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2 This retrospective, single site observational study aimed to delineate five abnormal 3 embryonic developmental phenotypes assessing their prevalence, implantation potential and suitability for inclusion in embryo selection models in an IVF 4 5 laboratory. A total of 15, 819 embryos from 4559 treatment cycles cultured in 6 EmbryoScope® incubators between January 2014 and January 2016 were included. 7 Time lapse images were assessed retrospectively for five abnormal embryo 8 phenotypes; direct cleavage, reverse cleavage, absent cleavage, chaotic cleavage and 9 cell lysis. The prevalence of each abnormal phenotype was assessed. The embryo 10 fate, embryo quality and implantation rate were determined and compared to a 11 control embryo cohort. The collective prevalence for the five abnormal phenotypes 12 was 11.39% where chaotic cleavage and direct cleavage together constituted 9.63%. 13 The implantation rate was 17.4%, 0%, 25%, 2.1% and 0% for direct, reverse, absent, 14 chaotic cleavage and cell lysis, respectively. The overall implantation rate for all 15 abnormal embryos was significantly lower compared to the control population 16 (6.9% vs 38.66%, p<0.0001, Fisher's exact). The proportion of good quality embryos 17 in each category never reached over 24%. Embryos exhibiting an abnormal phenotype may have reduced developmental capability manifested in both embryo 18 19 quality and implantation potential when compared to a control embryo cohort. 20 21 Keywords 22 Abnormal phenotype; embryo development; time lapse; morphokinetics 23

24 Introduction

25 Abnormal cleavage patterns exhibited by some embryos include, but are not limited 26 to; abnormal syngamy, direct cleavage (DC), reverse cleavage (RC), absent cleavage in the presence of karvokinesis (AC), chaotic cleavage (CC) and cell lysis (CL). 27 28 The first of five abnormal cleavage patterns investigated here is direct cleavage (DC). 29 This is the cleavage of one blastomere into three, instead of the expected two, 30 daughter cells (supplementary figure 1). The ability of these embryos to create a 31 pregnancy has been shown to be significantly reduced (Rubio et al., 2012) where 32 13.7% of all examined embryos and 6.6% of transferred embryos underwent DC. 33 with 1.2% resulting in a clinical pregnancy. These embryos have been shown to have 34 a markedly decreased blastocyst formation rate when compared to their normal 35 counterparts (Athayde Wirka et al., 2014).

36 The second abnormal phenotype to be considered is reverse cleavage (RC); the 37 phenomenon of blastomere fusion (supplementary figure 1). Of 789 embryos 38 assessed for RC, defined as blastomere fusion or failed cleavage, 27.4% of embryos 39 were found to exhibit this abnormal cleavage pattern and were shown to have a 40 reduced implantation potential (Liu et al., 2014). An examination of 1698 embryos detected a prevalence of RC of 6.8% however embryos appeared to have similar 41 42 fragmentation, cell evenness and morphokinetic profiles compared to their non reverse cleaved counterparts (Hickman et al., 2012). This research concluded that 43 44 RC does not seem to impair embryo development to the blastocyst stage supported 45 by the findings of others (Desai et al., 2014).

Absent cleavage (AC) is defined as the process by which a blastomere undergoes a
pseudo division (seen as a 'roll') that does not produce two discernable blastomeres
but a single, or multiple, extra nuclei within the single blastomere (supplementary

49 figure 1). AC has previously been categorised under RC, termed type II RC (Liu et al., 50 2014). Of those embryos that underwent RC (27.4%), 82% were classed as type II; 51 absent cleavage rather than blastomere fusion. Further evidence of this specific 52 developmental pattern has not yet been published. This is perhaps due to the 53 likelihood that these embryos will not be used for treatment thus circumventing a 54 clinical need to further define this phenomenon 55 Chaotic cleavage (CC) results when an embryo undergoes apparent cleavage but 56 does not create distinctive blastomeres (supplementary figure 1). A single 57 investigation studying this cleavage pattern in 639 embryos found an overall 58 prevalence of 15%, a blastocyst formation rate of 14% and an implantation rate (IR) 59 of 0% (Athayde Wirka et al., 2014). Interestingly, this investigation also found that 60 35.2% of those exhibiting CC had good cleavage stage quality. This was however, 61 markedly lower than the other abnormal phenotypes observed (DC and abnormal 62 syngamy). Again, as with AC, this phenomenon may be under investigated due to the 63 reduced likelihood that embryos exhibiting this phenotype will be used in treatment. 64 Finally, an abnormal embryo developmental phenomenon that has yet to be

