Prediction of embryo viability

by morphology and metabolomic profiling

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Cover illustration: A fully expanded blastocyst

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Dedicated to my beautiful family, I am truly blessed.

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ABSTRACT

The ultimate challenge for all *in vitro* fertilisation (IVF) clinics is to develop the ability to select for transfer the best single embryo first, from the patient's cohort of embryos, thereby maximising the chance of pregnancy while the incidence of multiple pregnancies is kept to a minimum and fewer transfer cycles are required. This ambition has driven extensive research and development into methods that can be used to predict embryo viability. The aims of this thesis were to investigate two non-invasive methods; one new method of metabolomic profiling using Near Infrared (NIR) spectroscopy to analyse spent embryo culture media and the most routine method of morphological grading at the blastocyst stage.

In our initial study we investigated metabolimic profiling by NIR spectroscopy and demonstrated that there were distinct differences between NIR spectral profiles of spent embryo culture media of implanting embryos and non-implanting embryos on day 5 of development. These differences were successfully used in a predictive model to calculate viability scores that were positively correlated (R^2 = 0.82, P = 0.03) to implantation rates. In addition, viability scores were not related to morphology indicating that this method could be used as an adjunct to current morphological selection criteria. We also showed, by a method of cross-validation, that a predictive algorithm was accurate even when used at different clinics using different blastocyst culture media. These findings, in addition to other published studies, suggest that selection of embryos with high NIR viability scores could potentially improve implantation rates.

Unfortunately, when the application of this technology was tested in a prospective randomized controlled trial (RCT) for selection of embryos on day 2 and day 5 for transfer, its use in adjunct to morphology did not

significantly improve the ongoing pregnancy rate when compared to morphology alone (34.8% versus 35.6%, P = 0.97). As such, NIR spectroscopy, in its current form, did not improve selection of the most viable embryo for transfer. These results demonstrate the importance of performing RCT's before committing to the clinical application of any new technology or treatment.

We also investigated the independent predictive strength of morphological parameters used to predict blastocyst viability in both fresh and frozen-thawed cycles. We found through our retrospective studies looking at blastocyst morphology and prediction of live birth found that trophectoderm (TE) morphology was the most important predictor after fresh single blastocyst transfer cycles and one of the most important predictors after frozen thawed transfer cycles. Expansion grade was found to be the other most important predictor of live birth after frozen-thawed transfer cycles. The inner cell mass (ICM) in both studies was not shown to be one of the most significant predictors of live birth. We have shown, for the first time, the predictive strength of TE grade over ICM for selecting the best blastocyst for embryo replacement. It may be that, even though ICM is important, a strong TE layer is essential at this stage of embryo development, allowing successful hatching and implantation. Furthermore, we found that for thawed blastocysts degree of re-expansion was the most important post thaw morphological predictor of live birth.

In conclusion, we have been able to show that morphology is a strong predictor of embryo viability and by understanding the predictive strength of each parameter being used in a grading system, we can better use these parameters when making our decisions. Furthermore, there is still a need for alternative methods to predict embryo viability, but these new methods should be validated in properly conducted studies before clinical implementation, as shown by the conflicting results in our two studies when testing the NIR technology platform.

Keywords: NIR spectroscopy/morphology/blastocyst/IVF/trophectoderm/ ICM/live birth/embryo/metabolomic

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SAMMANFATTNING PÅ SVENSKA

En stor utmaning vid behandling med *in vitro* fertilisering(IVF) är att bland många embryon välja ut ett optimalt att återföra. Detta för att maximeras chansen till graviditet och minimera risken för flerbörd. Denna ambition har varit drivkraften för omfattande forskning och utveckling av metoder som skulle kunna förutsäga ett embryos viabilitet. Huvudsyftet med detta avhandlingsarbete var att jämföra och utvärdera två icke invasiva metoder att bedöma embryots viabilitet; en ny metod där ”Near infrared spectroscopy”(NIR) använts för profilering av s.k. metabolomics i näringslösning embryot odlats samt den sedan länge rutinmässigt använda morfologiska graderingen av en blastocyst.

I vår första studie undersökte vi med hjälp av NIR metoden näringslösning från odlingsdag 5 och fann att denna tydligt skilde sig mellan blastocyster som resulterat i graviditet jämfört med de som ej implanterat. Skillnad kunde sedan användas i en prediktiv modell för att beräkna viabilitets scorer som visade sig vara positivt korrelerade (R^2=0.82, P=0.03) med implantation. Scorerna var inte relaterade till embryots morfologi vilket talade för att metoden borde kunna kombineras med nuvarande morfologiska urvalskriterier. Vid s.k. kors-validering visade det sig även att den prediktiva modellen var tillförlitlig när den användes vid olika kliniker med olika odlingsmedier. Dessa fynd tillsammans med resultat från andra studier antyder att NIR metoden borde kunna förbättra chansen att identifiera det mest viabla embryot. Metoden testades därefter kliniskt i en prospektiv randomiserad kontrollerad studie (RCT) för att bedöma om den i kombination med morfologi bättre kunde välja ut det embryo som skulle återföras på odlingsdag 2 eller 5 jämfört med enbart morfologi. Märkligt nog fann vi då att metoden i kombination med morfologin inte medförde en bättre chans till graviditet jämfört med att enbart använda morfologi (34.8% versus 35.6%, P=0.97). Den utformning av NIR metoden vi använde förbättrade således inte chansen att välja ut det embryo som hade störst chans att resultera i en fullgången graviditet. Studien understryker vikten av att alltid utföra RCT studier innan en ny teknologi eller behandling införs i kliniskt bruk.

Vi har vidare studerat ett antal morfologiska parametrars prediktiva styrka att förutsäga både den färska och den fysta/tinade blastocystens förmåga att resultera i en fullgången graviditet. Genom att retrospektivt analysera ett antal morologiska karakteristika hos återåterförda blastocyster fann vi att trofektodermets (TE) morfolog var den viktigaste parametern att förutsäga en fullgången graviditet vid återförande av färska blastocyster. Vid återförande fryst/tinad blastocyst visade sig båda TE garderingen och graden av

blastocystens expansion bäst prediktera fulgången graviditet. Förvånande vara att i båda dessa studier visade sig graderingen av blastocystens inre cellmassan (ICM) vara av mindre prediktivt värde att förutsäga fullgången graviditet. Vi har för första gången visat att graderingen av TE bättre predikterar fullgången graviditet än gradering av ICM. Det kan mycket väl vara så att även om ICM är viktig så är ett kraftigt TE väldigt viktigt vid denna tidpunkt i embryots utveckling för både kläckning och implantation. Efter tining av fysta blastocyster visade det sig att graden av re-expansion var den viktigaste morfologiska parametern att förutsäga födelse av levande barn. Sammanfattningsvis har vi kunnat visa att vissa mofologiska parametrar väldigt bra kan förutsäga embryots viabilitet och därmed vara av stor betydelse vid val av det embryo som skall återföras. Ytterligare metoder att bedöma embryots viabilitet behövs dock men det är viktigt att de testas noga innan de används kliniskt vilket framgår av våra två studier med NIR teknologin med motsägelsefulla resultat.

LIST OF PAPERS

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

I. **Ahlström, A., Wikland, M., Rogberg, L., Siques Barnett, J., Tucker, M. and Hardarson, T**. Cross-validation and predictive value of near-infrared spectroscopy algorithms for day 5 blastocyct transfer. RBMOnline 2011; 22:477-484.

II. **Hardarson, T., Ahlström, A., Rogberg, L., Hillensjö, T., Westlander, G., Sakkas, D., and Wikland**, M. Non-invasive metabolomic profiling of Day 2 and Day 5 embryo culture medium: A prospective randomized trial. Human Reproduction 2011; 27:89-96.

III. **Ahlström, A., Westin, C., Reismer, E., Wikland, M., and Hardarson, T**. Trophectoderm morphology: an important parameter for predicting live birth after single blastocyst transfer. Human Reproduction 2011; 26:3289-96.

IV. **Ahlström, A., Westin, C., Wikland, M. and Hardarson, T.** Prediction of live birth in frozen-thawed single blastocyst transfer cycles by pre-freeze and post-thaw blastocyst morphology. submitted manuscript.

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ABBREVIATIONS

CGH	Comparative genomic hybridization
CI	Confidence interval
DSMB	Data safety monitoring board
EBSS	Earle’s balanced salt solution
EDTA	Ethylenediaminetetracetic acid
FCA	Fetal cardiac activity
FSH	Follicle stimulating hormone
GQE	Good quality embryo
HPLC	High performance liquid chromatography
hCG	Human chorionic gonadotropin
HLA	Human leukocyte antigen
H-hCG	Hyperglycosylated hCG
ICM	Inner cell mass
ITT	Intention to treat
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertilization
LH	Luteinizing hormone
NIR	Near infrared
OR	Odds ratio
PP	Per protocol

PGS	Preimplantation genetic screening
RCT	Randomized controlled trial
SET	Single embryo transfer
TCA	Tricarboxylic acid
TE	Trophectoderm
ZP	Zona of pellucida

1 INTRODUCTION

Today, within the In Vitro Fertilisation (IVF) community, a large research effort is directed towards developing reliable methods for selection of the most viable embryo from the patient's cohort of embryos. The aim of this effort is to attain high pregnancy rates while keeping the incidence of multiple pregnancies at a minimum.

Historically, to attain high pregnancy rates IVF has been more reliant on multiple embryo transfer practices than embryo selection methods. Transfer of multiple embryos was initially considered acceptable, as live birth rates were relatively low and multiple birth rates moderate. However, with improvement of stimulation protocols and embryo culture conditions higher numbers of Good Quality Embryos (GQE) were attained per IVF cycle and around the late 1980's many studies reported unacceptably high multiple birth rates. These rates raised the need for reductions in the number of embryos being transferred. In Sweden, a retrospective study showed that multiple birth rates for IVF were approximately 27% compared to 1% for the general population during the same period, 1982-1995 (Bergh, et al., 1999). These results were comparable to those seen in other countries such as Finland (Gissler, et al., 1995), the US (Seoud, et al., 1992), the UK (Rizk, et al., 1991) and Belgium (Bollen, et al., 1991).

The adverse outcomes of multiple pregnancies for both the mother and the child are well documented. Mothers have increased risk of pre-eclampsia, gestational diabetes, post partum hemorrhage and caesarean sections and infants are at greater risk for premature birth, low birth weight, congenital malformations, fetal and infant death, and long-term morbidity and disability (Martin, et al., 2003, Martin, et al., 1999, Petterson, et al., 1998, Stromberg, et al., 2002). Higher incidences of these adverse outcomes are also associated to IVF newborns and are most likely caused by higher multiple birth rates (Beral, et al., 1990, Craft, 1990, Gissler, et al., 1995, Westergaard, et al., 1999). This has unfortunately translated into higher health care costs for IVF children and provoked discussions about the benefits of IVF in many countries (Kitchen, et al., 1993, Papiernik, 1990, Peters, et al., 1991).

To combat these concerns a growing number of countries have implemented legal restrictions and guidelines to minimise the transfer of multiple embryos (ASRM and SART 2008, Bergh, 2007, Karlstrom, et al., 2007, Kutlu, et al., 2011, Maheshwari, et al., 2011). These restrictions have not only increased

the use of single embryo transfer (SET), but have led many IVF laboratories to search for alternative approaches for identifying viable embryos.

