be 15%, with a blastocyst formation rate of 14% and an IR of 0% (Athayde Wirka et al., 2014). In the current analysis, one of the 48 transferred embryos implanted, a rate significantly lower than the IR of the control embryo cohort. Of the utilized embryos, just 18.2% were classed as GQE, 27.3% as AQE and 54.5% as PQE. Interestingly, it has previously been found that 35.2% of those exhibiting CC were classed as good quality, a result not synonymous with the current analysis. A possible explanation for this disagreement is the time-lapse technology used. In the current analysis, EmbryoScope® was the time-lapse technology of choice; however, in the analysis by Athayde Wirka et al. (2014) the Eeva™ system was used. The Eeva™ system uses dark field illumination to enable the software within it to track blastomeres, perhaps making the distinction of blastomeres from fragments more difficult. An investigation conducted on patients carrying a Robertsonian translocation (the fusion of two acrocentric chromosomes), revealed that a high proportion of embryos resulting from these patients underwent numerous chaotic cleavage divisions, and rather than the aneuploid segregation of the Robertsonian translocation being the only reason for the infertility, there may be a post-zygotic manifestation leading to uncontrolled chromosome segregation (Conn et al., 1998). The presence of chaotically dividing embryos has been noted elsewhere (Delhanty et al., 1997; Harper and Delhanty, 1996; Laverge et al., 1997) and has also been identified as a patient-related phenomenon (Delhanty et al., 1997), evident in the current investigation in which CC occurrred in up to 1.82 embryos per treatment cycle.

CL is largely discussed in the literature when considering frozen-thawed embryos and, as discussed previously, there is an associatively low IR (Tang et al., 2006): 59.3% of the embryos were classed as PQE with 55.6% of the total discarded; just 13.6% were considered GQE and 27.2% AQE. As very few embryos were shown to exhibit this phenotype, and fewer still were transferred, it is difficult to draw conclusions about its implications. It would be reasonable to use previous evidence regarding frozen-thawed embryos to attribute their potential for success. However, CL in frozen embryos is likely to be a result of cryodamage during the freeze-thaw process, whereas in fresh embryos the CL could be as a result of exposure to another stressor such as suboptimal pH, temperature or osmolality. Cells that lyse may have a heightened sensitivity to changes in the environment, or lack a cytoplasmic constituent that regulates cell volume, for example, leading to lysis.

Abnormal phenotypes as deselection criteria

Where possible, UDC and TDC embryos should not be selected for transfer if other embryos are available, even when embryo quality is considered. It is important to note at this point that embryos transferred at the cleavage stage undergoing DC (of which there were five in the current analysis) will inevitably be classed as UDC. These embryos may have resulted in FDC; thus caution is advised owing to a potential bias in the current results of UDC cleavage-stage embryos. For this reason, extended culture of DC embryos may be valuable to allow the classification into either FDC or TDC and thus aid further in embryo selection and management of patient expectation. CC, the most common abnormal phenotype in the current analysis, has been linked to severe chromosomal abnormalities in the literature (Delhanty et al., 1997), which could be patient specific. Therefore it is possible that the phenomenon could occur more than once in a patient cohort, indicating an underlying genetic condition. Where CC embryos are transferred, in the current analysis the IR is 2.1% regardless of embryo quality. For this reason, identification of CC as a deselection tool should be considered for laboratories utilizing time-lapse imaging

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technologies. Almost 92% of embryos that exhibited AC created PQE; thus they would be likely to be automatically discounted from clinical use. RC and CL each had an IR of 0%, albeit from low numbers of transferred embryos. However, the relative prevalence was low, the majority of embryos exhibiting these phenomenoa were PQE and were not able to implant; therefore, these embryos should not be selected for transfer where possible. These recommendations have been implemented at the study site to aid in embryo selection. In addition to the above, the need for accurate and consistent annotation of embryos is imperative for any centre utilizing time-lapse technologies. This issue was raised a number of years ago, resulting in the publication of suggested terminology in order to create consensus among users (Ciray et al., 2014). Consensus is paramount, and caution is advised when implementing or analysing time-lapse parameters discussed by others.

This preliminary investigation sought to determine the prevalence, implantation potential and suitability for inclusion in embryo selection algorithms of five abnormal cleavage events. To determine IR, only known implantation embryos were included, leading to a significant reduction in the number of embryos available for analysis. Nevertheless, this number would be difficult to achieve at another single site based on the study site using time-lapse imaging for all patients and performing over 2000 treatment cycles per year. In addition, the ability to track the implantation of these embryos is made more difficult with the increased likelihood of transferring two embryos in these cases, potentially due to reduced embryo quality in the available embryo cohort. Based on the results presented here, future analyses should focus on embryos undergoing more than one abnormal division event, the cell stage at which the abnormal cleavage event occurs, the effect of treatment parameters such as ICSI and day of transfer as well as the assessment of a relationship between the abnormal phenotypes and multinucleated blastomeres. In addition, the authors plan to perform an extension of this analysis to include embryo quality and outcome information regarding DC1–3 versus DC2–5. Finally, scrutiny should be paid to CL, with the specific timings of the CL event being assessed and linked to the relative impact on embryo viability.

In conclusion, embryos exhibiting an abnormal phenotype appear to have reduced developmental capability, expressed as both embryo quality and implantation potential. Time-lapse systems are bringing to light many unusual and, most likely, fundamentally complicated embryological phenomena, requiring in-depth analysis that could ultimately improve the outcome of treatment cycles.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.rbmo.2017.02.011.

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