Journal of Assisted Reproduction and Genetics (2021) 38:847–855
https://doi.org/10.1007/s10815-021-02071-x

ASSISTED REPRODUCTION TECHNOLOGIES

Clinical pregnancy is significantly associated with the blastocyst width and area: a time-lapse study

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Received: 15 July 2020 / Accepted: 11 January 2021 / Published online: 20 January 2021

Abstract

In order to maintain pregnancy rates following single embryo transfer, optimisation of embryo culture and selection is vital. Time-lapse monitoring (TLM) has the potential to play a crucial role by providing sequential images of embryo development and minimal disturbance. Therefore, in this study morphometric assessment of blastocyst area and maximum width was performed in order to evaluate if these parameters are associated with pregnancy outcomes in IVF/ICSI cycles. This is a retrospective study of 664 patients who had elective single blastocyst transfer (eSBT). The EmbryoScope drawing tools were used to measure specific variables such as the maximum blastocyst width and blastocyst area. Our results show that women who were pregnant had significantly ($P < 0.01$) larger blastocyst width [median (range) μm] 184 (125–239) versus non-pregnant, 160 (120–230)] and area [median (range) μm^2] 26099 (12101–45,280) versus non-pregnant women, 22,251 (10992–37,931)]. A univariate logistic regression performed showed that blastocyst width [(OR = 1.026, 95% CI = (1.019, 1.033)] was significant ($P < 0.01$) and for every μm increase of blastocyst width, the odds of clinical pregnancy increase by 2.6%. A univariate logistic regression performed showed that blastocyst area [(OR = 1.00008, 95% CI = (1.00006, 1.00011)] was significant with $P < 0.01$. For every μm^2 increase of blastocyst area, our data showed the odds of clinical pregnancy increase by 0.008%. Hosmer-Lemeshow tests of calibrations were performed to verify calibration. Although our findings show a clear correlation between blastocyst dimensions and the clinical pregnancy rate, further studies are necessary to confirm these observations.

Keywords Embryo culture · EmbryoScope time-lapse incubator · Maximum blastocyst width · Blastocyst area · Single blastocyst transfer · Pregnancy outcome

Introduction

Over 8 million in vitro fertilization (IVF) children have been born since 1978 when the first IVF baby was announced [1–3]. Worldwide, approximately 2.5 million medically assisted reproduction (MAR) cycles are performed, resulting in over 500.000 deliveries annually. IVF scientists are focusing on the selection of the most competent embryo for transfer, to reduce the incidence of multiple gestations, which is associated with higher risk of adverse perinatal and maternal outcomes [4, 5]. The higher degree of differentiation of the blastocyst, and the contribution of the embryo genome to development from cleavage stage (day-3) suggests, that features visible at the blastocyst stage may be more useful in evaluating viability and the potential to generate a pregnancy than features of day-3 embryos [6]. However, there is little consensus on blastocyst selection, and morphology assessments may have high inter-observer variability [2]. The introduction of continuous embryo monitoring using time-lapse technology has allowed embryologists to study the dynamic process of early embryo development, from fertilization to blastocyst formation. This technology marked a turning point since it allows embryos to be scored not only according to morphology but also by their kinetics and morphometric features [7]. However, relatively little is known about specific characteristics of the blastocyst that are indicative of implantation potential. A study published by Yoon and colleagues [8] noted that implantation and pregnancy rates were significantly higher in patients who received one or more hatching blastocyst on day-6 compared to women who received non-hatching blastocysts on day-5. A more recent study also

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Published online: 20 January 2021

reported that extending culture of expanded blastocysts by a few hours to allow transfer of spontaneously hatching blastocysts provides higher implantation and pregnancy rates [9]. These results suggested that the chance of success in IVF treatment can be increased if selection of the most viable embryos is based on hatching status [8, 9]. In addition, necrotic areas within the inner cell mass (ICM) have been associated with poor implantation potential [10, 11]. Some studies have focused on blastocyst expansion as a considerable aspect in embryo selection [12–14]. In contrast, another study showed that only patient's age and trophectoderm (TE) grade were significantly associated with implantation and live birth [15]. Therefore, the debate on which morphological parameters have the highest prognostic value is still unresolved and it seems that existing blastocyst grading needs to be further improved by incorporating more objective and reproducible features. Our clinic has carried out routine elective single blastocyst transfer (eSBT) since 2014 and therefore, we were able to analyse the outcome of treatment from our database. We hypothesised that blastocyst morphometric assessment; in particular, blastocyst area and maximum blastocyst width may be predictors of clinical pregnancy rates (CPR).