1.1 Human embryo development

First, successful fertilization is achieved by fusion of the sperm and the oocyte and within 4 to 7 hours the male and female chromatin decondense and appear as pronuclei; a zygote is formed (diploid cell formed from fusion of two haploid cells) (Figure 1a). During the next few hours the pronuclei will migrate towards the centre of the oocyte, movement is driven by the sperm centrosomes organizing the oocytes microtubules. The pronuclear membranes then disintegrate and the pronuclei fuse (syngamy). The mitotic metaphase spindles then align two sets of chromosomes along the equator. The first cell division occurs within the next few hours and the embryo continues to divide by mitosis approximately once every 24 hours (Figure 1 b, c, d). In humans, fertilization takes place in the ampulla region of the Fallopian tube and the first few cleavages occur as the embryos travels along the tube towards the uterus aided by tubal cilia activity and muscle movement. During cell division the size of the embryo does not change instead the cells, called blastomeres, become smaller and smaller. At the 4 to 8- cell stage the embryonic genome is activated and the embryo is no longer reliant on maternal mRNA for development. At the 8-cell to 16-cell stage, 4 days after fertilization, the embryo undergoes the process of compaction, to form a morula (Figure 1 e). During compaction, the blastomeres flatten upon each other, maximizing cell-cell contact and minimizing intercellular space. A compacting embryo has started the process of differentiation and cell fate is decided by cell localization within the embryo, i.e. inside cells and outside cells. The trophectoderm (TE) is formed from the outer polar cells, which develop extensive zonular tight junctions with one another to form an impermeable epithelial outer layer. Once the seal is established the TE actively pumps ions into the cavity, causing an influx of fluid to the centre of the embryo through osmosis. A fluid filled cavity is formed and is known as the blastocoele (Figure 1 f). Enclosed within the blastocoele is a small clump of 'inside' cells that compact together and form the inner cell mass (ICM). These cells will give rise to the fetus proper. The TE cells, upon successful implantation, will eventually form the placenta and extra-embryonic tissue. So, approximately 4.5 to 6 days after fertilization, the embryo is a blastocyst and consists of 50 to 150 or more cells, two thirds comprising the TE and the remaining third the ICM (Figure 2). At this stage, the embryo has completed its travel through the fallopian tube and has entered the uterus. In order to implant the blastocyst also needs to successfully hatch from its zona

pellucida (ZP). The process of hatching in vitro is observed to be the result of several cycles of blasotcoele expansion and collapse. These cycles cause the ZP to stretch and thin, and eventually rupture. Of course, proteolytic enzymes produced by the TE and present in the uterus, that can digest the zona also suspected to play a role in this process. Once the blastocyst is free from the zona the intricate process of implantation can begin.

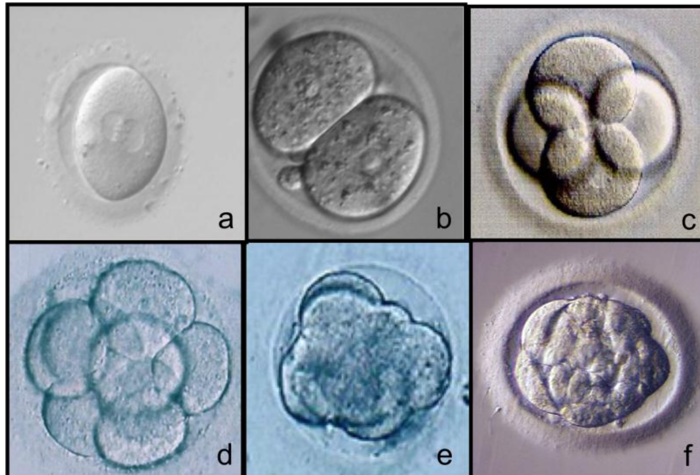

Figure 1. Embryo development, a) a fertilized zygote with a male and female pronuclei, b) first mitotic cell division into a 2 cell embryo, c) second mitotic division into a 4 cell embryo, d) third mitotic division into a 8 cell embryo, e) a morula d) early beginnings of a blastocoelic cavity.

1.2 Routine methods of embryo selection

1.2.1 Morphological observations

Morphology is the most traditional and routine method of embryo selection being used. The value of using morphological characteristics of embryos and timing of their development to predict pregnancy have been appreciated since the start of IVF and have arisen from studies observing the development of embryos in vitro (Cummins, et al., 1986, Edwards, et al., 1984, Fishel, et al., 1984).

Today, various scoring systems based on morphological observations have been established to predict the reproductive potential of embryos from the zygote to the blastocyst stage. At the zygote stage (Day 1, 16–18 hours post insemination), the number and size of the pronuclei and the number and symmetry of the nucleolar precursor bodies are indicators of normal embryo development (Scott, et al., 1998, Tesarik, et al., 1999). At the cleavage stage, embryos are assessed by the number, size and shape of their blastomeres; presence of multi-nucleation and degree of fragmentation (Antczak, et al., 1999, Tesarik, et al., 1987, Winston, et al., 1991). A recent consensus for embryo development agreed that an optimal day 2 embryo (44 ±1 hours post insemination) has 4 mononucleated evenly sized blastomeres and less than 10% fragmentation, and an optimal day 3 embryo (44 ±1 hours post insemination) has 8 mononucleated evenly sized blastomeres and less than 10% fragmentation (2011).

At the blastocyst stage, a morphological grading system first described by Gardner and Schoolcraft (1999) more than a decade ago is widely used. According to this system three parameters are graded; degree of blastocoele expansion and hatching status (1 up to 6) (Figure 2); size and compactness of ICM (highest score A, then B, and C) (Figure 3); and the cohesiveness and number of TE cells (A, B, and C) (Figure 4). This grading system has been validated by many investigators who have shown that transfer of two or more top-scoring blastocysts (high grades for all three parameters) achieve the highest implantation rates (Balaban, et al., 2000, Balaban, et al., 2006, Gardner, et al., 2000a, Gardner, et al., 2004). Even a recent consensus on embryo assessment based their criteria for blastocyst grading on Gardner and Schoolcrafts' system and agreed that an optimal blastocyst has a fully expanded blastocoelic cavity with a prominent and compact ICM and a cohesive trophectoderm epithelium, both structures composed of many cells (Alpha Scientists 2011, Racowsky, et al., 2010). Unfortunately, probably due to insufficient confounding reports, this consensus failed to discuss the

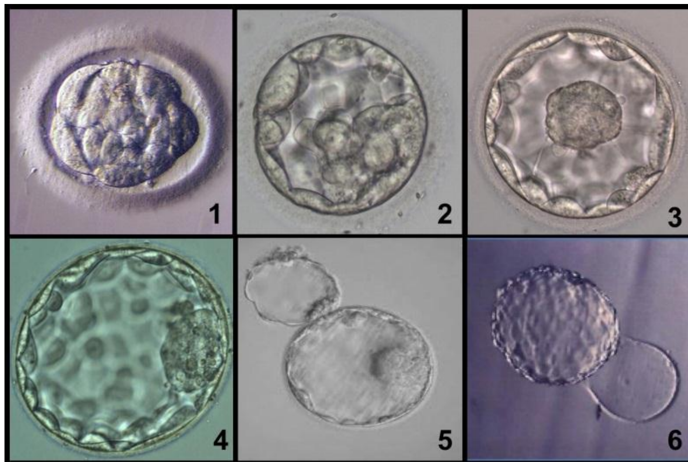

Figure 2. A numerical score from 1 to 6 based on degree of blastocoele expansion and hatching status: 1, early blastocyst with blastocoel that is less than half the volume of the embryo; 2, a blastocyst with a blastocoel that is half or greater than half of the volume of the embryo; 3, a full blastocyst with a blastocoel completely filling the embryo; 4, an expanded blastocyst with a blastocoel volume larger than that of the early embryo, with a thinning zona; 5, a hatching blastocyst with the trophectoderm starting to herniated through the zona; and 6, a hatched blastocyst, in which the blastocyst has completely escaped the zona..

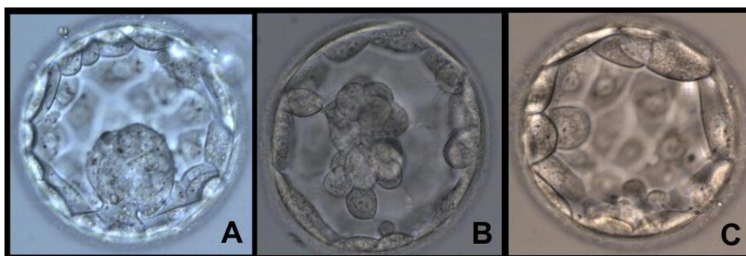

Figure 3. The ICM is assessed for blastocysts with expansion grades 3 to 6. A, tightly packed, many cells; B, loosely grouped, several cells; or C, very few or no cells.

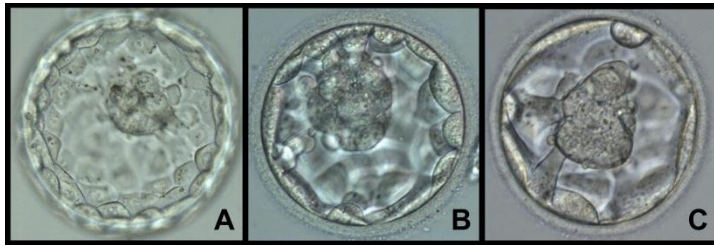

Figure 4. The TE is assessed for blastocysts with expansion grades 3 to 6. A, many cells forming a cohesive epithelium; B, few cells forming a loose epithelium; C, very few large cells.

independent predictive strength of each parameter and even rank their importance. Without this knowledge, selecting between sibling embryos with varying grades for each of the morphological parameters becomes slightly random. With this knowledge more precise decisions can be made to prioritise, if any, parameter(s) as having the highest grades when selecting the most viable blastocyst.

Alternative grading systems have been proposed for predicting blastocyst viability, but they have not been widely implemented (Dokras, et al., 1993, Kovacic, et al., 2004, Richter, et al., 2001). For example, Richter *et al* (2001) described a method of measuring the size and shape of the ICM. This study found that blastocysts with an ICM of a slightly oval shape and size greater than 4500μM^2 had the highest implantation rates.

1.2.2 Extended culture and blastocyst transfer

Blastocyst transfer is one approach being used to achieve higher implantation and live birth rates compared to cleavage stage embryos (Blake, et al., 2007, Gardner, et al., 1998b, Gardner, et al., 1998c, Papanikolaou, et al., 2006, Papanikolaou, et al., 2008). Delaying transfer and prolonging embryo culture to blastocyst stage is argued to improve uterine and embryonic synchronicity, and selection of the most viable embryo(s) (Gardner, et al., 1996).

Many observations show that morphologically normal day 2-3 embryos can fail to develop to blastocyst stage, while morphologically suboptimal day 2-3 embryos can develop to blastocyst stage and give rise to high pregnancy rates (Bolton, et al., 1989, Bolton, et al., 1991, Gardner, et al., 1998c). It is

postulated that a process of deselection occurs around day 3 of development, during embryonic genome activation, and that many embryos arrest at this stage due to major chromosomal abnormalities (Hardarson, et al., 2003, Sandalinas, et al., 2001). This in turn reduces the proportion of chromosomally abnormal embryos available for transfer at the blastocyst stage (Staessen, et al., 2004). Embryos that reach blastocyst stage are also considered to have an innately higher implantation potential due to exhibiting a functional embryonic genome that can control cell division and differentiation into the two different cell types; ICM and TE cells. There are of course a great number of embryos that fail to reach blastocyst stage of development and reasons may be linked to deficient culture conditions rather than non-functional genomes. Many IVF clinics prefer to restrict blastocyst culture for patients with a 'good' number of fertilized zygotes or a 'good' number of quality embryos on day 2 of development. The hope is to avoid cancelling embryo transfer.

