RBMO

REVIEW

Non-invasive selection for euploid embryos: prospects and pitfalls of the three most promising approaches

BIOGRAPHY

Santiago Munné developed the first preimplantation genetic testing for aneuploidy (1993), and pre-implantation genetic testing for chromosomal structural rearrangements, demonstrating that preimplantation genetic testing reduces miscarriages and increases implantation (1994, 1995, 1996 and 1998 SART prizes). He is an Adjunct Professor at Yale University (USA), has published >260 scientific publications, and is a co-founder of Reprogenetics (2001), Recombine (2011), MedAnswers (2013), Phosphorous (2016), Overture Life (2017) and HoMu inventis (2020).

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KEY MESSAGE

Three technologies were evaluated as alternatives to preimplantation genetic testing for aneuploidy (PGT-A). Artificial intelligence was found to be superior to morphological selection, but inferior to PGT-A. Non-invasive PGT-A has the potential to reach 100% PGT-A concordance. Metabolomics has the potential to identify euploid embryos that, metabolically, are incapable of implanting. Combining two or all these approaches is possible.

ABSTRACT

The objective of this review was to evaluate the efficacy of three promising technologies for assessment of ploidy status in IVF embryos [i.e. preimplantation genetic testing for aneuploidy (PGT-A)]: artificial intelligence (AI), non-invasive PGT-A (niPGT-A) and metabolomics. Publications where >80% correlation with blastocyst biopsies could be demonstrated in ≥50 cycles were prioritized. AI was found to classify the chance of an embryo implanting with an average area under the curve (AUC) of 0.7. AI is thus a superior selection method compared with morphological selection alone, but is still inferior to invasive PGT-A. Some niPGT-A studies have up to 100% concordance with PGT-A, but a multicentre study showed 78% concordance due to maternal contamination, which can improve with specific changes in culture conditions. niPGT-A has thus improved significantly and has the potential to reach 100% with PGT-A if the issue of maternal contamination is solved; however, >30% of euploid embryos never implant. Finally, metabolomics is the least developed technique of the three, but some preliminary data show >90% concordance with implantation and with PGT-A without changing culture conditions. Metabolomics thus has the potential to identify euploid embryos that, metabolically, are incapable of implanting. A combination of two or all of these approaches is possible.

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Declaration: Overture Life is developing metabolomic methods and tests, and Joson Horcajadas and Santiago Munne are shareholders. Life Whisperer is developing AI methods and products, and Michelle Perugini is a shareholder.

KEY WORDS

Pre-implantation genetic testing
Non-invasive pre-implantation genetic testing
Morphology
Morphokinetics
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INTRODUCTION

Conventional morphological evaluation, grading and selection of IVF embryos is currently considered to be the standard of care for IVF cycles worldwide. Despite this, IVF success rates for singleton births are still relatively low, at 30.8% per embryo transfer for women aged 35–37 years and approximately 50% overall (*SART National Summary Report, 2020*). Preimplantation genetic testing for aneuploidy (PGT-A) is applied in nearly 60% of all IVF cycles in the USA (*Munné and Griffin, 2024*). However, concerns remain regarding the outcomes of randomized controlled trials, the possible confounding effects of chromosome mosaicism, and fears that the biopsy process itself may impede the developmental potential of the embryo. As such, there are increasing calls for non-invasive approaches that are as effective as, or at least nearly as effective as, biopsy-based PGT-A. The three most advanced are machine vision facilitated by selection using artificial intelligence (AI), non-invasive PGT-A (niPGT-A) and metabolomics.

EMBRYO SELECTION USING ARTIFICIAL INTELLIGENCE

The introduction of time-lapse imaging (TLI) technology allowed for uninterrupted embryo culture and precise control of physiological conditions in the IVF laboratory. Furthermore, it empowered embryologists with the opportunity to evaluate tiny features of development without taking the embryos in and out of the incubator, thereby potentially improving embryo selection methodologies (*Hinojosa et al., 2021*), and thus the outcomes of assisted reproductive technology (*Armstrong et al., 2019*), if coupled with morphokinetic algorithms (*Alikani et al., 2018*; *Armstrong et al., 2019*; *Fishel et al., 2017*, *2018*, *2020*; *Pribenszky et al., 2017*). Progress in the field was reviewed recently by Bamford et al. (2022) in a systematic review and meta-analysis of 58 studies with >40,000 IVF embryos. They identified 10 morphokinetic variables that were delayed in chromosomally abnormal conceptuses. However, the authors were at pains to point out that the degree of variability between euploid and aneuploid embryos makes definitive classification impractical. Nonetheless, as a ranking tool, these criteria may find utility; they do, at least, suggest that aneuploid embryos are slower to divide.

The application of AI in the fertility sector has advanced significantly in recent years, with a significant body of research reporting it for oocyte assessment, sperm selection, optimization of patient stimulation protocols, donor matching, and embryo assessment (*Zaninovic et al., 2019*). The present review will focus on embryo selection with specific reference to the detection of aneuploidy. Morales et al. (2008) first applied Bayesian classifiers to a combination of clinical characteristics and features of embryo morphology to predict implantation outcome. However, more recent studies have described AI models trained specifically for embryo grading, aneuploidy assessment, and pregnancy and live birth prediction (*Curchoe and Bormann, 2019*). While each of these AI models can, potentially, influence embryo selection practices, it is essential to consider the data on which the AI have been trained, and how they relate to the primary intended purpose of that AI in clinical practice. Two studies (*Goodman et al., 2016*; *Storr et al., 2017*) described, and provided a powerful proof of principle, a convoluted neural network to differentiate, with >90% accuracy, between 2PN and abnormal zygotes 18 h post-fertilization. Zhao et al. (2021) demonstrated morphokinetic assessment of zygote cytoplasm, zona pellucida and pronuclei. *Khosravi et al. (2019)* employed a deep learning algorithm in static images to assess blastocyst quality, with expansion rates, inner cell mass (ICM) quality and trophectoderm quality prioritized, leading to a blastocyst ranking scheme called the 'BL score'. *Kragh and Karstoft (2021)* developed a reproducible manner of assessing embryo quality, similar to that of an embryologist, but with the AI demonstrating improved correlation between predicted embryo quality and implantation. *Conaghan et al. (2013)* and *Petersen et al. (2016)* asked whether blastocyst development on day 5 can be predicted using day 3 morphokinetic information. However, utility for this is limited in IVF clinics where day 5 transfers are the norm.

