

## Morphological and morphokinetic associations with aneuploidy

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1 **Morphological and morphokinetic associations with aneuploidy: a systematic review**  
2 **and meta-analysis**

3  
4 **Running title: Human embryo** morphology, morphokinetics and ploidy

5  
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59 **ABSTRACT**

60 **BACKGROUND**

61 A time lapse system (TLS) is utilised in some fertility clinics with the aim of predicting embryo  
62 viability and chance of live birth during IVF. It has been hypothesised that aneuploid embryos  
63 display altered morphokinetics as a consequence of their abnormal chromosome complement.  
64 Since aneuploidy is one of the fundamental reasons for IVF failure and miscarriage, attention  
65 has focused on utilising morphokinetics to develop models to non-invasively risk stratify  
66 embryos for ploidy status. This could avoid or reduce the costs associated with pre-  
67 implantation genetic testing for aneuploidy (PGT-A). Furthermore, TLS have provided an  
68 understanding of the true prevalence of other dysmorphisms. Hypothetically, the incorporation  
69 of morphological features into a model could act synergistically, improving a model's  
70 discriminative ability to predict ploidy status.

71 **OBJECTIVE AND RATIONALE**

72 The aim of this systematic review and meta-analysis was to investigate associations between  
73 ploidy status and morphokinetic or morphological features commonly denoted on a TLS. This

74 will determine the feasibility of a prediction model for euploidy and summarise the most useful  
75 prognostic markers to be included in model development.

## 76 **SEARCH METHODS**

77 Five separate searches were conducted in Medline, Embase, PubMed, and Cinahl from  
78 inception to 1<sup>st</sup> July 2021. Search terms and word variants included, among others, PGT-A,  
79 ploidy, morphokinetics and time lapse, and the latter were successively substituted for the  
80 following morphological parameters: fragmentation, multinucleation, abnormal cleavage and  
81 contraction. Studies were limited to human studies.

## 82 **OUTCOMES**

83 Overall, 58 studies were included incorporating over 40,000 embryos. All except one study  
84 had a moderate risk of bias in at least one domain when assessed by the quality in prognostic  
85 studies tool (QUIPS). Ten morphokinetic variables were significantly delayed in aneuploid  
86 embryos. When excluding studies using less reliable genetic technologies, the most notable  
87 variables were: time to 8 cells (t8, 1.13 hrs, 95% CI 0.21-2.05; three studies; n=742; I<sub>2</sub>= 0%),  
88 t9 (2.27 hrs, 95% CI 0.5-4.03; two studies; n=671; I<sup>2</sup>=33%), time to formation of a full  
89 blastocyst (tB, 1.99 hrs, 95% CI 0.15-3.81; four studies; n=1640; I<sup>2</sup>=76%), and time to  
90 expanded blastocyst (tEB, 2.35 hrs, 95%CI 0.06-4.63; four studies; n=1640; I<sup>2</sup>=83%). There is  
91 potentially some prognostic potential in the degree of fragmentation, multinucleation persisting  
92 to the 4-cell stage, and frequency of embryo contractions. Reverse cleavage was associated  
93 with euploidy in this meta-analysis, however this article argues that these are likely spurious  
94 results requiring further investigation. There was no association with direct unequal cleavage  
95 in an embryo that progressed to a blastocyst, or with multinucleation assessed on day 2 or at  
96 the 2-cell stage. However, owing to heterogenous results and poor-quality evidence,

97 associations between these morphological components needs to be investigated further before  
98 conclusions can be reliably drawn.

## 99 **WIDER IMPLICATIONS**

100 This first systematic review and meta-analysis of morphological and morphokinetic  
101 associations with ploidy status demonstrates the most useful morphokinetic variables, namely  
102 t8, t9 and tEB to be included in future model development. There is considerable variability  
103 within aneuploid and euploid embryos making definitively classifying them impossible,  
104 however, it is feasible that embryos could be prioritised for biopsy. Furthermore, these results  
105 support the mechanism by which algorithms for live birth may have predictive ability,  
106 suggesting aneuploidy causes delayed cytokinesis. We highlight significant heterogeneity in  
107 our results secondary to local conditions and diverse patient populations, therefore calling for  
108 future models to be robustly developed and tested in-house. If successful, such a model would  
109 constitute a meaningful breakthrough when accessing PGT-A is unsuitable for couples.

110 **Key words:** time-lapse, morphokinetics, ploidy, model, fragmentation, multinucleation,  
111 abnormal cleavage, contraction

112 **Introduction**

113 Pre-implantation embryo selection has historically relied upon morphological assessment using  
114 increasingly contested consensus guidelines (Gardner and Balaban 2016; Kemper et al. 2021;  
115 Gardner D. K. 1999; Alpha Scientists in Reproductive and Embryology 2011). Despite  
116 significant improvements since the inception of assisted reproduction, the average live birth  
117 rate in the UK remains low, at 32% per embryo transfer (for women < 35) (HFEA 2021). When  
118 one also considers the drive for single embryo transfers, advancing maternal age and higher  
119 associated aneuploid rates, the need for more advanced methods for assessing embryo viability  
120 is paramount.

121 A time lapse system (TLS) offers several advantages over static, basic morphological  
122 observations. This enclosed incubation system reduces the need to remove embryos from  
123 optimum atmospheric culture conditions by taking microscopic, multiplanar images at regular  
124 intervals. The retrospective analysis of these images allows the annotations of an embryo's  
125 developmental milestones (i.e., morphokinetics) to be compared to outcome variables, such as  
126 live birth or ploidy status. This allows embryos to be selected that display specific development  
127 patterns achieved at fixed times of development, for example blastocyst formation at 116hrs;  
128 usually recorded as hours post insemination (hpi). Unfortunately, due to poor quality evidence  
129 a Cochrane review was unable to conclude whether the use of a TLS increased live birth rates  
130 (Armstrong et al. 2019). In contrast, several large studies and randomised trials have reported  
131 improvements, therefore a TLS has become commonplace in many IVF laboratories worldwide  
132 (Pribenszky et al. 2017). A summary of definitions used for morphokinetic annotations and  
133 other morphological features denoted on a TLS can be found in Table I.

134 Aneuploidy is a major cause of implantation failure and miscarriage, however, there are  
135 barriers to accessing genetic testing. Aneuploidy arises from errors during mitosis or meiosis,  
136 such as non-disjunction. This increases with maternal age and therefore coincides with rapidly

137 declining success rates of IVF treatment in older women. For instance, in women under 35  
138 years an average aneuploidy rate of 30-50% has been reported, increasing to 80% in women  
139 aged 42 years or older (Ata et al. 2012; Franasiak et al. 2014). Modern methods for pre-  
140 implantation genetic testing for aneuploidy (PGT-A) provide an accurate assessment of embryo  
141 chromosome complement using biopsy techniques in the majority of cases (Munné et al. 2017;  
142 Munné et al. 2019). For some patients, however, this technology may be inaccessible because  
143 it is prohibited by legislation, or they may deem it ethically inappropriate. They may also not  
144 have embryos suitable for biopsy. Moreover, PGT-A can cost over £3000 in the UK and in the  
145 USA it can be as high as \$12,000, further limiting accessibility (Theobald et al. 2020). It is  
146 therefore not surprising that researchers have begun investigating methods to non-invasively  
147 detect aneuploidy.

