REPRODUCTION

REVIEW

Time-lapse imaging of cleavage divisions in embryo quality assessment

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Abstract

***In vitro* fertilization (IVF) is one of the most important procedures for treating infertility. As several embryos are usually produced in a single IVF cycle, it is crucial to select only the most viable ones for transfer to the patient. Morphokinetics, i.e. analysis of the dynamics of cleavage divisions and processes such as compaction and cavitation, has provided both biologists and clinicians with a new set of data regarding embryonic behaviour during preimplantation development and its association with embryo quality. In the current review, we focus on biological significance of morphokinetic parameters and show how they can be used to predict a reproductive outcome. We also explain the statistics behind the predictive algorithms and discuss the future perspectives of morphokinetics.**

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Introduction

Since 1978, when the first *in vitro* conceived baby was born (Steptoe & Edwards 1978), *in vitro* fertilization (IVF) has become one of the most important procedures for treating infertility. In the UK in the last two decades, the number of IVF cycles has increased over three times, from 18,338 in 1992 to 63,573 in 2014 (according to the Human Fertility and Embryology Authority (HFEA), www.hfea.gov.uk). A similar tendency has also been observed in the USA, where the number of IVF cycles increased from 64,681 in 1996 to 188,023 in 2014 (according to the Society of Assisted Reproductive Technologies (SART), www.sart.org). Although IVF procedures have been greatly improved over the years, their efficiency, measured as the live birth rate, is still below 50%. It ranges from 4.3% (UK)/3.7% (US) for patients older than 42 to 32.8% (UK)/48.7% (US) for women younger than 35 (data from 2013, HFEA and 2014, SART respectively). The efficiency of IVF can be elevated by transferring multiple (usually no more than 3) embryos in a single cycle, but this often results in multiple-pregnancies and as a consequence, in serious health complications for mothers and their babies (Petterson *et al.* 1993, Yokoyama *et al.* 1995, Pharoah & Cooke 1996, Walker *et al.* 2004, Ombelet *et al.* 2005). As a result, many countries have introduced policies limiting the number of embryos that can be transferred in a single cycle and facilitating an elective single embryo transfer (eSET). Therefore, scientists and the medical industry are under constant pressure to develop novel, reliable methods to select high-quality embryos for transfer.

Recently, novel selection procedures based on time-lapse imaging have been added to the embryo assessment toolkit. Although microscopic visualisation has been used for scoring embryos from the very beginning of IVF treatment, thus far embryos have been screened for specific morphological features only at certain time-points of their culture, and information about developmental dynamics (so-called morphokinetics) has been inaccessible (Ajduk & Zernicka-Goetz 2013, Basile *et al.* 2015*a*). Integration of high resolution imaging equipment into a fully functional incubator has finally enabled embryo-safe recording of the cleavage divisions (Nakahara *et al.* 2010, Pribenszky *et al.* 2010, Cruz *et al.* 2011, Kirkegaard *et al.* 2012, Park *et al.* 2015). Although time-lapse imaging involves periodic exposure to light, it is usually lower than light exposure associated with traditional morphology assessment (Ottosen *et al.* 2007, Wong *et al.* 2010, Chen *et al.* 2013). Moreover, in contrast to a scoring protocol based on a visual inspection of embryo morphology performed outside an incubator, time-lapse imaging enables embryo culture in stable, uninterrupted conditions, which, as some researchers suggest, may be beneficial for embryo viability and the final reproductive outcome of the IVF procedure (Meseguer *et al.* 2012, Kirkegaard *et al.* 2015).

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However, there is no evidence that culture conditions ensured by time-lapse monitoring do indeed improve developmental potential of embryos; currently we can only state that they do not diminish it in any way (Cruz *et al.* 2011, Kirkegaard *et al.* 2012, Park *et al.* 2015). Yet, there is evidence that additional information about the dynamics of embryonic divisions, provided by time-lapse imaging, may help to select more viable embryos for transfer, leading to better reproductive outcomes (implantation and pregnancy rates) than a selection based on the traditional morphology assessment alone (Meseguer *et al.* 2012, Aparicio *et al.* 2013, Herrero & Meseguer 2013, Findikli & Oral 2014, Rubio *et al.* 2014, Siristatidis *et al.* 2015, Aparicio-Ruiz *et al.* 2016). On the other hand, there are also reports showing that although morphokinetic parameters can predict embryo potential to achieve a blastocyst stage, neither implantation, nor pregnancy rate can be increased by morphokinetic analysis (Cruz *et al.* 2011, Kirkegaard *et al.* 2013, Polanski *et al.* 2014, Santos *et al.* 2014, Armstrong *et al.* 2015, Kirkegaard *et al.* 2015, Racowsky *et al.* 2015, Ahlstrom *et al.* 2016, Goodman *et al.* 2016, Kieslinger *et al.* 2016). Therefore, it is obvious that the usefulness of time-lapse imaging greatly depends on the accuracy of predictive models based on morphokinetic parameters. In the current review, we explain the biological significance of morphokinetic parameters and show how they can be used to model a reproductive outcome. We present the current state of research dedicated to establishing a morphokinetics-based embryo selection procedure. We compare the effectiveness of predictive models based on different statistical analysis methods and describe morphokinetic algorithms used in various IVF laboratories to predict preimplantation development, ploidy, implantation and clinical pregnancy. Finally, we discuss what is required to transform these locally-verifiable algorithms into a transferable and universally-effective embryo assessment protocol.

Morphokinetic parameters and their biological significance

Morphokinetic parameters include absolute timings of successive embryonic divisions, as well as relative timings, i.e. periods between divisions, reflecting either duration of the cell cycle or synchronisation of the cleavage rounds. The earliest time-points included in the morphokinetic analysis are tPB2, i.e. time of the 2nd polar body extrusion, marking a completion of 2nd meiotic division; tPNa, i.e. time when pronuclei appear, which reflects the beginning of the first embryonic interphase; tPNf, i.e. time of pronuclei fading and the entry into the first embryonic M-phase. The t2, t3, t4, t5, t6, t7, t8 and t9 parameters are defined as the times for achieving the stage characterized by the corresponding number of cells (t2 for 2 cells, t3 for 3 cells, etc.). The time tM is, on the other hand, defined as the first frame in which the embryo compacts (i.e. the clear boundaries between blastomeres disappear). The tSB time-point is marked by the appearance of a blastocoel cavity, tEB – by the onset of blastocyst expansion (i.e. increase in the overall embryo volume) and tHB – by the beginning of the blastocyst hatching, i.e. escaping from the zona pellucida encapsulation (Fig. 1A). Calculation of these absolute timings requires a starting time-point, set usually as the moment of fertilization (t0). In the case of intracytoplasmic sperm injection (ICSI), it is the moment of sperm injection. However, in the case of traditional IVF, when eggs are simply co-incubated with spermatozoa, determination of the exact time-point of fertilization is much more difficult. t0 is then often defined as the beginning of insemination, and thus times of embryonic divisions tend to be longer than in ICSI, because spermatozoa penetrate oocytes sometime after the onset of insemination (Lemmen *et al.* 2008, Cruz *et al.* 2013, Liu *et al.* 2015*b*, Kirkegaard *et al.* 2016). When the absolute times of embryonic divisions are calculated in relation to another time-point, such as tPNf, the differences between IVF and ICSI disappear (Cruz *et al.* 2013, Liu *et al.* 2015*b*).

The starting time-point is not required when periods between embryonic divisions are calculated. A period between t2 and t3 is called cc2 and reflects the length of cell cycle of the blastomere at the 2-cell stage. The length of the blastomere cell cycle at the 4-cell stage (cc3) is estimated as the difference between t3 and t5, and the length of the blastomere cell cycle at the 8-cell stage (cc4) – between t5 and t9. However, it must be pointed out that the last two parameters do not always reflect the real length of the cell cycle, because the first two blastomeres that originate from e.g. 2- to 4-cell transition are not always the first ones to divide in the next cleavage round. Periods between divisions can also provide information about cleavage synchronicity. For example, s2 is the difference between t3 and t4 and reflects synchronicity of the 2nd round of cleavage divisions. Analogically, s3, the synchronicity of the 3rd round of cleavage divisions, can be calculated as the difference between t5 and t8.