Material and methods

This was a retrospective study, which included 664 IVF/ICSI cycles carried out in the Unit, the Royal Infirmary of Edinburgh (RIE), between January 2014 and September 2019. All cycles were from patients using their own oocytes and fresh eSBT, which was scheduled on the morning of day-5, approximately 112 to 118 h after insemination. The pregnancy was confirmed by an ultrasound scan for gestational sac with confirmation of the fetal heartbeat at seven weeks of pregnancy. These embryos were defined as KID, known implantation data [16]. This retrospective observational study design did not require ethical approval. For the purposes of this study, all blastocysts were assessed using calibrated annotation tool of the EmbryoViewer (EmbryoScope, Vitrolife, Sweden), in order to measure specific variables such as the maximum blastocyst width and blastocyst expansion. All embryo measurements were carried out by a senior embryologist Romualdo Sciorio (RS). Data obtained was associated with clinical pregnancy rate (CPR).

Ovarian stimulation, egg retrieval, fertilization, embryo culture and scoring

All patients underwent controlled ovarian stimulation with gonadotropin-releasing hormone (GnRH) antagonist (subcutaneous Cetrorelix 0.5 mg daily, Merck Serono) or GnRH agonist (subcutaneous Buserelin 0.5 ml, Sanofi) treatment. Ovarian stimulation was carried out using either Gonal F (Merck Serono), Bemfola (Gedeon Richter) or Menopur (Ferring) and the dose of gonadotrophin was administered based on individual ovarian reserve. Follicular development was monitored by transvaginal ultrasound and ovulation was triggered when three follicles were 18 mm or above using Ovitrelle 0.25 mg (Merck Serono). Oocyte recovery was carried out under conscious sedation with transvaginal ultrasound guidance at 35 h after Ovitrelle injection. Details of oocyte collection has been previously described by Sciorio and colleagues [17]. Briefly, the cumulus-oocyte-complexes (COCs) were isolated from follicular fluid and then rinsed and cultured in 0.5 ml equilibrated G-IVF™ PLUS medium (Vitrolife, Sweden) and incubated at 37 °C in 6% CO_2 in atmospheric air in a Hera cell 240 incubator (Thermo Scientific), until ICSI or IVF insemination was performed approximately 38–40 h post Ovitrelle administration. Fertilization was identified by the presence of two pronuclei approximately 16–19 h after insemination or microinjection. At this stage, normally fertilized pronuclear stage embryos were allocated to the EmbryoScope time-lapse incubator for culture in an EmbryoSlide™ (Vitrolife). This slide has 12 individual wells for embryo culture, each well containing 25 μl of culture media with 1.4 ml overlay of mineral oil to prevent evaporation. The in vitro culture was performed in an atmosphere of 6.0% CO_2, 5.0% O_2 and nitrogen balance at 37 °C, using single step time-lapse medium (G-TL™ Vitrolife). In the EmbryoScope embryo culture, images were acquired every 10 min in 7 focal planes and morphological assessment was made by examining a video of development using the associated EmbryoViewer™ software, without moving embryos from the incubation. Embryos were assessed morphologically at cleavage stage according to British Fertility Society and Association of Clinical Embryologists guidelines, published by Cutting and coworkers [18]. Blastocysts were classified according to degree of expansion of the blastocoel cavity (1–6), quality and cohesiveness of the inner cell mass and trophectoderm cells (A-C), using the Gardner's scoring system [19]. Fertilization and embryo morphology were evaluated based on images obtained from the time-lapse system. Annotation of morphokinetics and morphometric parameters were performed on all embryos on culture days 1, 2, 3, and 5, and were carried out by a single operator (RS) to reduce the risk of inter-observer variability. The fertilization time was expressed as hours after ICSI or standard IVF insemination were performed, and was recorded as time zero on the EmbryoViewer™ software. Elective single embryo transfer was made at blastocyst stage (day-5) and using transfer medium containing hyaluronan and recombinant human albumin (EmbryoGlue™, Vitrolife).

