The Istanbul Consensus update: a revised ESHRE/ALPHA consensus on oocyte and embryo static and dynamic morphological assessment

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Title: The Istanbul Consensus update: a revised ESHRE/ALPHA consensus on oocyte and embryo static and dynamic morphological assessment

Running title: a revised ESHRE/ALPHA consensus on oocyte and embryo morphology assessment

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Introduction

Assessment of human embryo development is an essential but challenging task in the *in vitro* fertilization (IVF) laboratory. Embryos are assessed by embryologists in order to select the most likely to be viable for intrauterine transfer, cryopreservation, or biopsy for preimplantation genetic testing (PGT). Since the early days of IVF in the 1980's when embryos were optimistically viewed as 'nice, very nice, or very very nice' (Jacques Cohen, personal communication), a relatively large number of early embryo morphological features have been identified and investigated for their association with viability, implantation, live birth, and chromosomal status. Yet, morphology assessment remains largely subjective and prone to inter- and intra-observer and inter-laboratory variability (Arce et al., 2006, Baxter Bendus et al., 2006, Martínez-Granados et al., 2017, Storr et al., 2017).

In the past decade, the most significant advance in embryo assessment has been the introduction of sophisticated time-lapse microscopy technologies (TLT). This has led to the emergence of 'morphokinetics' assessment. As the term implies, morphokinetics represents the integration of morphology (the form and structure of embryos) with kinetics (the dynamics of their development), providing a comprehensive framework for understanding and evaluating embryo viability. These technologies allow continuous observation of embryo development, with minimal manipulation or disruption of culture (ESHRE working group on Time-lapse technology, 2020).

Hundreds of papers have been published on embryo assessment. The studies are mostly retrospective and heterogeneous with respect to some key parameters including patient population, outcome measures, control for confounders, laboratory procedures, and embryo culture conditions. Furthermore, morphokinetic studies, as well as classical morphological studies, may be influenced by maternal age, smoking status, ovarian stimulation protocols, and insemination methods, among other factors (Braga et al., 2015, Ubaldi et al., 2016, Grøndahl et al., 2017, Barrie et al., 2021a, Bamford et al., 2022). Nonetheless, TLT observations have significantly contributed to our understanding of developmental events, and morphology assessments are now enhanced by morphokinetics.

Over a decade ago, Alpha Scientists in Reproductive Medicine and ESHRE special interest group of Embryology collaborated to produce the Istanbul Consensus on assessing oocytes, zygotes and embryos (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011).

The Istanbul Consensus (2011) established common criteria and terminology for grading oocytes, zygotes and embryos that are now updated in this paper through close examination, compilation, analysis, and interpretation of data published in the intervening years. Most important, the new recommendations incorporate some embryo morphokinetic features that

have been elucidated since the introduction of TLT and can inform and complement the static observation approach.

Terminology

Embryologists routinely make decisions on disposition of oocytes and embryos, that is, whether they are clinically usable or should be discarded. Clinical use of oocytes and embryos is defined as use for an IVF/ICSI treatment, biopsy/PGT, cryopreservation, transfer, and donation.

In the updated set of recommendations provided in this manuscript, the working group used the terms embryo grading, ranking, and selection. **Embryo grading** is the evaluation of embryos using a specific set of criteria to assign a quality score: the number, size, and shape of blastomeres, the degree of fragmentation, the inner cell mass (ICM) and trophectoderm (TE) morphology and expansion, etc. **Embryo ranking** refers to the process of ordering clinically usable embryos based on grading and other assessment criteria, from most to least favourable for transfer. Embryos are ranked according to their perceived potential for implantation and development, which is determined by morphological and, when available, genetic factors. This is a prioritization of which embryo(s) to transfer first. **Embryo selection** for transfer involves consideration of ranking and other factors to select embryos for transfer into the uterus. The goal is to select the embryo(s) with the highest likelihood of resulting in a successful pregnancy and live birth.

Current data on oocyte and embryo assessment criteria

1. Expected timeline of embryo development

Development of the human embryo begins with fertilization and continues with a series of mitotic events each of which doubles the cell number as the embryo develops from a single cell into a multicellular blastocyst (Ciray et al., 2014). At fertilization, once the two pronuclei break down, paternal and maternal chromosomes are assembled into a bipolar mitotic spindle (syngamy), before sister chromatids are orderly segregated in the first two blastomeres at first cleavage. The resulting undifferentiated daughter cells are expected to be genetically identical. In the initial developmental phases, blastomere function is under the primary control of a delicate regulatory mechanism guided by maternal factors (Sha et al., 2020). However, recent studies have investigated the fine details of the first event of chromosome segregation in the human embryo, revealing a highly error-prone mechanism (Currie et al., 2022). Although exact timing is uncertain, embryonic genome activation should be well underway by the 8-cell stage, triggered by the degradation of the maternal transcripts (Braude et al., 1988, Vassena et al., 2011, Asami et al., 2022, Yuan et al., 2023).

Since the competence of the human embryo is reflected in its developmental timeline, assessment of morphology should be in accordance with predefined times.

The original Istanbul Consensus (2011) on embryo assessment proposed specific timings for observations of fertilized oocytes and embryos, and their expected stage of development at these time points. These timings were relative to the insemination time and aimed to reflect when the events of interest occur generally (Table 1). Times for observations were provided for the following stages: fertilization, syngamy, early cleavage, Day-2, -3, -4 and -5 embryo assessment. The Istanbul Consensus (2011) differentiated between ICSI- and IVF-derived embryos only for one stage of development - early cleavage. Specifically, the 2-cell stage was proposed to be checked two hours earlier post ICSI (26±1 hours post-insemination (hpi)), than IVF (28±1 hpi) (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011). The rationale behind this suggestion is that pronuclear formation post IVF is observed about 1 hour later than post ICSI (Nagy et al., 1998), where the cumulus-corona complex, zona pellucida and oolemma are bypassed, conserving the time required for the spermatozoon to traverse this path (Payne et al., 1997).

Studies have shown that early cleavage is an independent predictor of embryo quality (in terms of cell number and morphology at later cleavage stages), blastocyst formation, pregnancy and birth, although there were apparent differences between ICSI- and IVF-derived embryos (Shoukir et al., 1997, Lundin et al., 2001, Van Montfoort et al., 2004).

Several subsequent reports of the relative morphokinetic timings of IVF- and ICSI-derived embryos have been described in the literature and were considered in this revised version of the Istanbul Consensus. For example, several studies reported that only timing of the first cleavage was affected by fertilisation method, with IVF embryos reaching the 2-cell stage significantly later than their ICSI counterparts (Dal Canto et al., 2012b, Kirkegaard et al., 2016). Another study detected comparative delays in IVF embryo development beyond the 2-cell stage of 1.5 +/- 1.1 hours (Bodri et al., 2015). A recent randomised controlled study compared morphokinetics of 373 sibling IVF and ICSI embryos and reported that only t2 was significantly delayed in IVF embryos (De Munck et al., 2022). A large TLT study of 2376 embryos reported that time to 2-cell (t2) was 0.98 h earlier in ICSI-derived embryos (excluding those from donor sperm) while time to initiation of blastulation (tSB), and time to full blastocyst (tB) were 1.157 h and 1.510 h later, respectively, compared with IVF-derived embryos (Barrie et al., 2021a).

Furthermore, many morphokinetic-based studies have investigated the possible influence of other intrinsic and extrinsic factors on the timing of embryo development (ESHRE working group on Time-lapse technology, 2020). Two of the most studied patient variables are age of gamete providers and body mass index (BMI) albeit with varying findings and no meta-analyses or definitive studies yet available (Lebovitz et al., 2021, Setti et al., 2021, Bellver, 2022, Boucret et al., 2022, Hoek et al., 2022).

Whether ovarian stimulation protocol impacts embryo developmental timing has also been investigated using morphokinetic analyses with some apparent differences during early cleavage stages but no effect on overall embryo quality (Barrie et al., 2017a, Mumusoglu et al., 2017, Dietrich et al., 2020).

Culture medium type and oxygen tension have also been studied in relation to the timing of preimplantation developmental events, including a large analysis of over 10,000 embryos (Dietrich et al., 2020). This study compared two culture media and reported differences in the timing of compaction initiation but not the start of blastulation. Other factors known to affect embryo development, such as temperature and pH, can influence embryo morphokinetics; lower temperature and culture medium pH drift (typically in an alkaline direction) are associated with slower embryo development (Swain, 2015, Wale and Gardner, 2016). The impact of oxygen level during culture, a major influencer of embryo development, has not been extensively studied. However, development and implantation rates decrease when atmospheric oxygen level is employed, compared with lower, more physiological levels (Quinn and Harlow, 1978, Gardner and Kelley, 2017). Using TLT imaging, and similar to data in the mouse (Wale and Gardner, 2010), a prospective study compared the developmental timings of embryos according to oxygen tensions, reporting significantly slower development in embryos cultured in 20% oxygen compared with 5% (Kirkegaard et al., 2013).

Another area of scrutiny has been embryo chromosome status. A recent systematic review and meta-analysis incorporating over 40,000 embryos concluded that ten morphokinetic variables were significantly delayed in aneuploid embryos, most notably from t8 to the expanded blastocyst stage (Bamford et al., 2022). Irregularities of cleavage, such as prolonged or rapid cell cycles, may be associated with DNA repair activity, cellular rearrangement or failure to undergo cell cycle checkpoints (Regin et al., 2022).

Because TLT enables the precise recording of embryo developmental timings, and as some significant differences have been reported based on specific outcome measures such as clinical pregnancy and chromosome complement, morphokinetic selection algorithms are being proposed to improve embryo selection and thereby, shorten the time to pregnancy (Meseguer et al., 2011, Petersen et al., 2016, Pribenszky et al., 2017, Fishel et al., 2020). The potential of individual morphokinetic variables to predict clinical outcomes, has recently been assessed in two large analyses of over 30,000 embryos; the results show that peri-blastulation timings have more power to predict live birth than traditional TE or ICM morphology (Bamford et al., 2022, Campbell et al., 2022a). However, two recent randomized controlled trials (RCTs) found no improvement in ongoing pregnancy rate or cumulative live birth rate or live birth rate per transfer, when using TLT algorithmic selection (Ahlström et al., 2022, Kieslinger et al., 2023), corroborating the findings of the latest Cochrane review (Armstrong et al., 2019).

TLT studies can help inform and optimise static assessment timing windows in the IVF laboratory. However, many laboratories do not have this technology and the familiar, reliable daily descriptors remain practically applicable, although somewhat imprecise. Since the publication of the original Istanbul Consensus (2011), the convention of describing the timing of preimplantation development in terms of number of days (post insemination) has come to be viewed as simplistic, largely due to the facility to observe the developing embryo almost continuously, in minutes and hours, rather than days, using TLT imaging.

Consensus points

- Standardized timing of observations is critical to reliable comparison of results between different laboratories, culture conditions, patients, etc. This should be set relative to the time of insemination, and uniformly reported as hours post-insemination.

- There is an inherent variability in timing of all biological processes; the suggested observation times reflect those at which the associated developmental stages occur in most patients/cases, whilst accepting there are confounding and influencing factors, including human subjectivity.

- Culture media and culture systems in general are recognized as having a significant impact on embryo morphokinetics; accordingly, their impact should be considered in comparative studies.

- Each laboratory is encouraged to develop and analyse its own datasets to determine relevant timings. Data generated by other laboratories may or may not be generally applicable.

Table 1 Time lapse data generated reference timings related to specific embryo developmental stage assessments: Morphokinetic timings are obtained from manually annotated embryos in vitro (n=140,872 2PNs - 56,066 IVF and 84,806 ICSI-) (Unpublished Care Fertility multicentre data 2013-2022), fresh oocytes only. Nomenclature and definitions are based on (Ciray et al., 2014). Regarding day 6 and 7 observations, this dataset does not have sufficient data available to offer guidance for observation. However, see section 6 (blastocyst stage) regarding assessment of blastocysts beyond day 5.

Istanbul Consensus 2011			***2024***		
Type of observation	*Timing (hpi)*	*Expected stage of development*	*Median time to reach developmental stage (rounded to nearest hour)*	*Assessment time for each developmental stage to give highest chance of observation (hpi). Rounded. After fertilization check, all +/-1 hour*	*Proportion expected to be at stage required for specific assessment. Rounded.*
Fertilization check	*17+/-1*	*Pronuclear stage*	*N/A*	*16-17 (ICSI or IVF)*	*98% with visible pronuclei (Barrie et al., 2021)*
Syngamy check	*23+/-1*	*Expect 50% to be in syngamy (up to 20% may be at 2 cell stage)*	*tPNf (time to pronuclear fading)* *23 (ICSI)* *24 (IVF)*	 *25 (ICSI)* *26 (IVF)*	 *53%* *53%*
Early cleavage check	*26+/-1 (ICSI)* *28+/-1 (IVF)*	*2 cell stage*	*t2 (time to 2 cell)* *26 (ICSI)* *27 (IVF)*	 *31 (ICSI)* *32 (IVF)*	 *77%* *79%*
Day-2 embryo assessment	*44+/-1*	*4 cell stage*	*t4 (time to 4 cell)* *38 (ICSI)* *39 (IVF)*	 *43 (ICSI)* *45 (IVF)*	 *64%* *67%*
Day-3 embryo assessment	*68+/-1*	*8 cell stage*	*t8 (time to 8 cell)* *57 (ICSI)* *58 (IVF)*	 *63 (ICSI)* *65 (IVF)*	 *49%* *51%*
Day-4 embryo assessment	*92+/-2*	*Morulae*	*tM (time to morulae)* *89 (ICSI)* *91 (IVF)*	 *93 (ICSI)* *95 (IVF)*	 *47%* *44%*
Day-5 embryo assessment	*116+/-2*	*Blastocyst*	*tB (time to full blastocyst)* *108 (ICSI)* *107 (IVF)*	 *108 (ICSI)* *108 (IVF)*	 *47%* *52%*
			tEB (time to expanded blastocyst) *113 (ICSI)* *113 (IVF)*	 *111 (ICSI)* *112 (IVF)*	 *34%* *34%*

2. Oocyte

Oocyte morphology may be assessed with the aim of predicting the developmental competence of the resulting embryo. In the relevant literature, several extra-cytoplasmic - cumulus oocyte complex (COC), zona pellucida (ZP), perivitelline space (PVS), polar body (PB), shape, size) - and intracytoplasmic - vacuoles, refractile bodies (RFs), aggregates of smooth endoplasmic reticulum clusters (sER-a), central granularity, colour - oocyte dysmorphic features are reported.

In this section, the predictive value of oocyte morphological characteristics/dysmorphism for embryo developmental potential is assessed (**Table 2**). Moreover, the possible use of oocytes that are immature at the time of oocyte retrieval following standard controlled ovarian stimulation (COS) – so called rescue-IVM - is considered.

Oocyte morphological features relevant to oocyte scoring

The Istanbul Consensus (2011) described the optimal oocyte morphology as an oocyte with a spherical shape enclosed by a uniform zona pellucida, with a uniform translucent cytoplasm free of inclusions, and a size-appropriate polar body. Furthermore, it was noted that oocytes undergo both nuclear and cytoplasmic maturation, and that these processes are not equivalent, nor are they necessarily synchronous.

The survey results showed that 35% of respondents always apply the Istanbul Consensus (2011) recommendations to score oocytes, ranging from 22% for scoring the cumulus-oocyte complex (COC) to 53% scoring the polar body (**Supplementary data SII, figure 3.B**).

Cumulus oocyte complex

Most studies showed an association between COC morphology and biological and clinical outcomes (Daya et al., 1990, Ng et al., 1999, Lin et al., 2003, La Sala et al., 2009, Dal Canto et al., 2012a). More specifically, the presence of a compact COC and a very tight corona has been found to be negatively associated with fertilization and pregnancy rates. On the other hand, no association was observed in one study between COC morphology and fertilization rate or embryo cleavage (Rattanachaiyanont et al., 1999). Further evidence indicates that the presence of blood clots trapped in the COC has a negative impact on outcomes even if removed during oocyte collection (Daya et al., 1990, Ebner et al., 2008a).

These data suggest that such COC characteristics, if present in most of collected COCs from one patient, should be noted, especially if conventional IVF (cIVF) is used for insemination. However, further studies are necessary before establishing the potential predictive value of this assessment for embryo competence.

Zona pellucida

Different ZP phenotypes (increased thickness, irregularities of the surface and increased density) have been reported. Some studies showed that oocytes with indented, dark and/or

heterogeneous ZP had lower fertilization rate, embryo quality, embryo development, pregnancy, implantation and live birth rates (Shi et al., 2014, Sauerbrun-Cutler et al., 2015, Sousa et al., 2015, Pan and Zhang, 2020, Yang et al., 2022). On the other hand, in several studies, ZP with diverse phenotypes showed no association with fertilization rates, embryo quality, or implantation rates (De Sutter et al., 1996, Balaban et al., 1998, Esfandiari et al., 2006, Ten et al., 2007, Rienzi et al., 2008), embryo cryo-survival, and blastocyst and hatching rates (Balaban et al., 2008).

Only one study investigated the fertilization potential of oocytes without ZP (Ueno et al., 2014). Very rarely, two oocytes may share a single ZP. One live birth of dizygotic twins obtained from transfer of a pair of (zona-)conjoined blastocysts has been reported (Magdi, 2020). Moreover, two case reports described live births obtained from the transfer of embryos derived from insemination of (zona-)conjoined oocytes, one mature and the other immature (Fu et al., 2022a, Wang et al., 2022).

Evidence was insufficient to support any negative prognosis of zona pellucida characteristics on embryo developmental potential. Oocytes showing different ZP phenotypes are therefore considered suitable for clinical use.

Perivitelline space

Contradictory reports are found in the literature assessing different PVS phenotypes and developmental competence (De Sutter et al., 1996, Balaban et al., 1998, Hassan-Ali et al., 1998, Farhi et al., 2002, Chamayou et al., 2006, Ten et al., 2007, Balaban et al., 2008, Rienzi et al., 2008, Ashrafi et al., 2015, Sauerbrun-Cutler et al., 2015, Ferrarini Zanetti et al., 2018, Weghofer et al., 2019). Three studies have focused in particular on large PVS and fertilization rate, finding a significant negative association (De Sutter et al., 1996, Xia, 1997, Ten et al., 2007, Rienzi et al., 2008, Setti et al., 2011, Ashrafi et al., 2015)

On the other hand, evidence was insufficient to support a negative prognosis for embryo developmental potential. Oocytes showing different PVS phenotypes are therefore considered suitable for clinical use.

Polar body

Large or fragmented PB are commonly reported. No significant association was found between polar body fragmentation and fertilization. Although some studies showed an association between different PB phenotypes and early embryo development (Ebner et al., 2000, Chamayou et al., 2006, Fancsovits et al., 2006, Rienzi et al., 2008, Navarro et al., 2009, Zhou et al., 2016), no association with implantation or clinical pregnancy was reported (Verlinsky et al., 2003, Ciotti et al., 2004, De Santis et al., 2005, Ten et al., 2007).

Evidence was insufficient to support any negative prognosis of polar body size and fragmentation on embryo developmental potential. Oocytes showing fragmented or large PB are therefore considered suitable for clinical use. However, a disproportionately large polar body, although very rare, could be associated with abnormal meiotic spindle placement and deserves more attention.

Shape

Mature human oocytes generally have a spherical shape, nevertheless oocytes with ovoid shapes are reported. Overall, an ovoid shape, does not appear to affect laboratory and clinical outcomes (De Sutter et al., 1996, Balaban et al., 1998, Chamayou et al., 2006, Ten et al., 2007, Yakin et al., 2007). In case of an ovoid oocyte that leads to planar arrangement of blastomeres at 4-cell stage, further development up to blastocyst stage was found to be delayed (Ebner et al., 2008c).

Irregularly shaped oocytes are considered suitable for clinical use.

Oocyte size

Without consideration of the ZP thickness, small (<100 µm diameter) and large oocytes (≥125 µm diameter) have been reported to have very low developmental potential (Bassil et al., 2021).

Giant oocytes (e.g. >180 µm diameter) should be excluded from clinical use due to their possible tetraploid origin (Rosenbusch et al., 2002, Kitasaka et al., 2022). Presumably, these oocytes originally derive from the fusion of two primordial oocytes. This is suggestive of the presence of two diploid chromosome complements and an overall tetraploid oocyte constitution (Balakier et al., 2002, Rosenbusch et al., 2002, Munné et al., 2004). On the other hand, siblings of giant oocytes with normal diameter have been shown to have normal developmental potential (Machtinger et al., 2011, Lehner et al., 2015).

Vacuolization

Vacuoles are membrane-bound, translucent or fluid-filled cytoplasmic inclusions or SER vesicles (Otsuki et al., 2004, Sfontouris et al., 2018). Vacuoles can appear individually or in multiples (Fancsovits et al., 2011) and are assumed to originate as independent formations (Van Blerkom, 1990) or from the fusion of existing vesicles derived from the SER and/or Golgi apparatus (Veeck, 1999). Very large vacuoles (>25 µm) might distort the oocyte cytoskeletal structure, impairing sperm–oocyte signalling, sperm binding, meiotic resumption, and embryo development (Wallbutton and Kasraie, 2010, Dal Canto et al., 2017).

Different studies have shown that vacuolization is associated with lower fertilization rate, compromised embryo development, blastulation and cryo-survival rates (Ebner et al., 2005, Balaban and Urman, 2006, Ebner et al., 2006, Ten et al., 2007, Balaban et al., 2008, Rienzi et al., 2008, de Cássia et al., 2010, Sousa et al., 2016). In particular, the association between the

presence of vacuoles and lower fertilization was confirmed in a meta-analysis (Setti et al., 2011). However, in this analysis, evidence was insufficient to support any negative prognosis in relation to embryo developmental potential. Oocytes showing vacuoles are therefore considered for clinical use. In ICSI cases, however, care should be taken in avoiding injection of the sperm into a vacuole.