Crucial factors to consider in AI development include the type and number of input variables; these can vary widely from a single embryo image with associated clinical outcome data, to other embryo-related variables such as age post-fertilization, morphokinetic milestones, or manually determined morphological features. They can include patient-specific parameters such as maternal age, medical conditions affecting fertility, and/or prior implantation failure. While patient-specific parameters, particularly maternal age, are known to influence IVF outcome significantly, knowledge of these parameters is unable to improve AI performance when applied to the task of embryo selection, as they (by definition) affect all embryos in any given IVF cycle equally. Such a phenomenon is a critical consideration when developing and comparing AI for embryo selection. It is reassuring to observe that the AI fertility community is beginning to delineate between true embryo selection and prediction of IVF outcome (*Kragh and Karstoft, 2021*). It is also important to consider the testing paradigm: Of what does the test dataset consist? Is the ground truth outcome known for every record? What is the 'ground truth' (for instance, it could be live birth outcome or aneuploid versus euploid outcome following PGT-A)? There have been examples of embryo selection AI tested on datasets containing discarded embryos (*Huang et al., 2022*) that were labelled as a negative outcome (including non-viable, no live birth). It cannot be said with certainty, however, that none of these embryos would have led to a successful outcome, as there are clearly examples of poor-quality embryos leading to pregnancy (*Kemper et al., 2021*). While it may be tempting to increase the test dataset size by including these embryos, the performance on such test sets becomes artificially high, with receiver operating characteristic area under the curve (ROC-AUC) values of >0.9 quoted for such datasets (compared with approximately 0.6–0.7 on datasets where every outcome is known). This is because it tends to be easier for AI to differentiate between very-good-quality and very-poor-quality embryos. AI trained on such datasets may also demonstrate reduced capacity to aid in selecting from among embryos of relatively good quality, which remains the primary goal of any embryo selection method. At this point, it may be useful to define ROC-AUC in more detail: ROC-AUC is a metric that measures the performance of a classifier across all possible classification thresholds, applied to measure the performance of machine learning algorithms and calculated directly by measuring the area under the ROC curve. It is commonly used in classification tasks, plotting the true-

positive rate (sensitivity) against the false-positive rate at different threshold settings. A higher ROC-AUC value indicates better performance, with a perfect model having an AUC of 1, and a random model having an AUC of 0.5.

A key technical consideration therefore is 'What is actually being tested?' Is it the ability of the AI to predict whether a single embryo will or will not have a particular clinical outcome? Or is it the ability of the AI to rank all embryos in a cohort according to the likelihood of the desired outcome? Binary prediction models have a relatively limited contribution to the problem of embryo selection, and yet many studies still focus on accuracy, sensitivity and so forth. ROC-AUC is somewhat more appropriate to the issue of embryo ranking, as it evaluates binary accuracy metrics at every possible threshold (*Florkowski, 2008*), giving some idea of AI performance for this indication. However, a select few studies have gone to considerable lengths to develop a paradigm that mimics the real-world clinical use of these AI, which is to rank and select the best embryo from the available patient cohort. *VerMilyea et al. (2023)* devised a novel simulated cohort ranking approach, where simulated patient 'cohorts' consisting of embryos from different patients were ranked by the AI according to likelihood of clinical pregnancy. This was used to demonstrate that their viability AI could potentially reduce the time to pregnancy by up to 12% compared with standard morphological grading methods. The same group applied a similar simulated cohort approach to evaluate the ranking ability of their genetics AI for evaluating the likelihood of embryo euploidy (*VerMilyea et al., 2023*). The AI was able to select a euploid embryo as the top-ranked embryo in 82% of cohorts, which was 26% better than selecting an embryo at random from each cohort. *Chavez-Badiola et al. (2020)* utilized a normalized discontinued cumulative gain (NDCG) ranking metric system, previously published by *Järvelin and Kekäläinen, (2002)*. The metric uses a weighted relevance scale based on the position in the list to evaluate the quality of a ranking. They used NDCG to show that their genetics AI algorithm ranked a euploid embryo first in 79% of cohorts — a similar performance value to that of *VerMilyea et al. (2023)*.

The widespread introduction of TLI into IVF laboratories sparked the expansion of algorithms based on morphokinetic input to predict blastocyst development. Early commercialized AI based on decision trees included both Eeva (Early Embryo Viability Assessment system; Merck, USA) and KIDScore (Known Implantation Data Score; Vitrolife, Sweden). Embryologists are required to input selected kinetic milestones manually, such as pronuclear assessment, pronuclear fading, and time to two, three, four, five and eight cells. The algorithms then provide a score indicating the likelihood of blastocyst development at day 5. Eeva was shown to improve embryologists' selection of embryos that would reach blastocyst stage by combining day 3 morphological characteristics with the output of the Eeva system (*Conaghan et al., 2013*; *Diamond et al., 2015*). KIDScore was shown to predict blastocyst development with an ROC-AUC of 0.75 and, interestingly, could also predict implantation following day 3 transfer with an ROC-AUC of 0.65 (*Petersen et al., 2016*). The advent of more advanced deep learning methodologies has, however, turned the focus towards automated analyses based on image analysis.

There have been relatively few studies published to date describing AI algorithms for predicting blastocyst formation using imaging information obtained during the first few days of embryo development. *Liao et al. (2021)* developed two independent AI algorithms — one for prediction of blastocyst formation (STEM), and another for prediction of usable blastocyst formation (STEM+), both using time-lapse image series information obtained through to day 3 post-fertilization. Usable blastocysts were defined by embryologist evaluation with no specific grading criteria. The accuracies were 78.2% (ROC-AUC 0.82) and 71.9% (ROC-AUC 0.79) for the two algorithms respectively (*Liao et al., 2021*). *Coticchio et al. (2021)*, using data from multiple centres in Italy, described development of an AI algorithm for prediction of blastocyst development based on a single image at day 2 of development, together with time-lapse image series-based detection of cytoplasmic movement. When used in conjunction with embryologist assessment, the overall accuracy for predicting blastocyst formation was 83% (*Coticchio et al., 2021*).

When considering classifying (grading) blastocysts, a group from Cornell University developed an AI called STORK to identify blastocyst grade automatically according to a modified version of the Gardner and Schoolcraft scoring system (*Khosravi et al., 2019*). This AI showed very high accuracy of 97.5% for identification of good-quality embryos versus poor-quality embryos using time-lapse images obtained from the EmbryoScope system. Embryos labelled as 'fair quality' were excluded from the analysis, perhaps limiting the usefulness of the algorithm for these intermediate morphology embryos where guidance is often most valuable. A similar algorithm was developed by the IVI-RMA fertility group of Valencia, Spain, for automatic identification of good-quality blastocysts according to Spanish ASEBIR criteria. The AI was able to identify good-quality embryos by analysis of TLI series with accuracy of 80.0% at day 5 (*Payá et al., 2022*). Additional publications have described AI algorithms to identify or grade key morphological components and morphokinetic features of embryos based on analysis of standard or time-lapse images (*Kragh and Karstoft, 2021*). These approaches may be useful to assist with automated embryo grading methods. A recent study by *Illingworth et al. (2024)* reported a 10x increase in the speed of scoring embryos with AI compared with manual scoring. While these types of algorithms may be useful for standardizing embryo grading according to conventional morphological parameters, they are limited in their utility for selecting embryos based on the ultimate clinical outcomes of pregnancy and live birth. AI algorithms trained to replicate conventional grading methods to a high degree of accuracy may be useful for automation and standardization of grading, but they can, at best, only be as accurate as the grading method(s) itself. Perhaps the most advanced of the AI categories developed for embryo selection are those that have focused on predicting pregnancy. Here, it is important to note that clinical pregnancy can be measured using different parameters, which, although often used interchangeably, indicate slightly different measurements. These include blood levels of human chorionic gonadotrophin, the presence of gestational sacs, and the detection of fetal heartbeat(s) at first ultrasound.