148 It has been hypothesised that the morphokinetics of aneuploid embryos are delayed in  
149 comparison to euploid counterparts (Davies 2012; Campbell et al. 2013). Physiologically, this  
150 may be due to complex biochemical processes that occur when errors have been detected by  
151 the developing embryo (Coticchio, Barrie, et al. 2021). This results in slower cell division and  
152 is possibly a reason for the higher mitochondrial content seen in aneuploid embryos (Campbell  
153 et al. 2013; Ho et al. 2018). This has led to the development of several models using PGT-A  
154 and morphokinetic data aiming to risk-stratify embryos for euploidy (Campbell et al. 2013;  
155 Basile et al. 2014; Chawla et al. 2015; Mumusoglu et al. 2017; Del Carmen Nogales et al. 2017;  
156 Desai et al. 2018). At CARE Fertility a sophisticated time-lapse embryo selection model,  
157 “CAREmaps<sup>®</sup>”, has been successfully developed that can predict an individual embryo’s  
158 chance of resulting in a live birth. This was developed using a database of over 6000 transferred  
159 blastocysts with known live birth outcome data and has been shown to improve embryo  
160 selection (Fishel et al. 2018). Similar embryo selection algorithms have been developed by a  
161 variety of clinics internationally; some are commercially available (Petersen et al. 2016). It



162 remains unknown why embryos with higher scores should have better predicted outcomes; it  
163 would be sensible to hypothesise that the aetiology lies within delayed development as a  
164 sequela of chromosomal abnormalities. It would therefore prompt the assumption that if a TLS  
165 can identify embryos with the highest chance of live birth, it could be instrumented to enhance  
166 euploid embryo selection. This hypothesis is also supported by a recent meta-analysis that  
167 showed that the use of a TLS was associated with lower early miscarriage rates compared to  
168 traditional morphological assessment (Pribenszky et al. 2017). Other theories have also been  
169 suggested including partial compaction with or without cell extrusion or exclusion causing  
170 delayed cyto or karyokinesis, abnormal fertilisation, BMI, embryo sex, a failure of the embryo  
171 to undergo check points, and DNA repair mechanisms (Coticchio, Ezoe, et al. 2021; Coticchio,  
172 Barrie, et al. 2021; Bronet et al. 2015; Leary et al. 2015). It may therefore be feasible to utilise  
173 morphokinetics as a screening tool for ploidy status if this hypothesis becomes established by  
174 evidence.

175 The ability of morphokinetic models to predict ploidy status remains controversial and wide  
176 disparities exist in the morphokinetic events included in such models (Campbell et al. 2013;  
177 Kramer et al. 2014; Basile et al. 2014). This may be due to significant heterogeneity in study  
178 design and sample populations. For instance, the following have all been associated with  
179 altered morphokinetics: age, smoking status, biopsy techniques, stimulation protocols,  
180 insemination methods and culture conditions (Ciray et al. 2012; Muñoz et al. 2013; Lemmen  
181 et al. 2008; Bellver et al. 2013; Fréour et al. 2013; Kirkegaard et al. 2013).

182 Several morphological observations can be observed in greater detail when using a TLS,  
183 although historically there is limited correlation reported between ploidy status and these  
184 qualitative aspects (Magli et al. 2007; Minasi et al. 2016; Capalbo et al. 2014; Munné et al.  
185 2017). In fact, several authors have identified that it is possible for aneuploid embryos to  
186 achieve good morphology scores (Munné 2006; Alfarawati et al. 2011; Fragouli et al. 2014).

187 Nonetheless, it must be taken into consideration that most studies investigating associations  
188 between morphology and ploidy status were undertaken using standard morphology  
189 assessments and not using a TLS. This results in an inability to identify dynamic changes  
190 occurring between check points. Furthermore, many of these studies utilised older, less reliable  
191 techniques such as fluorescence *in situ* hybridisation (FISH) and blastomere biopsy. This  
192 results in a higher chance of misclassifying mosaics or failing to detect aneuploidy due to the  
193 limited number of probes used (Fragouli and Wells 2011). We aim to investigate the association  
194 of various morphological components commonly observed on a TLS with ploidy status.

195 The first variable to be explored is fragmentation. Fragmentation is often considered during  
196 embryo selection owing to associations with embryo viability, but it remains one of the most  
197 enigmatic features identified in early development (Edwards et al. 1984; Puissant et al. 1987).  
198 Origins of these anucleated structures have been correlated with many factors including culture  
199 conditions, poor quality oocytes or spermatozoon, increased maternal age, oxidative stress and  
200 aneuploidy (Kim et al. 2018; Delimitreva et al. 2005; Fujimoto et al. 2011; Munne and Cohen  
201 1998; Magli et al. 2007). It has even been associated with so called ‘self-correction’  
202 mechanisms whereby an embryo extrudes sequestered chromosomes in order to become more  
203 genetically normal (Coticchio, Barrie, et al. 2021). Considering that the causation is poorly  
204 understood, association with ploidy status will be explored further in this review.

205 The second factor to be investigated is abnormal cleavage, the occurrence of which has become  
206 more apparent through a TLS yet causality remains unproven (Zhan et al. 2016; Athayde Wirka  
207 et al. 2014). The prevalence of these atypical cell divisions ranges from 4.4 to 26.1% and the  
208 implantation rates of these untested embryos has been found to be as low as 1.2%-17% (Barrie  
209 et al. 2017; Rubio et al. 2012; Ozbek et al. 2021). There is, therefore, a tendency to deselect  
210 these embryos (Desai et al. 2018; Zhan et al. 2016; Balakier et al. 2016; Hashimoto et al. 2016).  
211 Previous theories for aetiology include multipolar spindles, surplus centrosomes, quality of

212 spermatozoa and chromosome aberrations (Kalatova et al. 2015; Ozbek et al. 2021). Similarly,  
213 it has been speculated that abnormal cleavage may also be involved in the process of ‘self-  
214 correction’. This is supported by the recent findings that abnormal cleavages are associated  
215 with partial compaction and the ‘excluded phenotype’ (Coticchio, Ezoe, et al. 2021). These  
216 excluded cells have also been shown to have a significantly higher abnormal chromosome  
217 content (Lagalla et al. 2017). We will assess the association between the most common types  
218 of abnormal cleavage and ploidy status: direct and reverse cleavage (Liu et al. 2014; Rubio et  
219 al. 2012).

220 Blastocyst contraction is the third feature to be examined that has been the focus of only a  
221 handful of studies. Physiologically this occurs through the inflow of liquid through aquaporin  
222 water channels and outflow through weak tight junctions (Watson et al. 2004; Marcos et al.  
223 2015). The reason for it remains largely unknown, and it has been suggested that this process  
224 may assist in embryo hatching and has been associated with lower implantation rates (Marcos  
225 et al. 2015; Bodri et al. 2016; Niimura 2003). Hypothetically, this may be secondary to  
226 aneuploidy, therefore this will be investigated in this review.

227 Finally, multinucleation has been associated with poorer implantation outcomes and possibly  
228 aneuploidy (Kligman et al. 1996; Royen et al. 2003). This dysmorphism has been hypothesised  
229 to be the result of errors in nuclear replication without cytokinesis, nuclear fragmentation or  
230 defective DNA packaging and migration during anaphase (Pickering et al. 1995). It is therefore  
231 possible that this could be linked to aneuploidy as a consequence of errors occurring in  
232 chromosome segregation.

233 The aim of this systematic review and meta-analysis is to determine the most reliable  
234 morphokinetic prognostic factors for future model development and investigate associations  
235 between morphology and ploidy status. Specifically, the degree of fragmentation, presence of  
236 direct and reverse cleavage, blastocyst contractions and multinucleation will be investigated in

237 association with chromosomal status. Incorporating these morphological parameters may  
238 improve the discrimination of a morphokinetic model with regards to ploidy.