Refractile bodies

RFs consist of a mix of lipids and dense granular material. They exhibit a yellow autofluorescence typical of lipofuscin (Sathananthan, 1994). A small number of publications have investigated the predictive value of refractile bodies and embryo developmental potential (Alikani et al., 1995, De Sutter et al., 1996, Balaban et al., 1998, Ebner et al., 2000, Otsuki et al., 2004, Setti et al., 2011, Takahashi et al., 2020). A lower fertilization rate is associated with the presence of such phenotype.

Although fertilization rate may be affected, the evidence was insufficient to support any negative prognosis of this phenotype for further embryo development. Oocytes showing refractile bodies are therefore considered suitable for clinical use.

Smooth endoplasmic reticulum clusters

From an ultrastructural standpoint, SER-a consist of tubular clusters surrounded by mitochondria that appear as more densely packed areas than the surrounding regions (Sá et al., 2011). SER-a have been described as potential biomarkers of oocyte quality. Numerous studies suggested lower fertilization (Sá et al., 2011, Massarotti et al., 2021), embryo quality (Ebner et al., 2008b, Sá et al., 2011, Braga et al., 2013, Massarotti et al., 2021, Wang et al., 2021) and pregnancy rates (Otsuki et al., 2004, Setti et al., 2016, Gurunath et al., 2019, Massarotti et al., 2021), and increased miscarriage rates (Otsuki et al., 2004, Ebner et al., 2008b, Braga et al., 2013). Moreover, in small studies, higher rates of perinatal complications and birth defects were reported as being associated with this dysmorphism (Otsuki et al., 2004, Ebner et al., 2008b, Akarsu et al., 2009, Sá et al., 2011, Mateizel et al., 2013, Sfontouris et al., 2018). Conversely, more recent studies and a meta-analysis reported no difference in fertilization rate, blastocyst formation rate, neonatal outcomes (Hattori et al., 2014, Shaw-Jackson et al., 2016, Itoi et al., 2017, Zhang et al., 2021, Fang et al., 2022) and euploidy rates (Xu et al., 2022, Mizobe et al., 2023, Wang et al., 2023); this body of evidence reinforces the recommendation, also supported by the Vienna Consensus (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017) that clinical use of SER-a positive oocytes may be considered.

Granularity

Oocytes with central granulation have been associated with defective pronuclear morphology, reduced embryo quality (Ebner et al., 2008a, Rienzi et al., 2008), decreased cryo-survival rate, compromised embryo developmental competence (Balaban et al., 2008, Ebner et al., 2008a,

Rienzi et al., 2008) and lower ongoing pregnancy rate (Kahraman et al., 2000). In contrast, other studies and meta-analyses suggest that centrally localized cytoplasmic granulation (CLCG) might be a normal/typical oocyte morphological feature (Wilding et al., 2007, Setti et al., 2011, Yi et al., 2019) . Currently, there are no studies investigating the potential of these oocytes to produce viable pregnancies. Available evidence is insufficient to support a negative prognostic value of this dysmorphism relevant to embryo developmental potential. Oocytes showing cytoplasmic granularity are therefore considered suitable for clinical use.

Colour

Few studies investigated translucency variation, often observed together with other anomalies. Some studies suggested an association between ooplasm darkness and poorer embryo quality (Loutradis et al., 1999, Ten et al., 2007). However, this finding was not confirmed by other investigations (De Sutter et al., 1996, Balaban et al., 1998, Esfandiari et al., 2006, Balaban et al., 2008, Shi et al., 2014). The highly subjective nature of these observations as well as heterogeneity of the data preclude any conclusions. Oocytes showing variations in translucency are therefore considered suitable for clinical use.

Immaturity

After standard COS, approximately 15-20% of oocytes fail to reach the metaphase II (MII) stage, arresting at the metaphase I (MI) or germinal vesicle (GV) stages (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017, ESHRE Clinic PI Working Group, 2021). Immature oocytes are usually not used for insemination and are discarded. However, in the case of poor prognosis patients and in patients with an unsynchronized follicle cohort, the use of immature oocytes that can mature after a period of *in vitro* culture (rescue IVM-oocytes) could contribute to the number of embryos obtained in each cycle, potentially increasing the chances of pregnancy (Shu et al., 2007). Several studies have shown that MI oocytes that mature within 2-6 hours from denudation may be injected and contribute to the number of available embryos (De Vos et al., 1999, Balakier et al., 2004, Shu et al., 2007). By contrast, overnight in vitro culture of MI and GV oocytes did not improve results. GV and MI oocytes that mature in vitro after 24 hours have compromised results in terms of fertilization and blastocyst formation rates (Yang et al., 2021), most probably due to a higher risk of being chromosomally abnormal (Strassburger et al., 2010). TLT analysis has also confirmed that rescue IVM oocytes differ from their sibling MII oocytes in the morphokinetic profile, showing a delay in the early stages of embryo development (Faramarzi et al., 2018, Margalit et al., 2019, Shani et al., 2023). However, the feasibility of the rescue-IVM approach is nevertheless supported by some studies reporting a contribution to embryo yield, and few live births obtained using those embryos (Rubino et al., 2016, Escrich et al., 2018, Moon et al., 2023, Shani et al., 2023).

Due to their lower developmental potential, immature oocytes could be considered for clinical use only in poor prognosis cases.

Oocyte morphology and morphokinetics

Some studies investigated a possible relationship between different cytoplasmic phenotypes and morphokinetics. Although not a standard procedure for oocyte assessment, ZP birefringence was shown in a recent study not to be correlated with embryo morphokinetics (Tabibnejad et al., 2018), while another study reported an early t5 in oocytes with high birefringence (Faramarzi et al., 2017). In the latter study, tPB2, t5 and t8 (time to extrusion of the second PB and development at the 5- and 8-cell stage, respectively), were associated with oocyte diameter, while PVS size showed no association with early development morphokinetics (Faramarzi et al., 2019). Finally, the incidence of failure of second polar body extrusion and the incidence of mitotic cleavage failure in oocytes with SER-a were found to be significantly higher than that in oocytes without SER-a (Otsuki et al., 2018).

Overall, individual dysmorphic features may not be strongly associated with viability and development potential or clinical outcomes. However, it is possible that occurrence of two or more of these features together exerts a negative influence on outcomes (Alikani et al., 1995, Bartolacci et al., 2022).

Consensus points

- Giant oocytes should be excluded from clinical use.
- The use of small/large oocytes and IVM-rescued oocytes should be documented for prognostic and traceability purposes due to their apparently lower developmental potential.
- Finally, embryos derived from MII oocytes free of large or multiple vacuoles, SER-a, and very large first PB should be prioritized for clinical use.
- Follow-up of babies born from oocytes with atypical phenotypes and rescue IVM demands attention.

373 *Table 2 Overview of all evidence and recommendations for oocyte assessment*

Overview of all recommendations on oocyte assessment							
Morphological feature	**Atypical patterns**	**Summary of review findings**				**Considerations**	**Recommendation**
		Fertilization rate	**Blastocyst formation rate**	**Implantation rate**	**Live birth rate**		
COC	**Compact COC**	**Association with lower fertilisation rate** **Very low⊕◯◯◯** 1 observational study (Rattanachaiyanont et al., 1999)	/	**Association with lower pregnancy rate** **Very low ⊕◯◯◯** 1 observational study (Dal Canto et al., 2012)	/	**Further studies are necessary before establishing the potential predictive value of this assessment on embryo competence**	**The presence of a dense COC and a very tight corona, if present in most of collected COCs from one patient, should be noted**
	Presence of blood clots	**Associated with lower fertilisation rate** **Very low ⊕◯◯◯** 2 observational studies (Daya et al., 1990; Ebner et al., 2008)	**Associated with lower blastocyst formation** **Very low ⊕◯◯◯** 1 observational study (Ebner et al., 2008)	/			
zona pellucida	**Dark/Thick zona pellucida**	**Contradictory results:** **No clear association with fertilisation rate** **Very low ⊕◯◯◯** 6 observational studies (De Sutter et al., 1996; Balaban et al., 1998; Esfandiari et al., 2006; Ten et al., 2007; Rienzi et al., 2008; Shi et al., 2014) **Associated with lower fertilisation rate** **Very low ⊕◯◯◯** observational studies (Bertrand et al., 1995; Shi et al., 2014; Pang and Zhang, 2020)	**No clear association with blastocyst formation** **Very Low ⊕◯◯◯** 1 observational study (Balaban et al., 2008)	**Contradictory results:** **No clear association with implantation rate** **Very low ⊕◯◯◯** 3 observational studies (Esfandiari et al., 2006; Balaban et al., 1998; Pan and Zhang 2020) **Association with lower implantation rate** **Very low ⊕◯◯◯** 3 observational studies (Shi et al., 2014; Sauerbrun-Cutler et al., 2015; Sousa et al., 2015)	**Association with lower live birth rate** **Very low ⊕◯◯◯** 3 observational studies (Shi et al., 2014; Sauerbrun-Cutler et al., 2015; Sousa et al., 2015)	**Evidence is insufficient to support any negative prognosis of zona pellucida characteristics/ dysmorphisms on embryo developmental potential**	**Oocytes showing different ZP phenotypes are suitable for clinical use.**
Perivitelline space	**Large PVS**	**Association with lower fertilisation rate** **Low ⊕⊕◯◯** 1 meta-analysis of 4 observational studies and 2 observational studies (Setti et al., 2011; Rienzi et al., 2008, Ashrafi et al., 2015)	**No clear association with blastocyst formation rate** **Very low ⊕◯◯◯** 1 observational study (Ferrarini Zanetti et al., 2018)	**Association with lower implantation rate** **Very low ⊕◯◯◯** 1 observational study (Kahraman et al., 2000; Ferrarini Zanetti et al., 2018)	/	**Evidence is insufficient to support any negative prognosis of atypical PVS phenotype/size on embryo developmental potential**	**Oocytes showing different PVS phenotypes are suitable for clinical use.**
	Granulated PVS	**No clear association with fertilisation rate** **Very Low ⊕◯◯◯** A meta-analysis of 3 observational studies (Setti et al., 2011)					
Polar body	**Fragmented PB**	**No association with fertilisation rate** **Low ⊕⊕◯◯** 1 meta-analysis of 7 observational studies (Setti et al., 2011; Ashrafi et al., 2015)	**Association with lower blastocyst formation** **Very Low ⊕◯◯◯** 1 observational study (Zhou et al., 2016)	**No clear association with implantation ate** **Very low ⊕◯◯◯** 4 observational studies (Verlinsky et al., 2003; Ciotti et al., 2004; De Santis et al., 2005; Chamayou et al., 2006; Ten et al., 2007; Zhou et al., 2016)	**No clear association with ongoing/ delivery rate** **Very low ⊕◯◯◯** 1 observational study (Zhou et al., 2016)	**Future quantitative studies are necessary to understand the potential negative impact of large polar bodies on embryo developmental potential**	**Oocytes showing fragmented or large PB are suitable for clinical use. Very large polar body could be associated with abnormal meiotic spindle configuration and deserve more attention**
	Large PB	**Association with lower fertilisation rate** **Low ⊕⊕◯◯** A meta-analysis of 4 observational studies (Setti et al., 2011)	/	/	/		
Vacuolization	**Presence of vacuoles**	**Association with lower fertilisation rate** **Low ⊕⊕◯◯** 1 meta-analysis 3 observational studies and 3 observational studies (Setti et al., 2011; Rienzi et al., 2008; De Cassia et al., 2010; Ashrafi et al., 2015)	**Association with lower blastocyst formation rate** **Very low ⊕◯◯◯** observational study (Ebner et al., 2005; Sousa et al., 2016)	/	/	Evidence was insufficient to support any negative prognosis on embryo developmental potential	**Oocytes showing vacuoles are suitable for clinical use**
Refractile bodies	**Presence of refractile bodies**	**No clear association with fertilisation rate** **Low ⊕⊕◯◯** 1 meta-analysis of 3 observational studies and 1 observational study (Setti et al., 2011; Takahashi et al., 2016)	**No clear association with blastocyst formation** **Very Low ⊕◯◯◯** 1 observational study (Takahashi et al., 2016)	**No clear association with implantation ate** **Very low ⊕◯◯◯** (Balaban et al. 1998, Takashaki et al., 2016))	/	**Evidence was insufficient to support any negative prognosis of this phenotype on further embryo developmental potential.**	**Oocytes showing refractile bodies are suitable for clinical use.**
	Large refractile bodies (>5µm)	**Association with lower fertilisation rate** **Very low ⊕◯◯◯** 1 observational study (Otsuki et al., 2007)	**Association with lower blastocyst formation rate** **Very low ⊕◯◯◯** 1 observational study (Otsuki et al., 2007)				
sERs	**Presence of smooth ER aggregates**	**No clear association with fertilisation rate** **Low ⊕⊕◯◯** 10 observational studies (Sa et al. 2011; Ebner et al., 2008; Wang et al., 2021; Otsuki et al., 2004; Setti et al., 2016; Gurunath et al., 2019; Xu et al., 2022; Hattori et al., 2014; Shaw-Jackson et al., 2016; Fang et al., 2022)	**No clear association with blastocyst formation** **Low ⊕⊕◯◯** 9 observational studies (Sa et al. 2011; Ebner et al., 2008; Wang et al., 2021; Setti et al., 2016; Gurunath et al., 2019; Xu et al., 2022; Hattori et al., 2014; Shaw-Jackson et al., 2016; Fang et al., 2022)	/	/	/	SER-a positive oocytes could be inseminated, based on a case-by-case evaluation
Granularity	**Central cytoplasmic granulation**	**Association with lower fertilisation rate** **Low ⊕⊕◯◯** 7 observational studies (Rienzi et al., 2008; Kahraman et al., 2000; Wilding et al., 2007; Serhal et al., 1997; Balaban et al., 1998; Chamayou et al., 2006; Yi et al., 2019)	**Association with lower blastocyst formation rate** **Very Low ⊕◯◯◯** 1 observational study (Balaban et al., 2008)	**Association with lower implantation rate** **Very low ⊕◯◯◯** 1 observational study (Kahraman et al., 2000)	/	**The difference was statistically insignificant, and the evidence was insufficient to support any negative prognosis of this phenotype on embryo developmental potential.**	**Oocytes showing cytoplasmic granularity are suitable for clinical use**
Shape	**Ovoid oocyte**	**No association with fertilisation rate** **Very low ⊕◯◯◯** 2 observational studies (Ebner et al., 2008; Braga et al., 2013)	**Association with lower blastocyst formation rate** **Very low ⊕◯◯◯** 1 observational study (Ebner et al., 2008)	**No association with implantation rate** **Very low ⊕◯◯◯** observational studies (Balaban et al., 1998; Chamayou et al., 2006; De Sutter et al., 1996; Ten et al., 2007; Yakin et al., 2007)	/		
Colour	**Ooplasm darkness**	**No association with fertilisation rate** **Low ⊕⊕◯◯** 1 meta-analysis and 2 observational studies (Setti et al., 2011; Esfandiari et al., 2006; Shi et al., 2014)	**Associated with lower blastocyst formation** **Very Low ⊕◯◯◯** 1 observational study (Balaban et al., 2008)	/	/	Few studies investigated colour variation, often observed together with other anomalies.	**Oocytes showing colour variation are suitable for clinical use.**

Immaturity	**Immature MI oocytes** **Immature GV oocytes**	**Association with lower fertilisation rate** **Low ⊕⊕◯◯** 6 observational studies (De Vos et al., 1999; Balakier et al., 2004; Shu et al., 2007; Strassburger et al., 2010; Shani et al., 2023; Yang et al., 2021) **No clear association with fertilisation rate** **Very Low ⊕◯◯◯** 2 observational studies (Escrich et al., 2018; Shani et al., 2023)	**Association with lower blastocyst formation** **Very low ⊕◯◯◯** 1 observational study (Yang et al., 2021) **No clear association with blastocyst formation** **Very Low ⊕◯◯◯** 1 observational study (Escrich et al., 2018)	/	**Few live births obtained from rescue IVM** **Very low ⊕◯◯◯** 4 observational studies (Escrich et al., 2018; Moon et al., 2023; Rubino et al., 2016; Shani et al., 2023)	**Due to their lower developmental potential, immature oocytes could be considered in case of poor prognosis individuals/couples and/or when alternatives are not available.**
Oocyte size	**Oocyte with small ooplasm (<100 µm diameter)** **Giant oocyte (>180 µm diameter)**	**Very low development potential** **Very low ⊕◯◯◯** 1 observational study (Basil et al., 2021) **Potential complications** **Very low ⊕◯◯◯** 2 observational studies (Kitasaka et al., 2022; Rosenbusch et al., 2002)	/	/	/	**Due to their lower developmental potential, very small oocytes could be considered only when alternatives are not available.** **It is recommended to exclude giant oocytes from all IVF/ICSI treatment programs due to their presumably possible tetraploid origin.**

COC: cumulus oocyte complex; sERs: smooth endoplasmic reticulum clusters sERs; GV: Germinal vesicle; Table colour code: Green: the oocyte can be clinically used; Yellow: the oocyte could be used with cautionary considerations. Red: the oocyte is not considered suitable for clinical use.

3. Zygote stage

TLT has revealed the complexity of morphokinetic changes occurring during normal (Payne et al., 1997, Mio and Maeda, 2008, Aguilar et al., 2014, Coticchio et al., 2018) and abnormal (Ezoe et al., 2022b, Wei et al., 2022) fertilization, leading to a more accurate and in-depth approach to fertilization assessment. Dynamic monitoring of this stage was previously inaccessible by static observation. PGT-A is also contributing to define the chromosomal constitution of zygotes with pronuclear abnormalities.

In this section, the optimal timing for zygote assessment and the significance of zygote characteristics for embryo developmental potential are reviewed.

Timing of zygote assessment

The Istanbul Consensus (2011) considered static fertilization assessment "*straightforward, based on the observation of two polar bodies (PBs) and two pronuclei (PNs)* at 17 ± 1 hpi."

The survey results showed that 68% of respondents always apply the Istanbul Consensus (2011) recommendations to assess the zygote stage at 17h ± 1 hpi (**Supplementary data SII, figure 3.A**).

Only one, but very large, TLT study attempted to optimise the timing of PN observation (Barrie et al., 2021b). Monitoring more than 54,746 ICSI and 26,302 cIVF embryos, the number of 2PN zygotes was annotated at 30-min intervals, between 15 and 20 hpi. In both insemination groups, the interval with the highest proportion (>98%) of visible 2PN zygotes was 16.0-16.5 hpi. At later intervals, this rate progressively decreased, due to early PN breakdown (PNBD) in some zygotes.

Morphological features relevant to zygote assessment

The Istanbul Consensus (2011) described that the optimal fertilized oocyte is a spherical oocyte with two polar bodies, and two centrally located, juxtaposed pronuclei that are even sized, with distinct membranes (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011). The pronuclei should have comparable numbers and size of nucleolar precursor bodies (NPBs) that are ideally clustered at the region of membrane juxtaposition of the two PN.

The survey results showed that 68% of the respondents always apply the Istanbul Consensus (2011) recommendation to score the pronuclear stage (**Supplementary data SII, figure 3.B**).

The predictive value of pronuclear stage features for embryo quality is discussed below (**Table 3**).

Zygote size

Oocyte and zygote size is usually reported as diameter, projected area or volume. Fertilized oocytes normally undergo progressive and moderate shrinkage during fertilization, also as a result of PBII extrusion (Liu et al., 2014). One study investigated this phenomenon, reporting a lack of association with live birth rate (Barberet et al., 2019). A more recent analysis suggested a negative correlation between zygote diameter/cytoplasmic volume observed at 17 hpi and blastocyst quality (Kljajic et al., 2023). Collectively, this evidence is insufficient and inconclusive on the hypothesis that zygote size can be a predictive parameter for embryo developmental potential.

Pronuclei

- ***Position***. Using TLT, two studies investigated PN position as a developmental biomarker. Although rarely observed, off-centre position annotated shortly before PNBD was associated with abnormal division, namely trichotomous cleavage (Coticchio et al., 2018). Off-centre position of PNs at the time of juxtaposition (8-9 hpi) was found to be associated with a two-fold decrease in live birth rate (Barberet et al., 2019), also after multivariate analysis. Notably, the feature observed in the latter study cannot be detected by single static observation at 16-17 hpi.

- ***Juxtaposition***. In one TLT report, lack of PN juxtaposition throughout fertilization was observed in 1-2% of zygotes. In this phenotype, cleavage, morulae and blastocyst formation rates were negatively affected (Ezoe et al., 2022a).

- ***Size***. PNs increase in size progressively as soon as they form, reaching their final size shortly before PNBD (Otsuki et al., 2017, Orevich et al., 2022). TLT investigation confirmed that the paternal PN is normally larger than its female counterpart (Barberet et al., 2019, Ezoe et al., 2022b, Orevich et al., 2022). Size difference between the two PN tends to progressively decrease as fertilization unfolds. If assessed in the 16-18 hpi interval or immediately before PNBD, this difference was smaller in zygotes that resulted in live births (Otsuki et al., 2017, Otsuki et al., 2019). Collectively, these studies suggest that abnormalities in PN position, juxtaposition and size are very rare and difficult, or impossible, to monitor by static observation.