TABLE 1 summarizes AI algorithms predicting pregnancy outcomes from embryo images using deep learning. Studies reporting on test datasets where the ground truth outcome was not known for all records were excluded (this included studies with multiple embryo transfers where the

TABLE 1 PERFORMANCE SUMMARY OF ARTIFICIAL INTELLIGENCE ALGORITHMS FOR PREDICTING IMPLANTATION

AI name (group)	Input	Output (ground truth)	Test dataset size (embryos)	Ploidy status	Performance	References
Embryo Predict (Alife)	*Standard images + patient age*	*Fetal heart- beat*	*~2600*	*Known euploid versus untested*	*ROC-AUC: 0.62–0.64* *ROC improvement over embryologists: 3–17%* *Accuracy: ND* *Accuracy improvement over embryologists: ND*	*Loewke et al., 2022*
FiTTE (Hanabusa Women's Clinic, Japan)	*Standard images*	*HCG + fetal heart-beat*	*~1400*	*Not stipulated*	*ROC-AUC: 0.68* *ROC improvement over embryologists: 5–10%* *Accuracy: 62.7%* *Accuracy improvement over embryologists: 5%*	*Enatsu et al., 2022*
Life Whisperer Viability (Life Whisperer)	*Standard images (or time-lapse still images)*	*Fetal heartbeat*	*~500–1200*	*Mixed euploid and untested*	*ROC-AUC: 0.61–0.68* *ROC improvement over embryologists: ND* *Accuracy: 61–64%* *Accuracy improvement over embryologists: 25%*	*Diakiw et al., 2022b; VerMilyea et al., 2020*
Unnamed AI (University of Miami)	*Standard images*	*Gestational sacs and/or fetal heartbeat*	*36*	*Not stipulated*	*ROC-AUC: 0.66* *ROC improvement over embryologists: 6%* *Accuracy: ND* *Accuracy improvement over embryologists: ND*	*Geller et al., 2021*
CHLOE EQ (Fairtility)	Time-lapse video (EmbryoScope and/or EmbryoScope+)	Gestational sacs	~1000	Mixed euploid and untested	ROC-AUC: 0.68–0.70 ROC improvement over embryologists: 9% Accuracy: ND Accuracy improvement over embryologists: ND	Erlich et al., 2022
iDAscore (Vitrolife)	Time-lapse video (EmbryoScope and/or EmbryoScope+)	Fetal heartbeat	~2200–3000	Mixed euploid and untested	ROC-AUC: 0.67–0.70 ROC improvement over embryologists: 5% Accuracy: ND Accuracy improvement over embryologists: ND	Berntsen et al., 2022; Ueno et al., 2021
Ubar (Embryonics)	Time-lapse video (EmbryoScope and/or EmbryoScope+)	Gestational sacs	272	Untested	ROC-AUC: 0.70 ROC improvement over embryologists: 15% Accuracy: 63% Accuracy improvement over embryologists: 20%	Fordham et al., 2022
Unnamed AI (Harvard University, USA)	Time-lapse still images (EmbryoScope and/or EmbryoScope+)	Not stipulated	97	Euploid only	ROC-AUC: ND ROC improvement over embryologists: ND Accuracy: 75% Accuracy improvement over embryologists: 12%	Bormann et al., 2020
Unnamed AI (Vitrolife)	Time-lapse video (EmbryoScope and/or EmbryoScope+)	Fetal heartbeat	287	Not stipulated	ROC-AUC: 0.66 ROC improvement over embryologists: 3% Accuracy: ND Accuracy improvement over embryologists: ND	Kragh et al., 2019
iDAScore (Vitrolife)	Time-lapse video (EmbryoScope and/or EmbryoScope+)	Fetal heartbeat, OPR and live birth	533 in each arm	Not stipulated	No difference found: clinical pregnancy rates were 46.5% for AI versus 48.2% for the manual arm	*Illingworth et al., 2024*

Static images are in italic type.

AI, artificial intelligence; ROC-AUC, receiver operating characteristic area under the curve; ND, not documented; OPR, ongoing pregnancy rate.

outcome for every transferred embryo could not be inferred). Some studies evaluated AI versions with and without additional clinical parameters — where possible, performance results for AI versions using images alone as input are quoted. A total of nine AI algorithms were identified. In general, the ROC-AUC ranged from approximately 0.6 to 0.7 for predicting pregnancy, with improvement over embryologists (using conventional morphological grading methods) ranging from approximately 0% to 15%. Where presented, binary predictive accuracy ranged from approximately 60% to 75%, representing a 0–15% improvement over embryologists. Many of the studies were performed using appropriately large test datasets; however, in some cases, the test datasets were <300 embryo images in total, with two assessed on <100 embryo images. Care should be taken, however, when interpreting results presented on small dataset sizes. The studies presented here did not consistently report whether embryos were tested by PGT-A, which may influence performance outcome. Additional testing of performance for known euploid embryos versus untested embryos is required. While it is difficult to directly compare AI algorithms tested on different datasets, these observations are helping to build a realistic expectation of AI performance for predicting pregnancy outcome from embryo images. Five of the nine AI algorithms were developed using images from the Embryoscope system or similar, limiting the use of these AI in clinical practice to laboratories that own an Embryoscope system. Two AI algorithms have shown the ability to work across both time-lapse and non-time-lapse systems, making them scalable to different laboratory environments and providing an opportunity to standardize embryo assessment across multiple imaging systems without further investment in imaging equipment (*Dimitriadis et al., 2019*; *VerMilyea et al., 2023*). However, further validation is needed to compare the performance of time-lapse- with non-time-lapse-based AI algorithms. While the applicability of AI developed simply to mimic known morphological evaluation schemes is somewhat limited in this context, it may be useful to characterize what AI algorithms for predicting pregnancy are focusing upon. Several groups began preliminary correlative and feature detection analyses for these AI, such as the investigation of iDAscore correlation with blastocyst morphokinetic parameters (*Ezoe et al., 2022*).

Investigations involving Embryo Predict and Life Whisperer Viability algorithms detected morphological components known to be associated with clinical outcome (ICM, trophectoderm) (*Conaghan et al., 2013*; *VerMilyea et al., 2023*). It may not ever be feasible to fully characterize how these AI are functioning or the biological significance of each feature being detected, but this additional knowledge can aid in understanding and building trust in AI for use in the IVF clinical setting.

To assess the value of deep learning in selecting the optimal embryo for IVF, *Illingworth et al. (2024)* conducted a multicentre, randomized, double-blind, non-inferiority parallel group trial across 14 IVF clinics in Australia and Europe. Women aged <42 years and who had at least two early-stage blastocysts (when examined on day 5 post-fertilization) were randomized to two study arms. The first was the control arm (533 patients), using standard assessment, and the second (also 533 patients) employed iDAScore (see above) to select the embryos. The study endpoint that was measured was clinical pregnancy rate (non-inferiority margin 5%). The control arm had 248 patients (46.2%) that became pregnant, compared with 257 (48.2%) patients in the test iDAScore arm. The statistical analysis reported a risk difference of -1.7% (95% CI -7.7 to 4.3; P=0.62). The curious phrase 'this study was not able to demonstrate noninferiority of deep learning for clinical pregnancy rate when compared to standard morphology and a predefined prioritization scheme' contained within their conclusions basically means that they reported no difference using iDAScore.