Blastocyst morphometric assessment

A blastocyst is composed of a spherical layer of outer trophectoderm (TE) cells, surrounding an inner blastocoel

cavity containing a tightly packed ICM. Before embryo transfer, morphometric assessments of all blastocysts were performed between 112 and 118 h post insemination. The EmbryoViewer™ software included a distance and ellipse tool that was used for measuring area and diameter of embryos, as already described by Almagor and collaborators [20]. Using the tool, the maximum blastocyst width was recorded (fig. 5a, b). Blastocyst maximum width (with zona pellucida excluded) was determined after four different measurements were taken on a single plane and the mean was calculated (in micrometres). Similarly, the EmbryoScope's elliptical measurement tool was used to annotate the cross-sectional area of the blastocyst, as described by Huang and colleagues [21]. A single measurement on one plane for each embryo was taken (in square micrometres). The blastocyst area was calculated between the outside borders of the TE, excluding the area occupied by the zona pellucida (fig. 6a, b). A small percentage of blastocysts 6.6% (44/664) could not be included for morphometric assessment because the blastocysts were out of the field for measurement, or the blastocyst shape was not perfectly round, or the blastocyst had collapsed during development. Therefore, these latter group of embryos were excluded from this study. These measurements were recorded for each transferred blastocyst and the data were analysed in relation to the clinical pregnancy rates (CPR).

Statistical analysis

Differences were considered statistically significance at the level of $P < 0.01$. Additionally, data obtained was assessed in terms of CPR at seven weeks gestation. Statistical analysis was performed with Mann-Whitney *U*-test and univariate logistic regression. Univariate logistic regressions were performed on the probability of clinical pregnancy in relation to blastocyst area, and probability of CPR in relation to blastocyst width, in order to determine if there was a statistically significant relationship between these variables. The calibrations of the logistic regressions were verified by Hosmer-Lemeshow tests.

Results

Data from 664 IVF/ICSI patients undergoing transfer of expanded blastocysts on day-5 were analysed, all of which included elective single blastocyst transfer. The overall median age of women in this study was 34 (22–44), and the mean number of oocytes collected was 11.7. IVF insemination was carried out in 69.1% (459/664) and ICSI 30.9% (205/664) of patients. 465 of those patients resulted in a positive beta hCG pregnancy test (70.0%). The implantation rate was 59.0% (392/664) and clinical pregnancy 56.5% (375/664). There was no significant difference ($P = 0.21$) in the mean (SD) age between pregnant [33.8 years (SD 3.8)] and non pregnant [(age 34.1 years (SD 4.2)] patients. There was also no significant difference in the number of oocytes retrieved in the pregnant versus non pregnant women. Our results (fig. 1) show that in women who had a clinical pregnancy, the width of the transferred blastocyst was significantly ($P < 0.01$) larger [median (range) μm] 184 (125–239) than in those who were not pregnant 160 (120–230). None of the parameters analysed, including age (33.7 years with width > 184 μm versus 33.8 in the group <160 μm) number of oocyte collected (11.7 oocyte; width > 184 μm versus 11.6; width < 160 μm), and cause of infertility were different between the two groups. The mean number of oocytes collected in women with blastocyst width > 184 μm was 11.7 versus 11.6 in women with transferred blastocyst width < 160 μm. A univariate logistic regression carried out showed that the probability of a clinical pregnancy increases with blastocyst width [(OR = 1.026, 95% CI = (1.019, 1.033)] and was found to be significant with $P < 0.01$ (fig. 2). This shows that for every μm increase of blastocyst width, the odds of clinical pregnancy increase by 2.6%. A significantly ($P < 0.01$) larger [median (range) μm^2] blastocyst area 26,099 (12101–45,280) was seen in pregnant versus non-pregnant women, 22,251 (10992–37,931). Age was similar in the two groups [33.7 years in the group of blastocyst with larger area (>26,000 μm^2), versus 33.8 in the group of smaller area (<26,000 μm^2)]. The number of oocyte collected was comparable [(mean) 11.8 oocyte in the group with larger area (>26,000 μm^2), versus (mean) 11.5 oocyte in the group of smaller blastocyst area (<26,000 μm^2)]. A univariate logistic regression carried out showed that blastocyst area [(OR = 1.00008, 95% CI = (1.00006, 1.00011)] was found to be significant ($P < 0.01$; fig. 3 and fig. 4). The latter showed that the probably of clinical pregnancy increases with a larger area. The lower odds ratio is explained by a larger range in blastocyst area. For every μm^2 increase of blastocyst area, the data showed the odds of clinical pregnancy increase by 0.008%. Hosmer-Lemeshow tests of calibrations were