1.3 Proposed methods of embryo selection

Internationally reported IVF results show that about eight out of ten transferred embryos fail to implant, two out of three IVF cycles fail to result in a pregnancy and cumulative pregnancy rates, combining results from fresh and subsequent frozen-thawed cycles, increase with each subsequent cycle (Bromer, et al., 2008, De Jong, et al., 2002, Kovalevsky, et al., 2005, Ubaldi, et al., 2004). Together, these reports confirm the inaccuracy of our current methods to predict embryo viability and select the best embryo first.

Today there is a large research effort being undertaken to develop and use the latest techniques of molecular biology to aid in the selection of viable embryos. These technologies can be roughly divided into invasive or non-invasive methods. Many of these methods are in their early stages of development and lack evidence based studies to support their wider implementation. In addition, most of these methods require large investments into laboratory resources; economic, personnel, training and equipment. All these factors play a determinative role in what method(s) are investigated and implemented into IVF clinics.

The following paragraphs will briefly describe some methods being used and investigated in the IVF community and give a more detailed description of the new method of selection we investigated as part of my doctorate.

1.3.1 Invasive techniques

Extensive research of preimplantation embryos has demonstrated that many morphologically normal embryos are not compatible with either implantation or a healthy pregnancy due the presence of chromosomal abnormalities (Magli, et al., 2001, Munne, et al., 1995, Munne, et al., 2005). It is estimated that between 40% and 85% of human preimplantation embryos obtained from IVF are aneuploid (Baart, et al., 2006, Delhanty, 1997, Munne, et al., 1995, Munne, et al., 1997, Vidal, et al., 1998). Most of these aneuploid embryos are lost before they reach a clinical stage of recognition (Egozcue, 1996, Munne, et al., 1993, Munne, et al., 1998).

With this knowledge, it became apparent that screening of embryos for aneuploidy could be used to select viable euploid embryos for transfer and improve IVF success rates, especially for high risk couples with advanced maternal age, multiple IVF failure and repeated miscarriages. This method became known as preimplantation genetic screening (PGS). PGS is an invasive method of embryo selection, as it requires the extraction of cell material in order to analyse the chromosomal complement of an embryo. Extraction of cell material can be performed at different stages: zygotes (polar body biopsy), cleavage stage embryos (blastomere biopsy) or blastocysts (trophectoderm biopsy). To analyse chromosomal content a multicolor fluorescence in situ hybridization (FISH) method is routinely used to label chromosomes most often shown to be aneuploid. Surprisingly, the few prospectively randomized controlled trials conducted were unable to show to a positive clinical outcome as measured by implantation and ongoing pregnancy for IVF patients after PGS. (Hardarson, et al., 2008, Mastenbroek, et al., 2007, Staessen, et al., 2004). These results were very much debated in literature. PGS advocates argue that failure to improve pregnancy rates is due to the technical limitations of FISH to screen all chromosomes simultaneously and detect all aneuploid embryos. Currently, PGS using more effective methods, such as comparative genomic hybridization (CGH) and microarray-CGH are being investigated and seem to be showing more promise (Gutierrez-Mateo, et al., 2011, Hellani, et al., 2008, Schoolcraft, et al., 2011)}. Importantly, these new microarray methods enable simultaneous analysis of the whole chromosomal set.

A major disadvantage to all genomic platforms being investigated, and even used in some laboratories, is their invasive nature. It is argued that removal of blastomeres can negatively affect embryo viability and if so, questions the wider application of these technologies to all IVF patients. This disadvantage may however be overcome by performing the biopsy at the blastocyst stage

instead of the more traditional 8-cell stage (Fragouli, et al., 2008, Jansen, et al., 2008).

1.3.2 Noninvasive Techniques

Morphokinetics

Rate of human embryo development in culture has long been considered an important predictor of embryo viability. Most notably, the timing of the first cell division to the 2-cell stage around 25-27 hours post insemination/ICSI, has been reported as a positive predictor of human embryo viability and is routinely used as for selection of embryos for day 2 and 3 transfers (Lundin, et al., 2001, Shoukir, et al., 1997). Thereafter, an optimal rate of cell division has been proposed from studies demonstrating that embryos with slow and fast cleavage rates have reduced implantation potential and a high incidence of chromosomal abnormalities (Almeida, et al., 1996, Gardner, et al., 1999a, Giorgetti, et al., 1995, Marquez, et al., 2000, Tao, et al., 2002, Ziebe, et al., 1997). From this work, we assess embryos at specific time intervals post insemination (PI) and favour embryos with a specific number of blastomeres and/or with specific morphological characteristics. For example, a 4 cell embryo at 44-46 hours, an 8 cell embryo at 66-68 hours, a compacting morula at 90-94 hours and an expanded blastocyst at 114-118 hours.

More recently, time-lapse photography has been used to continuously observe the embryos allowing embryologists to review the sequence of events occurring during an embryos development, from the zygote to the blastocyst stage. The aim of this technology is to map the most optimal set of developmental sequences and create a predictive model for selecting the best embryo for transfer.

Initial retrospective studies using this technology have nominated a number of morphokinetic parameters for selection of viable embryos. These include timing and synchrony of the first, second and third cell division, and uniform cleavage cycle patterns with short intervals in the 3 and 5-cell stage, and abrupt first cell division to three or more cells (Meseguer, et al., 2011, Wong, et al., 2010). More recently, preliminary data (interim analysis) from the ongoing prospective randomised controlled trials report improvement to clinical outcomes when using morphokinetic predictive models to select embryos for transfer (Mesenguer, ESHRE 2012).

Targeting components of embryo spent culture media

Although morphology and blastocyst development are good non-invasive methods to predict embryo viability many investigators are searching for

alternative, more quantitative, methods to use in adjunct. Many of these methods are targeting components in the spent culture media surrounding the embryo. The spent embryo culture media not only contains a great number of nutrients consumed by the embryo during development, but also many end products generated by the embryo. These end products are a result of many cellular processes or metabolic pathways driven by the embryo in response to genetic, nutritional and/or environmental factors. The complexity of some of the major pathways and their relationship to each other can be schematically viewed in Figure 5.

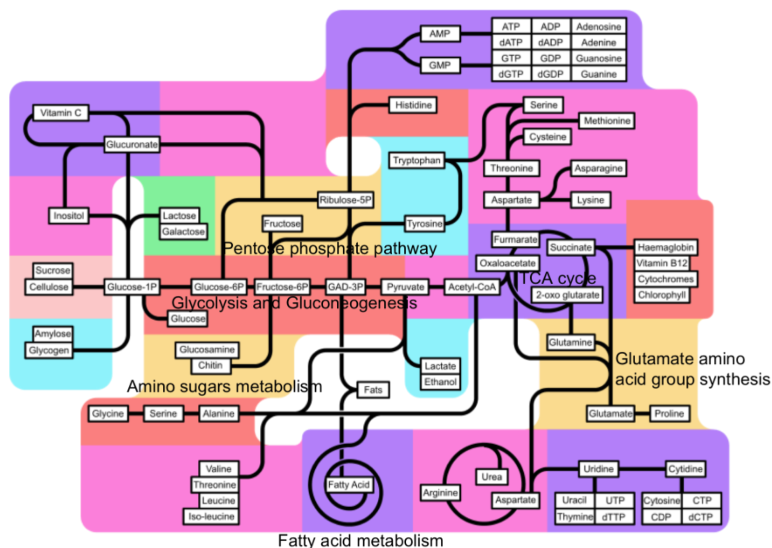

Figure 5. A schematic diagram of the networking between major metabolic pathways within a eukaryotic cell. This file is available from Wikepedia and is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license.

It is hypothesized that embryo consumption (uptake) and production of components within the culture media differ between embryos of high and low reproductive potential and measuring these differences can be used to predict embryo viability. Of course before we discuss these investigations, it is important to understand the evolution of IVF culture media and to know that there are many different schools of thought that have led to the development of different media with different formulations and these can affect the interpretation of data. Of concern to the IVF community, is the secrecy that surrounds the specific chemical composition of commercial media, while the

constituents are listed the concentrations are not given due to commercial confidentiality.

Culture media

Early IVF culture systems used simple media, such as Earle's buffered saline solution (EBSS) supplemented with pyruvate and serum (Fishel, et al., 1984). Although fertilization can be achieved in very simple media, embryo development was not so successful and development past early cleavage stages was limited. Today, our understanding of the embryos changing metabolic and nutritional requirements has led to the development of more complex culture systems using chemically defined media. There are many practical advantages for using chemically defined media in replacement of earlier serum supplemented media. Chemically defined media can be reproduced easily without significant batch to batch variation, changes to composition can be studied in a controlled manner, and they are free of unknown biological activities, such as enzymes and growth factors, which may have hidden affects on embryo development. To decide the exact chemical composition of media two approaches have been used; the so-called 'back to nature' principle, and the 'let the embryo choose' principle.

The 'back to nature' approach aims to mimic physiological conditions that the embryo is naturally exposed to, so that concentrations of constituents are close to those found in the oviduct and the uterus. Early work sampling the tubal and uterine fluids from non pregnant women during the luteal phase has provided information about the concentrations of Na+, K+, Cl-, Ca2+, Mg2+, glucose, pyruvate and lactate and has led to the development of human tubal fluids (HTF), synthetic oviductal fluid (SOF), M91 and P1 (Borland, et al., 1980, Casslen, et al., 1984, Gardner, et al., 1996, Gardner, et al., 2000b, Mortimer, 1986, Mortimer, et al., 1998, Quinn, 1995, Quinn, et al., 1995). Although these media have been tested extensively in animal models and are able to support human preimplantation development there is some debate about how close they mimic the concentrations of components found in the oviduct and uterus, due to technical/practical limitations when sampling and analysing biological fluids (Sturmey, et al., 2008, Summers, et al., 2003). Further works testing these media in animal models, mostly mouse and bovine embryos, and on spare human embryos have supported the notion that embryos have different nutritional requirements at different stages of development and these should be reflected in the culture media (Devreker, et al., 2001, Gardner, et al., 1994, Gardner, et al., 2000c, Lane, et al., 1994, Lane, et al., 1998, Leese, 1991). This extensive work has led to stage-specific modifications to culture media and the progression to two-step sequential media systems (Barnes, et al., 1995, Gardner, et al., 1997, Lane, et al., 1997a,

Lane, et al., 1997b). The most discussed differences at early cleavage stages include introducing EDTA, up to 8-cell stage, pyruvate and lactate as primary energy substrates and a limited supply of only seven non-essential (not required by diet) amino acids. Past the 8-cell stage, all 20 naturally occurring amino acids both essential (required by diet) and non-essential are included at physiological levels, a few vitamins (eg. Inositol and pantothenate), and glucose is the primary source of energy while pyruvate, lactate, taurine, EDTA concentrations are decreased (Gardner, 1998, Gardner, et al., 1998a, Lane, et al., 1998, Lane, et al., 2003). More recently, media companies have started to add essential amino acids and glucose to cleavage stage media as past studies are revised and new investigations show that their presence is more physiological and not detrimental as previously suggested. The biggest advocates for sequential media systems would have to be Gardner and colleagues, whose work with mouse embryos has led to the development of G1/G2 media now sold commercially by Vitrolife AB.