Several groups have published articles describing AI algorithms for analysis of embryo images to specifically predict live birth outcome. The first of these was a relatively early study (*Manna et al., 2013*) describing application of various AI deep learning techniques using a database of 269 embryos from 104 couples. Depending on the neural network methods used, ROC-AUC was between 0.65 and 0.83. While these pilot results are promising, the study did not differentiate between single and multiple embryo transfers, so ground truth outcome (in this case, the ground truth was 'was there a live birth?') was only known for 162 of the 269 embryos. In other studies, it is not always clear whether these were time-lapse based or used standard microscope mounted imaging systems. The accuracy for the first AI developed using a test set of 1139 images was 76.3%, with an ROC-AUC of 0.661 (*Miyagi et al., 2019a*). Despite the relatively high performance, this was not found to be significantly different to conventional embryo evaluation using a number of specific clinical and morphokinetic parameters (accuracy 74.0%, ROC-AUC 0.723). The authors went on to develop another AI deep learning algorithm combining image analysis with known clinical and morphokinetic parameters for prediction of live birth, which improved accuracy to 73.3% and ROC-AUC to 0.745 (*Miyagi et al., 2019b*). Performance increased incrementally with increasing patient age, likely due to reduced confounding medical factors in the older patient age groups. While live birth outcome is the gold standard for determining IVF success, it is heavily influenced by compounding factors outside of embryo quality (e.g. chronic health conditions such as diabetes, infections, weakened cervix, etc.) (*García-Enguídanos et al., 2002*). More accurate algorithms for predicting live birth may be developed by incorporating patient demographic data and clinical data as additional input. As described previously, however, these then become algorithms for overall prediction of IVF outcome, rather than useful tools for embryo selection. AI algorithms predicting the likelihood of pregnancy may therefore be of more clinical utility for embryo selection than those predicting live birth.

AI for the prediction of an embryo's genetic status is perhaps a standalone category in this field as it is not directly comparable to morphological selection. Prediction of euploid versus aneuploid embryos from static images may never reach the same level of accuracy or sensitivity of conventional PGT-A methodologies based on DNA sequencing; it nonetheless possesses alternative benefits over traditional PGT-A methodologies. That is, it is non-invasive, not subject to sample bias, provides results instantly, and may perhaps be less susceptible to issues associated with the phenomenon of embryo self-correction (*Rosenwaks et al., 2018*). To this end, several groups have published AI for predicting embryo ploidy, three of these using time-lapse-based input and two using standard microscope-mounted imaging systems (static images). The ERICA (Embryo Ranking Intelligent Classification Algorithm) system utilizes standard images,

and has been reported to have accuracy of 70% for predicting ploidy, with an ROC-AUC of 0.74 (*Chavez-Badiola et al., 2020*). The authors reported that ERICA correctly identified and ranked embryos more successfully than experienced embryologists for implantation potential levels. However, the test set in this study was relatively small, consisting of only 84 images obtained from 19 cycles. *Chavez-Badiola et al. (2020)* also established that there was a relationship between using ERICA and predicting the chances of pregnancy loss (of which aneuploidy is the leading cause), with accuracy of 67.4%. The Life Whisperer Genetics system was similarly trained utilizing standard microscopy images, demonstrating an ROC-AUC of 0.68 on a hold-back blind test dataset of 1001 images. Accuracies for predicting ploidy ranged from 60% to 80% depending on the test dataset evaluated (*VerMilyea et al., 2023*). This AI was assessed on independent test datasets comprising standard microscopy images and still images sourced from time-lapse imaging systems, demonstrating the generalizability of this algorithm. Two AI algorithms utilized Embryoscope time-lapse video as input. These reported similar performance to the AI developed using standard images — the first of these, EMA, demonstrated accuracy of 70% (*Bori et al., 2021*), and the second, developed by a group at Chung Shan University in Taiwan, demonstrated an ROC-AUC of 0.74 (*Lee et al., 2021*). However, the AI algorithm described by Chung Shan University was evaluated on a relatively small dataset of 138 images. STORK-A (*Barnes et al., 2023*) — see above — utilized single, static images extracted from the Embryoscope system at 110 h post-fertilization, together with maternal age and manually determined morphokinetic parameters. This AI was shown to generalize well over independent test datasets, with accuracies of 63–68% and ROC-AUC of 0.72–0.74. If, following PGT-A, all cells return an aneuploid result, the likelihood is that the perpetuating error is meiotic and a live birth will not ensue; alternatively, if a mosaic aneuploidy result is returned, the likelihood is that the perpetuating error was post-zygotic (mitotic), and thus the embryo would have a good chance of survival. AI algorithms should ultimately be able to discriminate between these.

Bamford et al. addressed the issue of machine learning models designed to predict ploidy status (in this case, where all cells biopsied are aneuploid, rather than a mosaic result). *Bamford et al. (2023)* asked whether such approaches were better than regular methods using morphokinetic and clinical biodata to predict chromosome abnormalities in blastocysts. Their findings in 8147 embryos (from 1725 patients) were that a mixed effects logistic regression model was superior, particularly when addressing the issues of morphokinetic timings and their relationships to aneuploidy. The main findings were that the likelihood of euploidy was significantly increased in correlation with the degree of expansion of the blastocyst ($P < 0.001$), and also with the trophectoderm grade ($P < 0.01$). In contrast, there was no correlation between aneuploidy level and cleavage stage fragmentation, nor morula fragmentation, morula grade, fertilization approach [IVF versus intracytoplasmic sperm injection (ICSI)], sperm concentration, progressive motility of spermatozoa, or multinucleation (two- to four-cell stage). In that study, the ROC-AUC was 0.61 for predicting the presence of chromosome abnormality, improving with increasing maternal age. Moving on to associating ploidy prediction by morphokinetics with pregnancy loss and live birth outcomes, in a multicentre cohort study, *Bamford et al. (2023)* concluded that the risk scores that they calculated were significantly associated with live birth and pregnancy loss. Finally, *Bamford et al. (2024)* asked whether morphokinetic models were better at prioritizing euploid blastocysts for transfer over embryologist-driven morphological selection alone. Here, their model 'PREFER' (Predicting Euploidy for Embryos in Reproductive Medicine) associated with pregnancy loss live birth outcome established that PREFER, and another model ('LB') predicted a euploid embryo 47% and 46% of the time, respectively, compared with a trained embryologist 39% of the time and a random selection 37% of the time. The ROC-AUC for the PREFER and LB models were 0.63 and 0.62, respectively. They concluded that morphokinetic algorithms lead to improved prioritization of euploid embryos compared with the subjective judgement of the embryologist, but use of the PREFER and LB models was far from 100% predictive of aneuploidy. These studies are promising for the use of AI algorithms in predicting ploidy status, with feasibly expected performance values of approximately 65–70% accuracy and approximately 0.70–0.75 ROC-AUC. Given that the primary purpose for identifying embryo ploidy is to improve clinical outcomes rather than to identify genetic disease definitively [being improved implantation potential, reduced miscarriages, increased likelihood of live birth (*Sciorio and Dattilo, 2020*)], these studies also provide evidence that such AI are likely to provide clinical utility in the IVF laboratory. *Xin et al. (2024)* presented a systematic review and meta-analysis of the value of AI algorithms starting with images of embryos for non-invasive aneuploidy detection. They identified 20 studies, with 12 of these (60%) going forward to the meta-analysis. The pooled results for sensitivity, specificity and ROC-AUC of AI for predicting embryonic euploidy indicated values of 0.71 (sensitivity), 0.75 (specificity) and 0.80 (ROC-AUC), with a total of 6879 embryos (3769 aneuploid, 3110 euploid) analysed. They concluded that AI algorithms had a somewhat promising performance in the ability to predict embryo ploidy, but short of that provided by PGT-A.