65 discussed in the literature, in terms of time lapse imaging of embryos from fresh

66 treatment cycles, is cell lysis (CL) (supplementary figure 1); a process often

67 visualized in frozen thawed embryos (Bottin et al., 2015; Rienzi et al., 2005; Tang et

al., 2006; Yeung et al., 2009). In an analysis of 891 frozen embryo transfer (FET)

69 cycles, no pregnancies resulted if CL occurred in over 50% of the embryo. However,

if CL accounted for 25 to 50% of the embryo the pregnancy rate was 3.2%;

significantly lower than if less than 25% CL had occurred (16.6%) (Tang et al., 2006)

supported by others (Bottin et al., 2015; Yeung et al., 2009).

73

Although these investigations are not entirely synonymous with the current analysis,
they provide evidence that embryos with lysed cells have a reduced implantation
potential.

77

78 As discussed above, there is disparity in the literature with regards to the prevalence 79 and implication of the presence of certain abnormal phenotypes. Further 80 investigation into these phenomena is required to determine if their presence is 81 severe enough to exclude these embryos from selection for use in treatment. Five 82 abnormal cleavage patterns exhibited by embryos (DC, RC, AC, CC and CL) are 83 explored in 15,819 embryos detailing their prevalence, implantation potential, and 84 the suitability for inclusion of these potential deselection criteria in embryo selection 85 models.

86

87 Materials and Methods

This investigation was a single site, retrospective observational design approved by
the North West Research Ethics Committee (ref: 14/NW/1043) as well as gaining
Institutional Review Board approval. All procedures and protocols complied with UK
regulation (Human Fertilisation and Embryology Act, 1990, 2008). Data were
obtained from 4559 treatment cycles including 15,819 embryos cultured in the
EmbryoScope® incubators between January 2014 and January 2016.

95 Ovarian Stimulation

96 Pituitary down regulation was achieved using either a gonadotrophin releasing 97 hormone agonist (buserelin, Suprecur®, Sanofi Aventis, UK) or antagonist (cetrorelix acetate, Cetrotide®, Merck Serono, Germany). Ovarian stimulation was 98 99 performed using urine derived or recombinant follicle stimulating hormone 100 (Progynova (Bayer, Germany), Fostimon, Merional (IBSA, Switzerland), Menopur® 101 (Ferring Fertility, Switzerland), Gonal f® (Merck Serono). Doses were adjusted 102 based on patient demographic and response. Patients were given 5000IU of 103 subcutaneous hCG (Gonasi® HP, IBSA Pharmaceuticals, Italy) 36 hours prior to 104 oocyte collection. Luteal support was provided using 400mg of progesterone 105 pessaries twice daily (Cyclogest[®], Actavis, UK) until the pregnancy test was 106 performed.

107

108 Oocyte retrieval and embryology

109 Ultrasound guided oocyte collection was performed transvaginally under sedation 110 (Diprivan, Fresenius Kabi, USA). Collected oocyte cumulus complexes were cultured 111 in 4 well dishes (Nunc[™], Thermo Scientific, USA) each well containing 0.65ml GIVF[™] 112 (Vitrolife, Gothenburg, Sweden) covered with 0.35ml OVOIL[™] (Vitrolife) in a 113 standard incubator (Sanyo Multigas MCO 18M). Sperm preparation was performed 114 using a standard gradient separation (ISolate®, Irvine Scientific, USA) at 0.3 relative 115 centrifugal force (rcf) for ten minutes followed by two washes at 0.6rcf for ten 116 minutes using GIVF[™]. Those oocytes destined for ICSI were prepared using 117 enzymatic (HYASE 10X[™], Vitrolife) and mechanical digestion. ICSI was performed on 118 all metaphase II oocytes (MII) approximately four hours following collection after 119 which time all injected oocytes were placed in individual culture drops of G1[™] (for