The'let the embryo choose' principle is a more traditional approach, whereby concentration-response experiments are conducted to test varying concentrations of only a single component to define the optimal response. This principle led to the design of sequential simple optimization (SOM) media (Lawitts, et al., 1991a, Lawitts, et al., 1991b), later modified to give a medium called KSOM (Lawitts, et al., 1993). Today, KSOM supplemented with amino acids is basis of the commercially available one-step single media formulation called Life Global media. The use of one-step single medium for culture of all stages of embryo development is not as widely adopted as the two-step culture systems.

Carbohydrates

During the early cleavage stages of development the level of metabolic activity is low (Leese, 1991). At this stage, energy is generated by the aerobic consumption of pyruvate, lactate and glutamine in the Krebs cycle (Tricarboxylic acid cycle, TCA) (Hardy, et al., 1989, Leese, 1991, Leese, et al., 1993)}. After the 8-cell stage, metabolic activity increases dramatically and glucose utilization via glycolysis (anaerobic metabolism) becomes the primary source of energy (Gardner, et al., 1987, Leese, et al., 1993, Sturmey, et al., 2003). In addition, many of the carbon metabolites in the glycolytic pathway are shuttled to other metabolic pathways, such as the pentose phosphate pathway, TCA cycle, and amino acid synthesis pathways. These pathways are especially important at this stage of development when the

embryonic genome is activated and is being replicated and transcribed to produce new DNA, RNA, proteins and enzymes

Studies of animal and human embryos have demonstrated that embryos of the same morphological grade have distinctive differences in energy metabolism. More importantly, these differences are related to developmental and reproductive potential.

Early animal studies of glucose uptake by day 10 bovine blastocysts (Renard, et al., 1980) and day 4 mouse blastocysts (Gardner, et al., 1987) were the first to demonstrate that elevated uptake was positively associated to better development and live birth outcome. Importantly, it was later demonstrated by Lane and Gardner that glycolytic activity, glucose uptake and conversion to lactate measured by ultramicroflourescence assay, could be used prospectively to select between morphologically identical mouse embryos before transfer (Lane, et al., 1996). Using glycolytic activity as a selection criterion increased the pregnancy rate four-fold compared to random selection. Similar retrospective studies have also been performed for human embryos (Conaghan, et al., 1993, Gardner, et al., 2001, Hardy, et al., 1989, Van Den Bergh, 2001). For example, Van den Bergh et al. showed that transferred blastocysts that successfully implanted had higher glucose uptakes and lower glycolytic rates than those that failed to implant (Van Den Bergh, 2001). In addition, Gardner et al. showed significantly higher pyruvate and glucose uptakes on day 4 by human embryos that went on to form blastocysts (Gardner, et al., 2001). More recently, Gardner et al. has revalidated earlier work demonstrating high glucose uptake on day 4 as a predictive marker of pregnancy (fetal heart activity at 8 weeks), although beneficially a new more rapid screening technique, microflourimetry was used. This method analysed small volumes (<10μL) of spent media using an enzyme linked reaction and quantitative fluorescence microscopy and has the capacity to analyse samples in real time with results obtained within a few minutes (Gardner, 2007). Even though it is unlikely that the turnover of only one metabolite will be able to predict embryo viability, this sampling methodology could prove to be very useful for other profiling technologies.

Amino acids and Proteins

Amino acids are another essential component supplied in the culture media during in vitro development (Brison, et al., 2004, Lamb, et al., 1994). A physiological mixture of 20 amino acids has been shown to be important from cleavage stage to blastocyst development (Brison, et al., 2004,

Houghton, et al., 2002, Jozwik, et al., 2006, Tay, et al., 1997). Primarily, amino acids are used to synthesize new proteins and nucleic acids. However, amino acid metabolism by the embryo and spontaneous break down of in the culture medium can lead to the accumulation of embryo-toxic ammonium. Ammonium accumulation has been shown to have a negative impact on embryo cleavage and blastocyst development and cell number (Lane, et al., 2003, Zander, et al., 2006) and alter metabolism and increase apoptosis (Lane, et al., 2003). These negative effects are the main reasons for limiting the number and concentrations of amino acids supplied in the culture media.

Using high performance liquid chromatography (HPLC) studies have showed that changes to amino acid turnover can predict the ability of early cleavage embryos to develop to blastocysts and implant (Brison, et al., 2004, Houghton, et al., 2002). For these studies, in-house culture media containing all 20 amino acids at physiological concentrations were used. Between days 1 and 2 of development turnover of three amino acids Asparagine, Glycine (non-essentail) and Leucine (essential) were significantly related to clinical pregnancy and live birth (Brison, et al., 2004). For cleavage stage embryos that developed to blastocysts only serine (non-essential), arginine, leucine, methionine and valine (all non-essential) were taken up from the mixture. A more recent study has even demonstrated that amino acid metabolism is positively related to DNA damage in human embryos suggesting that viable embryos can be selected by low amino acid turnover rates (Sturmey, et al., 2009). The authors of this work state that the HPLC technology used to complete an amino acid profile takes about 45 minutes to perform for each embryo. Considering that on any given day within the laboratory an average of 8 patients will receive embryo transfer, and each will probably have multiple embryos available for analysis, the time needed to analyse and obtain results is too long. If this time can be shortened, as the authors suggest, then amino acid profiling may become a useful technique in the future. Nevertheless, amino acid profiling will certainly be of considerable use for optimization of amino acid concentrations in culture media (Sturmey, et al., 2008).

A number of studies have also investigated the release of proteins into the culture media as markers of embryo viability. For example, embryo production of human leukocyte antigen-G (HLA-G) is thought to play an important role in embryo implantation and maternal tolerance of foetal tissue (Kanai, et al., 2001, Marchal-Bras-Goncalves, et al., 2001, Rajagopalan, et al., 1999). Detection of soluble HLA-G levels in spent culture media of day 3 embryos, by enzyme-linked immunosorbent assay (ELISA), was reported to be associated with higher pregnancy rates (Noci, et al., 2005, Sher, et al.,

2005). However, other investigators (Menezo, et al., 2006, Sageshima, et al., 2007) subsequently challenged these reports and the methods used. Another important protein secreted by blastocysts is human chorionic gonadotrophin (hCG). This secreted protein initiates early recognition of the preimplantation embryo and acts as a LH superagonist to rescue progesterone synthesis and secretion by the corpus luteum (Niswender, et al., 2000, Van De Sompel, et al., 2008). hCG has also been shown to modulate the receptive endometrium to facilitate implantation and induce immunological tolerance (Licht, et al., 2001a, Licht, et al., 2001b, Tsampalas, et al., 2010). More specifically, hyperglycosylated hCG (H-hCG) is produced by cytotrophoblasts during implantation. It has been suggested that poor implantation and even miscarriage is due to inadequate production of H-hCG by implanting blastocysts (Cole, 2012). Secretion of hCG has been detected in spent culture media of cleavage stage embryos as early as on day 2 of development (Fishel, et al., 1984, Ramu, et al., 2011). At the blastocyst stage, embryos of higher grades have been shown to secrete higher levels of hCG in culture, for both hatched and zona-enclosed blastocysts (Dokras, et al., 1993, Lopata, 1996). The specific detection of H-hCG in vitro has not yet been reported.

More recently, new methods enabling more sensitive protein profiling by mass spectrometry have revealed that embryos have stage specific protein profiles that are independent of morphology (Katz-Jaffe, et al., 2005, Katz-Jaffe, et al., 2006). These investigators have also identified an 8.5kDa protein biomarker that is expressed through all stages of development for embryos that develop to blastocyst, but is relatively absent in arresting and degenerating embryos (Katz-Jaffe, et al., 2006). Unfortnately, the advanced technique used to profile small media samples, surface enhanced laser desorption and ionization time of flight mass spectrometry (SELDI TOF-MS) and protein chips, is not going to be available to an IVF laboratory due to its complexity and cost. But may help to identify a combination of new biomarkers that can serve as targets for embryo selection

Metabolomic profiling

Recently, an interesting analytical technology, which is non-invasive requires and no reagents or sample preparation, has been introduced for evaluating a broad spectrum of metabolites, the metabolome, found within a biological system at one particular time point. The aim of this approach, called metabolomic profiling, is to quantify this dynamic inventory of metabolites and associate the resulting biochemical profile ("fingerprint"), with the functional status of the biological system, for example normal vs. abnormal.

This technology has now been proposed for analysis of spent embryo culture media. It is proposed that biochemical profiles of spent embryo culture media differ between embryos that implant and those that fail to implant and can thereby be used to prospectively predict reproductive potential.

To generate a biochemical profile this technology uses a type of vibrational spectroscopy, called Near-infrared spectroscopy (NIR). Briefly, NIR light is applied to a biological sample; the chemical bonds within functional groups absorb energy at specific wavelengths and begin to vibrate at characteristic frequencies, dependent on their chemical structure and bond strength. Applying a set of wavelengths between 920-1675nm (NIR light) generates a spectrum due to vibrations of N-H, C-H, O-H, S-H, C=C and C=O functional groups. The intensity of the light absorbed is directly proportional to the concentrations of biomarkers present in the sample and these biomarkers reflect changes in media constituents such as albumin, lactate, pyruvate, glutamate and glucose, due to oxidative and energy metabolism. Spectral profiles for implanted embryos and non-implanted embryos are comparatively analysed. The most parsimonious combination of spectral regions that are predictive of pregnancy outcome is determined by inverse least-squares regression and genetic algorithm optimization. The resulting multivariate algorithm is then used to calculate viability scores for unknown samples (Figure 6).

Recent proof of principle studies using Raman and NIR spectroscopy to analyze spent embryo culture media, collected after embryo transfer on day 2, 3 and day 5 have shown that spectral profiles exhibited discrete differences between embryos with positive and negative pregnancy outcomes (Scott, et al., 2008, Seli, et al., 2007, Vergouw, et al., 2008). Furthermore, these mean spectral differences could be successfully quantified in a multivariate algorithm to generate viability scores that are related to implantation potential. So, mean viability scores of embryos that implanted were significantly higher than mean viability scores for embryos that failed to implant.

This technology became of great interest to our clinic and we were given the opportunity to test this technology through observational studies and clinical studies. Of course we used morphology as our benchmark method, and it soon became apparent that morphology was not as optimized and simple as the textbooks suggest, but questions remain about how to best use the parameters we observe.