NON-INVASIVE PGT OF EMBRYONIC CELL-FREE DNA

Embryonic cell-free DNA was first detected in the blastocoele fluid of blastocysts by *Palini et al. (2013)*, and in spent culture media (SCM) by *Xu et al. (2016)* and *Shamonki et al. (2016)*. The amount of DNA in blastocoele fluid and SCM ranges from 3 to 36 ng/μl, compared with 25–46 ng/μl in trophectoderm biopsies. However, there is more DNA in frozen-thawed SCM (15 ng/μl) than in fresh SCM (22 ng/μl), as some cells lyse during the thawing process (*Jiao et al., 2019*). The origin of SCM DNA is multifactorial and still not fully determined. *Handayani et al. (2023)* suggested that the apoptotic pathway had been shown to remove aneuploid cells in developing mosaic embryos, and this might culminate in the release of cell-free DNA into the SCM. Apoptosis or controlled cell death would result in DNA fragments of a consistent size — multiples of 180–200 bp (*Zhang et al., 2016*) — meanwhile DNA from lysed or necrotic cells would have an irregular DNA fragment size. Indeed, it seems that DNA found in the blastocoele fluid is mostly of apoptotic origin as the DNA fragment sizes are 160–200 bp and 300–400 bp (*Zhang et al., 2016*), and some cells from both the ICM and trophectoderm cells go apoptotic during preimplantation development (*Hardy, 1999*; *Hardy et al., 1989*). On the other

hand, spent media DNA fragments are generally large and likely not apoptotic, but necrotic, or from other sources (*Kuznyetsov et al., 2020*). *Handayani et al. (2023)* also highlighted the need to explore other pathways including microDNA (60–2000 bp) and that of the cross-talk molecules known as extracellular vesicles (comprising tiny spherical objects with bilayered membranes). These are actively and physiologically expelled, and contain protein, microRNA and embryonic DNA. They are used for communication between cells that can traverse the zona pellucida (*Vyas et al., 2019*). There seems to be, in addition, more DNA in SCM in aneuploid embryo cultures compared with euploid embryo cultures, resulting in a higher karyotype concordance rate between SCM and blastocoele fluid with the trophectoderm (*Hammond et al., 2017*; *Hanson et al., 2021*; *Magli et al., 2019*). The implication is therefore that aneuploid embryos have a more active process of apoptosis and p53-mediated autophagy of abnormal cells (*Singla et al., 2020*).

Recovery of DNA from the blastocoele as a means of PGT-A, while not completely non-invasive, is minimally invasive compared with trophectoderm biopsy. *Magli et al. (2019)* is perhaps the seminal work in this area; they found that whole-genome amplification rates were significantly higher in blastocoele fluid from aneuploid blastocysts compared with chromosomally normal, finding concordance of 93.6% with trophectoderm biopsy results. *Campos and Nel-Themaat (2024)* recently reviewed the utility of blastocoele fluid as a source of DNA PGT (as well as a biomarker for embryo competence). Short DNA fragments can be combined with particular expression patterns of pro- and anti-apoptotic genes. Comparing the blastocoele fluid of aneuploid with aneuploid embryos provides evidence of a self-correcting mechanism that eliminates aneuploid cells from the embryo proper, thereby ejecting abnormal, poorly functioning cells. *Griffin et al. (2023)* took this a step further, providing evidence that chromosome abnormalities previously found at cleavage stage were seen at a higher frequency in the blastocoele fluid (and apoptotic cells at the periphery of the embryo) compared with the trophectoderm and ICM. The chromosomal comparison of blastocoele fluid with the whole embryo warrants further investigation and is by no means 100%, given the apoptotic origin.

Blastocoele fluid may nonetheless be used as a clinical biomarker for the competency of the embryo to prioritize those for transfer, and thus possibly improve implantation rates. These conclusions largely reflected those of *Hammond et al. (2016)*, who highlighted the consequences of contamination by mitochondrial and maternal DNA as well as the fragmented nature of the DNA. *Madjunkova et al. (2019)* asked whether blastocyst morphology influences the concentration and integrity of cell-free DNA recovered from blastocoele fluid, and concluded that it was not. Given the uncertainty of the diagnostic value of blastocoele fluid (and the semi-invasive nature of blastocentesis), attention has largely turned to SCM, with one of the key concerns being maternal DNA contamination.

A large source of SCM DNA is from maternal contamination from corona cells. Analysing methylation patterns, *Chen et al. (2021)* differentiated trophectoderm, ICM, polar body and cumulus cell DNA, determining that up to 50% of samples were contaminated with corona cell DNA and 28% with polar body DNA. Karyotype concordance between SCM and trophectoderm decreases with contamination (47% in contaminated versus 74% in uncontaminated), but day 6 embryos produced more DNA in the SCM and thus had higher karyotype concordance with the trophectoderm. On the other hand, there is no DNA contamination from spermatozoa, as the tightly packed sperm DNA does not denature during the sequencing process (*Xie et al., 2022*), and therefore a similar amount of maternal contamination is found in both ICSI and IVF procedures (*Rubio et al., 2019*; *Xie et al., 2022*). Another source of DNA could come from the culture media itself. DNA was detected in some types of unused culture media coming from protein supplements (*Hammond et al., 2016*). In order to minimize contamination, stringent corona cell denudation is recommended, although this process could harm the egg, followed by day 3 culture media changeover. Several companies have developed devices for automated denudation (*Guerrero et al., 2020*). Alternatively, some PGT platforms that also detect parent of origin in the DNA can detect the presence of maternal DNA contamination (e.g. the PGT complete test of CooperSurgical, CT or Reprobiogen Genetics), and determine the correct ploidy by phasing the different parental DNA complements present in the sample. Another strategy that has been followed is to culture the embryos to day 6 because there is more DNA in the SCM from the embryo and less chance of misdiagnosis due to maternal DNA contamination (*Chen et al., 2021*; *Christopikou et al., 2021*; *Jiao et al., 2019*; *Li et al., 2021*). However, vitrification of day 6 expanded blastocysts is challenging, and might be detrimental for embryo survival. In this case, the 'non-invasive' nature of the process is questionable.

TABLE 2 shows published studies examining the concordance between niPGT-A and trophectoderm biopsy, ICM or whole embryo analysis. Apart from *Rubio et al. (2019)*, which was a large multicentre study, all the others are small or single-centre studies. Not surprisingly, the best results were obtained in those small highly controlled studies that reach up to 100% concordance (*Chen et al., 2021*; *Madjunkova et al., 2019*). However, these do not take into account real-world variability in culture sampling, whereas *Rubio et al. (2019)* did.

Some of the studies combined the analysis of SCM with blastocoele fluid. Others sampled the media after embryo thawing. Both blastocoele fluid sampling and embryo thawing/warming boost the amount of embryonic DNA in the media, but their invasiveness is questionable. Some studies sampled the media on day 5 and others on day 6. **FIGURE 1** shows that freeze-thaw culture media has 94% concordance with the embryo, SCM collected between day 4 and day 6 has 83% concordance with the embryo, SCM collected between day 3 and day 5 has 71% concordance with the embryo, and SCM collected between day 1 and day 6 has 75% concordance with the embryo.