Nucleolar precursor bodies:

NPBs are intra-pronuclear aggregates of fibrillar material of largely unknown composition. Once condensed from amorphous material, they increase in size and finally cluster in the region of PN juxtaposition. NPB condensation and clustering reflects the distribution of zygotic chromatin (Cavazza et al., 2021). Chromatin remodelling may be a pre-requisite for optimal chromosome-spindle microtubules interaction and, ultimately, chromosome congression. TLT evidence on NPBs is not consistent. Studies focusing on implantation and live birth did not indicate a predictive value of NPB patterning (Azzarello et al., 2012, Aguilar et al., 2014,

Barberet et al., 2019), unless NPB speed was assessed with complex computational methodology (Inoue et al., 2021, Inoue et al., 2023). Another recent investigation (Cavazza et al., 2021) suggested a positive association between NPB clustering in both PN in the regions of juxtaposition and higher competence for blastocyst development, confirming previous data from static observation (Tesarik and Greco, 1999) . Such contradictions are expected. In fact, NPB clustering is a continuum that follows different kinetics in male and female PN (Mio and Maeda, 2008, Coticchio et al., 2018) and, once achieved, can even be lost due to active NPB dispersal in the few hours preceding NPDB (Cavazza et al., 2021). This complicates the use of NPB patterning as biomarker for embryo quality.

Cytoplasmic halo

The cytoplasmic halo is described as a cortical domain of the zygote denoted by reduced cytoplasmic granularity. Visible in most zygotes (82-98%), it can be symmetrically or asymmetrically positioned (Ebner et al., 2003). Usually, the halo forms 2-4 hours after PN appearance and disappears approximately one hour before PNBD (Coticchio et al., 2018, Ezoe et al., 2020). Its formation is probably due to centripetal displacement of mitochondria and other organelles towards the area surrounding the PNs (Squirrell et al., 2003). One TLT study including 1009 zygotes focused specifically on this feature and found that absence of the halo was strongly associated with abnormal cleavage and embryo attrition at cleavage and morulae stages. However, in single vitrified-warmed embryo transfers, halo-positive and halo-negative blastocysts produced comparable clinical outcomes (Ezoe et al., 2020). In the same study, halo position (symmetric or asymmetric) was not correlated with laboratory or clinical outcomes. Another TLT analysis confirmed that live birth rate is unaffected in transfers of halo-negative embryos (Barberet et al., 2019). This evidence disputes the significance of the halo, especially if embryo culture is extended to the blastocyst stage.

Nulli- mono- and tripronuclear zygotes

A designation of normal fertilization typically relies on observation of two pronuclei. However, in the past several years zygotes with other pronuclear patterns, discernible at the time of static fertilization assessment, have been considered for clinical use: no visible PN (0PN), one PN (1PN) or three PN (3PN). A fourth rarer profile showing 2PN with one (or more) extra micro-pronucleus, referred to as 2.1PN, has been also occasionally reported.

- ***0PN.*** Overall morphokinetic evidence does not confirm that embryo development can occur in the absence of formation of at least one PN. Rather, in all likelihood, “0PN zygotes” progressing to the first mitosis are 2PN, or rarely, 1PN/multi-PN zygotes undergoing PNBD before static fertilization assessment can detect PN presence (Barrie et al., 2021b). Therefore, it is not surprising that studies on “0PN zygotes” (all based on static fertilization assessment, here only a few cited) reported rates of development, euploidy, implantation and live births comparable – or higher – with those of 2PN zygotes (Liu et al., 2016, Destouni et al., 2018, Hondo et al., 2019, Paz et al., 2020, Fu et al., 2021, Li et al., 2021,

Kemper et al., 2023). In fact, in general, embryos displaying faster morphokinetics as early as the fertilization stage are also developmentally more competent (Coticchio et al., 2023).

- ***1PN***. The Vienna Consensus recommended that 1PN rate should not exceed 3% and 5% in cIVF and ICSI cycles, respectively (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). In unselected 1PN-derived ICSI embryos, all morphokinetic times and developmental rates are significantly affected (Ezoe et al., 2022b). However, in IVF/ICSI 1PN zygotes showing a relatively larger PN size (defined by projected area or diameter cut-offs of ≥710 μm^2 and ≥31 µm, respectively), cleavage and blastocyst formation rates are comparable with those of 2PN fertilization (Araki et al., 2018, Kai et al., 2018). It is plausible that a larger size of the single PN reflects a higher, possibly diploid, DNA content. Indeed, in approximately 50% of cases of monopronuclear fertilization following IVF, the presence of both maternal and paternal DNA inside the single PN was documented (Cohen et al., 1995, Kai et al., 2015). The genesis of biparental diploid 1PN zygotes may differ in cIVF and ICSI fertilization. A recent TLT investigation suggests a possible modality of formation of biparental 1PN zygotes in cIVF: if, at the very beginning of fertilization, the fertilizing sperm penetrates the oocyte near (within a radius of 18 µm) the presumed position of the maternal chromosomes, as suggested by the PBII localization, the paternal and maternal chromatin may be recruited together in the formation of a single PN (Wei et al., 2022). Consistent with this, several studies reported that 1PN blastocysts screened by PGT-A were diploid/euploid in significant proportions, in some cases similar to those of 2PN controls (Bradley et al., 2017, Capalbo et al., 2017, Destouni et al., 2018, Xie et al., 2018, Zhao et al., 2022). Documented use of 1PN zygotes for clinical purposes have been numerous (here only a few are reported). Overall, following blastocyst culture adopted to select more developmentally competent embryos, rates of implantation, pregnancy and live birth approached those derived from 2PN zygotes (Itoi et al., 2015, Hondo et al., 2019, Si et al., 2019, Li et al., 2020, Li et al., 2021, Fu et al., 2022b, Kemper et al., 2023).

- ***3PN***. According to the recommendations of the Vienna Consensus, polypronuclear (including 3PN) fertilization should be less than 6% (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). Morphokinetics and blastocyst development of 3PN zygotes is less affected compared with 1PN fertilization (Ezoe et al., 2022c). The origin of 3PN zygotes may be digynic or di/polyandric, also depending on the type of insemination technique. Reports on PGT-A analysis and clinical use of 3PN zygotes are very rare. In a study based on 30 3PN blastocysts the rate of diploidy/euploidy was 33% (Mutia et al., 2019). In a case report, an apparently healthy live birth was achieved from the transfer of one euploid 3PN blastocyst (Yalçınkaya et al., 2016). A very recent report described a healthy live birth and normal postnatal development up to four years from the transfer of a 4PN zygote (Bredbacka et al., 2023). However, in

presumptive 3PN/4PN zygotes the origin of the third/fourth PN – whether true extra PN or "larger than usual" micropronucleus – remains a matter of ambiguity.

- ***Micropronuclei***. At the time of PN assessment, one or more small extra PNs may be rarely observed. They may originate from assembly of one extra small nuclear compartment around one or more chromosomes of a diploid zygote (Currie et al., 2022). Specific TLT investigations are lacking. One study based on static observation and preimplantation genetic testing for aneuploidy (PGT-A) monitored >3,500 zygotes, among which only less than 1% (n=27) were 2PN showing one small extra PN (referred to as 2.1PN zygotes) (Capalbo et al., 2017). Although these zygotes show reduced first cleavage rate (74%), they can develop into biparental diploid blastocysts and produce apparently normal live births.

Consensus points

- Evidence reveals considerable plasticity of human fertilization and provides the basis for updated recommendations relevant to static fertilization assessment.

- *Timing of observation*: For static observations, assessment of PN number should be carried out at 16-17 hpi in both cIVF and ICSI cases, to minimise the probability that zygotes undergoing relatively early PNBD are incorrectly classified as unfertilized oocytes. Check of syngamy (disappearance of PN) by static observation, mentioned in the Istanbul Consensus (2011), is not recommended since timing of PNBD cannot be precisely assessed.

- *Morphological features.* Numerous zygotic attributes, including zygote size, PN size, PN position, and NPB patterning, may be associated with embryo quality and clinical outcome. However, their use as biomarkers is hindered by at least two factors: i) insufficient evidence (e.g., PN size), ii) intrinsic morphological mutability during short time periods (NPB patterning) not amenable to static observation. Lack of PN juxtaposition is very rare, but strongly associated with poor blastocyst development. The absence of the cytoplasmic halo affects blastocyst formation, but not implantation rate after blastocyst transfer. Therefore, the absence of the halo may be used to rank, but not de-select, embryos in Day-3 embryo transfers.

- *PN number*. By static observation, pronuclei may not be seen at fertilisation check, and yet embryo development can occur. This may be explained by TLT data, which show that a significant proportion of 2PN zygotes undergo PNBD at earlier times than the fertilization check interval recommended by the original Istanbul Consensus (2011). While these zygotes may be categorized as 0PN, if cultured, they may produce normal laboratory and clinical outcomes. Therefore, the term unfertilized or "0PN" should not be used in these cases. Instead, "PN not observed" may be a more suitable alternative for zygotes undergoing normal development without confirmation of fertilization.

Preliminary PGT-A data suggest that a significant proportion of 1PN and, some 3PN zygotes may be biparental diploid. In addition, a growing number of studies have reported normal live births from 1PN zygotes derived from both ICSI and IVF cycles. Collectively, this evidence supports cautious clinical use of 1PN zygotes, combining blastocyst culture and -if available- PGT-A technology appropriate for biparental diploidy assessment. The clinical use of 3PN zygotes is not recommended based on current evidence. 2PN zygotes with one extra micropronucleus (2.1PN) are relatively rare. However, they also may have a diploid genotype and lead to apparently normal live births. Their clinical use may be considered, especially if associated with PGT-A technology. In general, the possible clinical use of 1PN and 2.1PN zygotes should be discussed with the clinical team and the patient and governed by an internally approved policy.

566 *Table 3 Overview of all evidence and recommendations for zygote assessment*

Overview of all recommendations on zygote assessment								
Morphological feature	**Atypical patterns**	**Summary of review findings and level of evidence per outcome**					**Considerations**	**Recommendation**
		Abnormal cleavage rate	**Cleavage rate**	**Blastocyst formation rate**	**Implantation rate**	**Live birth rate**		
Zygote size	Diameter <113 µm	/	/	**Association with lower blastocyst formation rate** **Very low ⊕◯◯◯** 1 observational study (Kliajic et al., 2023)	/	**No clear association with live birth rate** **Very low ⊕◯◯◯** 1 observational study (Barberet et al., 2019)	**The evidence is insufficient and inconclusive on the hypothesis that zygote size can be harnessed as a predictive parameter for embryo developmental potential.**	
PN position	Off-centre position	**Association with higher abnormal cleavage rate** **Very low ⊕◯◯◯** 1 observational study (Coticchio et al., 2018)	/	/	/	/		
	Off-centre juxtaposition	/	/	/	/	**Association with lower live birth rate** **Very low ⊕◯◯◯** 1 observational study (Barberet et al., 2019)	**Abnormalities in PN position, juxtaposition and size are very rare and difficult, or impossible, to monitor by static observation.** **The evidence is insufficient for the application of the studied features as biomarkers.**	**Numerous zygotic attributes – zygote size, PN size, PN position, NPB patterning– might be associated with embryo quality and clinical outcome.** **Lack of PN juxtaposition is very rare, but strongly associated with poor blastocyst development.**
	Lack of PN juxtaposition	**Association with higher abnormal cleavage rate** **Very low ⊕◯◯◯** 1 observational study (Ezoe et al., 2022)	**Association with lower cleavage rate** **Very low ⊕◯◯◯** 1 observational study (Ezoe et al., 2022)	**Association with lower blastocyst formation rate** **Very low ⊕◯◯◯** 1 observational study (Ezoe et al., 2022)	/	**No clear association with live birth rate** **Very low ⊕◯◯◯** 1 observational study (Ezoe et al., 2022)		
	Interpronuclear Difference in male and female PN areas	/	/	/	/	**Association with lower live birth rate** **Very low ⊕◯◯◯** 2 observational studies (Otsuki et al., 2017; Otsuki et al., 2019)		
Nucleolar precursor bodies	NPB patterns (Z1-Z4)	/	/	**Association with higher blastocyst formation rate** **Very low ⊕◯◯◯** 1 observational study (Cavazza et al., 2021)	**No clear association with implantation rate** **Very low ⊕◯◯◯** 1 observational study (Aguilar et al., 2014)	**No clear association with live birth rate** **Very low ⊕◯◯◯** 2 observational studies (Azzarello et al., 2012; Barberet et al., 2019)	The intrinsic morphological mutability during short time periods (NPB patterning) is not amenable to static observation	
	Migration speed	/	/	/	/	**Association with higher live birth rate** **Very low ⊕◯◯◯** 2 observational studies (Inoue et al., 2021; Inoue et al., 2023)		
Cytoplasmic halo	Absence of cytoplasmic halo	**Association with higher abnormal cleavage rate** **Very low ⊕◯◯◯** 1 observational study (Ezoe et al., 2020)	**Association with lower cleavage rate** **Very low ⊕◯◯◯** 1 observational study (Ezoe et al., 2022)	**Association with lower blastocyst formation rate** **Very low ⊕◯◯◯** 1 observational study (Ezoe et al., 2017)	/	**No clear association with live birth rate** **Very low ⊕◯◯◯** 2 observational study (Ezoe et al., 2023; Barberet et al., 2019)	**The evidence disputes the significance of the halo, especially if embryo culture is extended to the blastocyst stage.**	**The absence of the cytoplasmic halo may be used to rank, but not de-select, embryos in day 3 embryo transfers.**
Number of PNs	0PN	/	**Association with lower cleavage rate** **Very low ⊕◯◯◯** 1 observational study (Fu et al., 2022)	**No clear association with blastocyst formation rate** **Very low ⊕◯◯◯** 1 observational study (Fu et al., 2022)	**No clear association with implantation rate** **Low ⊕⊕◯◯** 3 observational studies (Liu et al., 2016; Li et al., 2020; Fu et al., 2022)	**No clear association live birth rate** **Low ⊕⊕◯◯** 4 observational studies (Liu et al., 2016; Destouni et al., 2018; Li et al., 2021; Fu et al., 2022)	**The assumption that absence of PN formation (referred to as 0PN) is compatible with embryo development is not confirmed by morphokinetic evidence.**	**The term "0PN" should not be used, if based on static observation. "Not observed 2PN" or "not reported 2PN" may be alternative definitions of normal zygotes undergoing early PNBD and, for such a reason, not detected by static observation.**
	1PN	**Association with higher abnormal cleavage rate** **Very low ⊕◯◯◯** 1 observational study (Ezoe et al., 2020)	**No clear association with cleavage rate** **Low ⊕⊕◯◯** 2 observational studies (Capalbo et al., 2017; Fu et al., 2022)	**Association with lower blastocyst formation rate** **Low ⊕⊕◯◯** 3 observational studies (Itoi et al., 2015; Capalbo et al., 2017; Ezoe et al., 2022)	**No clear association with implantation rate** **Low ⊕⊕◯◯** 6 observational studies (Itoi et al., 2015; Hondo et al., 2019; Si et al., 2019; Li et al., 2020; Li et al., 2021; Fu et al., 2022)	**No clear association with live birth rate** **Low ⊕⊕◯◯** 6 observational studies (Itoi et al., 2015; Hondo et al., 2019; Si et al., 2019; Li et al., 2020; Li et al., 2021; Fu et al., 2022)	**It is plausible that a larger size of the single PN reflects a higher, possibly diploid, DNA content.** **The possible clinical use of 1PN and 2.1PN zygotes should be discussed with the clinical team and regulated by an internally approved policy.**	**The evidence suggests a possible cautious clinical use of 1PN zygotes, combining blastocyst culture and -if available- PGT-A technology appropriate for biparental diploidy assessment** **The clinical use of 2PN zygotes with one small micropronucleus (2.1 PN) may be considered, especially if associated with PGT-A technology appropriate for biparental diploidy assessment**
	2.1PN	/	**Association with lower cleavage rate** **Very low ⊕◯◯◯** 1 observational study (Capalbo et al., 2017)	**Association with lower blastocyst formation rate** **Very low ⊕◯◯◯** 1 observational study (Capalbo et al., 2017)	/	/		
	3PN	/	/	/	/	/	10/30 embryos with 3PN zygotes had a normal chromosomal array	**The clinical use of 3PN zygotes is not recommended, while pre-clinical studies should be encouraged**

Abbreviations: NBP: Nucleolar precursor bodies; PN: pronucleus; ; Table colour code: Green: the embryo can be clinically used; Yellow: the embryo could be used with cautionary considerations. Red: the embryo is considered not suitable for clinical use.

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4. Cleavage stage

Assessment of embryos at predefined times on Days 1, 2 and 3 has shown number of cells, fragmentation grade, blastomere size and multinucleation to correlate with pregnancy and live birth outcomes (Lundin and Ahlström, 2015).

The survey results indicate that the vast majority of clinics (95%) still perform early-stage embryo evaluations. However, the traditionally static "snapshot" assessments once or twice per day implies that no information regarding the development between these time points is obtained. Therefore, significant events such as abnormal cell divisions may be missed. Also, it has been shown that the morphology of an embryo may change in a couple of hours, for a better or a worse score (Montag et al., 2011), one reason being the dynamic occurrence and reabsorption of fragments during the cleavage process (Hardarson et al., 2001).

This section discusses morphological and morphokinetic attributes assessed at the early embryo cleavage stages and their potential impact on success rates for an embryo transferred or cryopreserved on Day 2 or Day 3 post fertilisation. It is important to consider that the same attributes may not be relevant or may have a different impact if the embryo survives extended culture and is transferred, fresh or after cryopreservation, at the blastocyst stage.

Timing of cleavage-stage embryo assessment

The Istanbul Consensus (2011) recommended static observation performed at 44 ± 1 hpi for Day-2 embryos and 68± 1 hpi for Day-3 embryos. The survey results showed that 41% and 63% of the respondents always assessed embryos on Day 2 or Day 3, respectively, applying these recommendations (**Supplementary data SII, figure 3.A**).

Assessment by TLT permits more detailed analysis of the traditional morphological parameters over time, as well as the incidence of abnormal cleavages. Several early, retrospective, TLT studies found that morphokinetic variables such as the timing of the first cell division, as well as the lengths of cell cycles, correlated with further embryonic development and subsequent pregnancy outcomes (Meseguer et al., 2011, Dal Canto et al., 2012b, Herrero et al., 2013). However, recent RCTs and meta-analyses have not found improvement in live birth rates following embryo selection using TLT algorithms (Armstrong et al., 2019, Ahlström et al., 2022, Kieslinger et al., 2023).

More recent TLT studies have shown timings with slight deviations from those reported in the Istanbul Consensus (2011), the differences becoming more pronounced and varied from the 4-cell stage onwards (**Table 1**).

Timing of first cleavage

The single most important indicator of embryo viability is cellular division. The occurrence of early cleavage, i.e. the first cell division occurring before 25-27 hpi, has been shown to

correlate positively with embryo quality on Day 2 and Day 3, blastocyst formation rate (Herrero et al., 2013, de los Santos et al., 2014, Milewski et al., 2015), and implantation and live birth rates after transfer on Day 2 or 3 (Lundin et al., 2001, Salumets et al., 2003). This is also more recently supported by TLT studies (Coticchio et al., 2018, Sayed et al., 2020). In addition, TLT has shown that the time from disappearance of pronuclei or pronuclei fading (PNf) to the start of the first cytokinesis was significantly related to ploidy (Vera-Rodriguez et al., 2015). A retrospective analysis of Day-2 single embryo transfers of ICSI embryos (n=207), including both traditional morphology variables as well as morphokinetic variables and patient characteristics, showed early cleavage, measured as more than one cell at 25-27 hpi, to be a significant predictor of live birth (OR 4.84, CI 2.14–10.96, P = 0.0002) (Ahlstrom et al., 2016). In addition, it was found that each increase in grade of fragmentation (to 5–10%, 11–20%, 21–50%, 51–100%) significantly decreased the probability for live birth (OR 0.46, CI 0.25–0.84, P = 0.012).

The same study also found that, for Day-2 transfers, early cleavage and fragmentation grade were better predictors of live birth outcome when compared with morphokinetic variables, and that no morphokinetic variables up to Day 2 improved prediction of live birth further (Ahlstrom et al., 2016). However, other studies have not found any correlation between early cleavage and implantation or live birth (Thurin et al., 2005, Sundström and Saldeen, 2008, de los Santos et al., 2014, Yang et al., 2015), and the data on potential importance of scoring early cleavage are currently inconclusive.

Still, the assessment of early cleavage in a TLT system can be used to select against abnormal early cleavages such as direct cleavage, reverse cleavage, and irregular chaotic division, which have been shown to be associated with lower blastocyst formation rate, implantation and live birth rate (Meseguer et al., 2011, Petersen et al., 2016, Zhan et al., 2016, Liu et al., 2020) as well as with aneuploidy (Arroyo et al., 2015, Yan et al., 2015, Desai et al., 2018) and multinucleation (Zhan et al., 2016). In a study by Barrie et al, the prevalence of these abnormal cleavages was found to be 11.4% per cleaved embryo (Barrie et al., 2017b).

At present, the use of early cleavage/early syngamy in scoring regimens varies greatly between laboratories. An important aspect to consider is the difference between zygotes originating from ICSI and cIVF, as discussed in section 1 (Expected timeline of embryo development and morphology) and section 3 (Zygote stage assessment) of this paper.

Number of cells on Day 2 and Day 3

The number of blastomeres at a specific time signifies the developmental rate of the embryo and is considered the most important parameter for embryo scoring (Machtinger and Racowsky, 2013, Yu et al., 2018). Many earlier studies already showed the number of cells at Day 2 or Day 3 to be highly predictive of laboratory and clinical outcomes (Giorgetti et al., 1995, Alikani et al., 2000, Holte et al., 2007, Racowsky et al., 2011).