## 239 **Methods**

### 240 *Registration*

241 This review was prospectively registered with PROSPERO (ID number: CRD42021260795).

### 242 *Data sources and search strategy*

243 Five separate literature searches were conducted for potential prognostic factors and their  
244 associations with aneuploidy in concordance with the Preferred Reporting Items for Systematic  
245 Reviews and Meta-Analysis (PRISMA) guidelines (Moher et al. 2009). Electronic searches  
246 were conducted in MEDLINE, PubMed, EMBASE and CINAHL (from inception to 1<sup>st</sup> July  
247 2021). Searches were conducted using the following MeSH key terms and word variants: ‘pre-  
248 implantation genetic testing for aneuploidy (PGT-A)’, OR ‘pre-implantation genetic screening  
249 (PGS)’, OR ‘ploidy’, OR ‘aneuploid’ AND ‘morphokinetics’, OR ‘time-lapse’. For the four  
250 subsequent searches ‘morphokinetics’ and ‘time-lapse’ were successively substituted for:  
251 ‘fragmentation’, ‘multinucleation’, ‘abnormal cleavage’ and ‘blastocyst contraction’.  
252 Similarly, word variants for each were included, such as ‘trichotomous mitosis’ for ‘direct  
253 cleavage’.

### 254 *Eligibility criteria*

255 Studies were limited to human studies and included if the primary or secondary outcome was  
256 the ploidy status of biopsied embryos in relation to the presence of any of the prognostic factors  
257 under investigation. No language restrictions were applied. Manuscripts on mosaicism were  
258 included if they also provided data on aneuploid and euploid embryos. Exclusions include:  
259 polar body biopsy, those reporting clinical outcomes only, where the outcome was

260 translocations not aneuploidy, those that focussed on a subset of embryos with a particular  
261 morphological feature (such as abnormal cleavage in multinucleated embryos) or from a  
262 subgroup of patients (for example, endometriosis). The blastocyst contraction literature search  
263 aimed to determine association of embryo contraction kinetics (number or frequency),  
264 therefore studies investigating the rate or volume of expansion in relation to ploidy were  
265 excluded. Similarly, authors that correlated blastocyst expansion grading or morphology scores  
266 with ploidy status but not the individual prognostic factors being tested were excluded.  
267 Validation studies for a prognostic model already developed were excluded from the meta-  
268 analysis.

#### 269 *Study selection*

270 Two reviewers initially screened all titles and abstracts independently for eligibility (T.B. and  
271 A.B.), and full length articles were then obtained and scrutinised. Any disputes were resolved  
272 by discussion with a third reviewer (S.M). Bibliographies of all relevant articles and review  
273 articles excluded were manually searched. Where more than 10 original articles met eligibility  
274 criteria, conference abstracts were subsequently excluded from the search. Otherwise, they  
275 were included due to a scarcity of published peer reviewed reports. Authors of all conference  
276 abstracts were contacted for additional information to assist with study selection, data  
277 extraction and quality assessments. Authors of original articles were contacted for further  
278 information where data presented was suboptimal. If data was not obtained or in a usable  
279 format, it was excluded from the meta-analysis but included in the systematic review.

#### 280 *Data extraction and study outcomes*

281 Outcome and prognostic factor data were extracted independently by two reviewers into tables  
282 (T.B. and A.B.). The primary outcome extracted was the prevalence of aneuploid and euploid  
283 embryos for each potential prognostic factor assessed. This included the mean or median time

284 taken for both aneuploid and euploid embryos to achieve each morphokinetic variable. Data  
285 was also collected for: overall aneuploid rate, study design, primary outcome measured,  
286 number of patients and embryos included, TLS assessment period, PGT-A technique  
287 (including stage and type of biopsy), atmospheric culture conditions, infertility diagnosis and  
288 indication for PGT-A. Additionally, details of any model development, including attempts at  
289 model discrimination, calibration and validation, were recorded. Importantly, we collected data  
290 on potential study participant factors that could act as confounders including age, BMI, and  
291 stimulation drugs used. A recently published article by Barrie et al. (2021) described how age  
292 and BMI are the most important factors to control for in morphokinetic studies. Data were  
293 extracted only on those embryos with PGT-A results available.

#### 294 *Risk of bias and quality assessment*

295 All articles meeting the selection criteria were quality assessed using the Quality in Prognosis  
296 Studies tool (QUIPS) (Grooten et al. 2019). It moves away from quantitative analysis of quality  
297 but rates the risk of bias in six domains (participation, attrition, prognostic factor measurement,  
298 outcome measurement, study confounding, and statistical analysis) as low, medium or high  
299 risk of bias (Higgins JPT 2021). The tool has been modified for use in this systematic review:  
300 an example can be found in Supplementary Table SI, including a summary of the bias domains  
301 and the criteria used to grade each category. Several items were removed from our adapted  
302 version of the tool. Firstly, the ‘adequate participation’, ‘drop out’ and ‘attempt to collect  
303 information on participants who dropped out’ prompting items were removed because they  
304 were less relevant to the study of embryos as research focuses on the retrospective analysis of  
305 existing PGT-A data sets. The original tool included a prompter within the confounding domain  
306 asking the reviewer to determine if the method used to measure confounding was reliable. This  
307 was removed because confounders for morphokinetics include readily available demographic  
308 data and standardised dosages.

309 There were a number of important factors to consider when undertaking the quality assessment.  
310 Firstly, if a particular study did not report on the proportion of embryos without PGT-A results,  
311 they were categorised as having a moderate risk of bias and if it was >5% they were deemed  
312 to have a high risk of bias. Using modern methods most genetic companies would now estimate  
313 that this occurs in up to approximately 2% of samples and this has been replicated in recent  
314 studies (Fiorentino et al. 2014; Neal et al. 2019; Tiegs et al. 2021). A significantly high  
315 proportion may lead to uncertainty regarding the validity of a study's conclusions and biopsy  
316 techniques. Secondly, if FISH was one of the genetic platforms used by a study it was  
317 considered a high risk of bias owing to the inaccuracies of this technique. Finally, due to the  
318 risk of inter-observer variability in morphological assessments of embryos, if there were no  
319 methods to account for internal validity then a publication was assessed as a moderate risk of  
320 bias. Similarly, if multinucleation was assessed as part of standard morphology assessment  
321 rather than the continuous observations enabled by a TLS, it was graded as a moderate risk of  
322 bias.

323 As per the Grading of Recommendations, Assessment, Development  
324 and Evaluation (GRADE) guidelines, publication bias was not assessed as less than 10  
325 studies were included for each prognostic factor analysed, rendering the interpretation of  
326 funnel plots unreliable (Schünemann, 2013). The quality of reporting was assessed using the  
327 Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist  
328 according to published criteria (von Elm et al. 2007).

### 329 *Data synthesis and analysis*

330 Morphokinetics of aneuploid and euploid embryos were compared using a weighted mean  
331 difference analysis in concordance with the Meta-Analysis of Observational Studies in  
332 Epidemiology recommendations (Stroup, 2000). Where studies did not provide a SD value it  
333 was calculated from the 95% CI if the sample size was >100 (using methods recommended by

334 the Cochrane Handbook) (Higgins et al. 2021). Alternatively, data from studies describing only  
335 medians were summarised graphically by prognostic factor analysis using the interquartile  
336 range as a measure of dispersion.

337 The relationship between the prevalence of aneuploid embryos and percentage fragmentation  
338 has been presented on a line graph. Abnormal cleavage, embryo contraction and  
339 multinucleation data was dichotomised and meta-analysed with forest plots and corresponding  
340 calculated relative risks (RR). The results for abnormal cleavage were pooled and analyses  
341 were conducted for each type of abnormal cleavage to determine their relative contribution.  
342 For all meta-analysed variables, heterogeneity was assessed using the  $I^2$  statistic, whereby a  
343 result  $>50\%$  was indicative of considerable heterogeneity. All analyses were undertaken using  
344 a random effects model by the Mantel-Haenszel method (DerSimonian and Laird 1986; Mantel  
345 and Haenszel 1959) using Review Manager (RevMan), version 5.4, The Cochrane  
346 Collaboration, (2020)

#### 347 *Sensitivity analysis*

348 Sensitivity analyses were conducted based on the quality assessment derived from QUIPS. The  
349 use of random versus fixed effect models were also compared. Analyses will be restricted by  
350 excluding studies with a high risk of bias in any domain, an approach also taken by other  
351 authors using QUIPS (Taylor-Rowan et al. 2021).