All the above studies were retrospective and differ significantly from the standard laboratory protocol used by most centres. *Hanson et al. (2021)* performed a prospective non-selection study in which SCM was collected before embryo vitrification and before embryo biopsy (two procedures that may increase DNA in the culture media). The Yikon niPGT test was compared with targeted next-generation sequecing of the trophectoderm biopsy. They found that there was 15% amplification failure in SCM samples collected on day 5, and 0% amplification failure in those collected on day 6. Ploidy concordance was unacceptably low, with

TABLE 2 KARYOTYPE CONCORDANCE BETWEEN NON-INVASIVE PREIMPLANTATION GENETIC TESTING FOR ANEUPLOIDY AND TROPHECTODERM BIOPSY PREIMPLANTATION GENETIC TESTING FOR ANEUPLOIDY OR WHOLE EMBRYO ANALYSIS

Studies	Amplification method	Sample	Days sampled	*n*	Amplify	Concordance	
						versus	Euploidy, aneuploidy
Rubio et al., 2020	Reproseq	SM	D4–D6	1108	89%	TE	78%
Xu et al., 2016	MALBAC	SM	D3–D5	42	100%	TE	86%
Navarro-Sanchez, 2021	Reproseq	SM	D4–D6	117	92%	ICM	86%
Li et al., 2021	MALBAC	BF+SM	1 day post thaw	41	95%	WB	87%
Christopikou et al., 2021	PerkinElmer PG Seq	SM	1 day post thaw	45	94%	WB	93%
Huang et al., 2019	MALBAC	SM	1 day post thaw	48	92%	WB	94%
Babariya et al., 2018	MDA	SM	D3–D5	118	90%	TE	94%
Lane et al., 2017	DOPlify	SM	D4–D5	178	94%	TE	95%
Kuznyetsov et al., 2018	Sureplex	BF+SM	1 day post thaw	28	100%	WB	96%
Kuznyetsov et al., 2020	Sureplex	BF+SM	D4–D5	91	100%	TE	96%
Jiao et al., 2019	MALBAC	BF+SM	1 day post thaw	62	100%	WB	97%
Madjunkova et al., 2019	Sureplex	BF+SM	D4–D6	71	100%	TE	100%
Chen et al., 2021	MALBAC	SM	1 day post thaw	26	100%	ICM	100%
Ardestani et al., 2024	Reproseq	SCM	1 day post thaw	135	100%	WB	92.3%

Only papers reporting ≥85% concordance with $n \geq 25$, or papers with large *n*.

SM, spent media; BF, blastocoele fluid; TE, trophectoderm biopsy; ICM, inner cell mass; WB, whole blastocyst.

only 60% of SCM coinciding with trophectoderm biopsy, which would imply that 13% of viable euploid embryos would be discarded.

A recent prospective randomized trial from *Sun et al. (2024)* addressed this question. It consisted of three arms: a niPGT arm, a biopsy PGT arm and a control arm. Studying >400 embryos in total showed cumulative live birth rates of 44.9%, 51% and 27.9%, respectively, with both PGT arms being significantly better

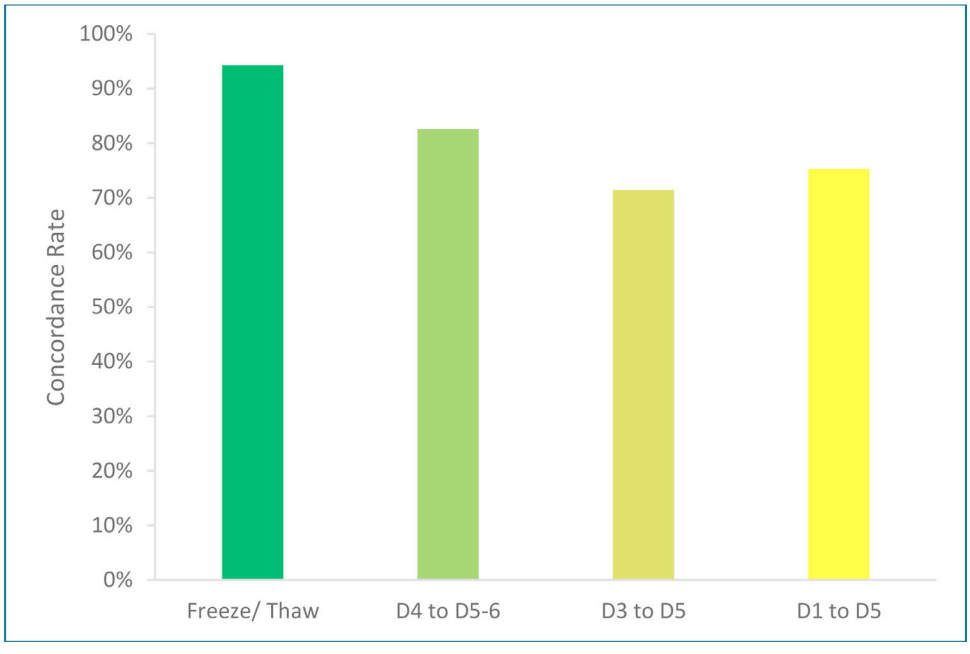

FIGURE 1 Concordance rate between non-invasive preimplantation genetic testing for aneuploidy and trophectoderm or whole embryo biopsy in relation to time of sampling. Freeze–thaw studies included 250 samples from Kuzyetsov et al. (2018), *Huang et al. (2019)*, *Jiao et al. (2019)*, *Chen et al. (2021)*, *Ardestani et al. (2024)*, *Li et al. (2021)* and *Christopikou et al. (2021)*. Day 4 to day 5–6 (D4 to D5–6) included 1565 samples from Lane et al. (2017), Majunkova et al. (2019), Navarro-Sanchez et al. (2021), Kuzyetsov et al. (2020) and Rubio et al. (2020). Day 3 to Day 5 (D3 to D5) included 280 samples from *Xu et al. (2016)*, Babariya et al. (2018), *Li et al. (2018)*, Vera Rodriguez et al. (2018) and *Xu et al. (2022)*. Day 1 to Day 5 (D1 to D5) included 89 samples from *Liu et al. (2017)*, *Feichtinger et al. (2017)* and Ho et al. (2018).

than the control arm. Some authors have argued that niPGT could be more accurate than trophectoderm biopsy results when compared with the ICM (*Huang et al., 2019*). However, this study was potentially flawed because the niPGT was performed with one PGT method, and the trophectoderm analysis was performed on four different PGT platforms. Reviewing the literature in that respect shows similar error rates of niPGT and trophectoderm versus ICM (**TABLE 2**). In 2024, a series of studies relevant to this question were published: *Nakhuda et al. (2024)* reported that, when practising niPGT-A, the negative predictive value for samples with no detected abnormality (euploidy) was 57.3% (43/75) and the positive predictive value of aneuploidy was 94.4% (17/18). An unequal sex ratio suggested that maternal contamination was a problem. *Ardestani et al. (2024)* found that culture for early day 6 was more informative than early day 5, finding concordance rates of 90.5% (day 5 short), 93.6% (day 5 long) and 92.3% (day 6 short) with whole blastocyst chromosome constitution. *Chow et al. (2024)* compared collection timings for SCM, rinsing protocols, and IVF versus ICSI in the context of niPGT-A (using regular PGT-A as a control). Rate of concordance to trophectoderm biopsy was higher in day 6 samples compared with day 5 samples, with a sequential method for rinsing (as opposed to a single step rinsing protocol) the superior approach. The more effective methods reached concordance rates of 82.5–85%, with no differences in IVF versus ICSI comparisons.