The Istanbul Consensus (2011) defined an optimal Day-2 embryo (44±1 hpi) as an embryo with 4 equally sized mononucleated blastomeres in a three-dimensional tetrahedral arrangement, with <10% fragmentation, and a Day-3 embryo (68±1 hpi) with 8 equally sized mononucleated blastomeres in a three-dimensional tetrahedral arrangement, with <10% fragmentation (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011). The survey results showed that 68% of the respondents apply these Istanbul Consensus (2011) recommendations to score Day-2 and Day-3 embryos (**Supplementary data SII, figure 3.B**).

There seems to exist an "optimal" development speed and many publications throughout the years have reported that too fast or too slow embryo cleavage rate has a negative impact on embryo development (Edwards et al., 1980, Kroener et al., 2015, Shebl et al., 2021). For example, it has been shown that fast growing embryos on day 3 (>8 cells) have a higher rate of aneuploidy and an increased incidence of abnormal cleavage patterns and are less likely to make blastocysts than 8-cell embryos (Kroener et al., 2015, Kong et al., 2016, Pons et al., 2019). However, once fast-growing embryos reach the blastocyst stage, their developmental potential is similar to or better than 8-cell embryos (see also Chapter 6 – Blastocyst stage). In contrast, concerning slow-developing embryos (<4 cells on day 2, <8 cells on day 3), there is clear evidence that these always perform worse and should only be used for transfer if better embryos are not available. These observations have been confirmed by embryo assessment using TLT (Meseguer et al., 2011, Montag et al., 2011, Herrero et al., 2013, Milewski et al., 2015).

Several studies using static observation have found speed of development to be predictive of live birth. In a prospective cohort study including 6252 Day-2 single embryo transfers, number of cells, number of mononucleated cells per embryo and fragmentation rate were found to be significant predictors of live birth, with 4 cells and low (<10%) fragmentation having the highest LBR (Rhenman et al., 2015). In the most recent analysis of SART data including 28 878 fresh Day-3 embryo transfers, it was shown that for women at 34 years of age, the highest LBRs were found after transfer of 8-cell embryos (24%), followed by >8 cell (23%), 7 cell (17%), 6 cell (8%), 5 cell (5%), and 4 cell (1%) embryos (Awadalla et al., 2022a). The 8-cell embryos with low degree of fragmentation (<10%) showed higher LBR compared to embryos with more than 10% fragmentation.

In addition, when looking at available evidence it should be taken into account that cell numbers on a specific day may be impacted by culture conditions and timing of assessments. It may also be challenging at times to distinguish between a cell and a large fragment. Obviously, assessment of Day-2 and Day-3 embryos by TLT permits more exact assessment timings, as well as detailed analysis of the developmental parameters over time, and the incidence of abnormal cleavages. For example, it is possible that some embryos with >8 cells

on day 3 are generated from trichotomous cleavages. This abnormal division can affect viability, but it is only detectable by TLT.

Fragmentation

A fragment can be defined as a membrane-bound extracellular cytoplasmic mass, often not including chromosomes. Fragments can vary in size and in distribution with different implications for the embryo (Alikani et al., 1999, Cecchele et al., 2022). The degree of fragmentation is difficult to evaluate, as it is first necessary to differentiate fragments from cells, and then estimate the relative proportion of the embryo that is fragmented. One study found that a majority of blastomeres of <45 µm diameter in a Day-2 embryo and <40 µm diameter in a Day-3 embryo did not contain nuclei (Johansson et al., 2003). The impact of <10% fragmentation in Day-3 embryos on implantation rate has been found to be negligible (Alikani et al., 1999, Ebner et al., 2001, Van Royen et al., 2001, Holte et al., 2007, Racowsky et al., 2011), while, as discussed above, both earlier and several more recent and large studies, including TLT studies, have shown negative correlation with increasing fragmentation on live birth rates after early transfer (Rhenman et al., 2015, Ahlstrom et al., 2016, Awadalla et al., 2022b). Interestingly, a study by Ahlstrom et al., indicated that for Day-2 and Day-3 embryos, AI score correlated significantly with cell number and fragmentation score (Ahlström et al., 2023).

In addition, correlation has been shown between the degree of fragmentation and the incidence of aneuploidy (Munné et al., 1995, Ziebe et al., 2003, Chavez et al., 2012).

Uneven cleavage and cell size

Uneven cellular cleavage, leading to unequal relative cell size, is commonly found in human embryos in vitro (Puissant et al., 1987). Unequal cell size has been defined as a 25% difference between the average diameter of the smallest cells compared to the average of the largest cells (Meseguer et al., 2011, Ziebe, 2013). Uneven cellular cleavage and its negative impact on pregnancy outcome for early transfer has been confirmed by several studies (Giorgetti et al., 1995, Ziebe et al., 1997, Hardarson et al., 2001, Racowsky et al., 2011), although some data are conflicting (Holte et al., 2007).

Interestingly, late-cleaving embryos have been reported to cleave more unevenly which in turn has been strongly correlated with an increased incidence of chromosomal errors (Hardarson et al., 2001, Shenoy et al., 2021), possibly due to uneven distribution of proteins, mRNA and mitochondria (Antczak and Van Blerkom, 1999).

It is important to consider that the relative cell sizes must be “cell stage appropriate”, i.e., assessed in relation to the number of cycles that cells have gone through. This means that the sister blastomeres representing the same cell cycle should be equally sized, i.e., only at the 2-, 4- and 8-cell stage should all the cells be of the same size.

Multinucleation

Multinucleation has been correlated with a higher degree of fragmentation and decreased number of blastomeres on Days 2 and 3 (Van Royen et al., 2003), as well as with uneven cell size (Kligman et al., 1996, Hardarson et al., 2001, Sayed et al., 2022). The presence of multinucleation is generally considered abnormal, however the reported incidence varies greatly. The term "multinucleation" can include different types of nucleation in one or more cells, including multiple (equally sized) nuclei, two nuclei (binucleation) and/or smaller size or micro nuclei (micronucleation). Most studies have not differentiated clearly between the different types, or in how many of the cells the condition is present, which may be a reason for some conflicting reports. For example, one study reported that 43% of patients had one or more embryo with multinucleation at the 2-cell stage, defined as ≥2 nuclei, which was reduced to 15% at the 4-cell stage (Balakier and Cadesky, 1997). Two other studies reported its occurrence in up to 87% of cycles, with 31–33% of the embryos affected at transfer (Jackson et al., 1998, Van Royen et al., 2003). Significantly slower development rate as well as lower implantation and live birth rates after early embryo transfer have been shown for multinucleated compared to mononucleated embryos (Ergin et al., 2014, Desch et al., 2017).

One recent TLT study, however, found that embryos that were binucleated at the 2-cell stage showed improved blastocyst formation rates and implantation rates, both compared to "true" multinucleated embryos (≥3) and non-multinucleated embryos (Talbot et al., 2022). This shows the importance of distinguishing between the different types of nucleation during embryo assessment. Nucleation has shown to be a dynamic process, and in all studies the rate of multinucleation seen at the 2-cell stage was significantly reduced at the 4-cell stage (Aguilar et al., 2016). It could also be that many of these embryos were binucleated but not "true" multinucleated (≥3 nuclei) on Day 2, and should not be considered compromised, as discussed in the study by Talbot et al. (2022).

Evidence collected via TLT, where the cells can be scored in much more detail, has shown an incidence of 29-43% in multinucleation in early (2-cell stage) embryos with a significant impact on implantation and live birth (Balakier et al., 2016, Goodman et al., 2016, Sayed et al., 2022). One study found an incidence of 6% multinucleated embryos with static scoring, compared to 23% using TLT (Ergin et al., 2014). Another study similarly found 7% and 35% using the two methods (Goodman et al., 2016).

In a further TLT study, it was shown that embryos with direct uneven cleavage or irregular chaotic divisions showed a lower developmental potential. However, for those that did develop to the blastocyst stage, the presence of a single abnormality (multinucleation, reverse cleavage, irregular chaotic division, or direct uneven cleavage) at an early cell stage was not associated with aneuploidy when analysed at the blastocyst stage (Desai et al., 2018).

Other morphological features of Day-2 and Day-3 embryos

There is no conclusive evidence that embryos with apparent spatial disorganisation, i.e., those that do not have the expected three-dimensional arrangement of blastomeres, should be considered abnormal (Ebner et al., 2012, Cauffman et al., 2014, Ebner et al., 2017, Desai and Gill, 2019).

Other morphological features, such as cytoplasmic granularity, membrane appearance and the presence of vacuoles can also be scored as part of the morphological assessment of Day-2 and Day-3 embryos (The Atlas of human embryology: from oocytes to preimplantation embryos, 2012). It is important to understand that these features can vary within and between cohorts.

Initiation of compaction

Compaction usually starts at the 8- to 16-cell stage. To be more precise, compaction spans the phase between the point in time when any two blastomeres of the multicellular embryo start to compact and the moment prior to the onset of blastocoel formation (Ciray et al., 2014). One study showed that almost 90% of embryos started compaction at the 8-cell stage or later (Iwata et al., 2014). Fifty percent of these developed into good quality blastocysts, while for embryos that initiated compaction before the 8-cell stage, less than 20% became good quality blastocysts. Several other studies showed that beginning compaction on Day 3 can be a positive feature (Alikani et al., 2000, Skiadas et al., 2006, Le Cruguel et al., 2013, Aslan Öztürk et al., 2022). It is noteworthy that compaction on Day 2 is atypical and of unknown biological significance.

Consensus points

- Cleavage-stage embryo scoring should include cell number, grade and reason for the grade (e.g., 4-cell, grade 2, fragmentation) as previously agreed in the Istanbul Consensus (2011).

- Two-cell embryos on Day 1, 4-cell embryos on Day 2 and 8-cell embryos on Day 3, showing <10% fragmentation, mononucleation, and stage-specific cell size, should be prioritized in case of cleavage stage transfer or cryopreservation.

- There is no significant body of evidence to support an impact on implantation potential for cleavage stage embryos with atypical features such as spatial disorganisation, vacuoles, cytoplasmic granularity, and zona abnormality, and these are therefore considered suitable for clinical use. However, extended culture of such embryos as a way of further selection for viability and evaluation should be considered.

- *Early cleavage*: The importance of scoring early cleavage for prediction of success rates has not been conclusively established. However, it may add information regarding other features such as binucleation/multinucleation and cell size. Assessment of early cleavage by TLT can be used to identify abnormal early cleavages such as direct cleavage, reverse cleavage, and irregular chaotic division.

- *Fragmentation*: The relative degree of fragmentation was defined as: No or minimal (<10%), mild (<25%) or severe (>25%). The percent values are based on the cell equivalents, so for a 4-cell embryo, 25% fragmentation would equate to one blastomere in volume.

- *Numbers of blastomeres on Day 2/3*: The current expected observation for embryo development is 4 cells on Day 2 and 8 cells on Day 3. However, this can be influenced by the exact time of observation and culture conditions. It is recommended that the time of assessment is documented.

- *Cell size*: For embryos at the 2-, 4- and 8-cell stages, blastomeres should be evenly sized. For all other cell stages, one would expect a cell stage appropriate size difference as the cleavage phase has not been completed.

- *Multinucleation*: True multinucleation (≥3 nuclei in one or several cells) is associated with decreased implantation potential, and with increased chromosome abnormality. Binucleation on Day 2, at the 4-cell stage, may not be necessarily a negative sign, but more evidence is needed. Laboratories should record the incidence and discriminate between binucleation, multinucleation and micronucleation in each embryo, and ideally, the nucleation status of each blastomere in each embryo. If available, multinucleation should be scored using TLT.

- *Time-lapse technology:* Large datasets including timing of certain developmental events have been analysed to design algorithms to predict implantation and live birth. However, there is currently limited good quality evidence of better clinical outcomes following TLT embryo selection (Armstrong et al., 2019, Kieslinger et al., 2023). TLT allow assessment of kinetic variables such as rapid cleavage, direct cleavage, and reverse cleavage. These data have been used for deselection of embryos and it has been demonstrated that certain atypical cleavage patterns such as direct cleavage to three cells negatively affect embryo development. These events would in most cases be missed using traditional culture without TLT.

- *Compaction:* Based on a few studies, the start of compaction before 8 cells seems to negatively affect blastocyst formation, while compaction from 8 cells and onwards may be a positive indicator and could potentially be used as an additional selection tool at this stage.

Ranking cleavage-stage embryo

Different morphological features can reflect the overall quality of Day-2 and Day-3 embryos and the combination of those morphological features can be used to define a ranking order for transfer or cryopreservation of Day-2 and Day-3 embryos. A proposed ranking scheme for Day-2 and Day-3 embryos is presented in **Table 4**.

817 *Table 4 Ranking Scheme for Day-2 and Day-3 embryo transfer*

Feature	Top ranking	Intermediate ranking	Low ranking
Number of cells	4 cells on Day-2 or 8 cells on Day-3	>4 cells on Day-2 or >8 cells on Day-3	< 4 cells on Day-2 or < 8 cells on Day-3
Early cleavage	Early cleavage	No early cleavage	
Cell size	Cell stage specific	Not cell stage specific	
Fragmentation	None or minimal fragmentation (<10%)	10-25% fragmentation	>25% fragmentation
Multinucleation	No multinucleation at any cell stage	No multinucleation at 4 cell stage	Multinucleated at 4-cell stage
Abnormal cleavage	-	-	Direct cleavage DC2 (2- to 5-cell)
Compaction	Compaction from ≥8-cell stage	No compaction	Compaction before 8-cell stage
Recommendation	Avoid transfer of Day 2/3 embryos with abnormal cleavage: direct cleavage DC1 (1- to 3- cell), irregular chaotic division or reverse cleavage. Extend culture of embryos with abnormal cleavage to blastocyst stage.		

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819 *Table 5 Overview of all evidence and recommendations for cleavage stage embryo assessment*

Overview of all recommendations on cleavage stage assessment							
Morphological feature	**Atypical pattern**	**Summary of review findings**				**Considerations**	**Recommendation**
		Embryo quality and development potential	**Ploidy**	**Implantation rate**	**Live birth rate**		
First cleavage	**Early cleavage (first division before 25-27 hours)**	**Association with higher embryo quality and blastocysts formation rate** **Very low ⊕◯◯◯** 3 observational studies (Herrero et al., 2013; de los Santos et al., 2014; Milewski et al., 2015)	**Association with higher aneuploidy rate** **Very low ⊕◯◯◯** 1 observational study (Vera-Rodriguez et al., 2015)	**Contradictory results:** **No association with implantation rate** **Low ⊕⊕◯◯** 5 observational studies (Lundin et al., 2001; Salumets et al., 2003; Coticchio et al., 2018; Sayed et al., 2020, Ahlström et al., 2016) **Association with higher implantation rates** **Low ⊕⊕◯◯** 1 RCT and 3 observational studies (De los Santos et al., 2014; Thurin et al. 2005; Sundstrom and Saldeen, 2008; Yang et al., 2015)	**Contradictory results:** **No association with live birth rate** **Low ⊕⊕◯◯** 1 RCT and 3 observational studies (De los Santos et al., 2014; Thurin et al. 2005; Sundstrom and Saldeen, 2008; Yang et al., 2015) **Association with higher live birth rates** **Low ⊕⊕◯◯** 5 observational studies (Lundin et al., 2001; Salumets et al., 2003; Coticchio et al., 2018; Sayed et al., 2020, Ahlström et al., 2016)	**Assessment of early cleavage embryos may add information regarding other features such as binucleation/multinucleation and cell size.** **An important aspect to consider is the difference between zygotes originating from ICSI or cIVF.**	**The importance of scoring early cleavage for prediction of success rates has not been conclusively established.**
	Abnormal early cleavage (direct cleavage, reverse cleavage, irregular chaotic division)	/	**Association with higher aneuploidy rate** **Low ⊕⊕◯◯** (Yan et al., 2015; Desai et al., 2018; Arroyo et al., 2015)	**Association with lower implantation rate** **Low ⊕⊕◯◯** (Meseguer et al., 2011; Petersen et al., 2016; Zhan et al., 2016; Liu et al., 2020)	/		**Assessment of early cleavage by TLT can be used to select against abnormal cleavage patterns such as direct cleavage, reverse cleavage, and irregular chaotic division.**
Cell numbers	**Cell number on Day 2/3**	**Association with embryo scoring** **Very low ⊕◯◯◯** 3 observational studies (Alikani et al., 2003; Machtinger and Racowsky, 2013; Yu et al., 2018)	**Correlation with chromosomal status** **Low ⊕⊕◯◯** 3 observational studies (Almeida and Bolton, 1996; Magli et al., 2007; Kroener et al., 2015)	**Correlation with implantation rates** **Low ⊕⊕◯◯** 4 observational studies (Giorgetti et al., 1995; Alikani et al., 2000; Van Royen et al., 2001; Renman et al., 2015)	**Correlation with live birth rates** **Low ⊕⊕◯◯** 5 observational studies (Giorgetti et al., 1995; Rhenman et al., 2015, Awadalla et al., 2022; Racowsky et al., 2011, Tian et al., 2022)		**The current expected observation for embryo development is 4 cells on Day 2 and 8 cells on Day 3.**
Fragmentation	**Degree of fragmentation**	**Association with lower embryo quality and development potential** **Very low ⊕◯◯◯** 2 observational studies (Alikani et al., 2000; Ebner et al., 2001)	**Association with lower euploidy rate** **Very low ⊕◯◯◯** 5 observational studies (Munné et al., 1995; Ziebe et al., 2003; Chavez et al., 2012)	**Association with lower implantation rate** **Very low ⊕◯◯◯** 4 observational studies (Alikani et al., 1999; Ebner et al., 2001; Racowsky et al., 2011; Van Royen 2001)	**Association with lower live birth rates** **Low ⊕⊕◯◯** 3 observational studies (Rhenman et al., 2015; Ahlstrom et al., 2016; Awadalla et al., 2022)	**The percent values are based on the cell equivalents, so for a 4-cell embryo, 25% fragmentation would equate to one blastomere in volume.**	**The relative degrees of fragmentation were defined as: No or minimal (<10%), mild (≤25%) or severe (>25%) .**
Cell size	**Uneven cellular cleavage**	/	**Correlation with chromosomal errors** **Low ⊕⊕◯◯** 2 observational studies (Hardarson et al., 2001; Shenoy et al., 2021)	**Association with lower implantation rate** **Very low ⊕◯◯◯** 1 observational study (Mugica et al., 2008)	/	**It is important to consider that the relative cell sizes must be "cell stage appropriate", i.e., assessed in relation to the number of cycles that they have gone through**	**For embryos at the 2-, 4- and 8-cell stages, blastomeres should be evenly sized. For all other cell stages, one would expect a cell stage appropriate size difference as the cleavage phase has not been completed.**
Multinucleation	**Multiple nuclei**	**Negative correlation with time of development** **Low ⊕⊕◯◯** 5 observational studies (Ergin et al., 2014; Desch et al., 2017; Goodman et al., 2016; Balakier et al., 2016; Sayed et al., 2022)	**No association with aneuploidy rates** **Very low ⊕◯◯◯** (Desai et al., 2018)	**Association with lower implantation rate** **Low ⊕⊕◯◯** 5 observational studies (Ergin et al., 2014; Desch et al., 2017; Goodman et al., 2016; Sayed et al., 2022)	**Association with lower live births rate** **Low ⊕⊕◯◯** 5 observational studies (Ergin et al., 2014; Desch et al., 2017; Goodman et al., 2016; Sayed et al., 2022)	**Multinucleation assessment on Day 3 would be complicated by the much smaller cell size, and therefore would be less reliable. If available, multinucleation should be scored using TLT.**	**True multinucleation (≥3 nuclei in one or several cells) is associated with a decreased implantation potential, and with an increased level of chromosome abnormality.**
	binucleation and/ or Micronucleation	**Association with higher blastocyst formation rate** **Very low ⊕◯◯◯** 1 observational study (Talbot et al., 2022)	/	**Association with higher implantation rate** **Very low ⊕◯◯◯** 1 observational study (Talbot et al., 2022; Aguilar et al., 2016)	/	**Laboratories should record the incidence and discriminate between binucleation, multinucleation and micronucleation in each embryo, and ideally, the nucleation status of each blastomere in each embryo.**	**Binucleation on Day 2, at the 4-cell stage, may not be necessarily a negative sign, but more evidence is needed.**
Other morphological features	**Spatial disorganization**	**No clear association with embryo development** **Very low ⊕◯◯◯** 2 observational studies (Ebner et al., 2012; Ebner et al., 2017)	/	/	/	**More research is required to identify which, if any, of these features are correlated with (or indicative of) implantation potential**	**Embryos with apparent spatial disorganisation should not be considered abnormal.** **There is no significant body of evidence to support a clear biological effect of cytoplasmic granularity, membrane appearance and the presence of vacuoles, these features on implantation potential.**
	cytoplasmic granularity, membrane appearance, vacuoles	**Negative correlation with Day 3 development (atypical early compaction)** **Low ⊕⊕◯◯** 3 observational studies (Skiadas et al., 2006; Le Cruguel et al., 2013; Osturk et al., 2022)	/	/	/		

Table colour code: Green: the embryo can be clinically used; Yellow: the embryo could be used with cautionary considerations. Red: the embryo is not considered suitable for clinical use.

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5. Morula stage

As indicated in the guidelines on the nomenclature and annotation of dynamic human embryo monitoring (Ciray et al., 2014), the term morula refers to the "end of the compaction process".

Timing of morulae assessment and scoring

Accordingly, a morula would be the expected developmental stage if embryo scoring is done on day 4 at 92 ± 2 hpi as recommended by the Istanbul Consensus (2011) (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011).

The survey results showed that 24% of the respondents always apply the Istanbul Consensus (2011) recommendations related to the timing of assessment of Day-4 embryos (**Supplementary data SII- figure 3.A**).