#### 352 *Subgroup analyses*

353 A subgroup analysis was performed by excluding studies using FISH, blastomere or day 3  
354 biopsy to produce a more reliable effect estimate. During the last decade trophectoderm biopsy  
355 at the blastocyst stage has become the preferred method of testing; fewer embryos have mosaic  
356 results and there is less risk of damage and diminishing the live birth rates (Los et al. 2004;  
357 Staessen et al. 2004; Cohen et al. 2007; Goossens et al. 2008; Tarín et al. 1992). FISH also has  
358 several limitations, primarily, the impossibility to screen all chromosomes and the risk of



359 misdiagnosis is significant when multiple probes are used (DeUgarte et al. 2008; Ruangvutilert  
360 et al. 2000; Scriven and Bossuyt, 2010). These studies were not excluded as part of the  
361 screening process as they may still provide valuable prognostic information. None of the  
362 studies included in the prognostic factor graphs used FISH or blastomere biopsy.

## 363 **Results**

### 364 *Search results*

365 A total of 1557 studies were identified from the initial searches; 137 duplicates were removed,  
366 1267 abstracts were screened, of which 123 were selected as being potentially relevant and 58  
367 met selection criteria after screening of the full texts. Overall, 65 studies were excluded for the  
368 following main reasons: a different prognostic factor being investigated (n=15), describing an  
369 alternative outcome to ploidy status (n=11), measuring clinical outcomes only (n=10), or the  
370 study population was a subset of embryos with a particular characteristic (n=7). Figures 1-5  
371 display the study screening process for each search and all exclusions are summarised in  
372 Supplementary Table SII (Moher et al. 2009). In total, 26 authors were contacted to attempt to  
373 identify missing information from their publication (Supplementary Table SIII). Two studies  
374 are ‘awaiting classification’ due to unanswered correspondence with the authors: this was  
375 intended to confirm whether their abstracts included different embryos than the later published  
376 articles (Desai, 2016; Lagalla, 2015). Responses were not received, therefore the publications  
377 with the most data were included in this systematic review (Lagalla et al. 2017; Desai et al.  
378 2018). Responses were not received from 19 authors in total; this did not result in exclusion  
379 from the systematic review for any of these studies but exclusion from the quantitative analysis  
380 in 10. The remaining were contacted for supporting information only. In total, 58 studies were  
381 included in the narrative synthesis, 43 of which had results suitable for meta-analysis.

382

383 *Characteristics of the included studies*

384 Overall, 7,004 embryos that underwent PGT-A from at least 1,058 patients were included from  
385 18 studies examining morphokinetics. Thirteen retrospective studies and one prospective  
386 cohort study provided morphokinetic data comparing euploid and aneuploid embryos, enabling  
387 their inclusion in the quantitative analysis (Chavez et al. 2012; Campbell et al. 2013; Basile et  
388 al. 2014; Chawla et al. 2015; Rienzi et al. 2015; Patel et al. 2016; Minasi et al. 2016;  
389 Mumusoglu et al. 2017; Zhang et al. 2017; Desai et al. 2018; Lee et al. 2019; Kimelman et al.  
390 2019; Martin et al. 2021; Yang et al. 2014). Three studies were excluded from meta-analysis  
391 because two were validation studies (Kramer et al. 2014; Campbell et al, 2013) and one study  
392 presented data in an unusable format (Del Carmen Nogales et al. 2017). The included studies  
393 for morphokinetics were from eight different countries in total (USA, UK, Spain, Italy, India,  
394 Turkey, Taiwan and China). A summary of the characteristics of the included studies are  
395 reported in Tables II-VI.

396 Subsequently in this review we have considered morphological associations of aneuploidy.  
397 Firstly, fragmentation was assessed using data from 10,008 embryos from 1,842 patients,  
398 extracted from five studies (Magli et al. 2001; Ziebe et al. 2003; Delimitreva et al. 2005; Magli  
399 et al. 2007; Minasi et al. 2016). The remaining studies provided no raw data for interpretation  
400 or when provided it was in an unusable format (Moayeri et al. 2008; Chavez et al. 2012; Vera-  
401 Rodriguez et al. 2015). All were retrospective cohort studies apart from one publication which  
402 was a consecutive case series (Minasi et al. 2016).

403 Secondly, abnormal cleavage was assessed in relation to ploidy status, and this included 4,788  
404 embryos from 1,100 patients from 10 retrospective cohort studies (Campbell et al. 2013; Rienzi  
405 et al. 2013; Vera-Rodriguez et al, 2015; Zhan et al. 2016; Lagalla et al. 2017; Zhang et al. 2017;  
406 Desai et al. 2018; Ho et al. 2018; McCoy et al. 2018; Ozbek et al. 2021). One study could not  
407 be included in the meta-analysis due to the limited provision of data (Davies 2012).

408 Thirdly, two cohort studies were meta-analysed to describe the relationship between embryo  
409 contraction and chromosome aberrations using data from 1,647 embryos from 460 patients  
410 (Vinals Gonzalez et al. 2018; Gazzo et al. 2020).

411 Finally, the presence of multinucleation was assessed in 18,676 embryos from 1,227 patients.  
412 Thirteen studies were included in the meta-analysis (Kligman et al. 1996; Magli et al. 2001;  
413 Agerholm et al. 2008; Ambroggio et al. 2011; Campbell et al. 2013; Mazur 2013; Munoz, 2014;  
414 Bayram, 2015; Balakier et al. 2016; Zhang et al. 2017; Hashimoto et al. 2016; Desai et al. 2018;  
415 Lee et al. 2019) and seven studies were included only as part of the narrative review (Scott,  
416 2010; Davies, 2012; Melzer et al. 2013; Yilmaz et al. 2014; Li, 2015; Goodman, 2016; Del  
417 Carmen Nogales et al. 2017). Eleven are cohort studies and the remaining nine are conference  
418 abstracts (Davies, 2012; Mazur, 2013; Melzer et al. 2013; Munoz, 2014; Bayram, 2015; Li,  
419 2015; Goodman, 2016; Del Carmen Nogales et al. 2017). The included manuscripts  
420 considering morphological prognostic factors were published from a broad range of countries  
421 (Tables II-VI).

#### 422 *Risk of bias and quality assessment results*

423 Overall, the quality assessment of the eligible studies demonstrated a moderate risk of bias,  
424 whereby all but one study was scored with a moderate risk of bias in at least one domain. In  
425 total, only 17 out of 58 studies (29%) appropriately addressed confounding. Similarly, few  
426 authors adequately described participant characteristics or the selection criteria used (n=18/58,  
427 31%). However, there was a low risk of bias for 'prognostic factor measurement' in most  
428 studies (n=45/58, 78%). The remaining studies had a moderate risk of bias within this category  
429 due to: unclear definitions of the prognostic factors (n=3), a lack of internal validation for the  
430 assessment of the morphological components (n=5), the use of standard morphology  
431 assessment at specific time points rather than the use of a TLS (n=4), or multiple methods used  
432 for prognostic factor measurement on the same cohort (n=1). Twenty-three studies were

433 considered a moderate risk of bias because they did not disclose the proportion of embryos  
434 with PGT-A results unavailable, and five studies had a high risk of bias since this was >5%.  
435 Furthermore, 11 studies were categorised as high risk of bias for the use of FISH and one study  
436 for 'statistics and reporting' as we consider their conclusions and results to be erroneous given  
437 the data presented (Davies, 2012). Finally, 17 studies within the 'statistics and reporting'  
438 domain were graded as a moderate risk of bias for: limited presentation of analytical strategy  
439 or data (n=10), poor modelling techniques and validation methods (n=5), and inappropriate  
440 statistical techniques (n=2). The results obtained using the QUIPS tool to determine the risk of  
441 bias are summarised in Supplementary Table SIV. The quality of reporting was assessed using  
442 STROBE (Supplementary Fig. S1).