TABLE 3 summarizes the error rate in studies where whole embryo studies were combined with both niPGT-A and trophectoderm biopsy PGT-A. Error rates are apparently slightly less using niPGT-A. All the above studies performed trophectoderm biopsy to compare results with SCM and determine concordance. However, they did not determine if niPGT can improve pregnancy or live birth rates to the same rate as trophectoderm biopsy PGT-A. Nor did they establish whether or not niPGT can improve such measures in the absence of any potential biopsy damage. There have been only a handful of studies using niPGT clinically. One study by *Fang et al. (2019)* obtained a pregnancy rate of 58%, but had no control for comparison. A very small study by *Franco Jr. et al. (2020)* obtained higher but non-significant differences in pregnancy outcome between niPGT (61%) and controls (49%). The most complete study to date is that by *Sun et al. (2024)*, who found that the cumulative live birth rate was significantly higher for both PGT-A and niPGT-A compared with ICSI with no testing (27.9% without, 44.9% niPGT-A, 51.0% PGT-A; $P < 0.003$ for all comparisons apart from the non-significant niPGT-A versus PGT-A). They concluded that, in women aged 35–40 years, euploid embryos can be selected effectively by either niPGT-A or PGT-A, providing evidence of concordance. niPGT may ultimately require changes in protocol that are somewhat invasive, such as boosting embryonic DNA in SCM by the following approaches: vitrification and warming, piercing the embryo to obtain blastocoele fluid, or culturing to day 6 to increase embryonic DNA content and minimize maternal contamination. However, these could all have an adverse effect on embryo development and the formation of expanded blastocysts. *Volovsky et al. (2024)* stress the importance of a robust three-phase validation process to ensure the clinical reliability of niPGT-A, highlighting issues of DNA amplification failure and diagnostic inaccuracy. *Moustakli et al. (2024)* concur, highlighting the need to enhance the overall effectiveness and accessibility of niPGT-A in the areas of genomic sequencing, bioinformatics and the integration of AI. Overall, it is necessary to ensure that niPGT-A is not only accurate and less invasive than regular PGT-A, but also seen to be less cumbersome than trophectoderm biopsy. The recent lawsuit in Australia pertaining to its effectiveness highlights the need for caution in its evaluation and adoption for clinical use. Clearly, all eyes are on this approach (*Cinnioglu et al., 2023*).

TABLE 3 ERROR RATE COMPARING TROPHECTODERM BIOPSY PREIMPLANTATION GENETIC TESTING AND NON-INVASIVE PREIMPLANTATION GENETIC TESTING WITH WHOLE EMBRYO ANALYSIS

References	Error rate versus whole embryo	
	niPGT-A	TE biopsy PGT-A
Huang et al., 2019	6% (3/48)	18% (9/50)
Kuznyetzov et al., 2018	11.5% (3/26)	12.5% (3/24)
Jia et al., 2019	3% (2/62)	5% (3/62)
Rubio et al., 2020	16% (10/64)	13% (8/64)
Li et al., 2021	11% (3/27)	15% (4/27)
Chen et al., 2021	0% (0/26)	12% (3/26)
Total	8.3% (21/253)	11.8% (30/253)

niPGT-A used spent culture medium.

n/N relates to number of errors/embryos tested.

niPGT-A, non-invasive preimplantation genetic testing for aneuploidy; TE, trophectoderm.

NON-INVASIVE EMBRYO SELECTION BY METABOLOMICS: A BRANCH OF REPRODUCTOMICS

Reproductomics was introduced and coined by *Bellver et al. (2012)*, and defined as the application of '-omics to the field of reproduction'. This term covers all the different 'omics' technologies that can be applied to a wide variety of molecules of the different cells and tissues involved in reproductive function (*Horcajadas and Gosálvez, 2018*). In reproductive medicine, omics studies can be classified into three different areas: preconceptional, pre-implantational and prenatal. The main pre-implantational studies carried out on humans are listed in **TABLE 4** and represent the state of the art in metabolomics in terms of embryo selection by this approach. That is, they provide a summarized snapshot of the current situation of the omics and embryo selection studies in a metabolomics context. Metabolites are defined as low-molecular-weight compounds that are products of cell and tissue metabolism. The metabolomics profile is, it is claimed, the closest link to phenotype. Metabolomics is used to find biomarkers that can aid in the diagnosis of pathologies; it thus can be used to discover changes related with depletion/

TABLE 4 STUDIES OF METABOLOMICS USING SPENT MEDIA IN IVF INDICATING CORRELATION WITH CLINICAL RATES

Citation	Technology	Number of embryos	Type of study	Main result
Seli et al., 2007	Raman and near-infra-red spectroscopy	69	Prospective	Correlation
Scott et al., 2008	Raman spectroscopy	41	Blind prospective	Correlation
Seli et al., 2008	Proton nuclear magnetic resonance	34	Retrospective	Correlation
Vergouw et al., 2008	Near-infra-red spectroscopy	333	Retrospective	Correlation
Seli et al., 2010	Near-infra-red spectroscopy	181+304	Retrospective	Correlation
Marhuenda-Egea et al., 2010	High-performance liquid chromatography and mass spectroscopy	25	Retrospective	Prediction
Seli et al., 2011	Near-infra-red spectroscopy	198	Retrospective	Correlation
Ahlstrom et al., 2011	Near-infra-red spectroscopy	137	Retrospective	Prediction
Vergouw et al.,2011	Near-infra-red spectroscopy	127	Retrospective	Correlation
Hardanson et al., 2012	Near-infra-red spectroscopy	164+163	Randomized controlled	No improvement
Vergouw et al., 2012	Near-infra-red spectroscopy	309	Double-blind randomized controlled	No improvement
Sánchez-Ribas et al., 2012	Liquid chromatography-mass spectroscopy and nuclear magnetic resonance	171	Retrospective	Partial correlation
Sfontouris et al., 2013	Near-infra-red spectroscopy	125	Randomized controlled	No conclusion
Zhao et al., 2013	Raman	57	Retrospective	Correlation
Kirkegaard et al., 2014	Nuclear magnetic resonance	161	Prospective	No correlation
Dominguez et al., 2015	Proteomics and time lapse	28	Prospective	Correlation
Iles et al., 2019	MALDI-TOF	401	Retrospective	Prediction

MALDI-TOF, matrix-assisted laser desorption/ionization time of flight.

appearance of small molecules in spent media, products of embryo metabolism that can be correlated with embryonic implantation potential and even, possibly, ploidy status. Studies are not necessarily hypothesis driven, as the studies often involve comparing the many metabolomic differences between two groups and observing what is different. Metabolomics in human embryos (and its possible diagnostic value) has its roots in the work of Henry Leese and colleagues (e.g. see *Hardy, 1999*), who measured glucose and pyruvate metabolism in a range of studies. *Gardner et al. (2001)* also measured ammonium production, alongside pyruvate and glucose consumption, by individual embryos to correlate their concentration with blastocyst formation and quality. They performed ultra-micro-fluorescence assays in a total of 60 cryopreserved pro-nucleate embryos and 13 non-cryopreserved blastocyst embryos. These studies demonstrated, for the first time in humans, that it was possible to identify, in spent media, molecules correlated with the highest developmental potential. Glucose intake had been measured previously in bovine (*Renard et al., 1980*) and murine (*Gardner and Leese, 1987*; *Lane and Gardner, 1996*) embryos.

Before the 'omics revolution', the significant advances involved high-performance liquid chromatography. Developmentally competent embryos showed a distinct amino acid profile in culture media compared with those which arrested in culture before blastocyst formation (*Houghton, 2002*). That is, measuring a mixture of 18 amino acids, researchers found significant changes in the concentrations of Ala, Arg, Gln, Met, Leu and Asn as a possible way to select developmentally competent single embryos for transfer, based on a short list of molecules. Following a similar approach, the turnover of three amino acids — Asn, Gly and Leu — was correlated significantly with clinical pregnancy and live birth (*Gardner and Leese, 1987*; *Lane and Gardner, 1996*). The evolution of proteomics and metabolomics technologies, the decreased cost of the assays, and easier access to instruments around the world perpetuated non-targeted analysis in the context of reproductive medicine. This became widespread from around 2010. Such a new strategy in the search for biomarkers was also applied to follicular fluid analysis to identify the most viable and competent oocytes (Lan *Xia et al., 2014*; *McRae et al., 2012*; *Petro et al., 2012*; *Piñero-Sagredo et al., 2010*; *Wallace et al., 2012*), and to endometrial fluid to investigate proteomics (*Casado-Vela et al., 2009*). The metabolomics profile of the menstrual cycle (*Vilella et al., 2013*) and the lipidomics profile were used to correlate with the implantation status of the endometrium (*Matorras et al., 2020*).