120 all cycles pre September 2014) or GTL[™] (all cycles post September 2014) (Vitrolife) 121 and cultured in the EmbryoScope® (Vitrolife). Those oocytes destined for standard 122 insemination (IVF) had this performed approximately four hours after collection and were replaced into a standard incubator until fertilisation check the following day. 123 124 Oocytes were then checked for fertilisation approximately 16 to 18 hours post 125 insemination (hpi) and all fertilised oocytes along with all unfertilised metaphase II 126 oocytes were placed in individual culture drops as with ICSI derived embryos and 127 cultured in the EmbryoScope®. Embryo selection was performed using the national 128 grading scheme (ACE/BFS guidelines (Cutting et al., 2008)) along with an internally 129 derived, embryo scoring algorithm (ESA). An ESA seeks to combine a number of 130 morphokinetic parameters that have been linked to an embryo's viability. The ESA 131 employed here was used as an additive to morphology with the latter remaining the 132 gold standard. This ESA included three morphokinetic parameters; s2 (time between 133 t3 and t4), cc3 (time between t4 and t5) and t5 with embryos graded in one of eight 134 categories from A+ to D-. Embryo transfer was performed using the highest grade 135 embryo(s) either three or five days post collection depending on the number of good 136 quality embryos the patient had on day three as well as how many were to be 137 transferred. Selected embryos were cultured in EmbryoGlue® (Vitrolife) for 10 to 30 138 minutes in a standard incubator prior to embryo transfer. Embryos were cultured at 139 37°C, 6% CO₂, 5% O₂, 89% N₂ throughout.

140

141 Analysis of time lapse information

142 The image interval on the EmbryoScope® was set to 10 minutes with seven focal

143 planes. Images were collected for the duration of culture immediately following ICSI

144 or fertilisation check (for IVF derived embryos) to utilisation. Images were assessed 145 by an embryologist for the abnormal embryonic phenotypes of interest. For DC, 146 embryos were classified into one of three categories: true DC (TDC, defined as all 147 three resultant cells cleaving on the subsequent cell cycle, each having a nucleus and 148 each included in the morula), false DC (FDC, one or more of the above criteria not 149 fulfilled) and unconfirmed DC (UDC, unable to classify as true or false). UDC embryos 150 were defined as such due to either obscurity preventing categorisation or the 151 cessation of culture before the morula stage was reached. A justification for the 152 choice of this classification, not reported elsewhere, lies in unit specific data 153 whereby two obviously distinct DC event patterns were visualized using time lapse 154 technology This, as well as previous reports of direct cleavage patterns (Kalatova et 155 al., 2015; Kola et al., 1987), led to the development of the three tiered classification 156 of DC events. With regards to the final criterion for TDC classification (inclusion of all 157 cells in the morula), this stage of development was used as an indicator that all cells, 158 abnormal or not, would contribute to the eventual blastocyst and would not be 159 excluded. Further to this, DC could be proposed as a correction mechanism whereby 160 the DC event is a means to remove surplus genetic material thus excluding the cells 161 from the eventual blastocyst, described here as FDC and a more favourable type of 162 DC event. . Direct cleavage from both one to three cells (DC1-3) and from two to five 163 cells (DC2-5) were included in the analysis. RC is defined simply as blastomere 164 fusion. AC is defined as the process by which a blastomere undergoes a pseudo 165 division (seen as a 'roll') that does not produce two discernable blastomeres but a 166 single, or multiple, extra nuclei within the single blastomere. CC is observed when an 167 embryo undergoes apparent cleavage but does not create distinctive blastomeres. CL

is defined as the loss of a blastomere through cell lysis (supplementary figure 1).
Although not exclusively a phenomena visualised through time lapse technology and
one that can be visulaised using standard embryo morphology assessments, CL is
predominantly seen in embryos following cryopreservation whereas here we
describe cell lysis in fresh embryos. Thus, this was included in the current
investigation to determine the effect of cell lysis on the viability of a fresh embryo.