It has been shown that both cleavage divisions that are too fast or too slow reflect poor developmental potential of the human embryo (Meseguer *et al.* 2011, Basile & Meseguer 2012, Cruz *et al.* 2012, Chamayou *et al.* 2013, Herrero *et al.* 2013, Milewski *et al.* 2015). It is likely that timely divisions reflect good quality of the cytoplasmic component of the embryo: we can assume that activation events accompanying fertilization, such as Ca^{2+} oscillations, occurred correctly, that mitochondria provide sufficient amounts of energy, and that the cytoskeleton is functional. Although there are no studies directly linking those factors with the cell cycle length in blastomeres, they regulate such a wide spectrum of cellular processes that their impact

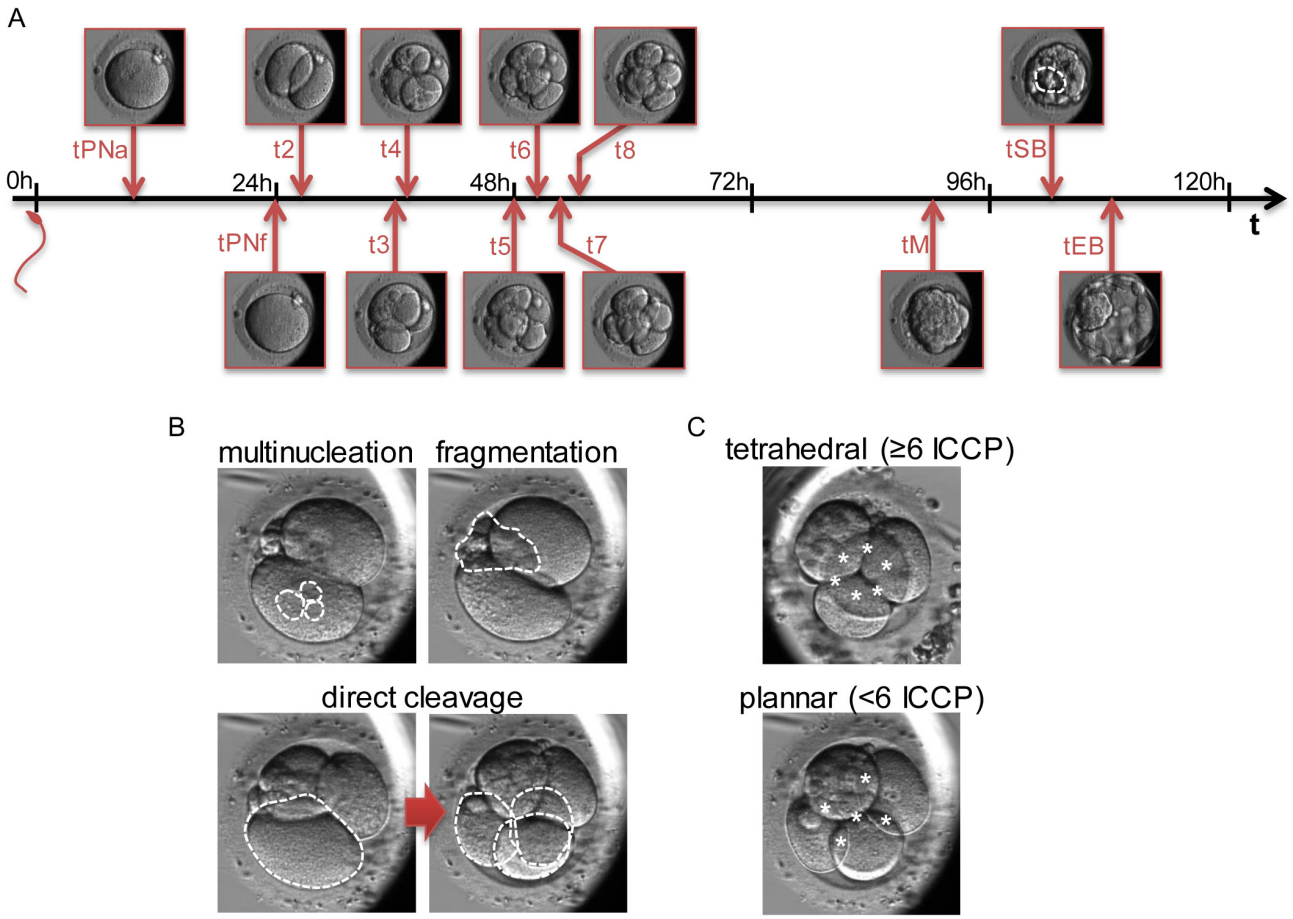

Figure 1 Morphokinetic parameters. (A) Morphokinetic timings and corresponding stages of human embryo development. Blastocoel cavity in a small blastocyst stage (tSB) marked with a white dashed line. (B) Examples of abnormal morphology in human embryos. White dashed line marks nuclei in the multinucleation panel, cytoplasmic fragments in the fragmentation panel and blastomeres in the direct cleavage panel. (C) Spatial arrangement of the blastomeres at the 4-cell stage. Intercellular contact points (ICCP) marked with asterisks.

on the cell cycle is inevitable. Ca^{2+} oscillations affect development of the embryo many days after fertilization, as they not only ensure monospermy, trigger completion of female meiosis and activate embryonic divisions, but also regulate mitochondrial activity, recruitment of maternal mRNAs and expression of embryonic genes (Dumollard *et al.* 2004, Ozil *et al.* 2005, 2006, Toth *et al.* 2006, Ducibella *et al.* 2006, Campbell & Swann 2006). Mitochondria provide energy required for DNA synthesis and chromosome segregation (Salazar-Roa & Malumbres 2017). The cytoskeleton, on the other hand, plays a key role in segregation of chromosomes, cytokinesis and cellular trafficking – each of them important for cell cycle progression (Tang 2012, D'Avino *et al.* 2015, Prosser & Pelletier 2017).

It has also been suggested that timely divisions correspond to high-quality nuclear apparatus. Divisions that are too fast may result in incorrect segregation of the genetic material and lead to aneuploidy. On the other hand, divisions that are too slow may be a sign of DNA damage or chromosomal aberrations that activate one of the cell cycle checkpoints that halt the cell cycle progression. The G1/S, intra-S and G2/M checkpoints halt the progression of the interphase in response to DNA damage, enabling its repair (Sancar *et al.* 2004), whereas an M-phase checkpoint (the spindle assembly checkpoint or SAC) ensures equal segregation of genetic material to daughter cells and becomes activated if sister chromatids are incorrectly attached to spindle microtubules (Musacchio & Salmon 2007, Nezi & Musacchio 2009). However, sometimes the checkpoints fail, giving rise to cells with compromised genetic integrity. Interestingly, recent results obtained for mouse embryos indicate that missegregation of chromosomes does not strongly affect length of the cell cycle, at least at the 8- to 128-cell stages (Bolton *et al.* 2016, Vazquez-Diez *et al.* 2016). On the contrary, in human 1- to 8-cell stage embryos aneuploidy seems to affect at least some division timings (Chavez *et al.* 2012, Vera-Rodriguez *et al.* 2015), indicating that there may be species- and/or stage-related differences in the regulation of blastomere reaction to ploidy defects.

Time-lapse imaging also allows for assessment of morphological parameters, such as size of the blastomeres, number of nuclei in a blastomere, degree of fragmentation, and occurrence of irregular cleavages (Fig. 1B). Healthy human embryos have evenly sized blastomeres, as cells of different sizes suggest uneven segregation of the genetic material during division, an activated checkpoint, or failure of cytokinesis (when one blastomere is significantly, and persistently, bigger than the rest). Such blastomeres contain an incorrect number of chromosomes, sometimes even multiple nuclei, and this typically diminishes developmental potential of the embryo (Giorgetti *et al.* 1995, Ziebe *et al.* 1997, Hardarson *et al.* 2001, Ergin *et al.* 2014). Fragmentation, i.e. formation of membrane-bound extracellular cytoplasmic structures, also tends to correlate negatively with the embryo developmental potential and implantation rate (Giorgetti *et al.* 1995, Alikani *et al.* 1999, Campbell & Fishel 2015, Yang *et al.* 2015). However, fragmentation is a part of programmed cell death, and as such it represents a way of eliminating blastomeres with severe genetic aberrations and does not necessarily have to be a bad prognostic (Jurisicova *et al.* 1996, Warner *et al.* 1998). Therefore, embryos with a low degree of fragmentation may still be considered as of good quality if other criteria, e.g. cleavage rate and blastomere shape, are fulfilled (Van Royen *et al.* 1999). Time-lapse imaging also detects direct or reverse cleavage divisions. Direct cleavage occurs when one blastomere divides directly into three or more daughter blastomeres. Sometimes this definition is extended to include so-called rapid cleavages, i.e. divisions from 2 to 3 blastomeres in less than 5 h (Rubio *et al.* 2012, Campbell & Fishel 2015). Such irregular cleavages strongly correlate with impaired preimplantation development and implantation capacity (Rubio *et al.* 2012, Zaninovic *et al.* 2013, Athayde Wirka *et al.* 2014, Zaninovic *et al.* 2015). It is likely that direct cleavage reflects faulty spindle structure (caused, for example, by incorrect ploidy of the gametes or defective sperm centrosomes) and as a consequence uneven chromosomal segregation during division (Kola *et al.* 1987, Sathananthan 1998, Campbell & Fishel 2015). Indeed, chromosomal abnormalities were detected in a majority of embryos that underwent direct cleavage (Chavez *et al.* 2012, Zaninovic *et al.* 2013, Vera-Rodriguez *et al.* 2015). Reverse cleavage, on the other hand, occurs when cytokinesis fails, or when two blastomeres fuse into a hybrid cell containing two nuclei (Balakier *et al.* 2000, Hickman *et al.* 2012, Campbell & Fishel 2015). Reverse cleavage is probably caused by a decreased functionality of the cytoskeleton, especially its actomyosin component responsible for cytokinesis progression, or by a defect in the cell membrane, and it has been reported that it can be induced by cryopreservation (Balakier *et al.* 2000). Depending on the number of fused cells per embryo and the time-point when a reverse cleavage occurs, the embryos become either entirely polyploid (complete fusion at the 2- or 3-cell stage) or mosaic (i.e. built of normal and polyploid cells). Therefore, the effect of reverse cleavage on the embryo quality may vary between embryos (Hickman *et al.* 2012, Liu *et al.* 2014).