Metabolomics uses different analytical techniques, with the most relevant being nuclear magnetic resonance spectroscopy, near-infra-red spectroscopy, Raman spectroscopy, gas chromatography-mass spectrometry, and high-performance liquid chromatography-mass spectrometry. Selection of the appropriate technology is crucial to obtain desired outcomes. Metabolomics and proteomics differ from genomics and transcriptomics in the variety of possibilities when carrying out an experiment. That is, metabolites and proteins are much more complex and numerous than nucleic acid molecules, making a fully detailed picture of a physiological or pathological situation in 'omics' terms challenging in an IVF embryo. In the meantime, several groups have continued studying metabolites from spent media in a targeted manner. *Kaihola et al. (2019)* investigated the different levels

of caspase-3 and histidine-rich glycoprotein, secreted by the embryo into the culture media, as biomarkers of embryo quality. They analysed a total of 334 samples of SCM collected from IVF treatments at three different clinics, and used a protein analysis method named 'multiplex proximity extension assay'. They concluded that caspase-3 levels were lower in secretomes from day 2 embryos, resulting in a significant correlation with pregnancy outcome ($P < 0.05$) (*Kaihola et al., 2019*).

The main limitations of these studies are the reproducibility of the technology and the different media composition used. A new approach selected biomarkers using ultra performance liquid chromatography followed by Fusion Orbitrap MS/MS technology. Different statistical techniques were applied to filter the vast number of metabolites found in order to identify the most informative. In the first approach, 90% aneuploid embryos ($n = 60$) were correctly ascertained (*Cabello-Pinedo et al., 2020a*). In a second approach using a different set of 60 metabolites, the model was tested using a different dataset (27 aneuploid and 13 euploid) for validation purposes. The results gave concordance with PGT-A results of 97.5% accurate prediction, with only one misclassified (*Cabello-Pinedo et al., 2020b*). In parallel, the same group identified metabolites with low implantation potential among 5500 metabolites detected in spent Vitrolife culture media. After identification of the metabolomics profile for pregnancy and non-pregnancy, the system was able to predict poor implantation potential for 30.95% of euploid embryos (*Cabello-Pinedo et al., 2020c*). A second analysis was performed with 68 Vitrolife media samples from two different clinics. With the data from the first clinic samples ($n = 37$: 20 pregnant and 17 non-pregnant), a Vitrolife-specific metabolite pregnancy index (MPI) was built with the most informative metabolites used to determine pregnancy potential. For the first clinic, the analysis showed an ability to predict embryo viability of 100% for pregnant samples and 88% for non-pregnant samples. A blind analysis on a third batch of Vitrolife samples from a second centre showed that 78% of pregnant samples and 61% of non-pregnant samples were identified (*Cabello-Pinedo et al., 2020c*). A final validation of these results was carried out in 2021 with more than 400 embryos. Considered to be a classification model, the method showed accuracy of 97% in the training subset and 74% accuracy in the test subset, with sensitivity of 100% and specificity of 65%. The results were also studied separately for euploid embryos and non-biopsied embryos, obtaining similar results as within the general group (*Cabello-Pinedo et al., 2021*). These results were independent of whether fertilization was performed by ICSI or conventional IVF (*McCaffrey et al., 2022*). These findings applied to routine fertility treatments resulted in improvement of up to 75% in pregnancy probability for patients undergoing their first embryo transfer. Knowledge of an MPI value when selecting an embryo for transfer would, potentially, result in valuable advanced information on the viability of the embryo. The non-invasiveness of the method, furthermore, avoids damage to the embryo that could further reduce implantation potential.

Two significant studies were published in 2024. The first, *Cabello-Pinedo et al. (2024)*, described the development of a new assay to predict the implantation potential of IVF embryos. This was a case–control study in two parts: the first (discovery) phase included 101 samples, whereas the second (validation) phase included 169 samples. SCM samples from PGT-A and non-PGT-A cases with known implantation outcomes were collected after blastocyst embryo transfer. Chromatography and mass spectrometry were the methodologies used, identifying biomarkers associated with embryo implantation. In the first stage, 148 embryo implantation biomarkers were identified, and 47 (31.8%) were characterized. The authors reported significant enrichment of tryptophan, arginine and proline metabolism, as well as lysine degradation. After transferring the method to lower resolution equipment, a model able to assign an MPI value to each embryo culture media was developed, taking the concentration of 36 biomarkers as input. Positive and negative predictive values of 88% and 77.78%, respectively, were reported. Moreover, mathematical combination of biomarker concentrations using AI techniques was used by the authors to predict embryo implantation outcome with accuracy of approximately 85%. In a pilot trial, *Sakkas et al. (2024)* asked whether fluorescence lifetime imaging microscopy (FLIM) metabolic imaging correlated with pregnancy outcomes. They found that FLIM did not distinguish consistent patterns of mitochondrial metabolism in blastocysts that resulted in pregnancy compared with those that failed to implant. However, the ploidy status of the blastocyst was highly distinguishable, with embryo regions and embryo day identified reliably using FLIM.

Overall, the evidence is that metabolomics provides some crucial information about embryo viability and genetic status. In combination with machine learning and morphokinetic data, it may ultimately provide a huge improvement in the prediction of embryo implantation potential (*Cheredath et al., 2023*). Moreover, the sampling of SCM, which is a necessary step, provides the opportunity to perform niPGT-A and metabolomics simultaneously.

CONCLUSIONS

AI is a faster selection method to screen embryos compared with morphological selection, but is still inferior to PGT-A, with average concordance with implantation of 0.7 AUC. However, AI is less involved and more conducive to wide implementation. niPGT-A has improved significantly in recent years and, with sequencing costs dropping continuously, has the potential to reach 100% concordance with PGT-A; indeed, some companies are now using it commercially. One issue here is that the problem of maternal contamination needs to be solved, which requires significant change in the standard IVF protocol. Even if niPGT-A reaches 100% concordance consistently in real-world studies, ≥30% euploid embryos never implant. Moreover, the steps required to achieve effective niPGT-A (removal of cumulus cells, thorough rinsing, culture to day 6) may well lead to it being almost as cumbersome and invasive as trophectoderm biopsy PGT-A. On the other hand, metabolomics has the potential to identify euploid embryos that are metabolically incapable of implanting, with minimal IVF protocol change, although attempts to commercialize this have, thus far, not been applied 'main-stream'. However, it does not seem beyond the bounds of feasibility that future non-invasive treatment regimes for embryo selection may involve a combination of two, or even all three, approaches. *Dominguez et al. (2015)* provide an early example of this, combining proteomics and TLI. However, there would be associated costs and risks with a three-pronged approach, and cost–benefit calculations need to be considered.

AUTHOR CONTRIBUTIONS

Santiago Munné set out the structure of the paper and the subject of the review, writing the section on niPGT-A and the original draft of the conclusions. Jose Horcajadas wrote the original draft of the section on metabolomics. Michelle Perugini wrote the original draft of the section on AI. Michelle Seth-Smith wrote the original draft of introduction and revised the manuscript for scientific consistency. Darren K. Griffin rewrote all sections of the manuscript and edited it in its entirety for accuracy, currency and flow.

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