However, TLT data have shown that there are considerable deviations in cleavage timings among a group of embryos of the same patient. At the extreme, a one-day delay or speed-up can be observed (Shebl et al., 2021), neither scenario being necessarily associated with a worse treatment outcome.

Morphological features to consider for morulae assessment

Due to this variation in developmental speed and cellular complexity, there is a lack of well-defined morphological markers of viability for this stage (Coticchio et al., 2019).

The survey results showed that 28% of the respondents always apply the Istanbul Consensus (2011) scoring criteria to score Day-4 embryos (**Supplementary data SII, figure 3.B).**

Timing of cavitation

Early cavitation of morulae is a good prognostic parameter related to better quality blastocysts with a higher potential to implant and higher ongoing pregnancy rates possibly due to a higher rate of euploidy (Hung et al., 2018). On the other hand, a delay in compaction and onset of cavitation was found to be associated with reduced blastocyst quality (Ivec et al., 2011, Desai et al., 2014) and reduced likelihood of live birth (Fishel et al., 2018).

Number of cells

Quality assessment at 92 ± 2 hpi usually takes both cell number and degree of compaction into consideration (Alikani et al., 2000, Tao et al., 2002, Feil et al., 2008, Ebner et al., 2009, Fabozzi et al., 2016). It has been found that the more cells and in particular the more compacting cells a Day-4 embryo shows the better its chance to form a blastocyst on Day 5 (Ebner et al., 2009, Iwata et al., 2014).

Since accurate evaluation of cell number is impossible once the majority of blastomeres is involved in the compacting mass, focus is placed on the proportion of cells involved in compaction. In principle, partly (PCM) and fully (FCM) compacted morulae can be

distinguished. The former group is characterized by a certain loss of embryonic mass either due to extensive cytoplasmic fragmentation or blastomere elimination. If the observed loss is substantial further development to blastocyst (Alikani et al., 2000, Ebner et al., 2009, Lagalla et al., 2017, Coticchio et al., 2021b) and formation of good quality blastocysts (Ebner et al., 2009, Coticchio et al., 2021b) will be affected, both of which could be associated with a lower live birth rate (Coticchio et al., 2021b).

Other morphological features

Beyond the degree of compaction, some studies also considered detrimental morphological features such as: excessive fragmentation, multiple excluded cells, "self-cavitation" of blastomeres and vacuolisation for morphological assessment of Day-4 embryos (Alikani et al., 2000, Feil et al., 2008, Ivec et al., 2011, Fabozzi et al., 2016). Of note, the first three abnormalities would reflect PCM, which implies that vacuolisation is the only abnormality that could be taken into consideration for quality assessment purposes. Indeed, spontaneous vacuole formation around the time of compaction was found to be a negative predictor of blastulation and top-quality blastocyst formation rates (Mayer et al., 2018, Chen et al., 2019), ongoing pregnancy rate (Feil et al., 2008, Mayer et al., 2018) and live birth rate (Mayer et al., 2018).

Recent TLT studies further shed some light on the phenomenon of blastomere loss around the morulae stage (Lagalla et al., 2020, Coticchio et al., 2021b). Two types of cleavage dynamics were identified, both of which were responsible for the elimination of blastomeres but differed in timing. One was the exclusion of blastomeres from the outset and the other was characterized by the extrusion of cells after full compaction had already occurred. The occurrence of the two phenomena together had the worst prognosis for live birth (Coticchio et al., 2021b, Hur et al., 2023).

Blastomere exclusion/extrusion at morulae stage is likely to be associated with abnormalities in the eliminated cells. It has been shown that excluded cells show E-cadherin (a key cell adhesion protein) expression profiles that are different from the expected membrane-localised pattern (Alikani, 2005). The degree to which blastomere loss reflects perturbations in key events in compaction and cell polarization of the morula (e.g., apical F-actin and PAR complex accumulation) remains speculative (Zhu et al., 2021). In relation to partial compaction, other studies reported "abnormal divisional behaviour" such as karyokinesis without cytokinesis or signs of degeneration (Zhan et al., 2016). The appearance of apoptotic nuclei following compaction further suggests that programmed cell death may play a role in eliminating affected blastomeres (Chatzimeletiou et al., 2005).

A more detailed annotation of the TLT sequences revealed that in comparison to FCM all patterns of PCM not only show a higher rate of irregular and asymmetric cleavage (Coticchio et al., 2021b) but also an evident delay in development starting with pronuclear fading (Lagalla

et al., 2020, Coticchio et al., 2021b, Hur et al., 2023). In particular, highly dynamic biological processes such as compaction and blastulation were deferred (Lagalla et al., 2020, Coticchio et al., 2021b, Ezoe et al., 2023).

A hierarchical classification model has found morulae formation (tM) within an optimal range (81.3-96.0 hpi) to be one of the strongest predictors of blastocyst formation (Motato et al., 2016). Similarly, a multivariate analysis has shown that tM was the only morphokinetic parameter that correlated with live birth rate after euploid blastocyst transfer (Rienzi et al., 2019).

While some studies showed no correlation between tM or starting blastulation (tSB) and aneuploidy (Minasi et al., 2016) others found a delayed initiation of compaction (tSC) in complex aneuploid embryos (Campbell et al., 2013).

There is evidence that PCM following irregular cleavages can develop into euploid blastocysts (Zhan et al., 2016, Lagalla et al., 2017). Those cells excluded from the morulae were shown to have a high rate of aneuploidy and degraded DNA (Lagalla et al., 2017). This, together with reduced aneuploidy rate in biopsied TE cells of the associated blastocyst, suggests that a self-check mechanism may reduce the relative abundance of aneuploid cells.

On the other hand, a recent study showed a high ploidy correlation between excluded cells and TE cells, suggesting that cell exclusion might be a consequence of compromised embryo development regardless the chromosomal constitution of excluded cells (Parriego et al., 2023).

Consensus points

- Day-4 embryos showing full compaction or early cavitation should be prioritized in case of Day-4 transfer or vitrification.

- Embryos with partial compaction can form blastocysts and should be considered for clinical use. Extended culture of these embryos for further evaluation should be considered.

916 *Table 6 Ranking for embryos selection of morulae with similar hpi*

Feature	Top ranking	Intermediate ranking	Low ranking
(Early) cavitation	Yes	No	No
Compaction	FCM	PCM	No compaction Compacting embryo with ≥8 cells PCM with significant cytoplasmic loss
Morphology	No vacuoles	No to minor vacuolisation	Heavy vacuolisation
Recommendation	Extend culture to blastocyst for embryos with atypical morphological features: self-cavitation of blastomeres, <50% compacted embryo, ≤8 cells without compaction, excessive fragmentation, widespread vacuoles.		
FCM: Fully compacted morulae; PCM: partially compacted morulae.			

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Table 7 Overview of all evidence and recommendations for Day-4 embryos assessment

Overview of all recommendations on morulae assessment							
		Summary of review findings				Considerations	Recommendation
Feature	**Atypical patterns**	**Embryo quality and development potential**	**Ploidy**	**Implantation rate**	**Live birth rate**		
Timing of cavitation	**Early cavitation**	/	**Association with higher euploidy rate** **Very low ⊕○○○** 1 observational study (Huang et al., 2018)	**Association with higher implantation rate** **Very low ⊕○○○** (Hung et al., 2018; Rienzi et al., 2019)	**Association with higher ongoing pregnancy rate** **Very low ⊕○○○** (Hung et al., 2018; Rienzi et al., 2019)	**Similar clinical pregnancy and live birth rates were achieved when transferring morulae on Day 5 rather than waiting for Day-6 blastocyst formation**	**Day-4 embryos showing full compaction or early cavitation should be prioritized in case of Day-4 transfer or cryopreservation.**
	Delay in compaction	**Association with lower blastocyst quality** **Very low ⊕○○○** 2 observational studies (Desai et al., 2014; Ivec et al., 2011)	**Contradictory results:** **No clear association with aneuploidy rate** **Very low ⊕○○○** 1 observational study (Minasi et al., 2016) **Association with higher euploidy rate** **Very low ⊕○○○** 1 observational study (Campbell et al., 2013)	**No clear association with implantation rate** **Very low ⊕○○○** 2 observational studies (Montjean et al., 2021)	**No clear association with live birth rate** **Very low ⊕○○○** 2 observational studies (Montjean et al., 2021;)		
Number of cells	**More compacting cells on day 4**	**Correlation with blastocyst formation rate** **Very low ⊕○○○** (Ebner et al., 2009; Iwata et al., 2014)	/	/	/	**Accurate evaluation of cell number is impossible once the majority of blastomeres is involved in the compacting mass, and the focus is placed on the proportion of cells involved in compaction.**	
Degree of compaction	**Partly compacted embryos (Excessive fragmentation, large number of excluded cells, self-cavitation of blastomeres)**	**Association with lower blastocyst formation rate and blastocyst quality** **Low ⊕⊕○○** (Alikani et al., 2000; Ebner et al., 2009; Lagalla et al., 2017; Coticchio et al., 2021; Parriego et al., 2023)	/	/	**Association with lower live birth rate** **Very low ⊕○○○** (Coticchio et al., 2021)	**Highly dynamic biological processes such as compaction and blastulation were deferred in partly compacted embryos**	**Embryos with partial compaction can form blastocysts and should be considered for clinical use. Extended culture of these embryos for further evaluation should be considered.**
Vacuolisation	**vacuole formation around compaction**	**Association with lower blastocyst formation rate and blastocyst quality** **Very low ⊕○○○** (Mayer et al., 2018; Chen et al., 2019)	/	/	**Association with lower ongoing pregnancy rate and live birth rate** **Very low ⊕○○○** (Feil et al., 2008; Mayer et al., 2018)	**No correlation has been found between the occurrence of vacuoles and patient parameters like age or baseline hormonal profile**	**Spontaneous vacuole formation around compaction was found to be a negative predictor for embryo development**
	Compaction of vacuolized blastomeres		**Association with higher mosaicism rate** **Very low ⊕○○○** (Chen et al., 2019)				
Cleavage dynamics	**Blastomere exclusion/extrusion**	/	/	/	**Association with lower live birth rate** **Very low ⊕○○○** (Coticchio et al., 2021; Hur et al., 2023)	**Blastomere exclusion/extrusion at morulae stage is likely to be associated with abnormalities in the eliminated cells.**	**Normally cleaving embryos result in euploid blastocysts less frequently than their irregular cleaving counterparts**

Table colour code: Green: the embryo can be clinically used; Yellow: the embryo could be used with cautionary considerations. Red: the embryo is not considered suitable for clinical use.

6. Blastocyst stage (Days 4 – 7)

Embryo culture to the blastocyst stage is routine in clinical embryology encompassing Days 4 to 7 and represents a significant shift in practice since the Istanbul Consensus was first published in 2011.

The survey results indicate that only 27% of the respondents follow the Istanbul Consensus (2011) recommendations on the timing and criteria for scoring blastocysts. The Gardner grading system (Gardner and Schoolcraft, 1999), remains the most common scheme utilized clinically, according to the survey results (63% of respondents) (**Supplementary data SII, figure 1.D**). Re-evaluation and modification of the Gardner grading system was to be expected and this has indeed occurred (Veeck and Zaninovic, 2003, Cuevas Saiz et al., 2018, Hammond et al., 2020, Pierson et al., 2023), and 30% of respondents indicated using an additional grade (either "D" or "X") or the term "non-classifiable" to denote blastocysts considered unsuitable for clinical use.

AI has been applied to both consecutive images of embryo development obtained through time-lapse (Khosravi et al., 2019, Tran et al., 2019, Berntsen et al., 2022), and to static images of blastocysts (Bormann et al., 2020, Chavez-Badiola et al., 2020, Diakiw et al., 2022), in an attempt to improve the ability to identify the most viable embryo in a cohort, while reducing the intra- and inter-operator variation associated with subjective evaluation of blastocysts using the grading systems discussed. Interestingly, a recent paper by Ezoe et al., indicated that AI score was tightly coupled to the morphological aspects of the Gardner grading system (Ezoe et al., 2022b). AI holds great promise to augment embryologist assessment of the blastocyst (Fitz et al., 2021, Sawada et al., 2021), but should not yet be considered as a replacement for conventional assessment. The survey results showed that only 14% of the respondents make use of AI mainly for embryo assessment in TL videos (in 71% of cases) (**Supplementary data SII, figure 6.C**).

Timing of Blastocyst Scoring

The recommended timing by the Istanbul Consensus (2011) for static observation of Day-5 embryos is 116 h ± 2 hpi (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011). However, formation and expansion of a blastocoel cavity in embryos leading to a live birth occurs over a wide timeframe, from as early as Day 4 (98.4 ± 0.4 hpi) to the "typical" timing of Day 5 (112.4 ± 0.1 hpi) or delayed until Day 6 (131.6 ± 0.1 hpi) or Day 7 (151.2 ± 0.5 hpi) (Coticchio et al., 2023). Maintaining a standardised window for embryo assessment can be beneficial for benchmarking, establishing and monitoring KPIs, although this should be balanced against workflow needs, particularly when TLT is not available. In terms of timing of assessment, even if daily assessment timings cannot be consistent, blastocysts within a cohort can be compared for developmental stage as well as morphology to aid selection – being mindful of reports that faster developing embryos, at each stage of

development, have greater potential for implantation and birth, than their slower counterparts (Campbell et al., 2022b).

Morphological features to consider for blastocyst assessment

Day of blastocyst formation

Developmental speed is directly correlated with blastocyst viability: slower growing blastocysts have lower implantation rates (Shebl et al., 2021). While blastocysts developing according to the expected timeline have high implantation rates when transferred during a fresh cycle (Shebl et al., 2021), slow growing blastocysts may miss the window of implantation, a problem that is partially alleviated with blastocyst vitrification and transfer in a frozen cycle (Day 5 vs Day 6, RR 1.74 for fresh transfer and 1.38 for FET(Bourdon et al., 2019), particularly for Day-6 blastocysts that were at the morulae stage on Day 5 (Tannus et al., 2019). Day 4 blastocysts, although rare, display a very high implantation rate in frozen embryo transfer cycles (Coticchio et al., 2023).

Live birth rates for untested blastocysts frozen on Day 6 are lower than those frozen on Day 5 (Bourdon et al., 2019; Yerushalmi et al., 2021; Coticchio et al., 2023); and this difference persists with the transfer of euploid blastocysts (Tiegs et al., 2019, Zhan et al., 2020, Cimadomo et al., 2022b, Lane et al., 2022). Day-7 blastocysts, which may represent 5-10% of all useable blastocysts (Hammond et al., 2018), have higher rates of aneuploidy and lower implantation rates compared to Day-5 and Day-6 euploid blastocysts (Tiegs et al., 2019, Cimadomo et al., 2022b, Lane et al., 2022). Nonetheless, healthy live births can be obtained with Day-7 blastocysts and these embryos may be of particular importance for patients with few embryos available (Du et al., 2018). Survey results indicated that a small minority (16%) of the respondents perform some fresh Day-7 blastocyst transfers, while most others (49%) transfer Day-7 blastocysts in frozen embryo transfer cycles.

Degree of expansion and ICM/TE grade

Implantation potential according to the Istanbul Consensus (2011) scoring system is related to expansion stage and ICM/TE grade, though the relative importance of each remains to be full resolved. The difference between ICM/TE grades A and B appears marginal, whereas grade C is considered non-useable by 44% of respondents. The remaining respondents use a modified Gardner grade or the term “non-classifiable” and consider blastocysts with grade C ICM or TE as useable. This marked difference in clinical practice indicates lack of consensus, an observation further supported by the finding that 8 of 10 respondents indicated that a universally accepted term for non-useable blastocysts is needed.

Fresh untested blastocyst transfers represent a significant proportion of treatment cycles and have helped establish relative importance of blastocyst characteristics. Multivariate analysis accounting for expansion stage, ICM grade and TE grade shows that grade of TE is the strongest predictor of live birth (Ahlström et al., 2011, Hill et al., 2013, Thompson et al., 2013, Ebner et

al., 2016, Bakkensen et al., 2019, Pons et al., 2023), followed by degree of expansion (Thompson et al., 2013, Du et al., 2016, Subira et al., 2016, Bakkensen et al., 2019). Few blastocysts with grade "C" ICM or TE were included in these studies; notably one study found Grade "C" ICM was associated with lower live birth rate (Subira et al., 2016). In general, expanded blastocysts with higher grade TE are associated with higher live birth rates in fresh transfers (Zou et al., 2023). Similarly, in a multivariate analysis of over 2,000 fresh blastocyst transfers, one study showed that both expansion stage and TE grade were associated with the probability of live birth (Storr et al., 2019). The impact of ICM grade on outcome is less clear. While ICM grade may be associated with pregnancy loss (Van den Abbeel et al., 2013), and birthweight (Licciardi et al., 2015), further evidence is needed to establish definitive links. Blastocysts showing marked signs of degeneration or without clearly discernible ICM may sporadically produce live births, but pertinent evidence is anecdotal (Kovacic et al., 2004).

Predictive features of untested fresh and frozen blastocysts compare favourably. TE grade was the most common variable associated with live birth from frozen blastocysts (Honnma et al., 2012, Ahlström et al., 2013, Chen et al., 2014), followed by expansion stage (Ahlström et al., 2013). None of these studies found an association between ICM grade and implantation, though similar to studies with fresh blastocysts, grade "C" ICM was not well represented in frozen embryo transfer cycles. Of note and in contrast to fresh transfers where only Day-5 embryos were transferred, none of the studies controlled for day of blastocyst formation in the multivariate analysis, thus limiting their applicability for using stage/grade when ranking slower growing blastocysts.

Though most studies have found that TE grade has the highest correlation with live birth, at least one multivariate analysis found that the grade of the ICM is the variable most commonly associated with implantation (Irani et al., 2017). However, most of the studies only found an association with grade "C" ICM, not between grade "A" and "B" (Zhao et al., 2018, Nazem et al., 2019, Abdala et al., 2022, Zhang et al., 2022). Some of these studies also found an association with TE grade (Zhao et al., 2018, Nazem et al., 2019) and expansion stage (Abdala et al., 2022). A recent study developed a composite blastocyst score where day, expansion stage, TE and ICM grades were all significantly associated with a clinical pregnancy, and blastocyst day had the largest impact, followed by ICM grade, expansion and TE grade (Zhan et al., 2020).

Early in the clinical application of blastocyst culture, a threshold for blastocyst useability was set at Gardner 3BB when slow freezing and variable cryosurvival influenced the decision (Langley *et al.*, 2001). Since the adoption of vitrification and PGT-A, several studies indicate that presumably low-grade blastocysts previously classified as non-viable (e.g. grade C) can produce healthy live births, albeit at greatly reduced rates (Morbeck, 2017; Kemper *et al.*,

2021). Similar to Day-7 blastocysts, these low-grade blastocysts may be useful for patients with few available embryos (Cimadomo et al., 2022b).

Abnormal chromosomal status reported

Human embryos with abnormal chromosomal status can develop as evidenced by the fact that specific trisomies are compatible with the formation of high scoring blastocysts, and some, such as trisomy 21, can go to term (Forman et al., 2013, Savio Figueira Rde et al., 2015). Importantly, blastocysts with abnormal chromosomal status will exhibit aneuploid stress, through which their transcriptome, proteome and metabolome will be affected, thereby compromising their physiology and development.

A relationship between blastocyst morphology and aneuploidy following TE biopsy was initially inferred by a retrospective observational study (Capalbo et al., 2014), which determined an incidence of aneuploidy of 6.8, 15.2, 17.4 and 27.5% in excellent, good, average and poor quality embryos in women >35 years old, respectively. Significantly, in blastocysts where both ICM and TE were abnormal, there was a doubling in the frequency of aneuploidy. Another case series study with analysis of 1,730 embryos reported that euploid blastocysts were characterised with high scoring ICM and TE, as well as a high degree of expansion, and a shorter time to the initiation of blastocoel formation (Minasi et al., 2016). Similarly, an analysis of 3,573 blastocysts showed that euploidy was correlated with the Gardner grade but did not report the relative contributions of the grading to ploidy (Kato et al., 2023).

Using time-lapse to determine the timing of blastocyst formation (reflected in the expansion stage), it was observed that kinetics and rate of embryo expansion are related to aneuploidy risks (Campbell et al., 2013, Huang et al., 2019, Cimadomo et al., 2022b). However, other groups failed to confirm these findings (Kramer et al., 2014, Yang et al., 2014, Rienzi et al., 2015). More recently AI has been applied to analysing embryo morphology correlation with blastocyst euploidy rates (Huang et al., 2021, Zou et al., 2022, Bamford et al., 2023, Barnes et al., 2023, Hori et al., 2023, Kato et al., 2023) with promising results. Interestingly, a study reported that the AI score closely associated with the Day-5 Gardner grade in aneuploid blastocysts (Kato et al., 2023). While certain aspects of blastocyst morphology and specific AI have been able to identify those embryos at highest risk of being chromosomally abnormal, the approach lacks diagnostic accuracy. However, these methods could be used to identify those blastocysts with greatest probability of being aneuploid and hence candidates for biopsy and genetic analysis.

Spontaneous Collapse

A benefit of time-lapse culture is the ability to assess poorly studied blastocyst features such as spontaneous blastocoel collapse. Approximately 1 in 4 blastocysts show spontaneous collapse and re-expansion and even fewer have more than one collapse (Marcos et al., 2015). The significance of a spontaneous collapse for ongoing pregnancy or live birth is unclear

(Marcos et al., 2015, Bodri et al., 2016, Sciorio et al., 2020), though most evidence suggests a negative impact. Blastocysts that collapse are more likely to be aneuploid; however, some reports indicate a history of collapse does not affect euploid embryo implantation (Cimadomo et al., 2022a, Bickendorf et al., 2023).