Additionally, time-lapse imaging provides data on the spatial arrangement of the blastomeres, which may also be predictive of the human embryo's developmental potential (Fig. 1C). At the 4-cell stage, a majority of embryos display tetrahedral geometry (i.e. have at least 6 intercellular contact points (ICCPs)), but some of them are planar instead (i.e. have less than 6 ICCPs). It has been reported that planar arrangement is associated with a low rate of blastocyst formation and poor implantation and pregnancy likelihood (Ebner *et al.* 2012, Paternot *et al.* 2014, Liu *et al.* 2015*a*). On the other hand, according to Cauffman *et al.* (2014), although tetrahedral and planar embryos differ in their ability to form high quality blastocysts, they do not differ in terms of pregnancy rate. Research conducted on mouse embryos indicates that the low developmental potential exhibited by planar embryos may be related to the separation of animal and vegetal material during the first two rounds of cleavage divisions. In mice, a certain pool of the planar embryos consist of blastomeres that inherited either only animal or only vegetal material from the oocyte (i.e. underwent two rounds of subsequent equatorial divisions, so-called EE embryos, Piotrowska-Nitsche & Zernicka-Goetz 2005). Such EE embryos gave rise to viable pups in only 35% of cases, as compared to 84–91% efficiency observed for the rest of the embryos. It is still unknown which molecules distributed differentially along the animal–vegetal axis of the oocyte/zygote could be responsible for the different cell fate of the blastomeres that inherited them. So far, the hormone leptin, transcription factor STAT3, growth factors TGFb2 and VEGF, and the apoptosis-associated proteins BCL-X and BAX have been proposed as possible candidates, but apart from the asymmetric localization of these proteins, there is no proof of a functional link between them and the developmental fate of the blastomeres (Antczak & Van Blerkom 1997, 1999, Schulz & Roberts 2011). There is, however, an increasing amount of evidence showing that mouse blastomeres at the 4-cell stage indeed differ between each other in their developmental fate and on a molecular level (Piotrowska-Nitsche *et al.* 2005, Torres-Padilla *et al.* 2007, Bischoff *et al.* 2008, Plachta *et al.* 2011, Burton *et al.* 2013, Tabansky *et al.* 2013, Goolam *et al.* 2016).

Types of morphokinetic algorithms

The morphokinetic parameters described above provide input information for reproductive heuristic models – algorithms that can predict chances of achieving a blastocyst stage, implantation, biochemical or clinical

pregnancy, or a full term development by each examined embryo. It has also been suggested that morphokinetic algorithms may distinguish euploid from aneuploid embryos. In preimplantation development, only embryo quality, related to the quality of gametes, matters, and therefore, heuristic morphokinetic models predicting an embryo's ability to cavitate are relatively efficient and reliable. Such models can be applied to select embryos for transfer at the 2nd–3rd day post fertilization and therefore minimalize the need for an extended, 5–6 day long *in vitro* culture. Importantly, it has been suggested that a prolonged embryo culture may have a negative influence on embryo quality, altering, among others, its epigenetic modification profile (Rinaudo & Schultz 2004, Rinaudo *et al.* 2006, Market-Velker *et al.* 2010). Thus, a shorter embryo culture is likely to facilitate effectiveness of the IVF treatment, not to mention that it also decreases costs of the procedure. Morphokinetic algorithms modelling chances for pregnancy, assessed biochemically (by a pregnancy test), by the presence of embryonic sacs, an embryonic heartbeat or by live birth, are less predictive. This is caused by the fact that apart from the embryo quality, pregnancy depends on other factors, such as hormonal levels or endometrium responsiveness, which are usually excluded from the model. Reliable morphokinetic algorithms predicting ploidy are also difficult to construct. Although it seems likely that prolonged cell cycles or delayed or irregular divisions reflect various chromosomal aberrations, the association with the embryo ploidy (as assessed by standard preimplantation genetic screening or diagnosis procedures) is not always clear. It has been believed that chromosomal mosaicism of the embryo (some blastomeres are aneuploid, while others are euploid) may contribute to this situation, as it renders genetic analysis of biopsied cells not representative of the whole embryo (Harper *et al.* 1995, Munne *et al.* 1995, Bielanska *et al.* 2002, van Echten-Arends *et al.* 2011, Ajduk & Zernicka-Goetz 2013). However, the mere correlation between ploidy status and length of the blastomere cell cycle has also recently been disputed (Chavez *et al.* 2012, Vera-Rodriguez *et al.* 2015, Bolton *et al.* 2016, Vazquez-Diez *et al.* 2016).

In terms of mathematical methods used in morphokinetic algorithms, there are four main approaches (see also Boxes 1 and 2). The most basic algorithms are based on the analysis of one parameter and usually rely on simple correlations. They provide information about the influence of an examined parameter on the reproductive outcome, but such information very rarely has any prognostic value. Hierarchical algorithms are more complex; they take into consideration numerous parameters and establish their optimal value range. Depending on whether the parameter value fits within or outside this range, the prediction is more or less positive. Such algorithms are often used in IVF practice and their reliability has been repeatedly reported (Meseguer *et al.* 2011, Basile & Meseguer 2012, Cruz *et al.* 2012, VerMilyea *et al.* 2014, Del Carmen Nogales *et al.* 2016, Motato *et al.* 2016, Patel *et al.* 2016). However, they have some disadvantages. First, the effect of each parameter on the final prediction strongly depends on their position in the hierarchy: parameters placed higher in the hierarchy affect the outcome to a greater extent than those with lower positions. A parameter's place in the hierarchy is

Box 1 Statistical methods used in reproductive modelling.

Regression – a statistical method that analyses associations between two or more variables and predicts unknown values of dependent variables (e.g. embryo's ability to achieve a blastocyst stage or to implant) based on known values of the independent (explanatory) variables (e.g. division timings). Depending on the number of explanatory variables used to predict the value of the dependent variable, we distinguish univariate (a single explanatory variable) and multivariate (at least two explanatory variables) regressions. Linear and logistic regressions are the most popular in biomedical sciences:

Linear regression estimates scalar value of a dependent variable (e.g. total number of embryo cells at the blastocyst stage) based on independent (explanatory) variables. Using this method, we assume a linear correlation between independent and dependent variables.

Logistic regression estimates binary value of a dependent variable (e.g. implantation/no implantation) based on independent (explanatory) variables.

Regression models require monotonic explanatory variables. A change of a monotonic explanatory variable in one direction (its increase or decrease) always leads to a change of the dependent variable in one direction also. Unfortunately, many morphokinetic parameters are non-monotonic, i.e. their effect on the dependent variable changes with their value. For example, cleavage divisions that are either too slow or too fast correlate with poor embryo quality, so an increase of the cleavage timings first increases the embryo quality, but beyond a certain value, it decreases it.