#### 443 *Morphokinetics and ploidy*

444 The following morphokinetic variables (Table I) were significantly delayed in aneuploid  
445 embryos: tPB2, t2, t4, t6, t7, t8, t9, tB, tEB, tHB. In contrast, tPNf, tM, tSB, cc3, S2, S3, and  
446 t5-t2 had no prognostic ability (Supplementary Fig. S2). Interestingly, euploid embryos were  
447 significantly delayed for cc2; however, this finding, t6 and tHB were no longer statistically  
448 significant when studies using FISH and/or blastomere biopsy were excluded. Additionally, t3,  
449 t5, and tPNf demonstrated significant differences exclusively in the subgroup analysis. The  
450 variables tPNf, t2, t3, t4, t5 were all delayed by up to 1 hour in aneuploid embryos. The  
451 variables tPB2 and t7 were delayed by > 1hour in aneuploid embryos, however, these results  
452 come from a subgroup analysis including only one study (1.3 hrs, 95% CI 0.88-1.72 and 1.8  
453 hrs, 95% CI 0.34-3.26, respectively). The following variables were the most delayed in  
454 aneuploid embryos: t8 (1.13 hrs, 95% CI 0.21-2.05; three studies; n=742; I<sup>2</sup>= 0%), t9 (2.27 hrs,  
455 95% CI 0.5-4.03; two studies; n=671; I<sup>2</sup>=33%), tB (1.99 hrs, 95% CI 0.15-3.81; four studies;  
456 n=1640; I<sup>2</sup>=76%), and tEB (2.35 hrs, 95%CI 0.06-4.63; four studies; n=1640; I<sup>2</sup>=83%) (Fig.  
457 6).

458 On visual inspection, these results were concordant with the prognostic factor graphs, apart  
459 from t8. Similarly, tSC, which was analysed solely by a prognostic factor graph, resulted in  
460 inconsistent differences. The only study excluded from the meta-analysis that was not a  
461 validation study analysed morphokinetics per chromosomal abnormality and found that  
462 complex embryos had shorter cleavage times (Del Carmen Nogales et al. 2017). Finally, the  
463 sensitivity analyses did not change our conclusions with the exception of tSB that became  
464 significant using a fixed effects model (Supplementary Figs S3 and S4).

#### 465 *Fragmentation and ploidy*

466 Fragmentation was associated with aneuploidy in six out of the eight included studies. The  
467 three most recent studies had the lowest risk of bias; two found no association and one found  
468 that a higher degree fragmentation was associated with aneuploidy (Minasi et al. 2016; Vera-  
469 Rodriguez et al. 2015; Chavez et al. 2012). Only four authors in total provided raw data that  
470 could be extracted into a line graph displaying a general trend of increasing prevalence of  
471 aneuploid embryos for increasing degrees of fragmentation (Supplementary Fig. S5).

#### 472 *Abnormal cleavage and ploidy*

473 Pooled direct uneven cleavage, DUC1 and DUC2 (Table I), had no association with  
474 chromosomal normality (RR 1.09, 95%CI 0.83-1.44; RR 1.26, 0.98-1.61; RR 0.74 95% CI  
475 0.26-2.1, respectively) (Supplementary Fig. S6). In contrast, reverse cleavage appears to  
476 provide some prognostic information specifically for euploidy (RR 1.36, 95% CI 1.14-1.63;  
477 five studies; n=3053;  $I_2=22\%$ ) (Fig. 7). There was a trend for more aneuploid embryos  
478 displaying DUC1 when studies using FISH and blastomere biopsy were excluded, however,  
479 this was not statistically significant (RR1.26, 95% CI 0.98-1.61; five studies, n=1917;  $I_2=27\%$ )  
480 (Supplementary Fig. S6). Only one study was not included in the meta-analysis owing to  
481 limited provision of data, and this concluded that embryos exhibiting DUC1 were more likely

482 be aneuploid (57%, n=21) versus euploid (30%, n=44),  $p=0.01$ ) (Davies 2012). Our findings  
483 were unchanged in a sensitivity analysis excluding studies with the highest risk of bias  
484 (Supplementary Fig. S7). A sensitivity analysis using a fixed effect model resulted in DUC2  
485 being significantly more prevalent in aneuploid embryos relative to euploid (Supplementary  
486 Fig. S8).

#### 487 *Contractions and ploidy*

488 Two studies examined the association between the presence of contractions and ploidy status  
489 and found that this observation was significantly more likely to occur in aneuploid embryos  
490 (RR 0.67, 95% CI 0.48-0.96; two studies, n=1,626,  $I_2=84\%$ ) (Fig. 8). These findings remained  
491 consistent in the sensitivity analysis (Supplementary Fig. S7).

#### 492 *Multinucleation and ploidy*

493 No association with ploidy was found for embryos assessed on day 2 or at the 2-cell stage for  
494 multinucleation (RR 0.69, 95% CI 0.29-1.63, four studies, n=3650,  $I_2=0\%$ ; RR 0.82 95% CI  
495 0.64-1.04, seven studies, n=2418,  $I_2=47\%$ , respectively), however there may be prognostic  
496 potential in multinucleation persisting to the 4-cell stage (RR 0.52, 95% CI 0.29-0.91; six  
497 studies, n=1703,  $I_2=82\%$ ) (Supplementary Fig. S9 and Fig. 9, respectively). This remains  
498 uncertain since the subgroup analysis was insignificant, albeit trending towards an increased  
499 prevalence in aneuploid embryos (RR 0.56, 95% CI 0.28-1.14; four studies, n=1106,  $I_2=88\%$ ).  
500 Furthermore, 4-cell multinucleation was significantly associated with ploidy using a fixed  
501 rather than random effects model (Supplementary Fig. S8). Multinucleation on day 2 and at the  
502 2-cell stage also had conflicting results in this sensitivity analysis; both were associated with  
503 aneuploidy but they remained insignificant in the subgroup analysis. Of the seven studies not  
504 included in the meta-analysis, two demonstrated association with ploidy when multinucleation  
505 was assessed during standard morphology assessments and one at the 4-cell stage (Melzer et

506 al. 2013; Yilmaz et al. 2014; Scott, 2010). In contrast, three studies reported no association  
507 with multinucleation when examined during daily morphology assessments (Davies, 2012;  
508 Goodman, 2016; Del Carmen Nogales et al. 2017) or at the 2-cell stage (Li, 2015). The findings  
509 of the main analysis were unchanged when excluding studies with the highest risk of bias  
510 (Supplementary Fig. S7).

## 511 **Discussion**

### 512 *Key findings*

513 Our study has found that aneuploid embryos are, on average, delayed by  $\geq 1$  hour in t8 and  $\geq$   
514 2 hours in the morphokinetic variables t9 and tEB. Overall, in the weighted mean difference  
515 analysis, seven morphokinetic variables were significantly delayed in aneuploid embryos  
516 (tPB2, t2, t4, t7, t8, t9, tEB). Blastocysts displaying contractions are associated with aneuploidy  
517 and reverse cleavages are more prevalent in euploid embryos, although these results should be  
518 interpreted with caution and investigated further before any conclusions can be drawn. In  
519 addition, although not statistically significant, there is a trend towards aneuploid embryos  
520 displaying multinucleation persisting to the 4-cell stage. The trend between increasing  
521 percentage fragmentation and aneuploidy needs confirming in future studies owing to very  
522 low-quality evidence.