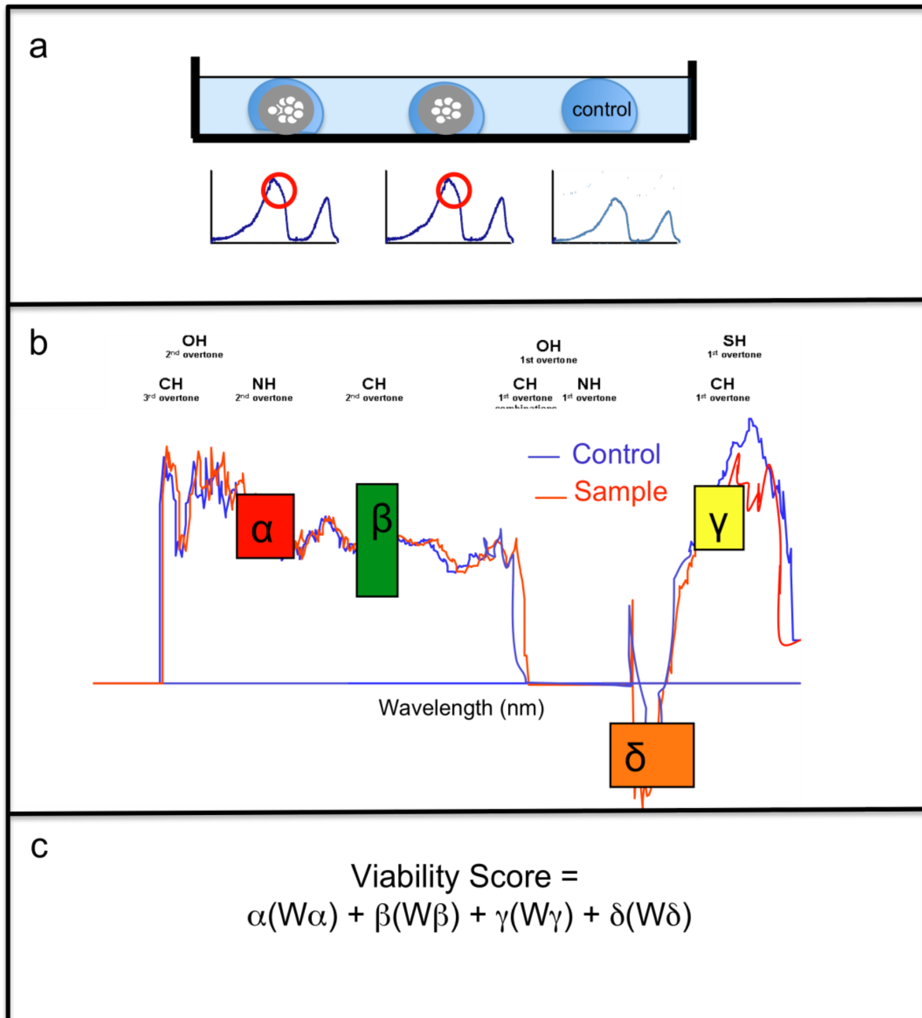

Figure 6. NIR spectroscopic analysis of spent embryo media samples. a) After the embryo is removed an aliquot of the spent culture media is sampled and analysed using NIR spectroscopy. A complementary control sample is also sampled and used as a reference to mean ratio the embryo spectrum. b) The most parsimonious spectral regions are quantified in a multivariate algorithm and used to calculate a viability score c).

2 AIMS OF THIS THESIS

The main objective of this thesis was to evaluate the predictive value of blastocyst morphology and Near Infrared spectroscopy for selection of the best single embryo for transfer.

Thesis aims were:

I. To assess the application of NIR technology in a clinical setting only performing SET and determine if an algorithm generated from NIR analysis of media samples at one clinical setting can blindly predict implantation potential of blastocysts cultured at another clinic in a different culture media and culture volume.

II. To investigate if NIR spectroscopic analysis of spent embryo culture medium in an on-site, prospective setting could improve the ongoing single embryo transfer (SET) pregnancy rate.

III. To determine the independent predictive power of each morphological parameter, grade of expansion, trophectoderm and inner cell mass, in relation to live birth.

IV. To determine if 1) pre-freeze morphology, 2) a three part post-thaw scoring system and 3) combination of pre-free and post thaw morphological parameters could be used to predict live birth outcome after frozen-thawed blastocyst transfer cycles.

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3 PATIENTS AND METHODS

More detailed descriptions of the different methods used in this thesis can be found in the respective papers. In this section the standard methods used are outlined, followed by more detailed discussion of the limitations and advantages of the methods used in each paper.

General Considerations

Ovarian stimulation

All patients were down regulated with either gonadotrophin-releasing hormone (GnRH) agonist (Suprecur; Hoecht, Germany or Synarela, Pfizer, USA) or GnRH antagonist (Olgalutran; MSD, USA or Cetrotide, Merck Serono, Germany) was used ina short protocol. Controlled ovarian stimulation was performed with recombinant FSH (Gonal-F, Merck Serono, Germany or Puregon, MSD, USA) or highly purified human menopausal gonadotropin (Menopur). Follicular aspiration was performed 36-38 hours after s.c. hCG (Ovitrelle, Merck Serono, Germany) administration.

Embryo culture

All culture dishes were pre-incubated and maintained during embryo culture in incubators controlled at 37°C under 6-7% CO^2, 5% O^2 and 90% N^2. Fertilization was assessed 16-18 hours after insemination/ICSI and blastocyst transfer was decided primarily by the presence of more than 5 fertilized zygotes. Fertilized zygotes were cultured individually in 25uL droplets of cleavage medium (Cook Medical, Australia) under mineral oil and then transferred on day 2 of culture to 25uL droplets of CCM medium (Vitrolife AB, Sweden) for culture to blastocyst stage. On day 5 all embryos were transferred to fresh pre-incubated culture dishes. Morphological assessment of embryos remaining in culture was performed on day 2, 5 and 6 according to the criteria described in paper I, II, III and IV. Embryo transfer at the blastocyst stage was only performed on day 5 of culture. All blastocyst transfers performed and analysed in this thesis were single embryo transfers.

Blastocyst cryopreservation and warming

All supernumerary blastocysts were cryopreserved using a method of vitrification as described in paper IV. Only blastocysts of grade 3BB or higher, according to Gardner and Schoolcraft's grading system, were vitrified. This three-step vitrification protocol uses increasing concentrations of ethylene glycol, DMSO and sucrose for dehydration and cryoprotection (Nidacon AB, Sweden). Individual blastocysts were then loaded on a cryoloop (Vitrolife AB, Sweden) and plunged into liquid nitrogen, followed by storage in nitrogen filled tanks.

Blastocysts were warmed in decreasing concentrations of sucrose in base medium before being transferred into culture dishes containing 1mL of CCM medium (Vitrolife AB, Sweden).

Blastocyst Grading

There were a total of 8 embryologists grading the blastocysts over the five year study period. All blastocysts were assessed by two embryologisits before assignment of the blastocysts grade, reducing the intra-variability of grading. Grading of morphology is a qualitative method and can be subjective. To avoid variability in our assessment of embryos, we annually perform an internal and external validation. Briefly, together with 4 other clinics we individually grade embryos (recorded by video) at all stages of development and of various grades. The results are then summarised and discussed to ensure that we are homogenous in our assessment of these embryos both within the group and in relation to other clinics. An acceptable level of agreement between embryologists and clinics is set at 80 %. That is for 80% of embryos graded by an embryologists, allocated grades for each parameter (expansion, ICM and TE) must be the same as the grades set by other embryologists. The past five years we have achieved this level of agreement, and passed the validation criteria.

Statistical Analysis (Paper III and IV)

A statistical consultant group who recommended the following methods for best elucidation of our aims has performed all statistical analyses. For prediction of the clinical outcome live birth, Generalized Estimating Equations (GEE) models were used as they allow for adjustment of within-individual correlation. This was important as within the sample population a number of patients had more than one single blastocyst transfer cycle, so for

some of the variables (i.e. patient characteristics) there are repeated measurements in the data analysed. Univariate GEE models were performed to identify the confounders that statistically significantly affect live birth outcome. This method simply looks at the relationship between each variable and the response (live birth) and ignores the relationship between variables. The variables we chose to include and test for significance were based upon what is known to affect pregnancy outcome and what information was available and documented accurately in the patients journal. Because multiple predictors contribute to live birth outcome and may even be related to each other a multivariable GEE analysis was then performed to determine the independent affect of each significant variable (predictor) on live birth outcome. In this model all predictors are included at the same time and the 'pure effect' of each predictor can be estimated by the coefficient. In comparison, in a linear (univariate) model the affect of a predictor on outcome may be exaggerated or reduced by its relationship to another predictor not included in the model. Stepwise logistic regression was used for selection of independent statistically significant predictors among the morphology variables and the confounders. Once variables were selected, the GEE models were performed including the selected variables to obtain the odds-ratios (OR), 95% confidence intervals (CI) and associated p-values. All significance tests were two-tailed and conducted at the 0.05 significance level

Specific Methodological Considerations

Paper I

Ethical considerations

The ethical committee at the University of Gothenburg did not review this study. This was decided for two main reasons. First, the sampling of the spent culture media, which is otherwise discarded when embryos are removed for transfer, would in no way impair or affect the treatment of the patient. Secondly, no information regarding the patients' identity was used in this study. The only information used was the morphological grade of the embryo and implantation and live birth outcome. This decision may have been presumptuous on our part, but when the same reasoning and methodology was reviewed for Paper II and IV the Ethics committee granted approval.

Sampling and data collection

The sampling of spent embryo culture media performed at Shady Grove Fertility Centre and Fertilitetscentrum occurred during the same time period during 2009. Sampling methods used were the same. Briefly, approximately 15 µL of spent culture media was sampled directly after removal of the blastocyst in preparation for transfer. Collected media was then snap frozen in liquid nitrogen and stored at -80ºC. Control samples incubated under the same conditions were also collected at the same time. Blastocysts cultured at Fertilitetscentrum remained within the same media droplet for approximately 72 hours of culture before collection. At the Shady Grove clinic blastocysts were cultured for 24 hours in the same media droplet before collection. Collected samples were sent to a central laboratory for analysis by NIR spectroscopy. Information regarding the embryo score and implantation outcome was also provided to the central laboratory.

Unfortunately, no validation of blastocyst grading was performed between the two clinics to assess possible variability in assignment of morphological scores. Both clinics use a standard blastocyst grading system described by Gardner and Schoolcraft (Gardner, et al., 1999b). For the purpose of this study these scores were reassigned into one of four grades, A, B, C or D. Although these four grades A, B, C, and D are clearly described and are broader categories than Gardner's grading system, some variability in allocation of grade may exist. If so, the most likely source of variability would have arisen from grading poor versus excellent/good ICM and TE. This may have affected the assignment between grade A or B to Grade C and vice versa.

Statistical Analysis

Parametric statistical methods were used to investigate the viability score and its relationship to pregnancy outcome and morphological grade. The normal distribution of the viability score was tested by the Kolmogorov-Smirnov (p-value 0.041) and Shapiro-Wilk test (p-value 0.004). The histogram (Appendix 1) shows the normal distribution of the viability score.

Paper II

Randomisation and study design

All patients that fulfilled the inclusion criteria and signed informed consent were included in this study. On the day of embryo transfer the patients' cohort of embryos were first morphologically graded to identify patients with two or more good quality embryos before randomisation into either the control or NIR study group. Only good quality embryos were included in this study and analysed by NIR spectroscopy. Although Paper I and other proof of principle studies showed that the viability score was related to pregnancy outcome irrespective of morphological grade we still decided to exclude all morphologically poor embryos in our study. This was decided as none of the proof of principle studies had looked at the variation of viability scores within a patients' cohort of embryos, only transferred embryos were retrospectively analysed. We therefore considered it unethical to test this new method independently of the routine morphological criteria we use to qualify and select a good embryo for transfer.

Patients were allocated into one of the two study groups by a computerized randomization program that balanced for several known confounders of pregnancy outcome that we chose, such as female age and number of good quality embryos on the day of transfer. We decided not to balance for the morphological grade of the transferred embryo during randomization knowing that poorer grades of embryos would be transferred in the NIR group compared to the control group. We predicted that balancing for this effect would have lead to allocation of a better cohort of embryos and patients into the NIR group and been unfavourable to our study design. Other approaches could have been used for this study. We could have limited the study to include only patients with embryos of the same morphological grade on the day of transfer, for example only 4 cell embryos with no fragmentation on day 2. However, this would have limited inclusion of patients and completion of this study would have required a multicentre collaboration. Alternatively, we could have balanced for the morphological grade of the cohort. Although, it was also balanced in our chosen study design.