Cytoplasmic strings

Cytoplasmic strings are dynamic structures connecting TE and ICM cells and are involved in cellular communication (Salas-Vidal and Lomelí, 2004). Appearing in 55-85% of expanded, transferred blastocysts, cytoplasmic strings are positively associated with implantation (Ebner et al., 2020, Eastick et al., 2021, Ma et al., 2022, Eastick et al., 2023, Joo et al., 2023). Since strings are also associated with higher blastocyst quality (Ma et al., 2022) and a multivariate analysis has not been performed for blastocyst grade and implantation, the utility of their inclusion as an independent predictor of viability for ranking is unknown.

Other morphological features

The presence of two ICMs in one blastocyst is a rare occurrence and warrants careful consideration. Monozygotic twinning is a complication more common following assisted reproductive technologies with significant risks to the offspring and the mother (Vitthala et al., 2009, Hviid et al., 2018, Busnelli et al., 2019, Kadam et al., 2023). Since few case reports exist of blastocysts with 2 ICMs in vitro (Veeck and Zaninovic, 2003, Payne et al., 2007, Noli et al., 2015), splitting of the ICM is unlikely to occur until after embryo transfer. Given the risks to the offspring and the mother, clinics may consider having a policy to not use blastocysts with suspected 2 or more ICM. Alternatively, when two ICM are visible prior to transfer, clinics should have a policy whereby the medical team is notified to allow for proper patient counselling.

Several other features beyond traditional morphology may also be used in ranking blastocysts. While many reports correlate early embryo developmental features with blastocyst implantation, most do not account for blastocyst morphology in the statistical analysis. The only pre-compaction variable associated with blastocyst live birth, when accounting for blastocyst quality, is the number of cells on Day 3, where slow cleaving embryos (<7 cells) have reduced implantation rates when transferred at the blastocyst stage (Wu et al., 2020, Zhao et al., 2020). Utility of this finding is uncertain, however, since it would only be applied when selecting between two blastocysts with similar Day/stage/grade.

Consensus points

- Ultimately, the goal of blastocyst grading is ranking for order of use.

- The Gardner scoring system for blastocyst scoring should be used (**Table 8; Supplementary data SIII, figure 1 and table 1**). This system is distinguished from the prior

Consensus grading by using letters for the ICM/TE grades and adding additional expansion stages (e.g. hatched blastocyst).

- Non-viable blastocysts should be graded as "D" as opposed to "C" based on degenerative features or absence of a distinct ICM.

- The common features that are clearly associated with implantation potential include day of blastocyst formation (Day 4-7), stage of expansion (3,4,5,6), and grade of ICM (A, B, C) and TE (A, B, C).

- Blastocysts with grade C ICM and/or TE and Day 7 blastocysts can be viable and could be considered suitable for clinical use.

- Blastocysts with 2 ICM indicating potential monozygotic twinning should not be transferred without thorough patient counselling.

- Assigning relative importance of each variable requires systematic multivariate analysis with a large dataset and is further complicated when assessing fresh versus frozen untested and euploid blastocysts.

1116 *Table 8 Consensus scoring system for blastocysts*

	Stage	Description
Stage of expansion	**1**	Early blastocyst: blastocoel less than half of the volume of the embryo.
	2	Blastocyst: blastocoel that is half of or greater than half of the volume of the embryo.
	3	Full blastocyst: blastocoel completely fills the embryo.
	4	Expanded blastocyst: blastocoel larger than that of the early embryo, with a clearly thinning zona.
	5	Hatching blastocyst: trophectoderm starting to herniate though the zona.
	6	Hatched blastocyst: blastocyst has completely escaped from the zona
	Grade	**Description**
ICM	**A**	Prominent, easily discernible, with many cells that are compacted and tightly adhered together.
	B	Easily discernible, with several cells that are loosely grouped together.
	C	Very few cells visible.
	D	No visible cells, or presence of degenerating cells.
TE	**A**	Many cells forming a cohesive epithelium.
	B	Moderate number of cells forming a loose epithelium.
	C	Few and larger cells with poor epithelia formation.
	D	Sparse or degenerating cells surrounding the ICM

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1118 *Table 9 Overview of all evidence and recommendations for blastocyst assessment*

Overview of all evidence and recommendations on blastocyst assessment							
		Summary of review findings				Considerations	Recommendation
Feature	**Atypical patterns**	**Embryo quality and development potential**	**Ploidy**	**Implantation rate**	**Live birth rate**		
Day of blastocyst formation	**Slow blastocyst (Day 5 vs Day 6)**	/	/	**Association with lower implantation rates** **Very Low ⊕◯◯◯** 1 observational study (Shebl et al., 2021)	**Association with lower live birth rate** **Low ⊕⊕◯◯** 3 observational studies (Bourdon et al., 2019; Yerushalmi et al., 2021; Coticchio et al., 2023)	**Slow growing blastocysts may miss the window of implantation, a problem that is partially alleviated with blastocyst vitrification and transfer in a frozen cycle.**	**Speed of development is one of the primary indicators of blastocyst potential and should be used for ranking of blastocysts for transfer**
	Day 7 blastocysts	/	**Association with higher aneuploidy rates** **Low ⊕⊕◯◯** 3 observational studies (Tiegs et al., 2019; Cimadomo et al., 2022; Lane et al., 2022)	**Association with lower implantation rates** **Low ⊕⊕◯◯** 3 observational studies (Tiegs et al., 2019; Cimadomo et al., 2022; Lane et al., 2022)	**Association with lower live birth rate** **Low ⊕⊕◯◯** 1 review and 3 observational studies (Hammond et al., 2018; Tiegs et al., 2019; Cimadomo et al., 2022; Lane at al., 2022)		**Day-7 blastocysts can be viable and could be considered for clinical use.**
Grade	**Degree of expansion**	/	**Contradictory results:** **Association with higher aneuploidy rates** **Very low ⊕◯◯◯** 3 observational studies (Campbell et al., 2013; Cimadomo et al., 2022; Huang et al. 2019) **No clear association with aneuploidy rate** **Very low ⊕◯◯◯** 3 observational studies (Kramer et al., 2014; Rienzi et al., 2015; Yang et al., 2014)	/	**Association with higher live birth rate** **Very low ⊕◯◯◯** 4 observational studies (Ahlstrom et al., 2013; Du et al., 2016; Subira et al., 2016; Thompson et al., 2013; Bakkensen et al., 2019; Storr et al., 2019)		**Degree of expansion is one of the primary indicators of blastocyst potential and should be used for ranking of blastocysts for transfer**
	ICM/TE grade	/	**Association with aneuploidy rate** **Moderate ⊕⊕⊕◯** 1 systematic review and 5 observational studies (Bamford et al., 2023; Barnes et al., 2023; Hori et al., 2023; Huang et al., 2021; Kato et al., 2023; Zou et al., 2022)	**Contradictory results:** **ICM grade associated with implantation** **Very low ⊕◯◯◯** 4 observational studies (Irani et al., 2017; Zhao et al., 2018; Nazem et al., 2019; Abdala et al., 2022; Zhang et al., 2022) **No clear association of ICM grade with implantation rate** **Very low ⊕◯◯◯** 3 observational studies (Ahlstrom et al., 2013; Chen et al., 2014; Honma et al., 2012)	**TE grade is the strongest predictor of live birth rates** **Low⊕⊕◯◯** 10 observational studies (Ahlstrom et al., 2011; Honma et al., 2012; Ahlstrom et al., 2013; Hill et al., 2013; Thompson et al., 2013; Chen et al., 2014; Ebner et al., 2016; Bakkensen et al., 2019; Storr et al., 2019; Pons et al., 2023)		**Trophectoderm is one of the primary indicators of blastocyst potential and should be used for ranking of blastocysts for transfer.** **Grade C blastocysts can be viable and could be considered for clinical use.**
Chromosomal status	**Aneuploid**	**Association with lower embryo quality** **very low ⊕◯◯◯** 3 observational study (Capalbo et al., 2014; Minasi et al., 2016; Kato et al., 2023)	/	/	/	**Identifying embryos at highest risk of being chromosomally abnormal is not a diagnostic approach but rather could be perceived as a mean to identify those blastocysts with greatest probability of being aneuploid and hence candidates for biopsy and genetic analysis.**	
Cytoplasmic strings	**Presence of cytoplasmic strings**	**Association with higher blastocyst quality** **Very low ⊕◯◯◯** 1 observational study (Ma et al., 2020)	/	**Association with higher implantation rate** **Low ⊕⊕◯◯** (Ebner et al., 2020; Eastick et al., 2021; Ma et al., 2020; Eastick et al., 2023; Joo et al., 2023)	/	**The utility of cytoplasmic strings presence as an independent indicator for ranking is unknown.**	**Blastocyst presenting cytoplasmic strings could be used clinically.**
Spontaneous Collapse	**Spontaneous Collapse**	**Association with lower blastocyst quality** **Very low ⊕◯◯◯** 1 observational study (Cimadomo et al., 2022)	**Association with lower euploidy rate** **Low⊕⊕◯◯** 1 meta-analysis of 3 observational studies (Bickendorf et al., 2023)	**No clear association with implantation potential** **Very low ⊕◯◯◯** 2 observational studies (Sciorio et al., 2020; Cimadomo et al., 2022)	**Contradictory results:** **Association with lower ongoing pregnancy rate** **Low ⊕⊕◯◯** 1 meta-analysis of 5 observational studies (Bickendorf et al., 2023) **No clear association with live birth rate** **Low ⊕⊕◯◯** 1 meta-analysis of two observational studies (Bickendorf et al., 2023)	**The significance of spontaneous collapse on pregnancy outcomes is unclear**	
ICM	**Presence of 2ICM**	**Potential complication** **Very low ⊕◯◯◯** 2 observational studies (Payne et al., 2007; Noli et al., 2015)	/	/	/	**Given the risks to the offspring and the mother, clinics may consider having a policy to not use blastocysts with 2 or more ICM**	**Blastocysts with 2 ICM indicating potential monozygotic twinning should not be transferred without thorough patient counselling.**
	Absence of ICM	/	/	**Association with lower implantation rate** **Very low ⊕◯◯◯** 1 observational study (Kovacic et al., 2004)	**Association with lower live birth rate** **Very low ⊕◯◯◯** 1 observational study (Kovacic et al., 2004)	**Transfer of blastocysts without ICM may lead to abnormal pregnancy or pregnancy loss.**	**Non-viable blastocysts should be graded as "D" as opposed to "C" based on degenerative features or absence of a distinct ICM .**
Table colour code: Green: the embryo can be clinically used; Yellow: the embryo could be used with cautionary considerations. Red: the embryo is not considered suitable for clinical use.							

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7. Duration of embryo culture and frequency of assessments: safety versus effectiveness

The Istanbul Consensus (2011) offers a broad spectrum of morphological parameters for oocyte and embryo assessment. In laboratories using TLT-equipped incubators, continuous culture allows flexibility in the frequency and level of detail of embryo evaluation, without disturbing the culture conditions. In laboratories performing static observations, however, the frequency of embryo assessment should be determined considering factors such as the type of incubators used (bench top or big box), the type of culture medium (single or sequential), the use of isolettes for embryo handling, and the duration of embryo culture (cleavage or blastocyst stage). The aim is to strike an optimal balance between acquiring the desired information on developing embryos and minimising the disturbance of the culture conditions (Swain, 2014, Wale and Gardner, 2016, ESHRE working group on Time-lapse technology, 2020).

Several ART centres still combine cleavage and blastocyst stage embryo transfers, as shown in our survey (**Supplementary data SII, figure 4**). The duration of embryo culture, embryo morphology assessment and embryo transfer policy- whether fresh or frozen- should primarily aim for the fastest, safest and most economically sustainable way to achieve the goal of fertility treatment. The choice of assessment methods, level of detail, and the duration and frequency of monitoring of embryo development under in vitro conditions should therefore be tailored to the available laboratory equipment.

Current practice of cleavage stage versus blastocyst transfer

Our survey showed that the blastocyst stage is commonly used in ART centres for performing embryo transfer. Fewer than 2% of ART centres did not perform blastocyst transfer at all while 17.4% performed blastocyst transfer nearly exclusively (in >95% of cycles) (**Supplementary data SII, figure 4.A**).

Interestingly, Day-2 and Day-3 embryo transfer were not practiced at all in 44% and 8% of ART centres, respectively. On the other hand, only 2-3% of ART centres exclusively practiced cleavage stage embryo transfer with 2.2% performing transfers on Day-3 and 0.7% on Day-2 (**Supplementary data SII, figure 4.A**).

Moreover, cryopreservation of blastocysts predominates over cleavage stage embryos. More than 50% of the respondents reported that embryos are exclusively cryopreserved at the blastocyst stage, while in the remaining cases mostly a combination of cryopreservation of Day-3 and Day-5/6 embryos is performed (**Supplementary data SII, figure 4.B**). Day-2 and Day-4 embryos are never cryopreserved by roughly 75% of ART centres (**Supplementary data SII, figure 4.B**). A similar trend with a higher percentage of blastocyst (73.9%) over cleavage stage (26.1%) frozen transfers can be found in the ESHRE report for 2018 (Wyns et al., 2022).

The transfer of Day-4 embryos occurred in less than 25% of the transfer cycles according to 36.3% of the respondents and only 19.9% of the respondents reported that they cryopreserve

Day-4 embryos in less than 25% of the transfer cycles (**Supplementary data SII, figure 4**). It is not clear whether the reason for the use of day 4 embryos is the earlier development of the blastocyst or the earlier scheduling of the day of transfer or cryopreservation at the convenience of the patient or the centre.

Reasons for increasing use of extended embryo culture

Several factors have contributed to the increasing use of blastocyst transfer. There is consistent evidence from a multitude of studies showing higher pregnancy and live birth per transfer using fresh blastocyst transfer, with this observation being more prominent in good prognosis patients (Practice Committee of the American Society for Reproductive Medicine, 2018). However, a retrospective analysis of more than 100 000 IVF/ICSI cycles showed that after adjusting for indication bias, there was not enough evidence to suggest a difference in the odds of live birth following blastocyst versus cleavage-stage embryo transfer in the first complete cycle (Cameron et al., 2020), although the majority of the cycles included were performed following culture in atmospheric oxygen, known to negatively impact blastocyst outcomes (Gardner, 2016). Although the cumulative live birth rate appears to be similar, blastocyst transfer is associated with a shorter time to pregnancy and to birth, but also higher cancellation rates compared to cleavage-stage transfer (De Vos et al., 2016).

The implementation of national strategies towards elective single embryo transfer to decrease multiple birth rates has resulted in increasing use of extended embryo culture (ESHRE Campus Course Report, 2001, ASRM, 2012, Knez et al., 2013, Harbottle et al., 2015, De Geyter et al., 2020, Fouks and Yogev, 2022, The ESHRE guideline group on the number of embryos to transfer, 2024).

The development of TE biopsy for PGT has also contributed to the increasing use of blastocyst culture (ESHRE PGT Consortium and SIG Embryology - Biopsy Working Group, 2020). Cleavage stage biopsy has been shown to have a negative impact on embryo developmental competence, especially when two blastomeres are removed (Scott et al., 2013). Blastocyst biopsy seems to be safer compared with Day-3 embryo biopsy, as some studies have suggested that removing a small number of TE cells does not affect the embryo implantation or fetal development (Van de Velde et al., 2000, Scott et al., 2013).

The increasing use of TLT in IVF laboratories, reported in more than 50% of all ART centres responding to our survey (**Supplementary data SII, figure 6.A**), also means that patients are increasingly offered continuous and detailed monitoring of embryo development to blastocyst stage.

Initial concerns about extended embryo culture due to the possible prolonged influence of environmental factors on embryonic epigenetics are decreasing (White et al., 2015, Ghosh et al., 2017, Ji et al., 2018), although follow-up studies of children conceived after ART point to

the possible influence of culture media, culture duration and other laboratory factors on infant health (Berntsen et al., 2019). Some studies have reported a significantly higher rate of preterm birth and very preterm birth after blastocyst compared to cleavage stage transfer, but the risk of small for gestational age infants was significantly lower for singletons born after blastocyst transfer (Martins et al., 2016, Wang et al., 2017, Alviggi et al., 2018). Analysis of 130,156 live births confirmed the reported association between blastocyst transfer and lower risk of small for gestational age and showed a reduced risk of congenital anomaly following blastocyst transfer (Raja et al., 2023). In a review of over 60,000 cycles in the UK, single fresh blastocyst transfer did not show a negative effect on gestational age at birth nor birth weight compared with cleavage stage embryo transfer (Marconi et al., 2023).

One remaining question is whether in poor responders with low zygote numbers, embryo transfer should be done on Day 2, Day 3 or Day 5/6. A retrospective study showed that transferring embryos on Day 2 versus Day 3 in this patient group does not affect early pregnancy outcomes and suggested the flexibility in scheduling the day of transfer at the convenience of both the patient and the centre (Sacha et al., 2018). According to another study, there is no difference in clinical pregnancy rates after fresh Day-3 or Day-5 embryo transfer in patients with 5 or fewer zygotes (Dirican et al., 2022). However, those with 6 or more zygotes can benefit from blastocyst transfer due to better selection options. Larger prospective studies are needed to provide a conclusive answer to the above question.

Technical considerations for extended embryo culture

The success of extended embryo culture relies on crucial parameters, such as reduced oxygen concentration, optimal pH, temperature and osmolality (Gardner and Lane, 1997). Blastocyst culture affects logistics and workflow, as well as technical requirements in the laboratory, such as incubator type and capacity, frequency of embryo assessment, and - if performed - annotation of morphokinetics and culture media renewal. Success also depends on stable culture conditions and an efficient blastocyst vitrification programme (Swain, 2019, Cairo Consensus Guidelines on IVF Culture Conditions, 2020). Therefore, the ART centre's capacity to ensure appropriate conditions for blastocyst culture should be proven. A blastocyst culture approach should be introduced starting first with good responder patients and, after appropriate blastocyst development rate and clinical outcomes are obtained, gradual wider application to other groups of IVF patients (Gardner and Lane, 2017, De Croo et al., 2020). The success of the blastocyst vitrification programme should be self-verified by the IVF laboratory by tracking key performance indicators. The reference rates for blastocyst cryosurvival are expected to be ≥90% for competency and ≥99% for benchmark (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). Due to greater experience with blastocyst vitrification, the rate of degeneration during warming is now lower than that estimated in a previous cryopreservation consensus (The Alpha Scientists in Reproductive Medicine, 2012).

Modern benchtop incubators with individual chambers represent a safer incubator design and provide a faster recovery time of all physico-chemical parameters after door openings compared to older 'big-box' incubators (Kovačič, 2021). However, in the case of prolonged and continuous culture of embryos, possible changes in osmolality and pH over time must also be monitored (Swain, 2019).

Incubators with integrated TLT allow continuous observation of the morphokinetics of developing embryos with uninterrupted incubation throughout the preimplantation period (Meseguer et al., 2012). A good practice recommendation paper including a systematic assessment of how to approach and introduce TLT for IVF was published to provide centres with technical advice (ESHRE working group on Time-lapse technology, 2020).

Due to the overwhelming evidence of the detrimental effect of atmospheric oxygen concentration on embryonic development (Gardner, 2016), the use of reduced oxygen is now considered standard practice, especially for extended incubation of embryos to blastocyst stage (Kovačič, 2012, De los Santos et al., 2016).

Frequency of embryo assessment: rationale

While the accuracy of assessing blastomere cleavages is important, laboratories with limited number of incubators should carefully consider certain limitations and prioritize the safety and quality of the embryo culture conditions. More frequent opening of incubators may have a negative impact on embryonic development (Gardner and Lane, 1996, Zhang et al., 2010, Nguyen et al., 2018). In such situations, assessing morphology only at the end of the culture period may be considered, with no or few intermediate checks on their development.

If it is decided to practice short-term embryo culture in IVF cycles with large numbers of zygotes, then a more detailed and frequent assessment of embryo morphology might improve selection of embryos by the ranking scheme given in this paper.

Consensus points

- Extended embryo culture is an accepted and standard practice.

- The length of embryo culture and frequency of static embryo observations must be adjusted to the equipment in the laboratory and staff skill, ensuring minimal changes in culture conditions that could affect embryo development.

Conclusion

This consensus paper provides updated recommendations on criteria and terminology for assessing oocyte, zygote, cleavage-stage embryo, morulae, and blastocyst development based on a thorough review of evidence accumulated over the past decade. Critical information gained from application of TLT has provided the impetus for revised timings of developmental

milestones, greater consideration of the influence of insemination methods on early embryogenesis, and presentation of a broader spectrum of atypical morphology detected with time-lapse imaging. The collated recommendations (Table 10) aim to promote standardized embryo evaluation practices to better predict viability and optimise embryo selection for transfer and cryopreservation. Notwithstanding the progress of the past decade, several knowledge gaps remain (Table 11) concerning the clinical value of specific morphological and morphokinetic parameters that warrant further investigation and scrutiny. Undoubtedly, the next decade will bring a more substantial incorporation of AI in the ART laboratory, offering solutions to the perpetually challenging problem of viable gamete and embryo selection.

Lastly, by combining expertise and experience across institutions and geographical regions, international collaborative efforts such as that represented by this consensus paper can contribute to improving research consistency, clinical practice, and most importantly, outcomes for patients seeking assisted reproduction.