### 523 *Morphokinetics and ploidy*

524 Since the development of the Campbell model there has been a plethora of attempts to test and  
525 create models for ploidy status, each with significant limitations (Campbell et al. 2013). This  
526 original model has been tested by several authors; only Desai et al. (2018) was able to reliably  
527 risk stratify for aneuploidy (Kramer et al. 2014; Rienzi et al. 2015; Zhang et al. 2017; Desai et  
528 al. 2018). This may be because the morphokinetics of embryos are so sensitive to laboratory  
529 conditions that models may not be translatable between clinics or patient populations. Indeed,

530 this variability may also account for why some models incorporate early cleavage parameters  
531 (Chavez et al. 2012; Chawla et al. 2015; Patel et al. 2016; Del Carmen Nogales et al. 2017) and  
532 some late, blastulation variables (Campbell et al. 2013; Kramer et al. 2014; Desai et al. 2018;  
533 Lee et al. 2019; Martin et al. 2021). There are several common limitations to the published  
534 models, including the lack of control of confounders and the use of apparent validation by some  
535 authors, leading to model overestimation (Basile et al. 2014; Chawla et al. 2015; Del Carmen  
536 Nogales et al. 2017; Desai et al. 2018). In fact, confounding variables were overlooked in over  
537 70% of the included studies (Supplementary Fig. S1). Four articles attempted to adjust for age,  
538 finding no association between age and morphokinetics (Rienzi et al. 2015; Mumusoglu et al.  
539 2017; Desai et al. 2018; Martin et al. 2021). Conversely, BMI was found to be associated with  
540 delayed morphokinetics, while yet another such study demonstrated no such association  
541 (Martin et al. 2021; Mumusoglu et al. 2017). The effects of stimulation dosages were only  
542 assessed by three authors; two concluded there were no dose dependant differences, whereas  
543 one reported higher dosages were associated in delayed development kinetics (Campbell et al.  
544 2013; Martin et al. 2021; Mumusoglu et al. 2017).

545 In comparison to t8, t9 and tEB, the variables tPB2, t2, t4, and t7 were less dramatically delayed  
546 in aneuploid embryos, therefore in the context of such wide CIs are less likely to reliably predict  
547 ploidy status. Of the most delayed variables, t8 and t9 had minimal heterogeneity ( $I_2=0\%$  and  
548  $33\%$ , respectively), whereas tB and tEB were substantially heterogenous ( $I_2=76\%$  and  $83\%$ ,  
549 respectively). The reasons for the heterogeneity are multifactorial including diverse patient  
550 populations, insufficient control for confounders, lack of standardisation of morphokinetic  
551 annotations, differences in laboratory and genetic testing techniques, and diverse embryo  
552 culture conditions. It must be highlighted that the results from tB and tEB are significantly  
553 heterogenous, therefore conclusions regarding these variables cannot reliably be drawn. That  
554 said, the heterogeneity for tEB is trending towards aneuploidy rather than traversing across the



555 line of no effect. Ordinarily we would be opposed to the meta-analysis of such heterogenous  
556 results, however the aim of this systematic review was not to provide a summary statistic to be  
557 translated directly into model development but to indicate potential prognostic markers for  
558 testing at local units. Whilst they are heterogenous, the results highlight the trend towards  
559 blastulation parameters predicting aneuploidy but, that said, we acknowledge that further  
560 research is needed to confirm our findings for tB and tEB.

561 The sensitivity analysis did not alter the results when studies with a high risk of bias were  
562 excluded, however tSB became significant with the use of a fixed effects model. This would  
563 indicate the need for more data to reliably conclude whether this variable could act as a  
564 prognostic marker. Interestingly, two morphokinetic studies were of higher quality and had  
565 comparable findings to our conclusions (Mumusoglu et al. 2017; Martin et al. 2021).

566 The association of day of blastocyst formation with aneuploid rates has been extensively  
567 studied, illustrating an increasing prevalence of aneuploidy from day 5 to day 7 blastocysts  
568 (Whitney et al. 2013; Minasi et al. 2016; Su et al. 2016; Kaing et al. 2018; Werland et al. 2017;  
569 Tiegs et al. 2019; Hernandez-Neito et al. 2019; McDaniel et al. 2021). Critics of time-lapse  
570 technology would argue that there is little to be gained from the study of cleavage parameters  
571 over the day of blastocyst formation using traditional monitoring. We argue that whilst day of  
572 blastocyst formation is a useful tool to counsel patients with limited access to time lapse, the  
573 accuracy and practicality that a TLS offers (for assessing readiness for biopsy whilst remaining  
574 in culture) is irreplaceable. Relying solely on traditional methods can lead to inaccuracies with  
575 the timing of blastulation in comparison to a TLS, where t0 is standardised to tPNf or time post  
576 insemination (hpi), allowing a more precise discrimination of a viable embryo despite slower  
577 development. The most successful morphokinetic logistic regression models for live birth are  
578 now much more complex than those using pre-defined thresholds, such as tSB <116 hrs or  
579 more traditional hierarchical models (Petersen et al. 2016; Zaninovic et al. 2017; Fishel et al.

580 2018). Time lapse therefore allows a statistical interpretation of embryo development whilst  
581 accounting for confounders that is not possible using traditional methods or univariate analysis.  
582 The variables more confidently associated with aneuploidy in this review are t8 and t9, factors  
583 that can only be considered through time lapse. It must also be considered that whilst tEB  
584 showed prognostic potential for ploidy, tSB and tB were not significantly associated,  
585 highlighting the precise nature of these associations rather than simply blastocyst formation.  
586 Finally, it has been suggested that there is some degree of multi-collinearity between cleavage  
587 and blastocyst kinetics, and this is illustrated by the fact that several authors have used earlier  
588 variables to predict blastocyst development (Wong et al. 2010; Cruz et al. 2012; Dal Canto et  
589 al. 2012; Hashimoto et al. 2012; Desai et al. 2014; Kirkegaard et al. 2013; Milewski et al.  
590 2016). Therefore, this raises the question as to whether cleavage variables add prognostic value  
591 over the later blastulation parameters. Unfortunately, this has not been directly compared as  
592 published models either incorporate early cleavage parameters (Chavez et al. 2012; Chawla et  
593 al. 2015; Patel et al. 2016; Del Carmen Nogales et al. 2017) or blastulation variables (Campbell  
594 et al. 2013; Kramer et al. 2014; Desai et al. 2018; Lee et al. 2019; Martin et al. 2021). This  
595 would be an interesting question to drive future research, and care would need to be taken to  
596 not ‘cherry-pick’ variables to be included in prognostic model development however, as this  
597 can introduce significant bias outside the context of prognostic factor research (Riley et al.  
598 2019).

599 More recently, artificial neural networks have demonstrated an impressive ability to evaluate  
600 images of pre-implantation embryos. Chavez-Badiola et al. (2020) developed a ranking system  
601 for ploidy status using this technology, with an impressive AUC of 0.70. Interestingly, two  
602 groups have investigated if there was an additive effect of using morphokinetic algorithms with  
603 artificial intelligence to improve diagnostic accuracy (Barnes et al. 2020; Huang et al. 2021).  
604 Barnes et al. (2020) demonstrated that both work synergistically to improve the AUC from

605 0.62 when solely image analysis is used to 0.76 (Barnes et al. 2020). Huang et al. (2021),  
606 similarly found the AUC increased from 0.57 to 0.77 with the addition of morphokinetics, age  
607 and full video analysis. This use of artificial intelligence in combination with morphokinetic  
608 models is a new direction of research that is evolving. Initial results appear promising and  
609 further studies are needed to demonstrate the application of this methodology. It would be  
610 beneficial for future work to include a prospective study design to validate these more complex  
611 models.