175 Outcome measures and statistical analysis

176 The overall prevalence of the five abnormal embryo phenotypes was defined per 177 embryo and per treatment cycle. The average patient age, oocytes collected and 178 previous attempts were calculated for each of the five categories. The fate (transfer, 179 freeze, discard) of each abnormal embryo was determined as well as their quality on 180 the day of utilisation defined as good, average or poor (supplementary table 1). The 181 IR for each abnormal phenotype was determined where the origin of the fetal heart 182 could be confirmed i.e. using known implantation data from an abnormal embryo or 183 not. The number of single and double abnormal embryo transfers and the stage at 184 which the abnormal embryo(s) was transferred was also determined 185 (supplementary table 2). Statistical analyses included the student t test for the 186 comparison of the abnormal phenotype baseline information (patient age, oocytes 187 collected and previous attempts) to the control embryo baseline data. The Fisher's 188 exact test was used to compare the IR of the abnormal embryos with normal 189 counterparts. Results were considered significant at p<0.05. Statistical analysis was 190 performed using the statistical package Prism® 5 (GraphPad Software©, USA).

191

192 Results

209

210

193 Data were obtained from 15,819 embryos from 4559 treatment cycles cultured in 194 the EmbryoScope® between January 2014 and January 2016. Of the 15,819 195 embryos, 14,008 were derived from 3273 treatment cycles where no abnormal 196 divisions of interest (DC, CC, RC, AC and CL) were observed and thus constituted the 197 control group. These embryos resulted in 3456 embryos transferred and 1336 fetal 198 heartbeats (IR= 38.66%) (table 1). The remaining embryos (1811) were found to 199 pertain to a treatment cycle (n=1286) exhibiting an embryo with one of the 200 abnormal division patterns of interest. 201 202 Abnormal phenotypes with the highest prevalence per embryo observed were DC 203 and CC at 4.38% (TDC, FDC, UDC, collectively) and 5.25%, respectively. The 204 remaining phenotypes had considerably lower prevalence ranging from 0.41 to 205 0.84% (table 3). The overall prevalence per embryo observed of abnormal division 206 patterns was 11.39% (table 3). The IR of abnormal embryos ranged from 0 to 33.3% 207 (table 3). Of the five abnormal division patterns the IR of UDC, CC and RC were

significantly lower than normal counterparts; 12.5% (2/16), 2.1% (1/48) and 0%

(0/9), respectively (table 3). Furthermore, the overall IR of all abnormal embryos

was significantly lower than normal counterparts (6.9% (6/86) vs 38.66%) (table 1

and 3) and of the six implanted embryos, five resulted in a live birth, with no birth
defects, and one remains ongoing. In all cases the percent of good quality embryos
resulting from those exhibiting abnormal division patterns never reached above
24% and the majority of embryos were classified as poor quality (table 3). This is

also reflected in the utilisation of these embryos where the highest proportion of

each group was discarded (supplementary figure 2). The proportion of embryos
undergoing either DC1-3 or DC2-5 in each of the DC categories was as follows,
respectively; TDC, 16 and 32; FDC, 26 and 43; UDC, 176 and 404.
Patient age was significantly lower for those undergoing DC, RC and CC to those not

exhibiting an abnormal division pattern. The number of oocytes collected was found
to be significantly higher in treatment cycles containing abnormal embryos than
those not containing embryos exhibiting an abnormal division pattern. Finally, the
number of previous attempts was not found to be significantly different between any
of the abnormal division categories and the control embryo cohort (table 2).
Baseline information from treatment cycles containing an abnormal embryo did not
contribute to baseline information for the control cohort.

228

229 Discussion

230 The prevalence of DC in the literature has been stated as 13.7% (Rubio et al., 2012) 231 and 18% (Hickman et al., 2012). In the current analysis the overall prevalence of DC 232 was 4.38% (UDC, FDC and TDC combined) occurring in 1.22 embryos per treatment 233 cycle. The implantation potential of embryos undergoing DC has been stated as just 234 1.2% (Rubio et al., 2012) however, in the current analysis the IR was found to be 235 17.4% (4/23) (TDC, FDC and UDC combined); not significantly lower than that of the 236 control embryo cohort although this could be attributed to the reduced numbers. A 237 classification system of DC was not adopted by other publications therefore if FDC 238 were not considered, the IR would be significantly lower than those not exhibiting a 239 DC. Of the three categories, those that were classed as FDC had the highest IR, as one