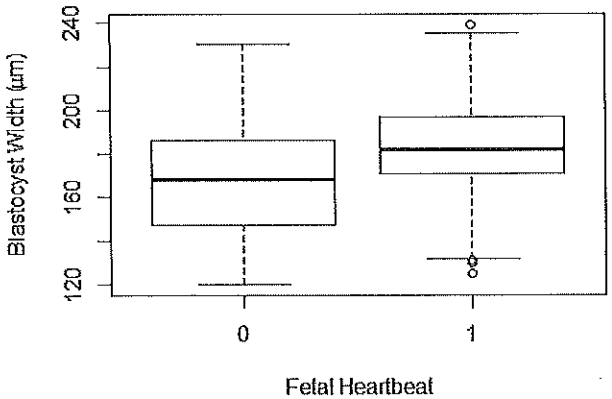


Fig. 1 Boxplots of Blastocyst width grouped by clinical pregnancy outcome. FH = 0 denotes non-pregnancy, FH = 1 clinical pregnancy

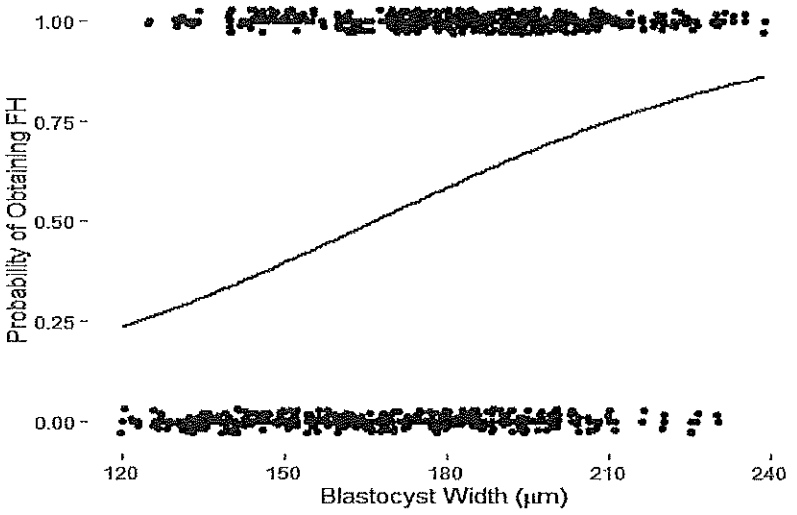


Fig. 2 Probability of Clinical pregnancy in relation to blastocyst width

performed to verify calibration. Figures 5a, b and 6a, b showed how the measurements were taken for each blastocysts.

Discussion

The results of the present study demonstrate a statistically significant ($P < 0.01$) relationship between clinical pregnancy and maximum blastocyst width [(OR = 1.026, 95% CI = (1.019, 1.033)] in the context of elective single blastocyst transfer. This was also the case with blastocyst area [(OR = 1.00008, 95% CI = (1.00006, 1.00011)], with a blastocyst area > 25,000 μm^2 associated with higher clinical pregnancy rates than following transfer of blastocysts with an area < 25,000 μm^2 ($P < 0.01$). Detailed analysis suggests that for every μm increase of blastocyst width, the odds of clinical pregnancy increase by 2.6%. The high rate of clinical pregnancy associated with the elective transfer of a single blastocyst with a width more than >184 μm or with an area > 26,000 μm^2 may assist optimisation of embryo selection procedures in order to improve clinical pregnancy from eSBT. Advances in embryo culture conditions have enabled IVF units to move towards a policy of single embryo transfer (SET) or eSBT to reduce the incidence of multiple pregnancy [4, 5, 22] and whilst such policies remain, robust embryo selection procedures are vital to a successful IVF programme. Traditional and subjective morphological evaluation is still the most common method applied to evaluate and select embryos for transfer, but in the last decade, data obtained from TLM has allowed identification of novel morphokinetic variables as additional potential markers of embryo viability and implantation potential [23–26]. Following the first report of human fertilization and early development events through TLM