Recommendations

Table 10 List of recommendations

	Recommendations
Oocyte assessment	• Giant oocytes should be excluded from clinical use. • The use of small/large oocytes and IVM-rescued oocytes should be documented for prognostic and traceability purposes due to their apparently lower developmental potential. • Finally, embryos derived from MII oocytes free of large or multiple vacuoles, SER-a, and very large first PB should be prioritized for clinical use. • Follow-up of babies born from oocytes with atypical phenotypes and rescue IVM demands attention.
Zygote state assessment	• Assessment of PN number should be carried out between 16 and 17 hpi in both conventional IVF and ICSI cases. • Zygotes with 2PN should be prioritized for clinical use. • 2.1PN and 1PN zygotes from IVF or ICSI may be considered for clinical use with appropriate counselling, especially if associated with PGT-A technology appropriate for biparental diploidy assessment. • The clinical use of 3PN zygotes is not recommended, while pre-clinical or pilot clinical studies should be encouraged. • Dynamic features such as PN size, PN position and juxtaposition, NPB pattern, and cytoplasmic halo cannot be accurately assessed during static observations. Thus, they cannot be consistently used as biomarkers of viability.
Day -1, -2 & -3 embryo assessment	• 2-cell embryos on Day-1, 4-cell embryos on Day- 2, 8-cell embryos on Day-3 showing <10% fragmentation, mononucleation, and stage-specific cell size should be prioritized in case of cleavage stage embryo transfer or cryopreservation. • Cleavage stage embryos with atypical features such as extensive fragmentation, multinucleation, vacuoles, cytoplasmic granularity, membrane, and zona irregularities, can be considered suitable for clinical use. However, extended culture of these embryos for further evaluation should be considered.
Day-4 embryo assessment	• Day-4 embryos showing full compaction or early cavitation should be prioritized in case of Day-4 transfer or vitrification. • Embryos with partial compaction can form blastocysts and should be considered for clinical use. Extended culture of these embryos for further evaluation should be considered.
Day-5, -6 & -7 embryo assessment	• Ultimately, the goal of blastocyst grading is ranking for order of use. • The Gardner scoring system for blastocyst scoring (Table 8) should be used. This system is distinguished from the prior Consensus grading by using letters for the ICM/TE grades and adding additional expansion stages (e.g. hatched blastocyst). • Non-viable blastocysts should be graded as "D" as opposed to "C" based on degenerative features or absence of a distinct ICM. • The common features that are clearly associated with implantation potential include day of blastocyst formation (Day 4-7), stage of expansion (3,4,5,6), and grade of ICM (A, B, C) and TE (A, B, C). • Blastocysts with grade C ICM and/or TE and Day 7 blastocysts can be viable and could be considered suitable for clinical use. • Blastocysts with 2 ICM indicating potential monozygotic twinning should not be transferred without thorough patient counselling. • Assigning relative importance of each variable requires systematic multivariate analysis with a large dataset and is further complicated when assessing fresh versus frozen untested and euploid blastocysts.
Duration of embryo culture and frequency of assessments	• Extended embryo culture is an accepted and standard practice. • The length of embryo culture and frequency of static embryo observations must be adjusted to the equipment in the laboratory and staff skill, ensuring minimal changes in culture conditions that could affect embryo development.

Knowledge Gaps and recommendations for future research

Table 11 List of knowledge gaps and recommendations for future research

	Knowledge gap	*Recommendations for future research*
Expected timeline	It is unknown how and whether artificial intelligence-based analyses and selection algorithms will evolve or deal with data heterogeneity.	Develop more advanced analytic tools to provide the facility to identify the most viable embryo(s) from a cohort and an estimation of the likelihood of each embryo leading to live birth.
Oocyte assessment	The body of evidence to date is based almost exclusively on qualitative (presence/absence) analyses and exclude an objective description of each dysmorphism. The impact of different ovarian stimulation protocols/responses on oocyte parameters has not been fully evaluated.	Future standardized and quantitative analyses should be conducted on oocyte morphology, thereby filling important gaps in knowledge. Further studies using artificial intelligence for oocyte assessment might be useful.
Zygote stage assessment	TLT has highlighted complex changes over time of the majority of relevant morphokinetic parameters, such as PN size and position, NPB patterning and cytoplasmic halo. Use of such parameters to predict embryo developmental competence remains elusive, probably because morphokinetic abnormalities occurring at fertilization may be compensated by the outstanding developmental plasticity of the human embryo (Coticchio et al., 2021a).	Use of TLT and allied technologies, namely image analysis and artificial intelligence to decrypt the developmental significance of fertilization biomarkers, such as NPB patterning. This is expected to lead to novel criteria for embryo ranking and perhaps for the prediction of blastocyst aneuploidy. Use of TLT and PGT-A to distinguish haploid/triploid from diploid 1PN and 3PN zygotes, thereby identifying potentially viable embryos that would be otherwise discarded.
Day -1, -2 & -3 embryo assessment	Insight into what may be considered optimal timing for cleavage stage embryo evaluation is still lacking. Questions surrounding the significance of multinucleation, the number of nuclei and the number of affected cells and the developmental stage when this condition appears remain largely unanswered. There is a crucial gap in knowledge concerning the criteria for exclusion of embryos from selection for clinical use.	Further studies using TLT are expected to provide a deeper understanding of the association between time of assessment, morphological features, and clinical outcomes.
Day-4 embryo assessment	It is currently unknown whether and to what extent type and composition of culture media (e.g. Ca^{2+}, Mg^{2+}) might influence compaction timing and phenotypes. Little information is available on premature compaction behaviour as early as the 2- to 4-cell stages.	Explore the underlying cellular mechanisms that can explain compaction timing and blastomere exclusion/extrusion processes.
Day-5, -6 & -7 embryo assessment	A best practice for establishing a clinic-specific ranking of blastocysts based on morphology and time of development and in-house validation of established algorithms before use is lacking.	Develop objective measures of blastocyst quality to improve the accuracy of blastocyst scoring and ranking, though early reports have not shown an improvement with either of these methodologies (Kato et al., 2023). Identify markers of viability beyond morphology and bright-field microscopy to improve non-invasive blastocyst assessment (Gardner and Balaban, 2016, Gardner and Sakkas, 2023).
Duration of embryo culture and frequency of assessments	Evidence is lacking on the effectiveness of non-selective use of extended embryo culture in all patients.	Assess whether more frequent observations of embryos during prolonged culture improves embryo selection or clinical efficacy of the procedure.

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Supplementary data SI – Survey questions

Section I. General information on your centre

* 1. Please select the country where your centre is located.

Section II. Current practice

Section II.1. Istanbul Consensus - Compliance

* 2. Do you, in your centre, follow the Istanbul consensus recommendations for embryo assessment?

- No
- Yes
- Yes, partly or with modifications.

* 3. How often do you apply the Istanbul consensus recommendations regarding **the timing of static observations** in your daily practice? Rate the frequency of applying the recommendations. (One option per row needs to be checked)

	Always (>90%)	Frequently (50% - 90%)	Sometimes (5% - 50%)	Rarely/Never (< 5%)	This stage of development is not assessed
Fertilization check: 17h± 1 post insemination	○	○	○	○	○
Syngamy check: 23h± 1 post insemination	○	○	○	○	○
Early cleavage check: 28h± 1 (post IVF) or 26h± 1 (post ICSI)	○	○	○	○	○
Day-2 embryo assessment: 44h ± 1	○	○	○	○	○
Day-3 embryo assessment: 68h ± 1	○	○	○	○	○
Day-4 embryo assessment: 92h ± 2	○	○	○	○	○
Day-5 embryo assessment: 116h ± 2	○	○	○	○	○

* 4. How often do you apply the Istanbul consensus recommendations regarding **oocyte and embryo scoring** in your daily practice? Rate the frequency of applying the recommendations. (One option per row needs to be checked)

	Always (>90%)	Frequently (50% - 90%)	Sometimes (5% - 50%)	Rarely/Never (< 5%)	This stage of development is not scored
Cumulus-oocyte complex	◯	◯	◯	◯	◯
Zona pellucida	◯	◯	◯	◯	◯
Perivitelline space	◯	◯	◯	◯	◯
Polar body	◯	◯	◯	◯	◯
Cytoplasm	◯	◯	◯	◯	◯
Vacuolization	◯	◯	◯	◯	◯
Pronuclear stage	◯	◯	◯	◯	◯
Cleavage stage	◯	◯	◯	◯	◯
Day-4 stage	◯	◯	◯	◯	◯
Blastocyst stage	◯	◯	◯	◯	◯

5. To what degree do you rely on **the Istanbul consensus recommendations** in your daily practice related to **the timing of static observations**? (One option per row needs to be checked)

	Little	Moderate	Completely	Not at all
Fertilization check: 17h± 1 post insemination	◯	◯	◯	◯
Syngamy check: 23h± 1 post insemination	◯	◯	◯	◯
Early cleavage check: 26h± 1 (post ICSI) or 28h± 1 (post IVF)	◯	◯	◯	◯
Day-2 embryo assessment: 44h ± 1	◯	◯	◯	◯
Day-3 embryo assessment: 68h ± 1	◯	◯	◯	◯
Day-4 embryo assessment: 92h ± 2	◯	◯	◯	◯
Day-5 embryo assessment: 116h ± 2	◯	◯	◯	◯

6. To what degree do you rely on **the Istanbul consensus recommendations** in your daily practice related to **oocyte and embryo scoring**? (One option per row needs to be checked)

	Little	Moderate	Completely	Not at all
Cumulus-oocyte complex	○	○	○	○
Zona pellucida	○	○	○	○
Perivitelline space	○	○	○	○
Polar body	○	○	○	○
Cytoplasm	○	○	○	○
Vacuolization	○	○	○	○
Pronuclear stage	○	○	○	○
Cleavage stage	○	○	○	○
Day-4 stage	○	○	○	○
Blastocyst stage	○	○	○	○

* 7. What grading system are you using for cleavage-stage embryos?

- Istanbul Consensus ASEBIR
- SART
- Another grading system. Please specify.
- N/A. We don't use a grading system for cleavage-stage embryos.

8. What grading system are you using for blastocysts?

- Istanbul Consensus Gardner
- ASEBIR
- SART
- UK/ACE
- Another grading system. Please specify.
- N/A. We don't use a grading system for blastocysts.

9. In your centre, at which stage are fresh embryos transferred? Please indicate an approximate percentage in the comment box (from 0 to 100% of the transfer cycles)

- Day-2:
- Day-3:
- Day-4:
- Day-5/6:
- Day-7:

10. In your centre, at which stage are embryos cryopreserved? Please indicate an approximate percentage in the comment box (from 0 to 100% of the transfer cycles)

- Day-2:
- Day-3:
- Day-4:
- Day-5/6:
- Day-7:

11. Which nomenclature do you use for blastocysts that are considered clinically non-usable?

- Non-classifiable

- ICM or TE Grade D
- ICM or TE Grade X
- Grade CC
- Grade 33
- Another nomenclature. Please specify

12. Would it be helpful in your daily practice if there is a universally accepted term to classify clinically non-usable blastocysts?

- Yes
- No

Section II.2. IVF laboratory technologies for embryo selection

* 13. Do you use Time-lapse (TL) in your centre?

- Yes
- No

14. For what proportion of IVF/ICSI cycles do you use TL? (From 0 to 100%)

15. For what purposes do you use TL in your centre? (Tick all that apply)

- For all patients
- For Day-5/6 cycles only
- For selected patients with a history of embryo cleavage problems
- For selected patients who choose this option
- For research
- Other purposes. Please explain in the comment box.

16. *Do you use an algorithm for embryo assessment and/or selection?

- Yes, we use an algorithm developed in-house.
- Yes, we use an algorithm developed by manufactures.
- Yes, we use an algorithm published by other professionals Other (please specify)

17. *Do you use Artificial Intelligence (AI) rather than using a morphokinetic algorithm for embryo assessment and/or selection

- Yes
- No

18. For which of these areas of daily practice related to gamete and embryo assessment and/or selection are you using AI? (Tick all that apply)

- Sperm selection
- Oocyte assessment
- Embryo assessment in static images
- Embryo assessment in TL videos
- Other (please specify)

19. * Is PGT-A offered in your centre (either in-house or outsourced)?

- Yes
- No
- Other (please specify)

20. In your centre, at which stage are **non-PGT-embryos** scored? One option per row needs to be checked.

	Always (>90%)	Frequently (50% - 90%)	Sometimes (5 - 50%)	Rarely/Never (<5%)
Day-2	◯	◯	◯	◯
Day-3	◯	◯	◯	◯
Day-4	◯	◯	◯	◯
Day-5/6	◯	◯	◯	◯
Day-7	◯	◯	◯	◯

21. In your centre, at which stage are **PGT-embryos** scored? One option per row needs to be checked.

	Always (>90%)	Frequently (50% - 90%)	Sometimes (5 - 50%)	Rarely/Never (<5%)
Day-2	◯	◯	◯	◯
Day-3	◯	◯	◯	◯
Day-4	◯	◯	◯	◯
Day-5/6	◯	◯	◯	◯
Day-7	◯	◯	◯	◯

22. When PGT-A is performed, do you still use **morphology** to rank/select euploid embryos for transfer?

- Yes
- No

Please explain in the comment box

23. When PGT-A is performed, do you still use **morphokinetics** to rank/select euploid embryos for transfer?

- Yes
- No

Please explain in the comment box

Section III. Quality assurance

24. * In your centre, is embryologist performance of the oocyte/embryo assessment monitored by internal/external quality assurance programs?

- Yes, internal quality assurance programs.
- Yes, external quality assurance programs.
- Yes, both internal and external quality assurance programs.
- No

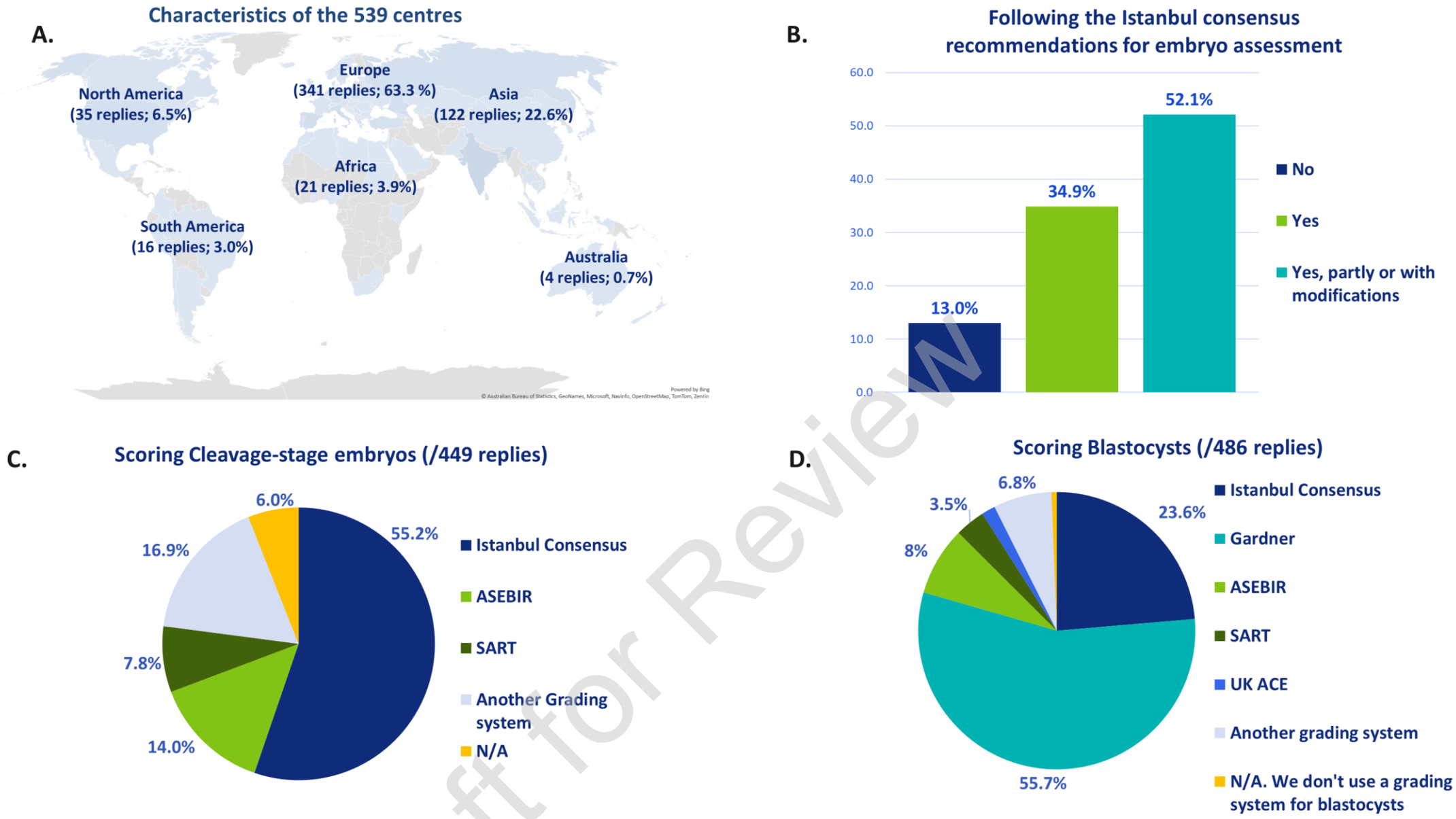
Please explain.

Supplementary Data SII – Survey results

Current practice in ART centres regarding the application of the Istanbul consensus recommendations on oocyte and embryo morphology assessment

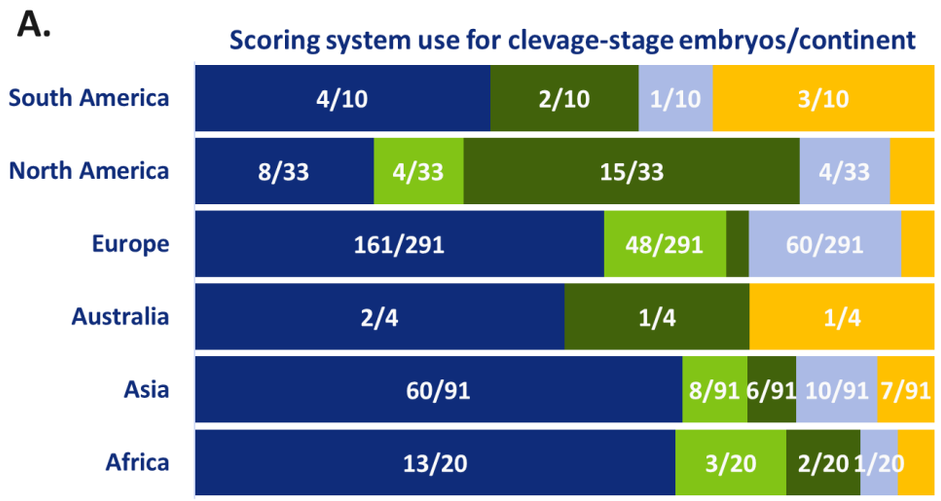
Eight hundred and thirty-three replies were received. After exclusion of irrelevant replies including only background information (e.g., replies including only information on the country or on whether the centre is following or not the Istanbul consensus recommendations), the resulting data set included 539 replies from 82 different countries.

2271 *Figure 1 Overview of the location of the 539 centres included in the survey (A), whether the centre is following or not the Istanbul consensus (B), and the different embryo scoring systems used for cleavage-stage embryos (C) and for*
2272 *blastocysts (D)*

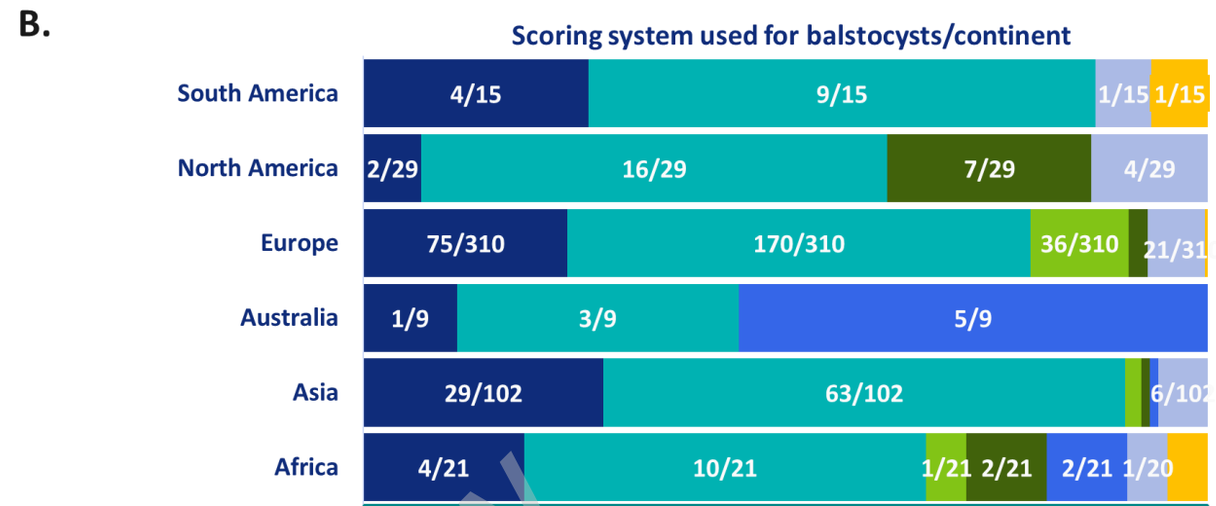


2273

Figure 2 Scoring systems used for cleavage-sage embryos (A) and blastocysts (B)/continent. Bars represent the absolute numbers of respondents using the respective scoring system/continent. Tables represent the percentages of use for each scoring system /continent.