240 might expect from the definitionThere is a paucity of literature regarding the exact 241 mechanisms underlying the phenomenon of DC however a recent comprehensive 242 review discusses both molecular and cellular mechanisms that could be related 243 (Kalatova et al., 2015). In particular, centrosome defects are suggested as possible 244 causes for DC facilitated through the lack of certain regulatory proteins such as p53. 245 The presence of surplus centrosomes leading to DC, as suggested by Kalatov et al 246 (2015), is reflected in an early investigation of tripolar oocytes. Genetic assessment 247 of tripolar DC oocvtes revealed three division patterns; DC to three cells (62%); 248 cleavage to a morphologically normal two cell 'embryo' (24%) and cleavage to a two 249 cell 'embryo' plus an extrusion (14%) (Kola et al., 1987). All triploid oocytes that had 250 undergone DC to three cells were chromosomally abnormal with each containing a 251 varied number of chromosomes (here considered a TDC). Those that cleaved to 252 morphologically normal two cell 'embryos' were found to be true triploid with each 253 blastomere containing a 69XXX/XXY chromosome complement. However, of those 254 oocytes that cleaved to a two cell 'embryo' plus an extrusion, 75% were found to 255 have two diploid blastomeres and a haploid extrusion. In the analysis presented 256 here, the IR of FDC, those embryos analogous to the two cell embryo plus an 257 extrusion, was 33.3% (2/6). Caution should be taken as the numbers are 258 considerably reduced in this group due to the need to use known implantation 259 embryos, however, this represents a result just over 5% lower than that of a 260 phenotypically normal embryo. Although speculative, the findings by Kola et al. 261 (1987) not only corroborate the aforementioned theory by Kalatova et al (2015) of 262 amplified centrosome material, but could also indicate that embryos have the 263 potential to correct genetic abnormalities. There are many studies detailing self

264 correction between the cleavage stage and the blastocyst stage of embryo 265 development (Barbash-Hazan et al., 2008; Li et al., 2005; Munne et al., 2005; Northop et al..2010; Voullaire et al., 2000). It has been noted that trisomy embryos correct 266 267 more often than other aneuploidies (Barbash-Hazan et al., 2008) possibly occurring 268 through the loss of a chromosome in trisomy cells (Munne et al., 2005). In addition, 269 in previous reports, CC could be misinterpreted as a DC thus causing the prevalence 270 of DC to appear falsely increased. The increased IR of DC seen in the present 271 investigation compared to previous reports may also be due to observers having 272 experience with the different categorisations of DC, making them proficient at 273 recognising patterns of FDC, such as blastomere behavior, allowing preferential 274 selection of a potential FDC in UDC cases. The reduced patient age and increased 275 number of oocytes collected may reflect a simple association between maternal age 276 and number of oocytes collected. However, it may also indicate that stimulation can 277 lead to reduced oocyte quality (Aboulghar et al., 1997) and high oocyte numbers 278 (>15) can reduce the chance of a live birth (Ji et al., 2013) which could manifest as an 279 abnormality such as DC.

280

RC occurred in 65 embryos (1.07 embryos per treatment cycle) of which 36 were either transferred or frozen where 26 were classed as good or average quality. It is likely that embryos classed as PQE were utilised due to unavailability of others. The R of embryos undergoing RC in the current investigation was 0% (0/9). The prevalence of RC has been reported as 6.8, 7 and as high as 27.4% in previous reports (Desai et al., 2014; Hickman et al., 2012; Liu et al., 2014). However, the rate of formation of usable embryos is in conjunction with others at approximately 40%

288 (Desai et al., 2014). There have been reports that RC is affected by other variables 289 such as ICSI and GnRH antagonists. Therefore a possible explanation for the 290 disagreement presented here could be due to the difference in baseline patient and 291 treatment variables, a consideration for further investigation. The phenomenon of 292 RC has been recognised previously with regards to frozen thawed embryos (Balakier 293 et al., 2000; Trounson, 1984). Balakier et al. (2000) sought to determine the 294 chromosomal changes in blastomeres that undergo fusion following thawing. This 295 analysis included 1141 embryos frozen on day two and 873 frozen on day three. RC 296 was found in 51 embryos of which 70% were classed as good quality. The overall 297 frequency of RC was 4.6% in day two embryos and 1.5% in day three embryos. A 298 slightly higher incidence of blastomere fusion was found in embryos created using 299 IVF when compared to ICSI. When a control group was observed (embryos not 300 subject to freezing and thawing) the prevalence of RC was 0.3%, a result not far from 301 that recorded in the present study (0.41%). The IR of embryos that underwent 302 blastomere fusion following thawing in the above investigation was very poor with 303 15 embryo transfers containing one abnormal and one normal embryo resulting in a 304 single live birth only. Again, a result similar to that seen in the present investigation 305 where no pregnancies resulted from nine embryos transferred that had undergone 306 RC. The chromosomal status of blastomeres resulting from fusion was also examined 307 where embryos affected by RC were transformed into either polyploidy or mosaics 308 embryos. The authors suggested that the occurrence of blastomere fusion could be 309 associated with existing membrane abnormalities that could promote fusion affected 310 by factors such as pH, temperature and osmolality differences. Interestingly, in some 311 fields of research the production of tetraploid embryos is advantageous and it has