Hierarchical algorithm – a statistical, tree-structured algorithm that is very popular in reproductive medicine. Explanatory variables form 'nodes' that are connected with 'branches.' There are usually two branches growing from each node, and a decision, which branch should be chosen, depends on the value of the variable: whether or not it falls in the predetermined range. Nodes situated higher in the tree structure (i.e. closer to the starting node) have a greater impact on the outcome (e.g. embryo's ability to cavitate or implant).

Data-mining – a computational process of discovering patterns in large data sets involving methods at the intersection of artificial intelligence, machine learning, statistics and database systems.

Artificial intelligence – a branch of computer sciences drawing upon neuroscience, psychology and cognitivistics. It creates machines and software mimicking the cognitive abilities of the human mind that cannot be subjected to algorithmization.

Artificial neural networks – a computational artificial intelligence approach mimicking the way a biological brain solves problems. It is built as a network of neural units connected with each other like neurons in a brain. These systems are not explicitly programmed, but by feeding data into them, one can train them to analyse problems difficult to solve for conventional statistical methods.

Box 2 Statistical significance of the reproductive models.

Odds ratio (OR) – a measure used, for example, in a logistic regression that quantifies the association between two variables. It is calculated as a ratio of odds that an outcome A (i.e. dependent variable A) occurs when a property B (i.e. explanatory variable B) is present to the odds of the outcome A occurring in the absence of property B. If the OR is greater than 1, then the presence of B raises (relative to the absence of B) the odds of having A, so these two parameters are associated. However, this does not necessarily mean that B is a contributing cause of A: it could be associated with A through a third property, C, which is the real contributing cause of both A and B. This situation is typical for morphokinetics, e.g. timely cleavage divisions are associated with the implantation capability of the embryo, but they are not a contributing cause of this capability. Timely divisions reflect other cellular properties (correct ploidy, functional cytoskeleton, efficient energy metabolism, etc.) that are the real contributing causes of the embryo capability to implant.

Sensitivity – proportion of positive outcomes that are correctly identified by the model, e.g. the percentage of embryos that manage to implant and that were correctly identified as being able to do so.

Specificity – proportion of negative outcomes that are correctly identified by the model, e.g. the percentage of embryos that did not manage to implant and were correctly identified as being unable to do so.

Receiver Operating Characteristic (ROC) analysis – a graphical method assessing how well a scalar explanatory variable (e.g. division timings) predicts a binary outcome. It allows one to find the optimal threshold value of the explanatory variable, i.e. the value that distinguishes the two possible outcome situations (e.g. implantation/no implantation) in the best way. The ROC curve is created by plotting the **true positive rate** (or **sensitivity**) against the **false positive rate** (i.e. proportion of all negative outcomes classified as positive ones, calculated as **1 – specificity**) at various threshold settings. **Area under the curve (AUC)** – a measure of the predictive power of a variable provided by the ROC (Receiver Operating Characteristic) method. It is calculated as an area under the ROC curve. AUC values range from 0.5 (when the explanatory variable is not at all associated with the analysed outcome) to 1 (when the variable with the chosen threshold value can distinguish two possible outcomes in 100% of cases). If AUC is lower than 0.5, then the explanatory variable influences the outcome in the opposite direction, e.g. higher values of the explanatory variable correlate not with implantation, but with lack of implantation.

95% confidence interval (CI) – an estimated range of values which is likely to include the real value of the parameter of interest (e.g. AUC). In other words, if the experiment was repeated, our parameter would fall inside this range in 95% of cases. If CI applies to AUC, it cannot include 0.5 value, otherwise it renders the association described by the algorithm statistically insignificant.

decided in a subjective way by a researcher. It usually does not have any deeper biological meaning, and it depends on its impact on the efficiency of the resulting algorithm. Second, in hierarchical algorithms, values inside the same value range (e.g. optimal or non-optimal range) have an equal power to affect the prediction. It does not matter whether the value is close to the minimum or maximum of the range, or whether it is close to its median. Moreover, values that are almost identical, but are situated at two sides of the range limit, have completely different impacts on the algorithm outcome. These flaws are not present in regression algorithms, in which the influence of each parameter on the outcome is not decided by a researcher, but depends on the obtained data. However, morphokinetic parameters are usually non-monotonic (i.e. both very low and very high values indicate poor embryo quality); therefore, they need to be mathematically transformed before being used in regression analysis. It has been proposed that the parameter values favoring the highest developmental potential are close to the median value calculated for the group of embryos with a positive outcome (i.e. achieving the blastocyst stage, implantation and live birth) (Milewski *et al.* 2015, 2016*a*, 2016*b*). Thus, non-monotonic parameters can be transformed to the distance between their value and the median, gaining the monotonic characteristics required for the regression models. The last type of morphokinetic algorithms is based on data-mining or artificial intelligence methods (e.g. artificial neural networks). These methods find associations between analysed parameters that cannot be easily uncovered by typical statistical procedures. Such algorithms provide outcomes without describing how each of the analysed parameters affects the result. They simply adapt to the data, but it is impossible to identify the power and 'direction' of the parameter influence. Despite this flaw, algorithms applying artificial neural networks are probably the most potent tools in explaining biological processes and predicting biological outcomes, including outcome of IVF treatment (Milewski *et al.* 2009, Siristatidis *et al.* 2011).

How to predict the ability to achieve a blastocyst stage?

Many studies show that timings of human embryo divisions correlate with preimplantation development potential. Wong *et al.* (2010) correlated the human embryo's ability to achieve the blastocyst stage with (i) duration of the first cytokinesis (the very brief last step in mitosis that physically separates the two daughter cells), (ii) time interval between the end of the first division and the initiation of the second (the length of the 2nd cell cycle, cc2 = t3–t2) and (iii) the time interval between the second and third divisions (synchronicity of the 2nd round of divisions, s2 = t4–t3). Ability of embryos to reach the blastocyst stage could be predicted, with a sensitivity and specificity of 94% and 93% respectively, by having a first cytokinesis of 0–33 min, cc2 of 7.8–14.3 h and s2 of 0–5.8 h (Wong *et al.* 2010). Further independent research (including a prospective multicentre study) has confirmed the usefulness of the cc2 and s2 parameters: they differed significantly between embryos that did or did not develop to the blastocyst stage and could be applied to predict implantation and pregnancy (Cruz *et al.* 2012, Conaghan *et al.* 2013, VerMilyea *et al.* 2014, Aparicio-Ruiz *et al.* 2016). Another prospective study

positively verified the duration of the first cytokinesis as a biomarker of an embryo's ability to form high-quality blastocysts (Kirkegaard *et al.* 2013).

Cetinkaya *et al.* (2015) found that although most absolute and relative timings up to Day 3 of development differed between human embryos that later would form good or bad quality blastocysts, more complex parameters, calculated as ratios of relative timings, namely: cleavage synchronicity from 4 to 8 cells (CS4–8 = (t8–t5)/(t8–t4)) and cleavage synchronicity from 2 to 8 cells (CS2–8 = ((t3–t2) + (t5–t4))/(t8–t2)), were better indicators of blastocyst formation and quality, with CS2–8 reaching the highest predictive power (AUC = 0.786, 95% CI: 0.772–0.800, sensitivity: 83.43% and specificity: 62.46%). In the case of the CS4–8 parameter, embryos that had a t8–t4 period significantly longer than t8–t5 (i.e. CS4–8 tending towards 0) were favoured. In other words, embryos with a relatively synchronous 3rd round of cleavage (i.e. short t8–t5 interval) and synchronous 2nd round of cleavage (i.e. divisions to 3- and 4-cell stage occurring at a similar time, which ensures the longest possible period between t4 and t5) had higher developmental chances. In the case of the CS2–8 parameter, embryos that spent a long time at the 2- and 4-cell stages (high values of t3–t2 and t5–t4 parameters), and therefore had CS2–8 tending towards 1, were favoured. In other words: embryos with 2nd and 3rd rounds of cleavage occurring relatively synchronously (i.e. occupying a small percentage of the t8–t2 period) had higher developmental potential.

A composite parameter Sc created as a mathematical transformation of absolute cleavage times (t2 and t5) and the duration of the 2nd cell cycle (cc2) has also been used to predict blastocyst formation in an algorithm applying logistic regression analysis (Milewski *et al.* 2015). In this algorithm, t2, t5 and cc2 timings were assigned different scores (0, 1, or 2), depending on their values and corresponding likelihood of blastocyst formation (0 for the lowest and 2 for the highest likelihood). Then, these individual scores were multiplied by their corresponding odds ratios (ORs), forming the composite Sc parameter. Sc was very effective in predicting blastocyst formation with the AUC reaching 0.806 (95% CI: 0.747–0.864). Importantly, the algorithm was validated on the independent data set and reached similarly high predictive power (AUC = 0.813, 95% CI: 0.746–0.880) (Milewski *et al.* 2015).