### 612 *Morphological features and ploidy*

613 It has been established that embryos with higher degrees of fragmentation have lower  
614 implantation rates; if the relationship suggested by our results is in fact true, the aetiology may  
615 be, in part, due to aneuploidy (Ziebe et al. 1997; Ebner et al. 2001). The quality of the evidence  
616 presented in all studies is poor, predominantly because of the use of unreliable genetic  
617 technologies (all used blastomere biopsy of intact cells and many adopted the use of FISH).  
618 Furthermore, the characteristics of the included patients are also extremely heterogenous. Some  
619 studies include couples with a good prognosis, in contrast to others focussing on patients with  
620 recurrent miscarriage or advanced maternal age, with no methods used to account for this  
621 (Tables II-VI). Notably, the fragmentation assessment method and timing were also  
622 inconsistent. This is important given that one author concluded that fragmentation was only  
623 associated with aneuploidy when assessed at the 7 and 8 cell stages and others when assessed  
624 at 48 hours (Magli et al. 2007; Ziebe et al. 2003). Other authors categorised fragmentation as  
625 ‘high’ or ‘low’; these arbitrary thresholds make testing association more unreliable and to our  
626 knowledge there is no evidence to support such an approach (Vera-Rodriguez et al. 2015;  
627 Chavez et al. 2012). All considered, we cannot reliably conclude whether percentage  
628 fragmentation is associated with aneuploidy. There is, therefore, a need for future adequately

629 powered studies to examine fragmentation using time-lapse, next generation sequencing, and  
630 with adequate control of confounding.

631 Reverse cleavage has been associated with euploidy in our results, but it should be considered  
632 that these findings come from the contribution of one study and all other authors concluded  
633 that there was no significant difference (Ozbek et al. 2021). Whilst this was the largest study  
634 with 8% of embryos (n=78/1015) displaying reverse cleavage, the event rate remains low. For  
635 instance, we have calculated that for a power of 80% and a value of 0.05 for alpha you would  
636 need a sample size of 1617 embryos with at least 147 displaying reverse cleavage in order to  
637 find a difference when one truly exists. This is presuming a difference of 12% in the euploid  
638 rate between embryos displaying reverse cleavage and those that did not (extrapolated from the  
639 studies in this meta-analysis) and assuming a 1:10 ratio for the presence of this dysmorphism  
640 to normal cleavage. This illustrates a significant limitation of studies investigating  
641 dysmorphisms with such low prevalence. Ozbek et al. (2021) provide no explanation why  
642 embryos displaying reverse cleavage may have a higher incidence of euploidy, particularly in  
643 the context of the dramatically inferior live birth rates stated in their study when compared to  
644 normally cleaved euploid embryos (23% versus 56%). This association between reverse  
645 cleavage and inferior implantation rates has been replicated by several other authors, therefore  
646 we highly doubt that a relationship between euploidy and reverse cleavage truly exists (Barrie  
647 et al. 2017; Liu et al. 2014; Desai et al. 2018), particularly considering the underpowered nature  
648 of this study and the fact that reverse cleavage is often associated with compromised embryo  
649 development and quality. In fact, studies of bovine embryos have demonstrated an association  
650 with aneuploidy, strengthening the argument that these results are likely spurious (Magata et  
651 al. 2019). There have also been multiple factors independently associated with reverse  
652 cleavage, such as antagonist cycles, low progressive sperm motility and the use of ICSI (Liu et  
653 al. 2014).

654 While our main analyses indicate that direct uneven cleavage is not associated with ploidy,  
655 there is a significant limitation to the designs of the included studies. Aneuploid embryos may  
656 have been inadvertently excluded, either because only good quality embryos were biopsied or  
657 because a significant proportion (up to 87%) arrested in their development before biopsy (Zhan  
658 et al. 2016; Lagalla et al. 2017). It would be safer to conclude that embryos that have displayed  
659 direct cleavage that make it to the blastocyst stage could still be considered for biopsy or  
660 transfer: it has been demonstrated that they can result in live births, however, the patient must  
661 be warned of the increased likelihood of adverse outcome (Zhan et al. 2016; Fan et al. 2016;  
662 Ozbek et al. 2021). What causes these abnormal cleavages remains largely unknown, although  
663 it has previously been associated with the follicular environment of oocytes, poor-motility  
664 sperm and GnRH antagonists (Liu et al. 2014). Considering this, and the fixed effects  
665 sensitivity analysis that demonstrated DUC2 to be significantly associated with aneuploidy,  
666 further investigation is required to confirm or refute these findings.

667 Embryo contraction is a common phenomenon observed in a TLS (42% of embryos in the  
668 included studies), yet despite an understanding of the physiology, causality remains  
669 controversial. It has been hypothesised that contractions may assist in embryo hatching,  
670 although recent evidence does not support this theory (Gazzo et al. 2020). Future research  
671 should exclude studies that have undergone assisted hatching on day 3 as this has been related  
672 to altered frequency of contractions, a limitation of the included studies in the current analysis  
673 (Gazzo et al. 2020; Vinals Gonzalez et al. 2018). Embryos displaying contractions were more  
674 likely to be aneuploid, however this data comes from only two studies therefore further research  
675 is recommended to investigate this association.

676 The relationship between multinucleation at the 4-cell stage and ploidy is yet to be established  
677 given the significantly heterogenous results ( $I_2=88\%$ ) and contradictory findings in the  
678 subgroup and sensitivity analysis. It has been described how the presence of multinucleation

679 and associated aneuploidy can ‘self-correct’ by exclusion of cells during compaction or  
680 blastulation (Kligman et al. 1996; Ambroggio et al. 2011; Balakier et al. 2016; Desai et al.  
681 2018). This complicates our understanding but may explain why only embryos displaying  
682 multinucleation at the 4-cell stage may be associated with aneuploidy and how healthy babies  
683 have been born from such embryos (Yilmaz et al. 2014; Meriano et al. 2004). Furthermore,  
684 multinucleation is only visible at interphase during conventional culture, therefore is likely to  
685 be underreported in the five included studies not utilising a TLS (Kligman et al. 1996; Magli  
686 et al. 2001; Agerholm et al. 2008; Scott, 2010; Ambroggio et al. 2011). In addition to  
687 aneuploidy, the presence of multinucleation has been related to the use of agonist down-  
688 regulation (perhaps associating it with poor ovarian reserve), high FSH dosages, high oestrogen  
689 levels and excessive oocyte numbers (Scott, 2010; De Cássia Savio Figueira et al. 2010; Desai  
690 et al. 2018). Despite this, across all the morphological studies only two manuscripts reporting  
691 the use of statistical modelling to adjust for age and no other confounders were considered  
692 (Minasi et al. 2016; Desai et al. 2018). In contrast to embryos displaying abnormal cleavage,  
693 there has been no difference demonstrated in the development of multinucleated embryos to  
694 expanded blastocyst, therefore our results are unlikely to be affected by arrested embryos  
695 (Goodman, 2016).

#### 696 *Strengths and limitations of this systematic review and meta-analysis*

697 The findings of our study should be interpreted with caution due to an overall moderate risk of  
698 bias and significant heterogeneity of the included studies. Attempts have been made to control  
699 for sources of heterogeneity in our study design. This was primarily through subgroup analysis  
700 by excluding studies using older, unreliable technologies. In some variables the heterogeneity  
701 was calculated to be worse in the subgroup than in the main analysis, and this highlights the  
702 manifestation of other factors contributing to the diversified results. Heterogeneity may also  
703 exist in the way studies classified mosaics; this definition remains ambiguous in several studies.