been concluded that tetraploidy does not prohibit preimplantation development
(Eglitis, 1980); corroboration for the development of approximately 40% G/AQE in
the present investigation. This investigation could conclude similarly to others
where the presence of RC did not seem to affect an embryos ability to create a GQE
but does impair an embryos ability to implant.

317

318 Absent cleavage has been characterised as a type of RC in a previous report (Liu et 319 al., 2014) however, in the current report it is classed as a distinct phenotype. The 320 prevalence per embryo of this abnormality compared to RC is more than double 321 (0.84 vs 0.41%) and of the four embryos that were transferred with this phenotype. 322 one implanted. However, in a previous report, of 22 embryos, none implanted that 323 underwent type I or type II RC (defined here as AC) (Liu et al., 2014). In another 324 investigation using disaggregated human embryos, blastomeres were scored for the 325 number of nuclei present after 16 to 20h culture and a small proportion of 326 mononucleated blastomeres exhibited two nuclei after culture. It was hypothesised 327 that approximately 30% of these occurred through AC (Pickering et al., 1995). Here, 328 AC was shown to occur in 1.08 embryos per treatment cycle and of the 133 embryos 329 exhibiting AC, 122 were classed as PQE and 116 were discarded. Unlike DC, RC and 330 CC however, the patient age was not shown to be significantly different when 331 compared to the control embryo cohort.

332

333 CC has an overall prevalence per embryo of 5.25%; by far the highest of the five
334 abnormal phenotypes. Occurring in 1.82 embryos per treatment cycle suggestive of a
335 patient, treatment or environmental effect rather than a spontaneous event. One

336 comprehensive analysis identified the prevalence of CC to be 15%, with a blastocyst 337 formation rate of 14% and an IR of 0% (Athavde Wirka et al., 2014). In the current 338 analysis, the IR of these embryos was 2.1% (1/48); significantly lower than the IR of 339 the control embryo cohort. Of the utilised embryos, just 18.2% were classed as GQE, 340 27.3% as AQE and 54.5% as PQE. Interestingly, it has previously been found that 341 35.2% of those exhibiting CC were classed as good quality, a result not synonymous 342 with the current analysis. A possible explanation for this disagreement is the time 343 lapse technology used. In the current analysis, EmbryoScope® was the time lapse 344 technology of choice however, in the analysis by Athayde Wirka et al. (2014) the 345 Eeva[™] system was used. The Eeva[™] system uses dark field illumination to enable the 346 software within it to track blastomeres. The EmbryoScope® does not use dark field 347 illumination which could make distinction of blastomeres from fragments more 348 straightforward. An investigation conducted on patients carrying a Robertsonian 349 translocation (the fusion of two acrocentric chromosomes), revealed that a high 350 proportion of embryos resulting from these patients underwent numerous chaotic 351 cleavage divisions and rather than the aneuploid segregation of the Robertsonian 352 translocation being the only reason for the infertility, there may be a post zygotic 353 manifestation leading to uncontrolled chromosome segregation (Conn et al., 1998). 354 The presence of chaotically dividing embryos has been noted elsewhere (Delhanty et 355 al., 1997; Harper and Delhanty, 1996; Laverge et al., 1997) and has also been 356 identified as a patient related phenomenon (Delhanty et al., 1997) a statement 357 synonymous with CC occurring in up to 1.82 embryos per treatment cycle. 358