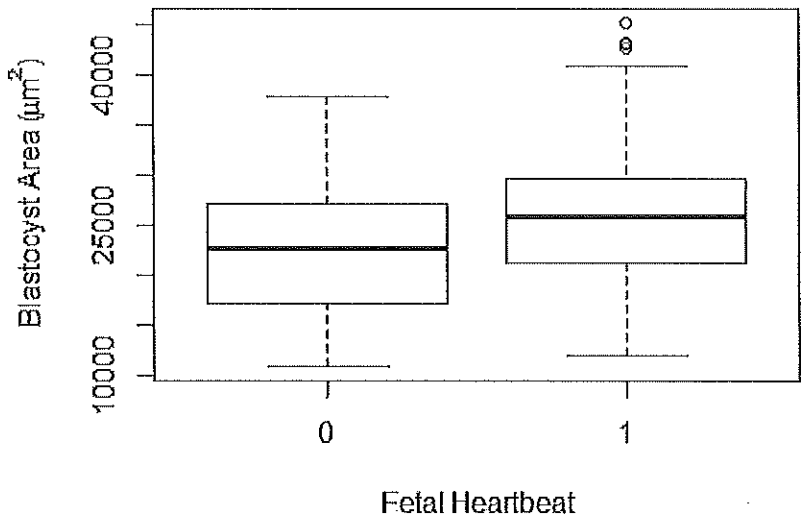


Fig. 3 Boxplots of blastocyst area grouped by clinical pregnancy outcome. FH = 0 denotes non-pregnancy, FH = 1 clinical pregnancy

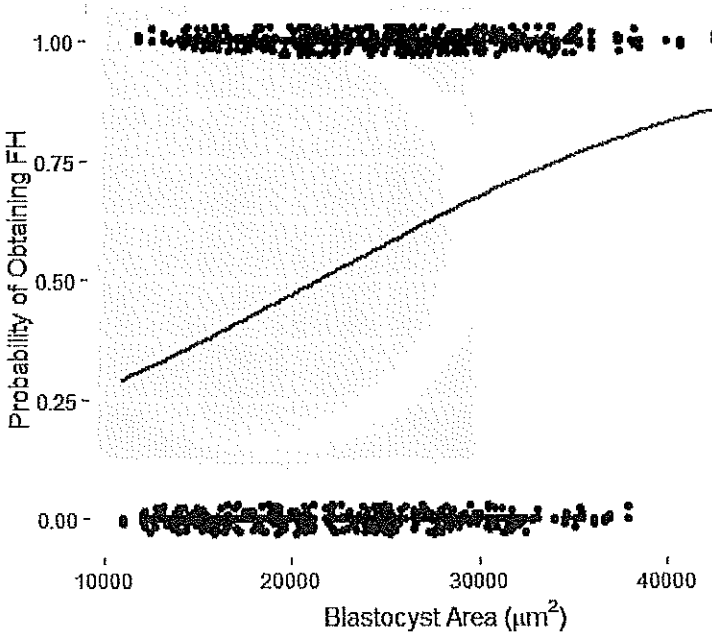


Fig. 4 Probability of Clinical pregnancy in relation to blastocyst width

described by Payne and co-workers [27], TLM has evolved considerably, and currently allows analyses of morphological changes during embryo development, which would previously have gone largely undetected, such as abnormal or reverse cleavages. TLM now enables embryologists to evaluate the whole sequence of embryonic development, from fertilization to the blastocyst formation [7, 25, 28]. Cruz and collaborators [29] found that development of human embryos to the blastocyst stage was linked to key timing events in early embryo development, such as the duration of the first cleavage (cytokinesis), and the length of the interval between divisions in the first stages of embryonic development. There are also specific reported morphometric observations from TLM which suggest a negative or positive impact on the chance of implantation and pregnancy. For instance, blastocyst collapse during in vitro embryo development has been suggested as a novel marker of embryo quality and that this negatively affects implantation and pregnancy outcomes [21, 30–32]. In the mouse model, Niimura (2003) reported that blastocysts showing consecutive weak contractions reached, mostly, to the stage of hatching, while those showing strong collapse(s) failed to hatch. The author suggested that weak contractions (less than 20% volume reduction) may be normal and play an important role in the hatching process, whereas strong contractions (20% or more) may inhibit hatching [33]. Despite demonstration of the collapse episode in several mammalian species, the role and the mechanism of blastocyst collapse in vitro remains unclear. Huang and colleagues [21] analysed