For approximately 6% of embryos randomized to the NIR group there was a technical failure that prevented analysis and calculation of a viability score. For this there were two main reasons. The first was the inability to obtain enough sample volume to run the analysis. Although the recommended volume for analysis was 10µL, we soon realised that at least 15µL was

required to ensure a fully loaded sample cell. This was sometimes impossible due to small volumes lost during transfer of the embryo from the spent culture media droplet. The other reason for technical failure arose when loading the sample cell. This procedure required precision to prevent the presence of air bubbles and mineral oil within the sample chamber, which inhibited NIR analysis and calculation of a viability score. By increasing the culture volume we could have solved these problems, however this would most likely dilute the metabolic changes being measured and possibly the threshold of signal required for detection.

At the start of this controlled trial we were using a prototype NIR spectrometer that was entrusted to our lab for research purposes by Molecular Biometrics (MB). This instrument was not commercially available. At the end of this study (at 287 randomized patients) this early prototype was replaced and we were given a commercially available instrument to analyse our samples (from randomized patient 288). For a short period of time we run samples parallel to obtain spectral data, which were later sent MB. It became apparent from this spectral data that there were some inconsistencies between the two instruments (prototype versus commercial) and the spectral data obtained. Because of slow patient recruitment to the study we had already planned an early Data Safety Monitoring Board (DSMB) analysis, so we continued the study in spite of some growing concerns about the stability of the instrument. At the end of the study we performed a sub group analysis to determine if the commercially available instrument was a better instrument, and a time analysis to determine if the predictive value of the NIR analysis changed during the time course of the study. Refer to results.

Statistical Analysis

A statistical consultant group has performed all statistical analyses. Demographics and baseline variables were analysed by treatment group and subsequently corrected for in the statistical analysis as possible confounders. All data was analysed from all of the randomised couples, i.e. intention-to-treat (ITT) analysis and from couples that were without any protocol violations that might have had a significant effect on the analysis, i.e. a per protocol (PP) analysis. The main analysis performed compared between the two groups of the ITT population. Unadjusted comparison between the two groups was analyzed using the Fisher's exact test for dichotomous variables, Mann-Whitney U-test for continuous variables, Mantel-Haenszel Chi-square test for ordered categorical variables and Chi-square test for non-ordered categorical variables. The two groups could be either the two study groups or

pregnancy/not pregnancy. Complementary unadjusted analyses were also performed between the two groups on the PP population. All significance tests were two-sided and conducted at the significance level of 0.05.

To study if the Viability Score increases pregnancy rate over time (6 month time intervals) a complememtary analysis was performed and pregnancy outcome between study groups compared using Fisher exact test.

Paper III

Study design

To investigate the odds of live birth for each morphological parameter and each grade we conducted a retrospective study of all patients who received a fresh single blastocyst transfer on Day 5 from January 2006 through to June 2010 at our clinic. The advantage of performing a retrospective study was that all the data was immediately available for analysis. As such, we were able to obtain our results quickly. We selected a time period we believed to be most consistent and representative of how we work today in our IVF lab. Even so, we are aware that this study design can also minimise the ability to control for known confounding and unknown variables during the time period selected. For example, changes in methods, improvement of blastocyst grading precision, trends for optimizing IVF treatment, such as reasons for selection of patients who receive blastocyst culture.

Another disadvantage of this design is the inability to control for the distribution of each characteristic, i.e. each grade of each morphological parameter, when fairly evaluating the odds of live birth outcome. For example, when selecting blastocysts for transfer we prioritized higher grades of ICM compared to higher grades of TE. As such, blastocysts with grade A TE were more often transferred in companionship of grade A ICM, or when no grade A ICM was available. Furthermore, grade C TE and ICM were only ever transferred when no other blastocyst of higher grade was available indicating a poor treatment cycle or patient group. An alternative study design would have been to test our aim prospectively by randomly selecting the blastocyst grade to be transferred balancing for all known confounding factors. This study design would not include selecting grade C ICM and TE in favour of grades above as this would be unethical.

Paper IV

Study design

Soon after introducing blastocyst vitrification (2005) to our clinic, we began to search for parameters that could be used to indicate cryosurvival and predict pregnancy outcome. This information would then help us to decide if a thawed blastocyst is 'good enough' to be transferred or if another sibling frozen stored blastocyst should be thawed, if available. The following criteria were chosen: 1. Blastocoele re-expansion that was already in use for determination of cryosurvival, although the degree of expansion and time needed for re-expansion was ill defined. 2. Degree of cellular transparency/viability. 3. Cell contour was chosen because of observations that the structure of the ICM and TE was compromised in many of the thawed blastocysts. Around 2008 we began to systematically grade thawed blastocysts for each of these three parameters. However, for this paper we chose to limit the study period from March 2008 to October 2011. This was decided because it was at this time we had changed the intervals (10%) we use to grade the degree of each parameter, greater intervals were used before this period. Previous intervals were 100%, 75%, 66%, 50%, 33%, 25% and 0% for degree of re-expansion and for degree of cell contour and viability 100%, 66% 33% and 0% were used. These intervals were too large and there was a tendency to grade up and grade down for the different parameters.

Measurement of hCG

Sandwich ELISA

Spent embryo culture media samples were collected daily, from day 2 through to day 10, and the embryo transferred to a new culture dish. Control media samples were also collected. These samples were frozen stored at -80ºC until analysis. A sandwich ELSIA method described by Ramu et al. was used to quantify the concentration of hCG in the samples (Ramu, et al., 2011). Briefy, ELISA microtiter plates were coated with goat polyclonal antibody to hCG (Abcam). This antibody binds to common variants of hCG found in urine and serum in pregnancy. Synthetic hCG protein (Abcam) was serially diluted to generate a standard curve. A pure media blank control was included to measure background noise. Spent media culture samples were added to the plate to allow for any free hCG to be bound by the coating

antibody. Bound hCG was detected using a mouse monoclonal antibody (α-hCG-β). A horseradish peroxidase conjugated antibody (goat-α--mouse IgG-HRP, Leinco Technologies Inc.) was then used to bind to the antibody complex and colorimetric substrates, SureBlue reserve TMB (KPL, Inc), was added. The substrate reaction was allowed to proceed until colour intensity was detected at the lower standard concentrations. The optical density of the colour was measured using an ultra micro plate spectrophotometric plate reader at 450nm. hCG concentrations for unknown samples were calculated using the standard curve.

Measurement of hyperglycosylated hCG by ultramicroflourescence

Measurement of samples was performed at Quest Diagnostics Nichols Institute (San Juan, CA) using a highly sensitive microflourescence assay. Briefly, this assay uses a capture antibody B207 that is specfic for the hCG-β subunit, followed by labelling with B152 antibody, specific for the H-hCG isoform detection antibody. B152 is the only antibody available for detection of H-hCG and is the property of Quest diagnositics. This method is the property of Quest diagnostics and therefore Commercial-in-confidence applies.

4 RESULTS

Paper I

This paper demonstrated that metabolomic profiling by NIR spectroscopic analysis of day 5 spent embryo culture media from 137 transferred blastocysts could predict implantation and live birth outcome. Calculated viability scores were found to be significantly higher for blastocysts resulting in FCA positive (0.63 ± 0.31) and live birth outcomes (0.63 ± 0.32) compared to FCA negative outcomes (0.36±0.31). It was also demonstrated that the morphological grade of top versus good scoring blastocysts (grade A; ≥ 4BB versus grade B; 3AA ≥3BB) could not predict pregnancy outcome, whereas the viability score of these blastocysts were significantly related to their outcome. This predictive algorithm was further developed and refined through analysis of subsequent day 5 samples and then used prospectively in our RCT, paper II. Four spectral regions were found to be important predictors of pregnancy outcome.

Furthermore, in this paper a blinded cross-validation of a predictive algorithm trained at one clinical site showed that it could maintain its predictive value when assessing viability of blastocysts cultured at another clinic using different culture media. It was encouraging to know that this technology was tolerant to some changes within the IVF culture system.

Paper II

This study was a prospective randomised controlled trial (RCT) to test the ability of the NIR technology to select the best single embryo for transfer and thereby increase the ongoing pregnancy rate. Unfortunately, after a DSMB analysis we were advised to stop the study before completion (performed after 287 patients had been randomized). Final analysis of the 327 randomized patients, 164 in the NIR group and 163 in the control group, showed no significant improvement of the ongoing pregnancy rate (34.8%

versus 36.2%) using the NIR spectroscopic technique. Furthermore, no significant improvement was found for ongoing pregnancy rate when looking at only day 2 or day 5 transfers.

Subgroup analysis was performed for all samples analysed by the new commercial instrument (patients randomized 288 to 327) on the ITT population. Similar to the total population no significant differences between the NIR and control group were found for patient and cycle characteristics analysed. However, unlike the total population, no significant differences were also found for morphological grades of embryos transferred. Of interest, no significant difference in the ongoing pregnancy rate was found between the control group (40.0%) and the NIR group (56.0%, p=0.5145) for the subgroup ITT population.

In order to study if the ongoing pregnancy rates between the two treatment groups changed over time, a multiple logistic regression analysis was performed with pregnancy as the dependent variable and time in months and treatment groups as independent variables. An interaction term was included to account for a possible relationship between time and treatment group. The regression model was not significant (AUC 0.518) and ongoing pregnancy rate did not significantly change over time (Table 1 and Figure 7).

Time_Group_2	Time Group	NIR (n=164)	NIR Control (n=163)	p-value	Difference between groups Mean (95% CI)
0-6 months		n=41	n=31		
	Pregnancy	14 (34.1%)	10 (32.3%)	1.0000	-1.9 (-24.6; 21.5)
6-12 months		n=52	n=59		
	Pregnancy	21 (40.4%)	26 (44.1%)	0.8425	3.7 (-14.9; 22.1)
12-18 months		n=55	n=62		
	Pregnancy	15 (27.3%)	19 (30.6%)	0.8453	3.4 (-14.7; 21.4)
18-24 months		n=16	n=11		
	Pregnancy	7 (43.8%)	3 (27.3%)	0.6470	-16.5 (-50.8; 22.8)

For categorical variables n (%) is presented.
For comparison between groups Fisher's Exact test was used for dichotomous variables. The confidence interval for dichotomous variables is the unconditional exact confidence limits. If no exact limits can be computed the asymptotic Wald confidence limits with continuity correction is calculated instead.

Table 1. Time course analysis, grouped in 6 month intervals (ITT population).