704 This is important as mosaic embryos have previously been shown to have independent  
705 morphokinetic characteristics (Martin et al. 2021). It is also worth considering that whilst it is  
706 generally accepted that PGT-A biopsy results are concordant with the rest of the embryo in  
707 most cases, it is not absolute and sceptics exist (Victor et al. 2018; Esfandiari et al. 2016;  
708 Gleicher and Orvieto, 2017). There have been reports and suggested models of so called ‘self-  
709 correction mechanisms’ whereby mosaic embryos become more chromosomally normal as  
710 development progresses, although the existence of this phenomenon remains debatable  
711 (Capalbo and Rienzi, 2017; McCoy, 2017; Bolton et al. 2016; Munné et al. 2017; Coticchio,  
712 Barrie, et al. 2021).

713 Of the studies included in the meta-analysis of morphokinetic variables, all used ICSI, thus  
714 timing development from insemination apart from two groups, namely Lee et al. (2019) and  
715 Chavez et al. (2012). Unfortunately, t0 remains ambiguous in the study by Chavez et al. (2012)  
716 due to unanswered correspondence. Lee et al. (2019) used both standard IVF and ICSI for the  
717 included embryos, therefore this is a significant confounding factor to consider as they time  
718 conventional IVF embryos from the addition of spermatozoan to the oocyte; the accepted  
719 standard would be from tPNf. The exclusion of Chavez et al. (2012) for cc2 would make this  
720 variable not associated with ploidy status rather than associated with euploidy. That said, this  
721 study is not included in the subgroup analysis therefore the findings for this variable and S2  
722 remain unchanged when considering the studies using the most reliable genetic technology.

723 While the conclusions drawn from this study are taken from data of over 40,000 embryos, the  
724 quality of evidence is low due to imprecision and large CIs. Only a limited number of studies  
725 tested each variable, leading to low event rates for some variables and the inclusion of only a  
726 handful of studies of those reporting usable data. As discussed previously, this is even more  
727 profound when the sample size of patients rather than embryos is considered. Furthermore, if  
728 the true population mean lies on the lower boundary of the 95% CI, we would be unable to

729 predict ploidy using tB and tEB. A final limitation is our inability to test for publication bias.  
730 That said, embryological studies tend to report a whole array of potential prognostic factors for  
731 ploidy per manuscript, therefore there is less risk of reporting only positive findings.  
732 Our review does have multiple strengths, firstly the thorough methodological approach and  
733 comprehensive search of multiple variables and their association with ploidy status will be the  
734 first of its kind. Secondly, our meta-analyses of morphological and morphokinetic variables  
735 provide a strong argument for the local development of morphokinetic algorithms for ploidy  
736 and suggest those most likely to be included. Finally, we have provided an extensive critique  
737 of existing research and the quality of evidence in order to inform future prognostic  
738 methodologies.

### 739 **Conclusion**

740 In this first systematic review and meta-analysis of morphological and morphokinetic  
741 associations with ploidy, we have reported the most reliable prognostic markers to be t8, t9,  
742 and tEB. These results support the mechanism by which algorithms for live birth have  
743 predictive ability, suggesting that aneuploidy causes delayed cytokinesis. That said, we have  
744 demonstrated considerable variability within aneuploid and euploid embryos making  
745 definitively classifying them impossible. Time-lapse is, therefore, not suitable as a method to  
746 diagnose the ploidy status of pre-implantation embryos. Considering recent reports, it may be  
747 that morphokinetic algorithms can be used as a tool to risk stratify embryos for ploidy status,  
748 and more accurately by instrumenting artificial intelligence. Further research is needed to  
749 determine the suitability of machine learning for embryo assessment and selection.

750 Owing to the limited number of studies, heterogenous results and poor-quality evidence the  
751 suggested association between aneuploidy and multinucleation at the 4-cell stage, frequency of  
752 embryo contractions and fragmentation needs to be investigated further. Adequately powered  
753 studies should be conducted to test our hypothesis that reverse cleavage is not associated with



754 euploidy. We propose that incorporating associated morphological factors into a prognostic  
755 model may work synergistically to improve euploid embryo selection. On the other hand,  
756 multinucleation assessed on day 2 or at the 2-cell stage and direct unequal cleavage in an  
757 embryo that progresses to a blastocyst do not appear to be associated with ploidy.  
758 Differing clinical and laboratory practices and inadequate control for confounders in previous  
759 research is most probably why TLS is rated as ‘amber’ by the UK regulatory body (HFEA  
760 2021). There have been calls for multi-centre randomised controlled trials heard for many years  
761 (Armstrong et al. 2019). Instead, we argue that since embryos are so significantly affected by  
762 local conditions it may be more appropriate to robustly test models developed in-house.  
763 While this review concludes that a TLS cannot be used to definitively diagnose ploidy status,  
764 further research is needed to comprehend the potential of morphokinetic algorithms to prioritise  
765 embryos for biopsy, or to use morphokinetics to select between euploid embryos. Therefore,  
766 we will test this hypothesis in a cohort study at CARE Fertility using a morphokinetic dataset  
767 of over 8,000 embryos with known PGT-A outcomes. This model will be trained, tested and  
768 validated geographically and, if successful, a prospective study will determine its  
769 discriminative ability. If successful, this has the potential to be a meaningful improvement for  
770 patients, aiming to make more advanced and costly reproductive technologies more accessible.

#### 771 **Data availability**

772 The data underlying this article are available in the article and in its online supplementary  
773 material.

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### 783 **Authors' roles**

784 The study was conceived by Prof. Arri Coomarasamy and A.B. as part of a PhD programme of  
785 research undertaken by T.B at the University of Birmingham. The study protocol was designed  
786 by T.B., S.M., and supervised by Prof. Arri Coomarasamy. Study selection and extraction of  
787 data was performed by T.B. and A.B., followed by a quality assessment by T.B. All authors  
788 analysed and interpreted the data. T.B. drafted the first manuscript, this was subsequently  
789 approved by all authors before publication. C.E. provided statistical support and A. Campbell  
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793 University of Birmingham

### 794 **Conflict of interest**

795 Alison Campbell is a minor shareholder at CARE Fertility. No other conflicts of interest  
796 exist. To note, CAREmaps<sup>®</sup> is a technology for which patients are charged extra for at  
797 CARE.

798

799

800 **Figure legends**

801

802 **Figure 1** Flow diagram for study selection process for human embryo morphokinetics search.

803

804 **Figure 2** Flow diagram for study selection process for human embryo fragmentation search.

805

806 **Figure 3** Flow diagram for study selection process for human embryo abnormal cleavage

807 search.

808

809 **Figure 4** Flow diagram for study selection process for human embryo contraction search.

810

811 **Figure 5** Flow diagram for study selection process for human embryo multinucleation search.

812

813 **Figure 6** Weighted mean difference and prognostic factor analysis graphs of aneuploid vs.

814 euploid human embryos for morphokinetic variables:

815 t8 **(A)**: time from insemination to 8 cells (hpi)

816

817 t9 **(B)**: time from insemination to 9 cells (hpi)

818

819 tB **(C)**: time from insemination to the formation of a full blastocyst (hpi)

820

821 tEB **(D)**: time from insemination to expanded blastocyst (hpi)

822

823 **Figure 7** Relative risk of euploidy in a human embryo displaying reserve cleavage (RC).

824

**Figure 8** Relative risk of euploidy in a human embryo displaying contractions.

825

**Figure 9** Relative risk of a multinucleated (MN) human embryo being euploid when assessed

826

at the 4- cell stage.

827

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