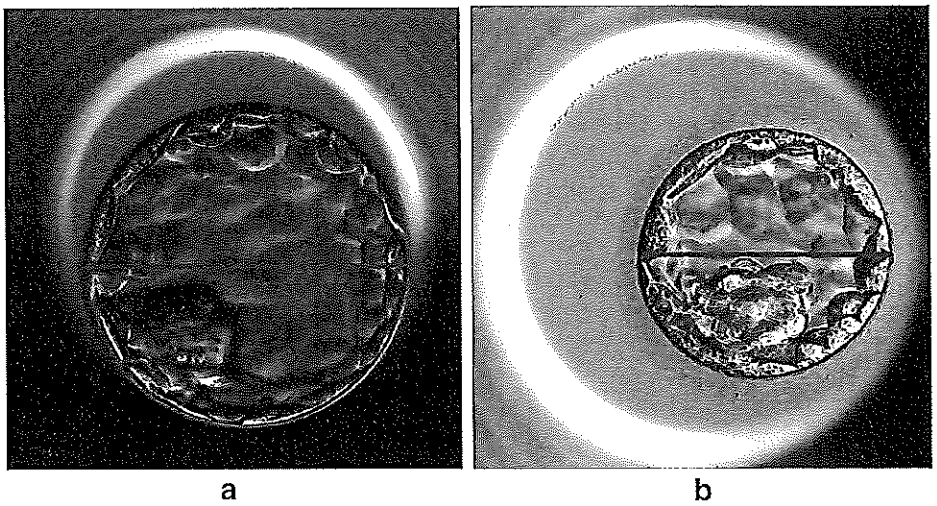
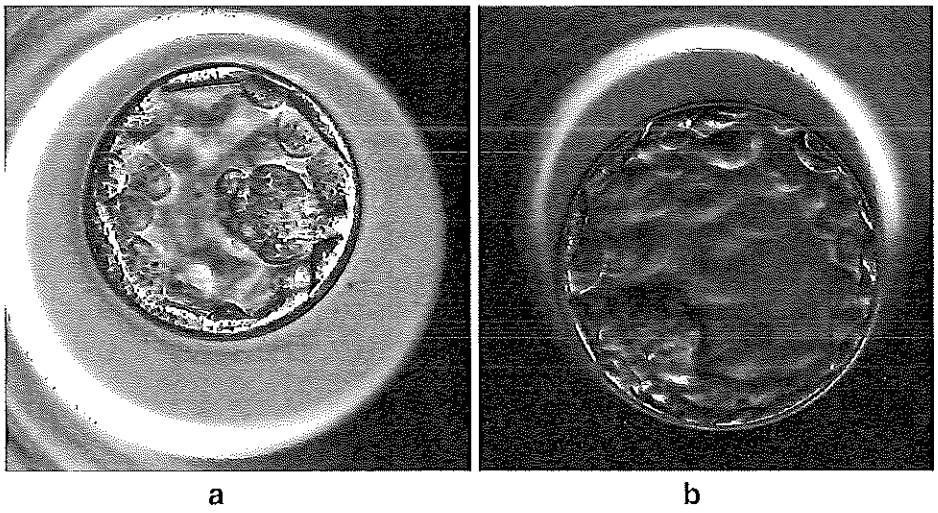


Fig. 5 a) Blastocyst maximum width 205 μm Fig. 5: b) Blastocyst maximum width 148 μm