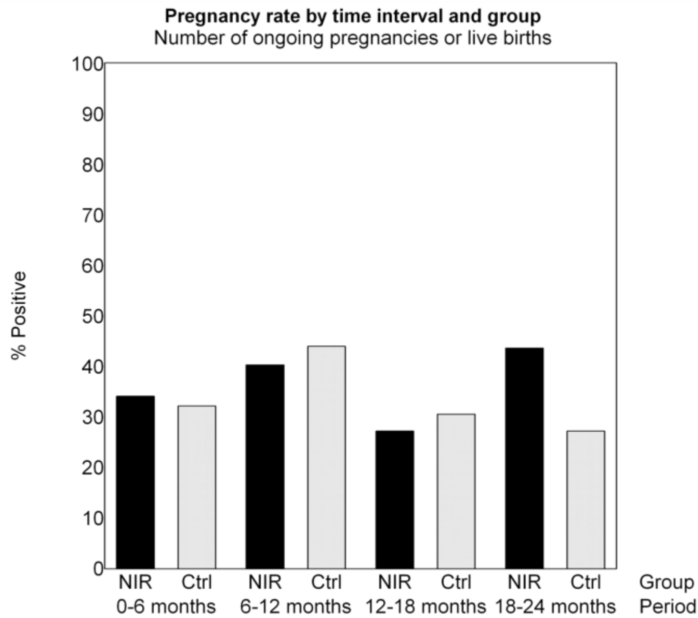

Figure 7. Ongoing pregnancy rates at 6 month intervals and treatment group

Paper III

This paper found that of the three morphological parameters graded at the blastocyst stage, trophectoderm morphology was the most important predictor of live birth outcome after day 5 SET. Although all three morphological parameters were found to be significant predictors of live birth outcome it was found that a higher grade of trophectoderm should be prioritized for transfer instead of ICM and grade of expansion when looking at good quality embryos. For example a 4BA should be selected instead of a 4AB, and a 3AA should be selected instead of a 4AB.

Paper IV

In this study we found that blastocoele expansion and trophectoderm grade were the most significant pre-freeze morphological predictors of live birth. Furthermore, we showed that degree of re-expansion was the best post-thaw parameter for prediction of live birth. As such, for frozen-thawed blastocyst transfer cycles blastocysts with higher pre-freeze grades of expansion and TE, irrespective of day of cryopreservation, should be given priority when thawing. Furthermore, re-expanding blastocysts, assessed within 2-4 hours, with greater than 60% viability should be transferred. For thawed blastocysts with slower rates of re-expansion, degree of cell contour can be used to determine the fate of the embryo.

Measurement of hCG

To determine the possible relationship between trophectoderm grade and production of hCG. We hypothesized that blastocysts with higher grades of trophectoderm produce higher concentrations of hCG and at earlier time points. This assumption may explain the predictive value of TE grade on live birth outcome.

When spent culture media samples collected from blastocysts cultured in fresh IVF cycles were analysed using the sandwich ELISA method, no hCG was detected for all trophectoderm grades (A, B or C) on day 5 or day 6 of development. Ten cryopreserved day 5 blastocysts (5 blastocysts with grade A TE and 5 with grade B TE, donated to research) were then thawed to culture until day 10. Spent media samples were collected daily to test for the onset of hCG. Using the sandwich ELISA method hCG onset was detected on day 8 and day 9 in 5 out of 10 blastocysts and by day 10, hCG was detected in 9 out of 10 spent media samples. No association was observed between TE grade and hCG onset. Unfortunately this method was not sensitive to hCG concentrations below 80pg/mL.

To increase sensitivity and specificity of H-hCG testing we approached Nichols Institute for collaboration to test spent media samples. Their ultra sensitive microflourescence method can detect H-hCG concentrations as low

as 2pg/mL. Ten spent media samples collected on day 5 and 6 from blastocysts (5 of grade A TE and 5 of grade B TE) of fresh IVF cycles were tested for H-hCG. No H-hCG was detected. Again ten cryopreserved blastocysts (8 with grade A TE and 2 with grade B TE, donated to research) were thawed and cultured to day 8. Spent culture media samples collected on day 7 and then on 8 were tested for H-hCG. Results are shown in Table 2. Earliest detection of H-hCG in spent embryo culture media was on day 7 for both grades of TE; A and B.

TE grade	Day 7 H-hCG pg/mL	Day 8 H-hCG pg/mL
A	355	NA
A	970	24883
A	260	1238
A	1600	24613
A	1558	6465
A	383	54073
A	81	34925
A	53730	4410
B	1400	14820
B	2263	4218

Table 2. Earliest detection of H-hCG in spent embryo culture media was on day 7 for both grade A and grade B TE. NA; not enough sample to analyse.

5 DISCUSSION

Near infrared spectroscopy and prediction of embryo viability (Paper I and II)

Metabolomic profiling of spent embryo culture media by NIR spectroscopy was proposed as a tool to predict reproductive competence of individual embryos. It was hypothesized that defining mean differences between NIR spectral profiles of spent embryo culture media of implanting embryos and non-implanting embryos, could be used to predict the probability of pregnancy for unknown samples. This probability estimate could then be used to select from a patients cohort of embryos.

Before our investigations of NIR spectroscopy, two other studies using this methodology demonstrated that NIR spectral profiles of day 2 and day 3 spent embryo culture media were related to pregnancy outcome (Seli, et al., 2007, Vergouw, et al., 2008). Furthermore, it was demonstrated that distinct spectral regions could be used in predictive models to calculate viability scores for blinded samples that were significantly associated with implantation outcomes. Subsequently, our study (Paper I) produced similar results for NIR spectroscopic analysis of spent embryo culture media on day 5 of culture (Ahlstrom, et al., 2011). Importantly, our study also showed, by a method of cross-validation, that a predictive algorithm was accurate even when used at different clinics using different blastocyst culture media. This had been previously demonstrated for different types of cleavage stage media (Seli, et al., 2007). This is an important attribute for any technology attempting to predict viability from analysis of spent culture media. Within most IVF laboratories commercial brands of media are used for optimal stage-specific embryo development and eventhough it is assumed that most media have similar constituents, the exact compositions of these media are unknown and are the proprietary of the commercial companies. It is therefore important that the metabolic differences being measured are not dependent on the starting composition of the media but are dependent on the metabolic activity of the embryo.

Of benefit to the application of this technology was evidence that viability scores were not related to embryo morphology on either day 2, 3 or 5. In fact, NIR spectroscopy detected metabolic differences between morphologically

equivalent embryos, confirming the findings of earlier target metabolite studies. Importantly, these differences, expressed as a viability score, were significantly related to implantation potential. All of the above findings gave positive indications for the clinical application of this technology.

Unfortunatley, when the application of this technology was tested in a prospective randomized controlled trial (RCT) for selection of embryos on day 2 and day 5 for transfer, its use in adjunct to morphology did not significantly improve the ongoing pregnancy rate when compared to morphology alone (Hardarson, et al., 2012). These conclusions were drawn from an interim analysis of the data after 37% of the patients were recruited. This interim analysis showed that the study had very little chance of showing any improvement to the ongoing pregnancy rate by using NIR spectroscopy, even if we had completed the study. As a consequence we ended the study and minimized the possible negative affects of applying this technology to more IVF cycles. These results were then supported by another RCT, fully completed soon after our study, which also failed to show improvement to the live birth rate after day 3 transfer when using NIR spectroscopy (Vergouw, et al., 2012).

The failure of this technology to improve ongoing pregnancy and live birth rates when tested in two RCT's was surprising. The proof of principle studies suggested that NIR analysis should substantially improve embryo selection and subsequent pregnancy rates. It is unlikely that the study design of both RCT's were unfavourable to testing this technology, but more likely that the faults lay within the technology platform itself.

To generate predictive algorithms a genetic algorithm optimization method is used to select the most parsimonious combination of spectral regions required to best separate implantation and non-implantation groups. It is important that the number of regions selected in the model is correctly optimized as too many can lead to "over-fitting" and too few can lead to "under-fitting". Over fitting can lead to a predictive model that fits the training data set too well, but is unable to predict on a new data set. Both result in failure to predict reliably. In our study, the predictive algorithm used for analysis of day 2 spent embryo culture media included four predictive regions while only three predictive regions were included in the day 5 predictive algorithm. The number of regions may explain why the algorithms performed poorly on a new data set, i.e. samples analysed in the RCT.

The accuracy of this predictive model is also dependent upon the accuracy of the training data set. A training data set consists of samples with known

positive and negative pregnancy outcomes to build a predictive algorithm and a blinded data set to then validate the predictive model. The number of samples included and the size of the spectral differences being measured determine the accuracy of the training data set to generate a predictive model. So, it is presumed that larger signal differences will require smaller training data sets to establish a predictive model and vice versa. Although, as a rule the more samples included the more reliable the model. It is hard to evaluate if the size of the training set used to configure the predictive models we used were enough as the science of this technology was out of our hands and in the hands of a commercial company. However, we are aware that for the day 5 algorithm only spectral data from our clinic and one other clinic (a total of 137 samples) were used to train and validate the predictive model. It would seem that this might have been too few to obtain a reliable model. Another important factor that can affect the reliability of the training data set is the presence of outliers. Outliers are samples and their outcomes that don't fit the 'norm'. Unfortunately, in IVF outliers are in abundance because there are so many different factors that we know about and more importantly don't know about that can dictate over the success of a pregnancy. Embryo viability is not solely responsible.

A presumed advantage of NIR spectroscopy and other metabolomic profiling technologies is that they can rapidly analyse and characterise the chemical profile made by an embryo, with little need for metabolite identification. As such, the cellular processes, which result in a 'viable' embryo profile versus a 'non-viable' embryo profile, are not considered. Had this technology worked, knowledge about these cellular processes may have been overlooked. But, with little information about the metabolites that were responsible for prediction of embryo viability it is hard to speculate possible reasons why this technology failed to be clinically useful. By referring to Figure 5, insight into the complexity of the metabolic pathways taking place within the cell at any given time. This diagram shows how many of the metabolites being measured by the NIR technology, and the other profiling technologies are shuttled from one pathway to the next, and these enzymatic reactions occur at very fast and different rates so the metabolome changes from one millisecond to the next. So, without background understanding of the metabolites and metabolic pathways that are being measured, the profiling information will be difficult to link to definite functional activities of the cells and how they impact embryo viability. It is like we went on a fishing experiment and threw out our nets not knowing what we were catching in these nets, compared to fishing with a rod for one defined metabolite like glucose. To move forward it may be better to choose technologies that enable biomarker identification such as nuclear magnetic resonance, mass spectroscopy and HPLC (Brison,

et al., 2004, Katz-Jaffe, et al., 2006, Seli, et al., 2008). At least with these technologies changes in profiles can be linked to specific metabolites and cause and effect relationships may be elucidated.

It is important to mention that NIR technology tried to do what the above-mentioned technologies have not yet succeeded and that is to provide a laboratory friendly instrument that is easy to use and at low cost to the patient. An ‘industrial’ NIR spectrometer is usually about the size of a washing machine. The bench top we had working in our lab was the size of a small centrifuge. This conversion or minimization has been the biggest challenge for the manufacturers of this instrument. Along the way the accuracy and stability of the instrument may have been compromised just enough to affect the spectral data and thereby the ability of the NIR platform to predict embryo viability.

Blastocyst morphology and prediction of Live birth

Our studies looking at blastocyst morphology and prediction of live birth found that trophectoderm morphology is the most important predictor after fresh single blastocyst transfer cycles and one of the most important predictors after frozen thawed transfer cycles. Expansion grade was found to be the other most important predictor of live birth after frozen-thawed transfer cycles. ICM in both studies (Paper III and IV) was not shown to be one of the most significant predictor of live birth. Very few reports have demonstrated similar results for fresh (Zaninovic, et al., 2001) and frozen thawed cycles (Honnma, et al., 2012) after SET. In fact, TE has repeatedly been considered to be of lowest significance when looking at the three morphological parameters. However, many of these conflicting studies fail to consider these parameters independently in logistic regression models and/or grouped blastocysts with different scores (Richter, et al., 2001, Shapiro, et al., 2008b, Shapiro, et al., 2000, Yoon, et al., 2001). This has thereby impaired the ability of these studies to fully elucidate the predictive strength of each parameter.