Another algorithm predicting blastocyst quality has been based on a hierarchical analysis of the timing of division to the 5-cell stage (t5; the primary variable) and the synchrony of the 2nd round of cleavage (s2 = t4–t3; the secondary variable) (Cruz *et al.* 2012). The algorithm divided embryos into four categories (A–D) with decreasing likelihood of cavitation (from 77% for A to 53.6% for D) and formation of a good quality blastocyst (from 61.3% for A to 34.5% for D). If the value of the t5 parameter fell inside the optimal range (48.8–56.6 h), the embryo was graded as A or B. If the value of t5 fell outside the optimal range, the embryo was graded as C or D. If s2 was within the optimal range (≤0.76 h), the embryo was graded as A or C, depending on the t5 value; if s2 was >0.76 h, the embryo was graded B or D, also depending on the t5 value (Fig. 2A). Unfortunately, A–D embryos did not differ in their ability to implant, demonstrating that algorithms predicting preimplantation development do not necessarily have a clinical significance (Cruz *et al.* 2012).

Another hierarchical classification procedure has been proposed by Motato *et al.* (2016). Researchers set timing of morula formation (tM) as the primary variable and synchronicity of the 3rd round of cleavage (s3 = t8–t5) as the secondary one, and divided embryos into 4 categories (A–D) with decreasing capacity to form blastocysts (from 84.4% for A to 13.8% for D). If embryos exhibited tM values falling inside the optimal range of 81.28–96.00 h, then they were categorized as A or B; if tM was outside the optimal range, the embryos were graded as C or D. If s3 was within the optimal range (≤8.78 h), embryos were graded as A or C, depending on tM. Analogically, if s3 was outside the optimal range, embryos were scored as B or D (Fig. 2B). Again, similar to the model by Cruz *et al.* (2012), the algorithm efficiently predicted the ability to cavitate (AUC = 0.849; 95% CI: 0.835–0.854), but was not effective in predicting implantation rates (AUC = 0.546; 95% CI: 0.507–0.585) (Motato *et al.* 2016).

Yang *et al.* (2015), on the other hand, focused on morphological information obtained from time-lapse imaging. They created a hierarchical algorithm dividing embryos into 6 groups (A–F) with a decreasing likelihood of cavitation (from 94.8% for A to 21.2% for F, $P < 0.001$) and forming good quality blastocysts (from 70.8% for A to 0% for E and 3.8% for F, $P < 0.001$). Category A embryos had only normal divisions in the first three cleavage rounds. B embryos exhibited minor abnormalities, such as distorted cytoplasm movement during division, formation of big cytoplasmic fragments and formation of uneven blastomeres, in all three cleavage rounds. C embryos displayed minor abnormalities in the first two cleavage rounds and major abnormalities, such as a developmental arrest of a blastomere, a direct cleavage, a disordered division (i.e. division of one blastomere was delayed and occurred only after the rest of the blastomeres completed the next round of cleavage) and extensive fragmentation, in the 3rd round of cleavage. D embryos showed minor abnormalities in the 1st round of cleavage and one of the two blastomeres showed major irregularities in the 2nd round. In E embryos, the 1st cleavage occurred with minor abnormalities, but both the 2-cell blastomeres displayed major abnormalities in the 2nd round of cleavage divisions. Finally, F embryos showed major irregularities in the 1st cleavage (Fig. 2C). Interestingly, a prospective observational study showed that embryos from the A–D categories also differed in

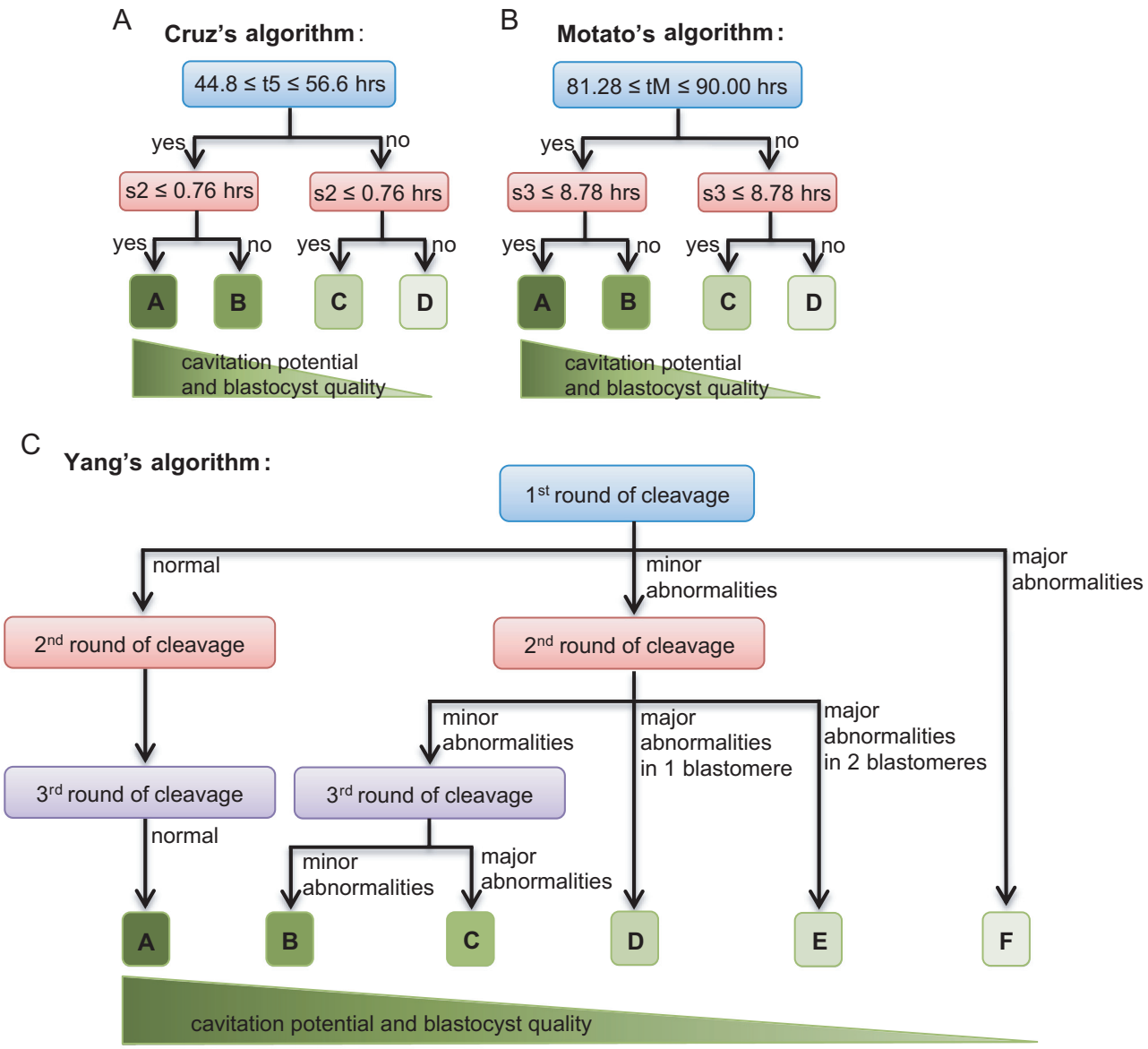

Figure 2 Morphokinetic algorithms predicting blastocyst formation and quality. Algorithms described by Cruz *et al.* (2012) (A), Motato *et al.* (2016) (B) and Yang *et al.* (2015) (C).

their ability to implant (from 67.0% for A to 0% for D, $P<0.001$) (Yang *et al.* 2015).

To sum up, algorithms predicting blastocyst formation and quality focus on relatively early developmental events. Parameters describing length of a cell cycle in a 2-cell stage blastomere (cc2) or synchronicity of the 2nd round of cleavage (s2) seem to be particularly informative (Wong *et al.* 2010, Cruz *et al.* 2012, Conaghan *et al.* 2013). Indeed, if morphokinetic irregularities that, as we assume, reflect suboptimal condition of the cytoplasm and/or nuclear apparatus of the cell are detected in the early embryonic stages, the number of descendant blastomeres that may inherit the faulty components increases, compromising viability of the embryo more heavily. The same rule applies to morphological parameters such as fragmentation, irregular cleavages or developmental arrest: the earlier they occur, the greater their negative impact is on embryo quality (Yang *et al.* 2015). It is also important to note that morphokinetic algorithms strongly favour embryos that cleave synchronously, which, from the biological point of view, reflects undisturbed progression of the cell cycle in all blastomeres and, in consequence, minimizes the risks of, for example, DNA damage and chromosomal abnormalities (Wong *et al.* 2010, Cruz *et al.* 2012, Conaghan *et al.* 2013, Cetinkaya *et al.* 2015, Motato *et al.* 2016). Sadly, algorithms predicting preimplantation development of embryos are not very

effective in selecting embryos with the highest likelihood of implantation or pregnancy, clearly demonstrating that a successful pregnancy requires more than just a morphokinetically faultless embryo.