359 CL is largely discussed in the literature when considering frozen thawed embryos 360 and, as discussed previously, there is an associatively low IR (Tang et al., 2006). 361 59.2% of the embryos were classed as POE with 55.6% of the total discarded. Just 362 13.6% were considered GQE and 27.2% AQE, a result similar to other abnormal 363 phenotypes. As very few embryos were shown to exhibit this phenotype, and fewer 364 still were transferred, it is difficult to draw conclusions about the implications of this 365 abnormal phenotype. It would be reasonable to use previous evidence regarding 366 frozen thawed embryos to attribute their potential for success. However, CL in 367 frozen embryos is likely as a result of cryodamage during the freeze thaw process 368 whereas, in fresh embryos, the CL could be as a result of exposure to another 369 stressor such as suboptimal pH, temperature or osmolality. Cells that lyse may have 370 a heightened sensitivity to changes in the environment, or lack a cytoplasmic 371

371 constituent that regulates cell volume, for example, leading to its lysis.

372 Abnormal phenotypes as deselection criteria

373 Where possible, UDC and TDC embryos should not be selected for transfer if other 374 embryos are available, even when embryo quality is considered. . It is important to 375 note at this point that embryos transferred at the cleavage stage undergoing DC (of 376 which there were five in the current analysis) will inevitably be classed as UDC. 377 These embryos may have resulted in FDC thus caution is advised due to a potential 378 bias in the current results of UDC cleavage stage embryos. For this reason, extended 379 culture of DC embryos may be valuable to allow the classification into either FDC or 380 TDC and thus aid further in embryo selection and management of patient 381 expectation. CC, the most common abnormal phenotype in the current analysis, has 382 been linked to severe chromosomal abnormalities in the literature which could be

383 patient specific therefore it's possible that the phenomenon could occur more than 384 once in a patient cohort indicating an underlying genetic condition. Where CC 385 embryos are transferred the expected IR is 2.1% regardless of embryo quality. For 386 this reason, identification of CC as a deselection tool should be considered for 387 laboratories utilising time lapse imaging technologies. Just fewer than 92% of 388 embryos that exhibit AC create PQE thus they would likely be automatically 389 discounted from clinical use. RC and CL each have an IR of 0%, albeit from low 390 numbers of transferred embryos. However, the relative prevalence is low, the 391 majority of embryos exhibiting these phenomenoa are POE and they are not able to 392 implant therefore these embryos should not be selected for transfer where possible. 393 These recommendations have been implemented at the study site to aid in embryo 394 selection. In addition to the above, the need for accurate and consistent annotation 395 of embryos is imperative for any centre utilising time lapse technologies. This issue 396 was raised a number of years ago resulting in the publication of suggested 397 terminology in order to create consensus among users (Ciray et al., 2014). 398 Consensus is paramount and caution is advised when implementing or analysing 399 time lapse parameters discussed by others.

400

This preliminary investigation sought to determine the prevalence, implantation
potential and suitability for inclusion in embryo selection algorithms of five
abnormal cleavage events. To determine IR, only known implantation embryos were
used leading to a significant reduction in the number of embryos available for
analysis. Nevertheless, this number would be difficult to achieve at another single
site based on the study site using time-lapse for all patients and performing over

407 2000 treatment cycles per year. In addition, the ability to track the implantation of 408 these embryos is made more difficult with the increased likelihood of transferring 409 two embryos in these cases, potentially due to reduced embryo quality in the 410 available embryo cohort. Based on the results presented here, future analyses should 411 focus on embryos undergoing more than one abnormal division event, the cell stage 412 at which the abnormal cleavage event occurs, the effect of treatment parameters 413 such as ICSI and day of transfer as well as the assessment of a relationship between 414 the abnormal phenotypes and multinucleated blastomeres. In addition, the authors 415 plan to perform an extension of this analysis to include embryo quality and outcome 416 information regarding DC1-3 versus DC2-5 in the DC classifications presented here. 417 Finally, scrutiny should be paid to CL where the specific timings of the CL event 418 should be assessed and linked to the relative impact on embryo viability.

419

In conclusion, embryos exhibiting an abnormal phenotype appear to have reduced
developmental capability expressed as both embryo quality and implantation
potential. Time lapse systems are bringing to light many unusual and, most likely,
fundamentally complicated embryological phenomena requiring in depth analysis
that could ultimately improve the outcome of treatment cycles.

425

426 Appendix: Supplementary material

427

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