Our findings also showed that the live birth rates after fresh day 5 blastocyst transfer (37.8%) and frozen-thawed transfer cycles (38.9%) were not significantly different. Furthermore, live birth rates of blastocysts cryopreserved on day 5 (39.3%) were not significantly different to day 6

(38.2%), suggesting that the rate of development important for success of fresh blastocyst transfers is not crucial to post thaw success. Similar findings have been reported previously (Behr, et al., 2002, Veeck, et al., 2004). However, it is important to consider that a much higher standard of blastocyst quality (>3BB) is required for cryopreservation compared to fresh blastocyst transfer. Therefore, a more equal comparison would be to look at the live birth rate found in the NIR control group (Paper II). In this study, only good quality blastocysts were transferred (>3BB) and patients were required to have more than two good quality embryos available before randomization, mimicking the conditions required for cryopreservation on day 5. The reported live birth rate for this study was 45.6%, approximately 7% greater than day 5 frozen-thawed transfers (all ongoing pregnancies resulted in a live birth after the study was completed). These results suggest a difference in the live birth rate between fresh and frozen-thawed day 5 transfers. As we do not perform transfers on day 6 no comparisons can be made between fresh and frozen transfers. However, a very recent report showed that day 6 frozen-thawed blastocysts have a significantly higher ongoing pregnancy rate than fresh day 6 blastocyst transfers (Shapiro, et al., 2012). Importantly, this study performed SET and compared morphologically equivalent blastocysts, unlike many other studies. This study supports suggestions that impaired results of day 6 transfers are due to poor embryo-endometrium synchrony (Barrenetxea, et al., 2005, Shapiro, et al., 2008a, Shapiro, et al., 2001).

In paper IV, we also studied the predictive nature of three post thaw morphological parameters; degree of re-expansion, degree of viability and degree of cell contour. This study found that degree of re-expansion is the most important post thaw morphological predictor of live birth. This was comforting as re-expansion is one of the most extensively used indicators of blastocyst cryosurvival for both slow-freeze and vitrification methods (Cho, et al., 2002, Son, et al., 2007, Vanderzwalmen, et al., 2003, Vanderzwalmen, et al., 2002). Post thaw cell contour was also shown in a multivariate analysis to significantly predict live birth. In contrast to re-expansion, this parameter has not been used in previous reports. The difficulty when introducing and analyzing the significance of a new morphological parameter is the inability to compare and validate our findings with other published data. Furthermore, because the number of blastocysts graded for each parameter and available for analysis is a limitation of this study, we plan to continue using all three post thaw parameters to better assess their predictive value in the future.

To explain the predictive strength of TE we speculate that TE quality is reflecting the functional ability of TE and at this stage of embryo development a blastocyst ready to start the complex process of implantation,

needs healthy TE cells that have the capacity to signal and invade the endometrium (Norwitz, et al., 2001). In contrast to the ICM which can develop more independently of the maternal endometrium during the early stages of implantation. A key function of TE is secretion of human chorionic gonadotrophin (hCG), a critical glycoprotein that has been studied for almost a century(Aschner, 1912, Fishel, et al., 1984, Handschuh, et al., 2007, Kovalevskaya, et al., 2002b). The earliest study showed that hCG rescued progesterone synthesis and secretion by the corpus luteum (Hirose, 1920). The extensive variety of roles hCG plays in the maternal-embryo crosstalk and the reproductive system has been eloquently reviewed by Cole (Cole, 2009, Cole, 2010). Interesting to our findings, a report looking at hCG levels produced by blastocysts after TE biopsy showed that hCG levels significantly decreased when more than 10 cells were biopsied (Dokras, et al., 1991). So, a lower number of TE cells, as found in lower grades of TE, translates into fewer hCG producing cells and a weakened signalling capacity of the unimplanted blastocyst. More recently, another study showed that hCG could be detected in day 2 culture media (Ramu, et al., 2011). This was much earlier than older reports where earliest detection was found on day 7 of culture (Dokras, et al., 1993, Lopata, 1996). Our interest in detection of hCG to explain the predictive strength of TE and even identify a possible target biomarker for selection of blastocysts on day of transfer (day 5) lead us to explore hCG secretion. Unfortunately, when these experiments were repeated in our laboratory we were unable to detect hCG in day 2 spent embryo culture media using the same sandwich ELISA method. Earliest detection of hCG using this method was detected on day 8 of culture. The discrepancy in results between our two groups is hard to explain and personal communication with this group did not lead to any possible explanations.

To ensure that this was not a technical failure on our part, we even tested specifically for H-hCG, the principal form of hCG produced at the time of implantation and in the first week of pregnancy (Bersinger, et al., 2008, Kovalevskaya, et al., 2002a, Sasaki, et al., 2008). However this time we only tested day 5 and 6 from blastocysts of fresh IVF cycles and day 7 and 8 from donated day 5 frozen-thawed blastocysts. Interestingly, using a more specific ultra-microflourescence assay to detect H-hCG (Nichols Institute) we found earliest detection on day 7 of culture. From this small sample size, the onset and concentration of H-hCG did not seem to be related to TE grade. Of interest to frozen-thawed transfer cycles is the knowledge that cryopreservation and subsequent thawing does not delay H-hCG production when compared to fresh blastocysts (Dokras, et al., 1993, Dokras, et al., 1991, Lopata, 1996). However, to better determine if there is a relationship between TE grade and hCG production a larger study with donated day 2

frozen embryos is planned and has been approved by the Ethic committee. Unforunately, the donation of embryos is a slow process and sufficient numbers were not accumulated before the completion of this thesis.

Lastly, it is important to discuss the drawbacks of using morphology. Morphology is a qualitative method and some variation can be expected within individuals (intra-variability), between individuals (inter-variability) and even between different IVF labs. These sources of variability can affect interpretation of study data. (Arce, et al., 2006, Baxter Bendus, et al., 2006, Paternot, et al., 2009). Most importantly, inter-variability has been shown to significantly affect embryo scores given on day 2 and 3. In these studies, better scoring agreement was observed for experienced embryologists and when training sessions were carried out. So far, no published studies have assessed the level of inter-variability when scoring blastocysts. Our clinic annually performs an internal (all embryologists within our lab) and external (embryologists at 4 other clinics) embryo assessment validation. The results of these validations indicate a high degree of intra-agreement for blastocyst scoring. However, we have found that agreement between laboratories can differ between those with and without experience. So that those laboratories with experience, (our lab included) have high level of agreement with other experienced laboratories.

Some studies have tried to quantify the morphological parameters used to assess blastocysts by measurement of the blastocoele cavity, ICM dimensions and a cross-sectional number of TE cells (Richter, et al., 2001, Shapiro, et al., 2008b). However, these measurements have not been widely applied. A suggestion would be to combine these methods with the Gardner and Schoolcraft scoring system to more accurately describe the different grades (A, B, and C) of ICM and TE. For example, a grade A TE is described as many cells forming a cohesive epithelium. Alternatively, it could be described as a cohesive epithelium with a cross-sectional cell count of more than 20 cells. Furthermore, ICM dimensions could be used to describe the different grades A, B and C together with degree of compaction/adhesion. For example, a grade A ICM having a size $>4500 \mu M^2$ and cells tightly packed, as according to Richter et al (Richter, et al., 2001).

6 CONCLUSION

In summary, this thesis work showed that although NIR technology showed promise for use in selecting the most viable embryo for transfer, in its current form we were unable to use it to improve ongoing pregnancy rates after SET when assessed in a prospective randomised controlled trial. Unfortunately, there is still a need for new non-invasive technologies to improve our selection methods.

Furthermore, the findings of this thesis work suggest that a new hierarchy should be put in place when selecting top quality blastocysts for transfer. Although separate strategies should be used for fresh and frozen thawed cycles. For fresh IVF cycles and transfers on day 5, our study data suggest that TE grade should be prioritised when selecting between good quality expanding and expanded blastocysts. For frozen-thawed transfer cycles higher expansion and TE grades should be prioritised, irrespective of day of cryopreservation. Of course to fully evaluate if our new selection hierarchy can improve the live birth rate a prospective randomized controlled study is needed. In addition, post thaw morphology is important for prediction of live birth after frozen thawed cycles, and most importantly degree of re-expansion.

7 FUTURE PERSPECTIVES

Grading embryo morphology is a mainstay method for selection of the most viable embryos for transfer. However, morphology is like looking at the summary of many other genetic, transcriptional and metabolic functions that are required for successful embryo development. Only through better understanding of these cellular processes and how they relate to each other, can we identify potential biomarkers of embryo viability and improve current IVF success rates. It is unlikely that one biomarker will be enough, instead groups of biomarkers that can describe different functional status of the embryo, such as chromosomal abnormalities, epigenetic defects or even stress imposed by the culture condition, will be required. The best methods, I believe, are going to be those that limit their impact on the well being of the embryo, such as non-invasive sampling of the spent culture media. The biggest challenge will be to develop an easy to use, fast, relatively cheap method to measure these biomarkers. From our involvement with the NIR technology, we gained an insight into the amount of effort required to first do the basic research that leads to the commercial idea and design of a technology, followed by testing over and over again before reaching the level at which it can be trialed for clinical use. Unfortunately, NIR didn't make it to daily clinical use and neither have any of the other technologies being researched to test spent embryo culture media. However, this is not to say they wont eventually get there but it will take time. Many researchers are close to the trial stages; amino acid profiling and protein profiling are promising new technologies and it looks like the 'old' glycolytic studies are being revived as possible pathways to measure. Of significant importance, these methods could not only help us predict the most viable embryo, but also help us to 'produce' a viable embryo so that we provide the best possible circumstances for all stages of an IVF cycle, from the acquiring of gametes to the return of the embryo to the uterus. Needless to say, this is a very exciting time in the IVF world with all the new technologies and multitude of parameters being investigated and developed to quantify embryo viability. A bit like the Cambrian explosion, 500 million years ago, and the explosion of weird and wonderful new species, some will survive and some wont measure up to the fitness level required.

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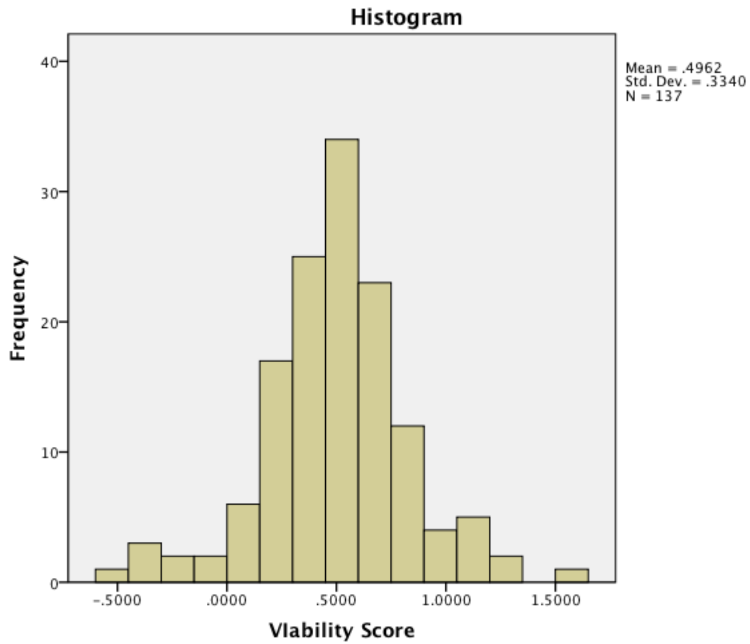
APPENDIX



Appendix 1. Normalization test.