Going further: how to predict which embryo will successfully implant?

The simplest algorithms predicting pregnancy use single parameters, both absolute and relative, such as time of nucleus appearance in the two blastomeres for 4-cell embryos, synchronicity of nuclear formation in both 2-cell blastomeres, or synchronicity of the 2nd round of cleavage division (s2 = t4–t3) (Lemmen *et al.* 2008). Parameters such as duration of 2-cell stage (cc2 = t3–t2) and synchronicity of the 2nd round of cleavage (s2 = t4–t3) were also used for the selection of embryos with the highest implantation and pregnancy potential (VerMilyea *et al.* 2014). However, this approach has been questioned in a retrospective study by Kirkegaard *et al.* (2015) as generating false rejection rate that is too high (i.e. frequently disqualifying embryos that actually could have resulted in a pregnancy).

More complex algorithms apply a hierarchical analysis of multiple parameters. Meseguer *et al.* (2011) combined static evaluation of embryos with an assessment of dynamic parameters and divided embryos into ten categories (A+, A–, B+, B–, C+, C–, D+, D–, E and F) with a decreasing likelihood of implantation (from 66% for A+ to 8% for E grade; F embryos were not transferred, AUC = 0.72, 95% CI: 0.645–0.795). The hierarchical classification procedure started with a morphological screening of the embryos: those that were clearly not viable (i.e. highly abnormal, attretic, etc.) were discarded and not considered for transfer and fell into the F category. The next step excluded embryos that fulfilled any of the three exclusion criteria: (i) uneven blastomere size at the 2-cell stage; (ii) direct division from one to three or more cells or (iii) multi-nucleation at the 4-cell stage (category E). Subsequently, the algorithm followed a strict hierarchy based on the timing variables t5, s2 (t4–t3) and cc2 (t3–t2). First, if the value of t5 fell inside the optimal range (48.8–56.6 h), the embryo was categorized as A or B. If the value of t5 fell outside the optimal range, the embryo was categorized as C or D. If the value of s2 fell inside the optimal range (≤0.76 h), the embryo was categorized as A or C, depending on t5; similarly, if the value of s2 fell outside the optimal range, the embryo was categorized as B or D, depending on t5. Finally, the embryo was categorized with an extra plus if the value for cc2 was inside the optimal range (≤11.9 h) and was categorized with a minus if the value for cc2 was outside the optimal range (Fig. 3A). The usefulness of this algorithm was validated by the same authors in a retrospective study on an independent data set (Meseguer *et al.* 2012) and in a randomized controlled trial (Rubio *et al.* 2014). However, subsequent external retrospective studies did not confirm its high predictive power and even questioned whether any predictive algorithm can be used universally in IVF units (Freour *et al.* 2015, Liu *et al.* 2015*c*). Interestingly, a simplified version of Meseguer and coworkers's algorithm, utilizing only t5 and s2 parameters, has been positively validated by Freour *et al.* (2015).

The study conducted by Meseguer *et al.* (2011) was followed up by a modified hierarchical algorithm based on a larger data set (Basile *et al.* 2015*b*). This time the t3 parameter was set as a primary variable, followed by cc2 and t5. As a result, 10 categories (A+, A–, B+, B–, C+, C–, D+, D–, E and F) with decreasing likelihood of implantation were distinguished (from 32% for A+ to 17% for E grade; F embryos were not transferred, $P < 0.001$, AUC = 0.61, CI 95%: 0.574–0.638). As in Meseguer and coworkers's algorithm, the first two steps were based on morphological assessment of the embryos, and only the embryos that had passed the assessment were subjected to the hierarchical classification. First, if the value of t3 fell inside the optimal range (34–40 h), the embryo was categorized as A or B. If the value of t3 fell outside the optimal range, the embryo was categorized as C or D. Next, if the value of cc2 fell inside the optimal range (9–12 h), the embryo was categorized as A or C, depending on t3; similarly, if the value of cc2 fell outside the optimal range, the embryo was categorized as B or D, depending on t3. Finally, the embryo was categorized with an extra plus if the value for t5 was inside the optimal range (45–55 h) and was categorized with a minus if the value for t5 was outside the optimal range.

A combination of morphological assessment and morphokinetic data has also been used in a hierarchical algorithm described by Liu *et al.* (2016). They divided embryos into 7 categories (A+, A, B, C, D, E, F) with decreasing implantation rates (from 52.9% for A to 0% for F, AUC = 0.762, 95% CI: 0.701–0.824). First, Day 3 embryos were scored according to their morphology: poor-quality embryos were categorized as F. The rest of the embryos were subjected to cleavage pattern analysis: if embryos underwent direct or reverse cleavages, or exhibited less than 6 intracellular contact sites (i.e. were planar) at the 4-cell stage, they were classified as E; otherwise, they were subjected to further examination. If embryos had less than 8-cells at 68 h post insemination, then they were categorized as D. In the rest of the embryos, synchronicity of the 2nd round of cleavage (s2 = t4–t3) was assessed and if it was longer than 0.84 h, embryos were graded as C. In the rest of the embryos, the time between disappearance of pronuclei and the 5-cell stage (t5–tPNf) was measured and if it was longer than 28.01 h, embryos were categorized as B; if it fell inside the range of 24.67 and 28.01 h – they were graded as A; and if it was shorter than 24.67 – they became A+ (Fig. 3B). The algorithm was also prospectively