a b

Fig. 6 a) Blastocyst area 19,704 μm^2 Fig. 6: b) Blastocyst area 33,549 μm^2

38 embryos from egg-donation programme with known implantation outcome and reported abnormal kinetics of blastocyst expansion and slower blastocyst formation in embryos that show collapse events and further a negative effect on implantation and pregnancy rates. Our group has also investigated this aspect, and the results recently published in a retrospective multicentre study [31] support the findings reported by Huang and collaborators [21]. However, all blastocysts analysed in the current study did not show sign of a collapse event at any stage during the development. Therefore, the main aim of this study was to investigate whether measured blastocyst dimensions following quantitative assessment from TLM could also impact on clinical outcome for patients. The importance of blastocyst expansion status has been previously documented in several other studies using grading systems as a method of assessment rather than morphometric measurement [34, 35]. Thompson and colleagues [36] reported that in fresh cycles, blastocyst expansion status was an independent predictor of live birth rates. Since expansion grade relates directly to blastocyst size, these results are in line with our own results which showed that the median width of blastocysts in patients with a clinical pregnancy was significantly larger ($P < 0.01$); 184 μm (125–239) versus 160 μm (120–230) in those who did not conceive, when assessed with morphometric tools. Similarly, a significantly ($P < 0.01$) larger [median (range) μm^2] blastocyst area 26,099 (12101–45,280) was seen in pregnant versus non pregnant women, 22,251 (10992–37,931). Both findings are suggestive that a larger, more expanded blastocyst results in a higher clinical pregnancy rate. A direct association between blastocyst diameter and clinical pregnancy has also previously been demonstrated in vitrified single blastocyst transfer cycles [37]. In this study, only blastocysts with an inner diameter between 175 μm and 184 μm were vitrified and after warming and embryo transfer, pregnancy rates progressively increased according to blastocyst diameter, with optimal results obtained when blastocyst expansion (175 μm and 184 μm) was reached in less <120 h after insemination. This study reported that blastocyst expansion could be used as a robust predictive marker for pregnancy following freezing and thawing, aligning well with the results from fresh transfers that we report here. It seems clear that blastocyst expansion status is an important indicator of embryo growth and viability. As discussed by Ahlstrom and collaborators [12] blastocyst expansion is likely to be associated with the quality and functionality of the trophectoderm layer, specifically the number of cells present and their cohesive properties. The TE layer plays an important role in osmotic regulation by preventing outflow of fluid and sodium ions from the blastocoel and pumping ions efficiently into the cavity. This leads to an accumulation of intracellular water, leading to further blastocyst expansion, followed by hatching. Since hatching is vital for implantation, it is perhaps not surprising that blastocyst expansion should be associated with clinical pregnancy. However, morphological grading systems do not only take into account the expansion status of the blastocyst. Traditional microscopic morphological evaluation has for many years been used to assess embryo quality at various stages of development [18]. The three part scoring system developed for blastocysts by Gardner and Schoolcraft [19] has historically been the cornerstone of blastocyst assessment and selection procedures and consists of detailed evaluation of the three main structural components: the ICM, the TE and the expansion status. However, despite >20 years of application, the relative impact of each of these parameters on pregnancy outcome(s) is still the subject of debate. Several authors have tried to quantify the relative importance of the three main scoring criteria and the data has sometimes been conflicting. In some studies, the morphological grade of the ICM has been found to be important in predicting clinical outcome and live