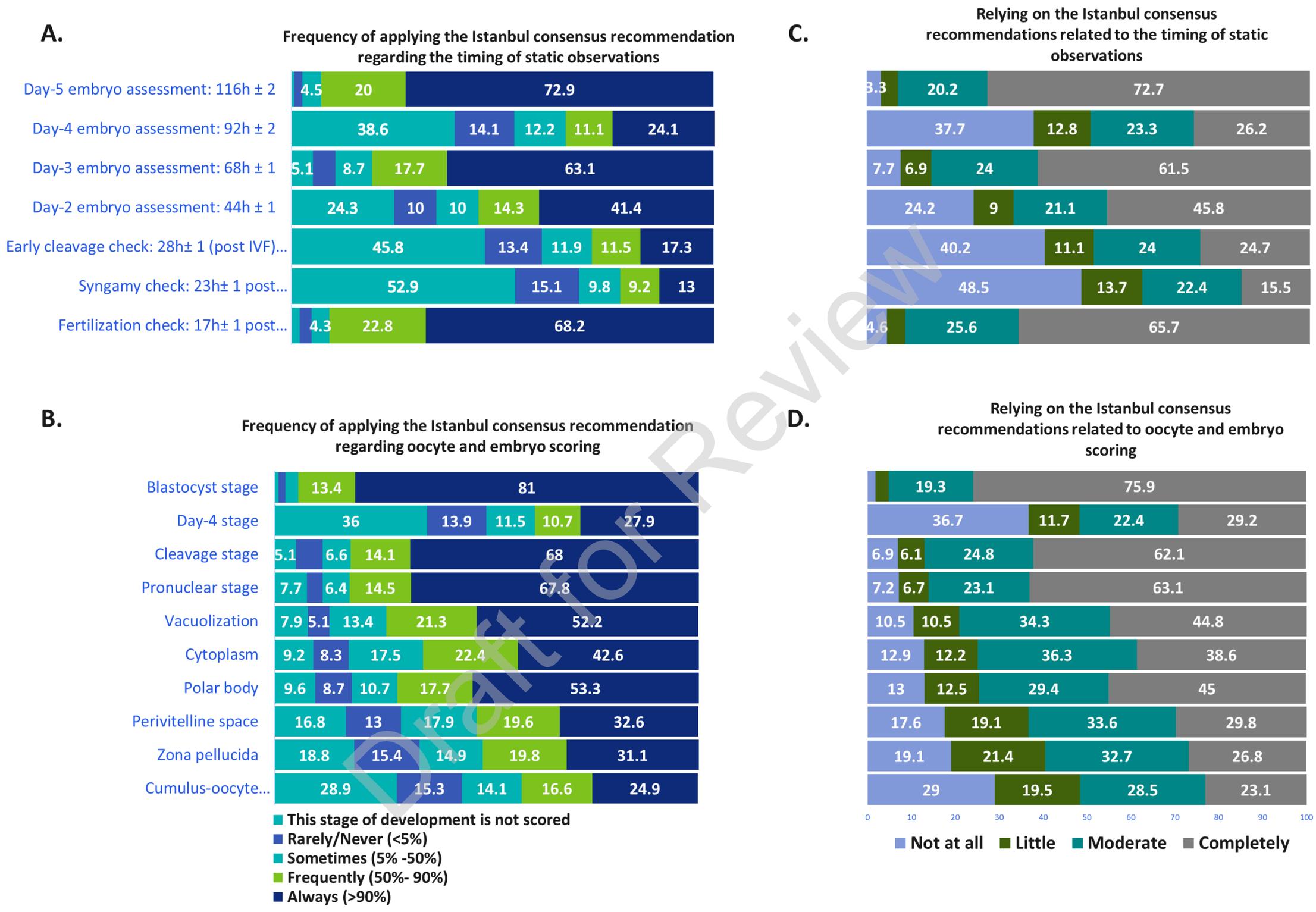


	Africa	Asia	Australia	Europe	North America	South America
Istanbul Consensus	65.0	65.9	50.0	55.3	24.2	40.0
ASEBIR	15.0	8.8	0.0	16.5	12.1	0.0
SART	10.0	6.6	25.0	3.1	45.5	20.0
Another grading system	5.0	11.0	0.0	20.6	12.1	10.0
N/A. We don't use a grading system for cleavage-stage embryos	5.0	7.7	25.0	4.5	6.1	30.0



	Africa	Asia	Australia	Europe	North America	South America
Istanbul Consensus	19.0	28.4	11.1	24.2	6.9	26.7
Gardner	47.6	61.8	33.3	54.8	55.2	60.0
ASEBIR	4.8	2.0	0.0	11.6	0.0	0.0
SART	9.5	1.0	0.0	2.3	24.1	0.0
UK ACE	9.5	1.0	55.6	0.0	0.0	0.0
Another grading system	4.8	5.9	0.0	6.8	13.8	6.7
N/A. We don't use a grading system for blastocysts	4.8	0.0	0.0	0.3	0.0	6.7

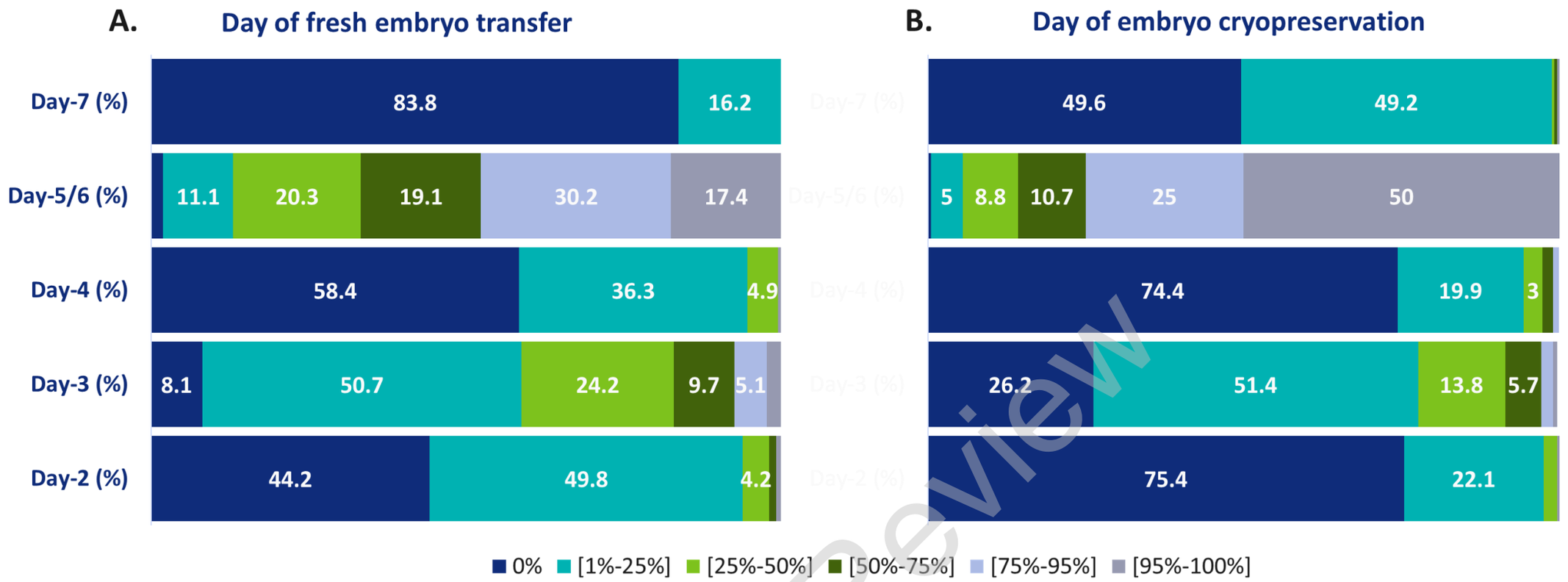
2278 *Figure 3 Percentage of applying the Istanbul consensus recommendations regarding the timing of static observations (A) and oocyte and embryo scoring (B) and the degree of relying on those recommendations regarding the timing of*
2279 *static observations (C) and oocyte and embryo scoring (D) in the centre daily practice.*



2280

2281 *Days of fresh embryo transfer and cryopreservation*

2282 *Figure 4 Stage of fresh embryo transfer (A) and Day of embryo cryopreservation (B)*

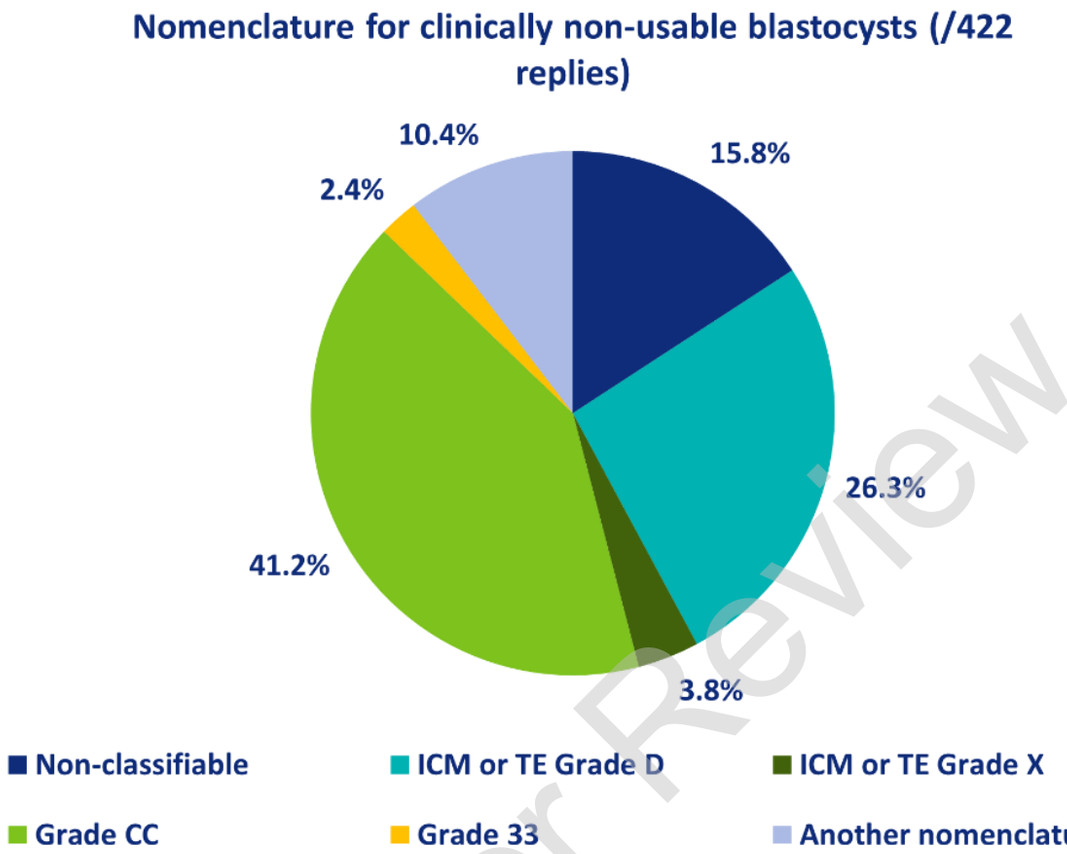


2283

2284 *Clinically non-usable blastocysts*

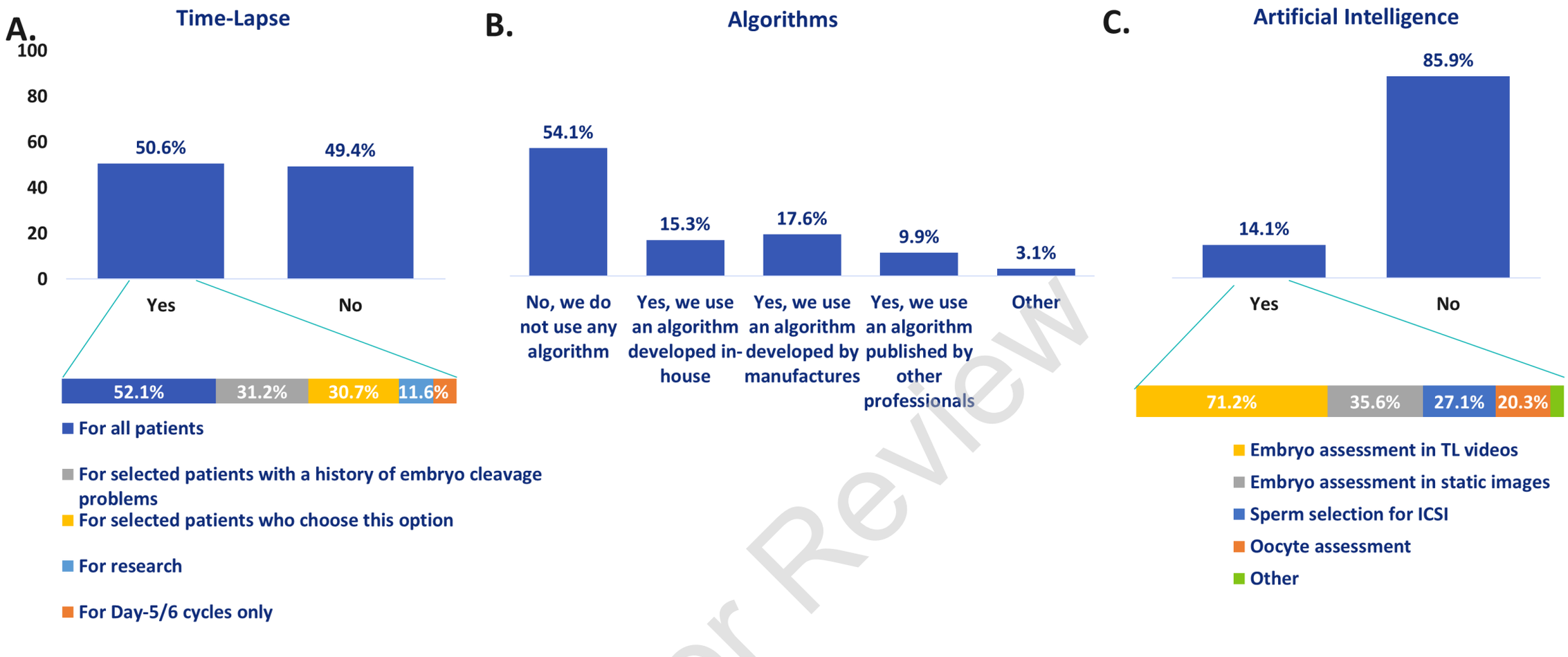
2285 81% of the respondents consider it helpful to have a universally accepted term to classify clinically non-usable blastocysts

2286 *Figure 5 Preferable nomenclature for clinically non-usable blastocysts.*



2287

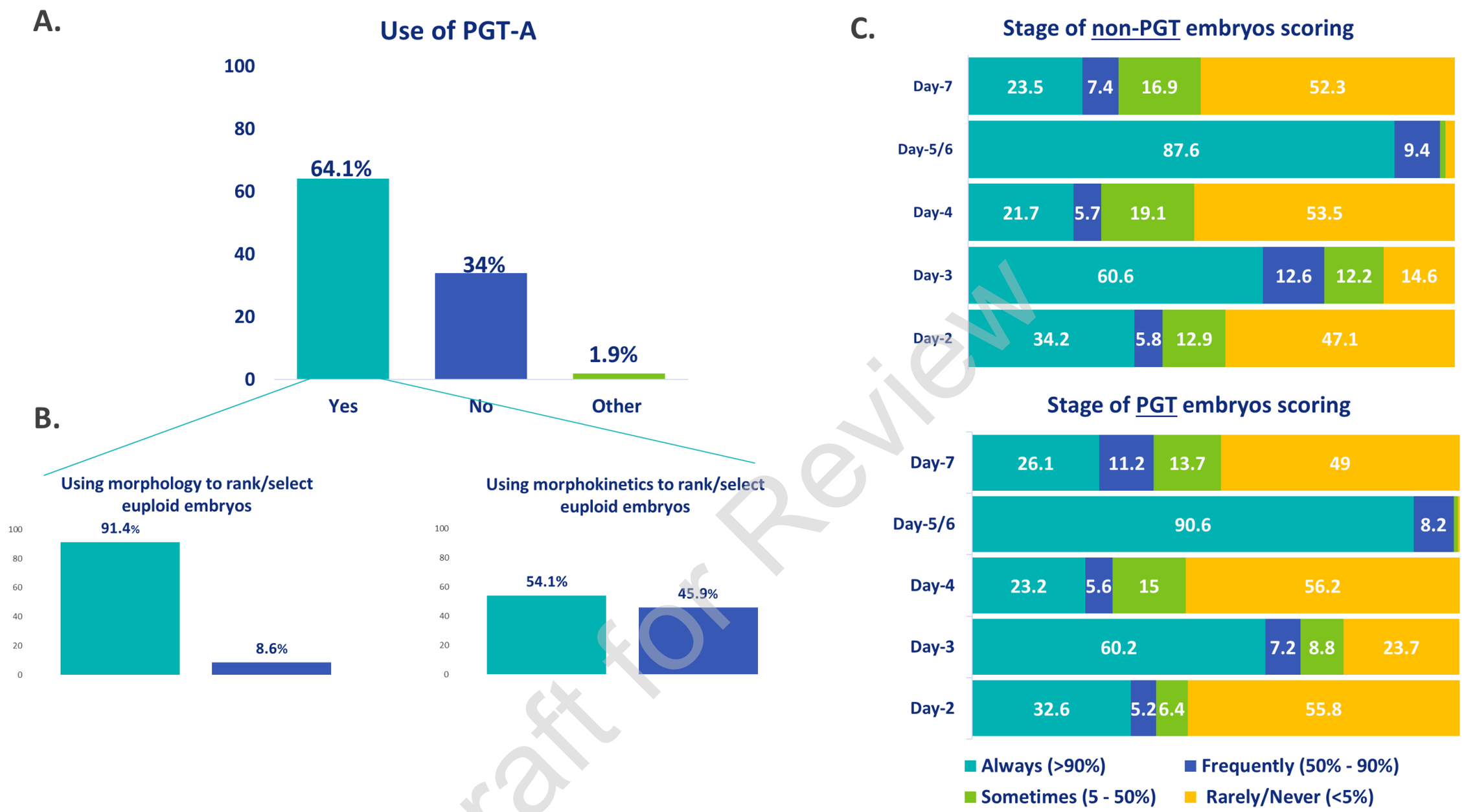
2289 *Figure 6 Using of Time-lapse (A), algorithms (B) and artificial intelligence (C)*



2290

2291 *Euploid embryos*

2292 *Figure 7 PGT-A use (A) and ranking of euploid embryos using the morphology or morphokinetics (B) and the day of PGT- and non-PGT-embryo scoring (C).*



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Supplementary Data SIII – Ranking tables for blastocysts

The following tables (**Supplementary Data SIII. figure 1.A.** and **B.** and **Supplementary Data SIII. table 1**) provide a general guideline on how to apply the Gardner grading system to rank embryos for transfer. However, the literature does not provide a consensus on the best method to do this, for example some studies (Ahlström et al., 2013) and (Honnma et al., 2012) concluded that TE grade was more important than expansion grade, and other studies (Van den Abbeel et al., 2013) concluded that expansion grade was more important than day of freezing. Further, there is not a linear relationship between embryo grade and pregnancy outcome, and in fact many grades have similar likelihood of pregnancy (e.g. 3AA and 4AB).

Figure 1 A general guideline on how to apply the Gardner grading system to rank blastocysts for Day-5 fresh embryo transfer(A) and Day-5 and Day-6 frozen embryo transfer. A numerical order is followed for grading (1 to 40).

A.

		Day 5 Fresh ET							
	Expansion	**1**	**2**	**3**			**4-6**		
	TE			**A**	**B**	**C**	**A**	**B**	**C**
ICM	**A**	10	9	5	7	17	1	3	15
	B			6	8	18	2	4	16
	C			13	14	20	11	12	19

Colour code:
Good quality blastocysts
Poor quality blastocysts
Early blastocysts

B.

	Day of freezing	Day 5 FET								Day 6 FET							
	Expansion	**1**	**2**	**3**			**4-6**			**1**	**2**	**3**			**4-6**		
	TE			**A**	**B**	**C**	**A**	**B**	**C**			**A**	**B**	**C**	**A**	**B**	**C**
ICM	**A**	18	17	5	7	31	1	3	29	20	19	13	15	35	9	11	33
	B			6	8	32	2	4	30			14	16	36	10	12	34
	C			23	24	38	21	22	37			27	28	40	25	26	39

C. **Priority**

1. Grade A/B > Grade C
2. Grade 1/2 > Grade C
3. Grade C ICM > Grade C TE
4. Day 5>Day 6
5. Expansion 4-6 > 3 > 2 > 1
6. TE grade A > B
7. ICM grade A>B

Table 1 Example of a blastocyst ranking scheme for fresh or frozen Day 5 embryo transfer.

Rank	Day 5
1	Expansion 4-6; ICM/TE Grade A/B
2	Expansion 3; ICM/TE Grade A/B
3*	Expansion 1,2
4	Expansion 4-6; TE Grade C
5	Expansion 3; TE Grade C
6	Expansion 4-6; ICM Grade C
7	Expansion 3; ICM Grade C
*: the culture of early blastocysts (expansion 1,2 on day 5) should be extended to day 6 before vitrification (Kovacic et al., 2004, Wirleitner et al., 2016)	

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Honnma H, Baba T, Sasaki M, Hashiba Y, Ohno H, Fukunaga T, Endo T, Saito T, Asada Y. Trophectoderm morphology significantly affects the rates of ongoing pregnancy and miscarriage in frozen-thawed single-blastocyst transfer cycle in vitro fertilization. Fertility and sterility 2012;98: 361-367.

Kovacic B, Vlaisavljevic V, Reljic M, Cizek-Sajko M. Developmental capacity of different morphological types of day 5 human morulae and blastocysts. Reproductive biomedicine online 2004;8: 687-694.

Van den Abbeel E, Balaban B, Ziebe S, Lundin K, Cuesta MJ, Klein BM, Helmgaard L, Arce JC. Association between blastocyst morphology and outcome of single-blastocyst transfer. Reproductive biomedicine online 2013;27: 353-361.

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