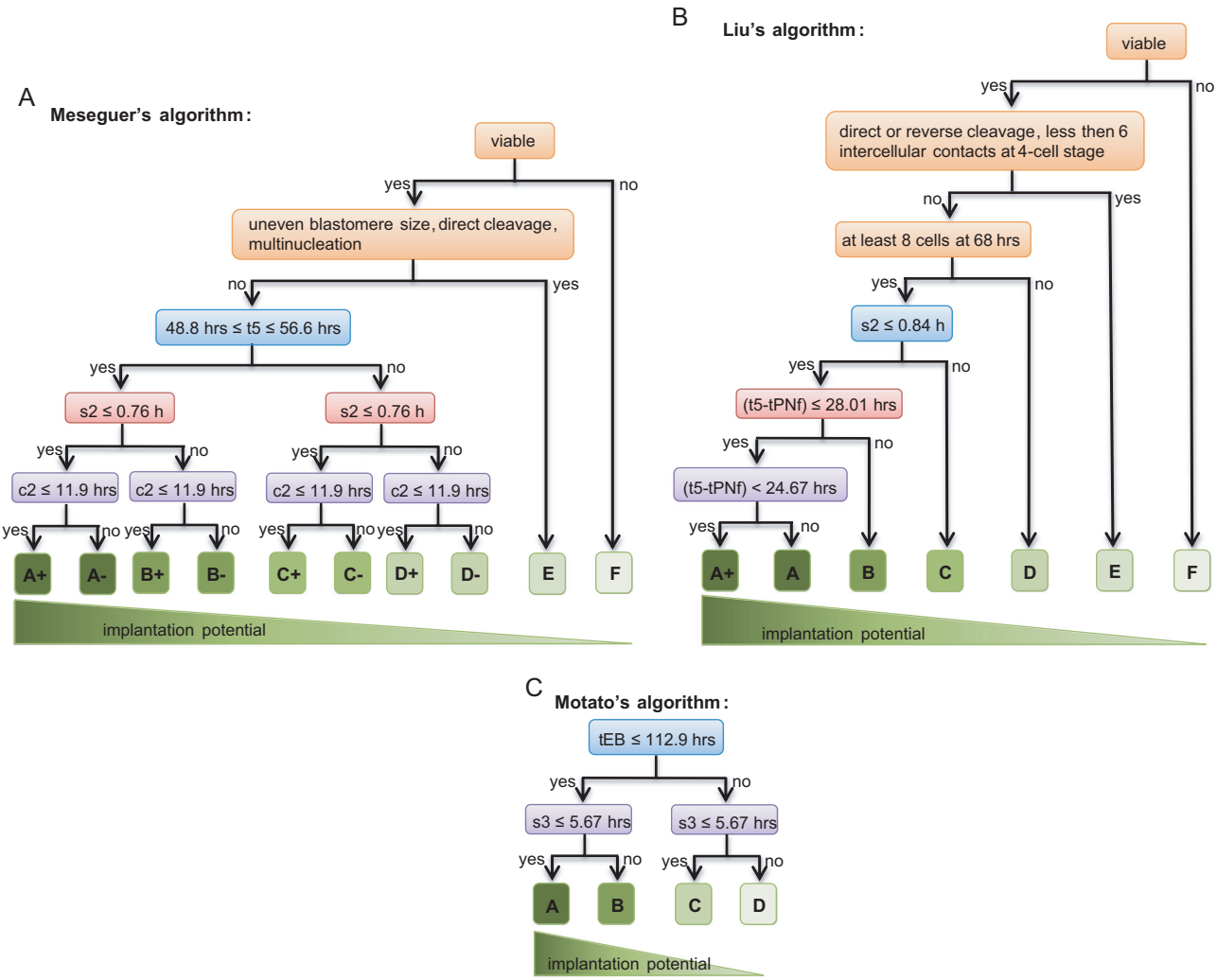

Figure 3 Morphokinetic algorithms predicting implantation and pregnancy. Algorithms described by Meseguer *et al.* (2011) (A), Liu *et al.* (2016) (B) and Motato *et al.* (2016) (C).

validated and, depending on the culture media used in the study, reached AUC = 0.750 (95% CI: 0.588–0.912) or AUC = 0.820 (95% CI: 0.671–0.969).

A simpler hierarchical algorithm was proposed byMotato *et al.* (2016). They set the timing of expanded blastocyst formation (tEB) as a primary variable with the optimal range of ≤112.9 h, and synchronicity of the 3rd round of cleavage divisions (s3 = t8–t5) as the secondary variable with the optimal range of ≤5.67 h. This approach divided embryos into 4 categories (A–D), with a decreasing implantation potential (from 72.2% for A to 39.7% for D, AUC = 0.591, 95% CI: 0.552–0.630) (Fig. 3C). While the algorithm was extended to include blastocyst morphology, oocyte donation, and age (patients' for their own oocytes and donors' for the donated oocytes), the AUC reached 0.602 (95% CI: 0.559–0.645). The algorithm was positively validated on an independent data set (AUC = 0.596, 95% CI: 0.526–0.666) (Motato *et al.* 2016)

Another approach utilized morphokinetic parameters such as division timings (from t2 to t5), the length of the 2nd cell cycle (cc2), and synchrony of the 2nd round of cleavage (s2) that were transformed to a set of monotonic, uncorrelated variables, and combined them with a fragmentation rate at t3 and female age (as an adjusting variable) (Milewski *et al.* 2016a). A multivariate regression analysis based on these parameters predicted pregnancy with a very high probability (AUC = 0.70, 95% CI: 0.64–0.75). Importantly, the algorithm was validated on an independent data set and the obtained results were very similar (AUC = 0.70, 95% CI: 0.59–0.80), proving that it is reliable and applicable in clinical practice (Milewski *et al.* 2016a). t2–t5 division timings, s2 and cc2 were also used, this time together with morphological parameters such as fragmentation, multinucleation, blastomere size at t2 and t4, and female age, in a model applying the artificial neural network method (Milewski *et al.* 2017). In this case,

the predictive power of the algorithm (AUC) reached 0.75 (95% CI: 0.70–0.80) – a result that was confirmed by validation on an independent data set (AUC = 0.71, 95% CI: 0.59–0.84).

In summary, algorithms predicting implantation or pregnancy tend to be more complex than models selecting embryos according to their ability to form good quality blastocysts. However, surprisingly, they usually use the same parameters – cc2, s2, t5, often accompanied by another parameter referring to the early embryonic cleavages – t3 (Meseguer *et al.* 2011, Basile *et al.* 2015*b*, Liu *et al.* 2016, Milewski *et al.* 2016*a*, 2017). It is therefore difficult to explain, at least from the biological point of view, why they reflect implantation or pregnancy potential better than the simpler 'preimplantation algorithms'. It seems plausible that their higher efficiency towards detecting implantation/pregnancy likelihood originates simply from the fact that they include more parameters and therefore set stricter criteria for embryo selection. Interestingly, implantation/pregnancy algorithms usually do not apply time parameters describing later developmental events, such as blastocoel formation or blastocyst expansion, and those that do (Motato *et al.* 2016) are not at all more efficient compared to the typical algorithms utilizing early morphokinetic parameters.

Going deeper: how to predict ploidy of developing embryos?

Several groups have attempted to create algorithms predicting aneuploidy in embryos based solely on non-invasive time-lapse monitoring instead of expensive and invasive genetic diagnostics. The rationale of this research is that embryos display different cleavage dynamics depending on their ploidy. However, the relationship between morphokinetic parameters and embryo ploidy is currently disputed (Chavez *et al.* 2012, Lammers *et al.* 2014, Chawla *et al.* 2015, Grau *et al.* 2015, Vera-Rodriguez *et al.* 2015, Bolton *et al.* 2016, Vazquez-Diez *et al.* 2016) and more research is definitely needed to solve this issue.

Basile *et al.* (2014), using a logistic regression analysis, showed that a combined duration of the 2-, 3- and 4-cell stage (t5–t2) and the length of the cell cycle in a 4-cell stage blastomere (cc3 = t5–t3) were the most relevant variables related to normal chromosomal content (OR = 2.853; 95% CI: 1.763–4.616 and OR = 2.095; 95% CI: 1.356–3.238 respectively). A hierarchical algorithm based on t5–t2 and cc3 variables divided embryos into four categories (A–D), with an increasing percentage of chromosomal abnormalities (from 64.1% for A to 90.2% for D category, $P < 0.001$, AUC = 0.634, 95% CI: 0.581–0.687). If the t5–t2 value fell into the optimal range, defined as >20.5 h, the embryos were graded as A or B; if the t5–t2 value was outside the optimal range, they were graded as C or D. If the cc3 value was inside the optimal range (11.7–18.2 h), the embryos were graded as A or C, depending on t5–t2. Similarly, if cc3 fell out of the optimal range, the embryos were scored as B or D, depending on t5–t2 (Fig. 4A). The t5–t2 variable is very interesting from the biological point of view, as it may distinguish embryos that cleaved to the 5-cell stage in a typical way (longer t5–t2 interval) from those that underwent direct cleavages (from 1- to 3-cells and/or 2- to 5-cells) and, therefore, probably contain aneuploid blastomeres (shorter t5–t2 interval). Of course, the t5–t2 interval can also be prolonged if blastomeres progress through the cell cycle too slowly (e.g. due to activated cell cycle checkpoint) and then a longer t5–t2 period would be a bad prognostic. This contradiction was solved by addition of the cc3 parameter, which ensures that the best quality embryos have standard cell cycle length. The assumption that morphokinetic parameters in aneuploid embryos often display values outside the narrow range typical for euploid embryos has been also confirmed by Chavez *et al.* (2012), although different morphokinetic variables (length of the 1st cytokinesis, length of the cell-cycle at 2-cell stage (cc2 = t3–t2) and

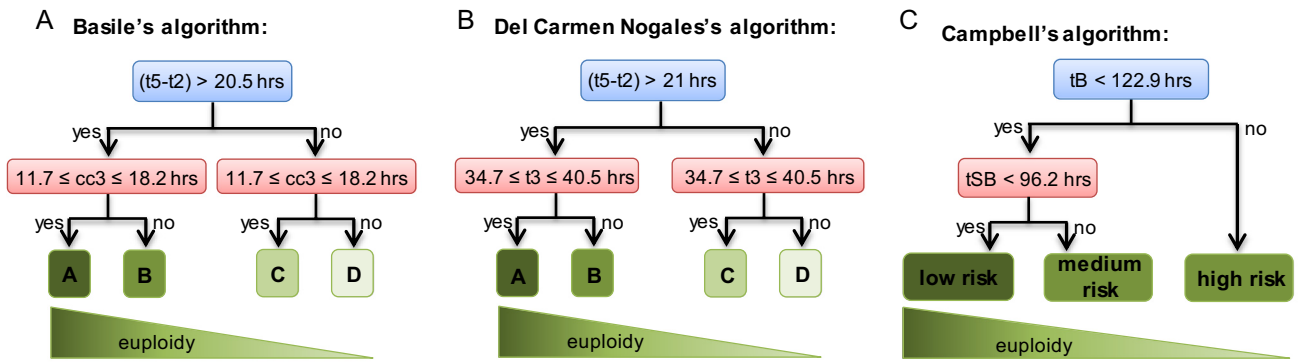

Figure 4 Morphokinetic algorithms predicting ploidy. Algorithms described by Basile *et al.* (2014) (A), Del Carmen Nogales (2016) (B) and Campbell *et al.* (2013*a*) (C).