birth [13, 14, 38] whereas in contrast, recent studies have reported that the TE grade may be a better predictor [39, 40]. Hill and co-workers [15] found that the TE grade was the most important parameter when predicting the live-birth rates for fresh single blastocyst transfer cycles, with similar results seen in vitrified-warmed cycles. Goto and colleagues [41] assessed relative importance of blastocyst morphology and expansion status in vitrified-warmed blastocyst cycles and found that blastocyst expansion rather than ICM or TE morphology was significantly associated with clinical pregnancy rate. Another recent study confirmed that blastocyst expansion is the most important parameter in predicting live-birth after both fresh and vitrified-warmed single blastocyst transfer cycles [42]. Considering that at this early stage of development, it is essential that the embryo hatches from the zona pellucida (ZP), and starts the process of adhesion and invasion of the endometrium [43–45], it seems logical that blastocyst expansion should be associated with pregnancy, but the studies outlined above show there is no current consensus on this. The possible explanations for such discrepant conclusions include potential differences in the relative distribution of blastocysts with different morphology grades between the studies: the vast majority of blastocysts included were of good or excellent quality, with only few (5% or less) blastocysts with C grades for ICM or TE as well as potential differences in the way blastocysts were graded. A work published by Van den Abbeel and associates [13] found that high grades of blastocyst expansion, ICM and TE grade were all significantly associated with increased pregnancy and live-birth rates after fresh transfers. However, when including all three morphology parameters in a logistic regression model of live birth rate, only the TE morphology remained as a significant independent predictor. It may also be the case that ICM and TE quality are closely linked with expansion status. The authors found that high ICM and TE grades were more frequently associated with larger expansion grades. In addition, better ICM quality was frequently associated with better TE quality, indicating a likely interdependency between developmental stage, ICM and TE of the blastocyst [13]. Such an association would likely increase the difficulty of interpreting the impact of the individual blastocyst grading factors leading to potentially discrepant results in different studies. Due to these factors and while blastocyst assessment is based on subjective morphological evaluation, the relative importance of the three morphological parameters (ICM, TE and blastocyst expansion) to predict pregnancy outcome(s) is complex [46]. The simple quantitative embryo assessments that we propose here could reduce inter-observer variation leading to a more objective assessment of embryo quality, thereby improving specificity and reproducibility of embryo selection process. Indeed, several studies have suggested that automated or semi-automated morphological evaluation using image analysis may be of considerable use in the future of clinical embryology, although some concerns remain, especially regarding assessments at the blastocyst stage [47–49]. Meanwhile, for those using TLM, it would relatively straightforward to incorporate an additional simple quantitative assessment (such as blastocyst width) at the final grading stage before embryo transfer in order to further refine current grading systems and help select the single best quality embryo available for transfer, especially in patients with several good quality blastocysts. No biopsied embryos were included in this study, therefore our findings presently could not be applied to biopsed embryos. In addition, our data are only applicable for day-5 blastocyst and not day-6 blastocyst. All treatment cycles in this trial were from women using their own oocytes and fresh single embryo transfer on day-5. In this study, morphometric blastocyst assessment was performed on the morning of day-5, approximately between 112 and 118 h after insemination. The fertilization time was expressed as hours after insemination, which was recorded as time zero on the EmbryoViewer™ software. Therefore, when ICSI was performed (30.9% of the cycles), the time zero indicated the time when the ICSI procedure was completed, which was identical to sperm penetration. On the other hand, for IVF embryo, time zero indicated the time when an aliquot of motile sperm was added to the dish containing the cumulus-oocyte-complexes, but the accurate moment of sperm penetration could not be documented. Our study may be useful for future research to investigate if there is any relationship between morphometric blastocyst assessement and chromosomal abnormalities or aneuploidy, or error on mitotic chromosome segregation. It is evident that chromosomal complement plays an essential role in early embryogenesis and would be important to determine if there is any relationship between the euploid state of an embryo and the blastocyst area and or maximum width. The latter was investigated in a recent study by Huang and colleagues [50]. The authors reported that euploidy and aneuploidy rates were more obvious in the regions of higher and lower blastocyst expansion, respectively. They reported that the number of euploid blastocysts was significantly higher ($P = 0.0039$) with higher blastocyst expansion (>20,000 μm^2). In contrast, the number of aneuploid blastocysts was significantly higher ($P = 0.0030$) where there was lower blastocyst expansion (<15,000 μm^2). This is in agreement with our study in which we found that in women who were pregnant, blastocyst width and area were significantly larger than in those who were not pregnant.

Conclusions

In this study, we report that the chance of clinical pregnancy progressively increases with increasing blastocyst width and area based on morphometric measurements. This study shows that objective morphometric assessment is a simple, non-invasive and effective tool to improve the chance of clinical pregnancy. A limitation of our study is that the blastocyst

assessment was performed retrospectively; therefore, our preliminary observational findings should be validated with a prospective or larger multicenter randomized trial. Although, this is a retrospective study with associated limitation(s) this is, to our knowledge, the first observational study to associate morphometric blastocyst assessment as a predictor of implantation and pregnancy outcome. The statistically significant correlation of blastocyst width and area reported in this study should stimulate discussion about novel morphometric parameters and their predictive effect on outcome from treatment. Our preliminary findings suggest that morphometric assessment of blastocyst width and area on day-5 may be used in addition to the grading system for embryo selection for transfer. It may be useful for predicting implantation and clinical pregnancy rate, particularly in cases where there are several blastocysts available to choose for transfer. Our results may be used to establish a novel and objective blastocyst grading system that may predict outcome following eSBTin IVF/ICSI treatments.

Funding Project support was provided by the Edinburgh Assisted Conception Programme, EFREC, Royal Infirmary of Edinburgh, 51 Little France Crescent, Old Dalkeith Road, Edinburgh, Scotland, EH16 4SA, UK.

Compliance with ethical standards

Competing interests We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Human and animal rights All procedures performed in studies involving human participants were in accordance with the ethical standards of the institution and with the 1964 Helsinki declaration and its later amendments. The Local Ethics Committee did not require an ethical approval for this retrospective study.

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