synchronicity of the 2nd round of cleavage (s2 = t4–t3)) were examined in their study. The predictive power of both t5–t2 and cc3 parameters was also confirmed by an independently performed univariate logistic regression analysis (OR = 1.06; 95% CI: 1.03–1.09 and OR = 1.07; 95% CI: 1.03–1.10 respectively). Embryos with t5–t2 longer than 20 h or with cc3 longer than 10 h were almost twice more likely to be euploid than embryos with t5–t2 or cc3 shorter than these threshold times (Chawla *et al.* 2015). Patel *et al.* (2016), on the other hand, showed that although embryos having t5–t2 and cc3 parameters inside the optimal range proposed by Basile *et al.* (2014) are more likely to be euploid than embryos with those parameters outside the range, the differences were not statistically significant.

The t5–t2 parameter, this time combined with the t3 variable, has also been used in a hierarchical algorithm proposed by Del Carmen Nogales *et al.* (2016). Researchers divided embryos into 4 categories (A–D) with a decreasing likelihood of euploidy (from 70.6% for A to 14.6% for D, $P < 0.01$). According to the algorithm, if the value of t5–t2 was inside the optimal range, i.e. >21 h, the embryo was categorized as A or B; if the value of t5–t2 was outside the range, it was graded as C or D. If t3 value was inside the optimal range of 34.7–40.5 h, embryos were categorized as A or C, depending on t5–t2. Analogically, if the t3 value was outside the optimal range, embryos were graded as B or D, depending on t5–t2 (Fig. 4B). Here, the t3 parameter played a role similar to cc3 in the Basile and coworkers's algorithm: it ensured that the cell cycle (this time at the 2-cell stage) had a standard, 'healthy' length.

Ploidy has also been predicted based on later developmental time-points, such as tSB (start of blastulation) and tB (formation of a full blastocyst) (Campbell *et al.* 2013*a*). Embryos with tB <122.9 h and tSB <96.2 h were found to be at a low risk of aneuploidy (37%); embryos with tB <122.9 h and tSB >96.2 h were at a medium risk (69%); and with tB >122.9 h – at a high risk (97%) (AUC = 0.72; Fig. 4C). Campbell and coworkers continued their studies in order to evaluate the effectiveness of this algorithm for pregnancy prediction in unselected IVF patients and found that embryos classified according to the abovementioned criteria as having low aneuploidy risk had a significantly higher potential to undergo foetal development compared to embryos classified as medium or high risk (AUC = 0.74, Campbell *et al.* 2013*b*).

The algorithm created by Campbell *et al.* (2013*a*, 2013*b*) has raised a vivid discussion among clinicians and has been criticized for small sample size, lack of randomized control trial and a likely bias caused by maternal age (Ottolini *et al.* 2014). Even though researchers increased the sample size and refuted the objection that differences in tSB and tB timings among embryos reflect the maternal age rather than the ploidy status (Campbell *et al.* 2014), the dispute has continued. Recently, the relevance of all published morphokinetic algorithms predicting aneuploidy has been questioned (Rienzi *et al.* 2015). In this new study, researchers examined a selected group of patients prone to aneuploidies and investigated a correlation between morphokinetic parameters obtained for individual embryos and their molecular karyotype assessed by trophectoderm biopsy and quantitative PCR screening of 24 chromosomes. Their results indicated that neither classification relying on tSB and tB timings (Campbell *et al.* 2013*a*, Campbell *et al.* 2013*b*, Campbell *et al.* 2014), nor hierarchical algorithm based on the interval between the two and five-cell stage (t2–t5) and the duration of the 3rd cell cycle (cc3) (Basile *et al.* 2014, Chawla *et al.* 2015) allows one to distinguish chromosomally normal and abnormal embryos. This corresponds to the recent observation that aneuploid and euploid human embryos differ only in two morphokinetic parameters (up to the 8-cell stage): duration of the 1st mitotic phase ($P = 0.025$) and the synchrony of the 2nd round of cleavage divisions (s2 = t4–t3) ($P = 0.048$) (Vera-Rodriguez *et al.* 2015). A lack of clear association between ploidy status and length of the blastomere cell cycle has also been reported for mouse embryos, although later cleavage divisions (from the 8- to 128-cell stage), which are usually not included in a standard morphokinetic analysis, were examined here (Bolton *et al.* 2016, Vazquez-Diez *et al.* 2016). Although these discrepancies may simply be caused by variations between laboratories and embryo culture systems (and in the case of mouse studies – by a difference between species), they emphasize the need for caution in interpretation of the data existing thus far and urge further, large-scale multicentre studies to clarify the possible relationship between cleavage dynamics and embryo ploidy.

Challenges and perspectives of the morphokinetic models

The last decade has brought a great advancement in the embryo-safe time-lapse imaging technique and use of predictive algorithms based on morphokinetics. Researchers have tested numerous morphokinetic parameters, used either individually or combined, and established which ones are strongly associated with good embryonic development, either to the blastocyst stage or full term. These results have been discussed in the present review. However, we are still at the beginning of our journey leading to the establishment of a universal, clinical morphokinetic algorithm for embryo selection. We have taken the first step: we have created algorithms that allow for effective embryo assessment in individual laboratories. Many of them fit the original set of data very well (AUC > 0.7), but they are often not transferable to other embryo cohorts and fail the independent validation (see discussion between Meseguer *et al.* (2011) and Freour *et al.* (2015) and Liu *et al.* (2015c),

or Campbell *et al.* (2013*a*, 2013*b*, 2014), Basile *et al.* (2014), Chawla *et al.* (2015) and Rienzi *et al.* (2015)). Therefore we need to make another step forward and focus our efforts on creation of an algorithm that would be effective in different clinics and that would take into consideration differences in IVF procedures (hormonal stimulation procedures, fertilization methods, culture media, etc.). To achieve this, we need to conduct extensive multi-centre validation studies of the morphokinetic algorithms and focus on finding parameters that have the highest inter-laboratory transferability.

Additionally, in order to maximize the predictive power of the reproductive outcome models, we should be continuing an intensive search for new biomarkers of embryonic quality. Studies in humans should be preceded by research on animal (preferably mammalian) models that allows for more extensive examination of molecular mechanisms governing embryo development. However, it must be emphasized that results obtained for animal models do not necessarily transfer directly to humans and that they should always be treated more as a mere indication of certain tendencies than a ready-to-use solution. We should explore the possibility of combining time-lapse information with data provided by other diagnostic methods, such as genetic diagnostics, transcriptomics, proteomics or metabolomics (Yang *et al.* 2014, Dominguez *et al.* 2015, Tejera *et al.* 2016). Our data, obtained using a mouse model, indicate that morphokinetic parameters can also be combined with a time-lapse-based analysis of fertilization-induced rapid cytoplasmic motion (Ajduk *et al.* 2011) to predict quality of the resulting blastocysts (Ajduk & Milewski, in preparation). In the case of pregnancy prediction, it is especially important to incorporate information related to the hormonal levels and endometrium condition, as they are important factors affecting implantation efficiency (Weimar *et al.* 2013, Atwood & Vadakkadath Meethal 2016). Morphokinetic heuristic models can also be improved by replacement of simple regressive or hierarchical algorithms with algorithms based on data-mining and artificial intelligence methods (such as e.g. artificial neural networks), allowing more extensive utilization of the time-lapse data. Indeed, current biomedical research indicates that application of the artificial neural network method can maximize the predictive power of reproductive algorithms (Milewski *et al.* 2009, 2017).

In summary, heuristic models utilizing morphokinetic parameters in order to predict blastocyst formation, embryo ploidy or pregnancy are a promising non-invasive alternative to diagnostic methods used currently in clinical practice. Time-lapse imaging provides a broad spectrum of information related to embryo morphology and dynamics of embryonic divisions and undoubtedly gives clinicians new insights into the process of embryo preimplantation development. Although the models are still waiting for a thorough validation, and therefore need to be treated with caution, they unquestionably have great potential to support clinical assessment of human embryo quality.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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