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OF TURKU

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Assessment of Blastomere Multinucleation
and Luteal Phase GnRH Agonist Support
in Relation to Reproductive Outcome

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To my family

ABSTRACT

Jaana Seikkula

Factors contributing to frozen embryo transfer outcomes

– assessment of blastomere multinucleation and luteal phase GnRH agonist in relation to reproductive outcome

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Of all embryo transfers in Finland, approximately half are now performed as frozen embryo transfer (FET). The effectiveness of FET is dictated by patient characteristics, embryo quality, and implantation environment. The significance of one of the embryo quality markers, blastomere multinucleation, and the effects of modified FET protocols on pregnancy outcomes, course of pregnancy, and health of the newborns are not thoroughly clarified.

The first part of this thesis focused on blastomere multinucleation in frozen-thawed embryo cohorts: *de novo* occurrence, associations to factors known to contribute to controlled ovarian hyperstimulation (COH) and pregnancy potential. Multinucleation was frequent in embryo cohorts before cryopreservation, but *de novo* multinucleation after thawing neither reached the level detected before freezing nor was associated with *de novo* multinucleation in sibling embryos. Young female age and higher ovarian response to COH were associated with the occurrence of multinucleation, especially before freezing. The pregnancy potential of binucleated (BN) and, notably, multinucleated (MN) frozen-thawed embryos seemed to be reduced, but this study demonstrated that transfer of BN/MN FET could result in acceptable live birth rate (LBR), healthy pregnancies, and newborns.

In the second part of this thesis, the effect of single dose triptorelin as an adjuvant to standard luteal support in natural and artificial cycle FETs was assessed in prospective pilot studies. The clinically relevant difference in LBR, pointing to the benefit of triptorelin, was statistically insignificant. These results underline a need for larger multicenter studies before implementing the use of triptorelin in clinical practice of FETs.

KEYWORDS: In vitro fertilization, intracytoplasmic sperm injection, frozen embryo transfer, multinucleation, binucleation, luteal phase, adjuvant therapy, gonadotropin releasing hormone agonist, triptorelin

TIIVISTELMÄ

Jaana Seikkula

Pakastealkiosirtojen ennusteeseen vaikuttavat tekijät

– blastomeerien monitumaisuuden ja luteaalivaiheen GnRH agonistituen vaikutus raskausennusteeseen

Turun yliopisto, Lääketieteellinen tiedekunta, Synnytys- ja naistentautioppi, Turun kliininen tohtoriohjelma, Turku, Suomi

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Kaikista Suomessa tehdyistä alkionsirroista noin puolet ovat pakastealkion siirtoja (PAS). Monet tekijät, kuten potilaan ominaisuudet, alkion laatu ja implantaatioympäristö vaikuttavat PAS:n tuloksellisuuteen. Kuitenkaan kaikkien alkio-ominaisuuksien, kuten blastomeerien monitumaisuuden tai PAS:ssa käytettävien hoitomenetelmien vaikutusta raskausennusteeseen, raskauden kulkuun tai syntyvien jälkeläisten terveyteen ei tarkkaan tiedetä.

Tutkimuksessa selvitettiin alkion monitumaisuuden esiintyvyyttä pakastealkio-kohorteissa, yhteyttä koeputkihedelmöityshoidon aikaisen munasarjastimulaation vasteeseen vaikuttaviin tekijöihin sekä monitumaisten (MN) alkiodien raskauden todennäköisyyttä ja syntyvien lasten terveyttä. Monitumaisuuden esiintyminen alkio-kohorteissa ennen alkio-pakastusta oli tavallista, mutta sen ei havaittu lisääntyvän alkio-pakastuksen myötä tai lisäävän yksitumaisten sisaralkiodien muuntumista monitumaisiksi. Monitumaisten alkiodien esiintyvyys oli yhteydessä erityisesti naisen nuoreen ikään ja korkeaan munasarjastimulaatio-vasteeseen. Raskauden todennäköisyys MN PAS:n jälkeen oli alentunut, varsinkin blastomeerien sisältäessä enemmän kuin kaksi tumaa. Raskaustulos oli kuitenkin hyväksyttävä, eivätkä raskaudenkulku tai vastasyntyneiden terveys eronneet yksitumaisista alkioista käynnistyneistä raskauksista.

Tutkimuksen toisessa osassa tutkittiin kerta-annoksena annetun triptoreliinin vaikutusta raskaustulokseen luonnollisen kierron ja hormonaalisesti ohjatun PAS:n luteaalivaiheen tukihoidon lisänä. Prospektiivisissä pilottitutkimuksissa syntyneiden lasten määrässä havaittiin kliinisesti merkittävä ero triptoreliinia käyttävän ryhmän eduksi, mutta ero ei ollut tilastollisesti merkitsevä. Laajemmat tutkimukset triptoreliinin vaikutuksesta PAS:n raskaustulokseen ovat tarpeen.

AVAINSANAT: koeputkihedelmöitys, mikroinjektio hedelmätyt, pakaste-alkionsiirto, monitumaisuus, luteaalivaihe, adjuvanttihoito, gonadotropiineja vapauttava hormoni, triptoreliini

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ABBREVIATIONS

AC	artificial cycle
ART	assisted reproductive treatment
BN	binucleated
CA	congenital anomaly
COH	controlled ovarian hyperstimulation
CPR	clinical pregnancy rate
CT	conjoined twins
eSET	elective single embryo transfer
ET	embryo transfer
FISH	fluorescence in situ hybridization
FET	frozen embryo transfer
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
hCG	human chorionic gonadotropin
HRT	hormonal replacement therapy
ICSI	intracytoplasmic sperm injection
IR	implantation rate
IVF	in vitro fertilization
LBR	live birth rate
LPS	luteal phase support
mNC	modified natural cycle
MN	multinukleaarinen/multinucleated
NC	natural cycle
OI	ovulation induction
PGT-A	preimplantation genetic testing for aneuploidies
PMD	placental mesenchymal dysplasia
PR	pregnancy rate
RCT	randomized controlled trial
TE	trophoectoderm
WOI	window of implantation

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-V.

- I Mankonen H, Seikkula J, Järvenpää T, Jokimaa V. A case of conjoined twins after a transfer of a multinuclear embryo. *Clin Case Rep.* 2015;3:260-5.
- II Seikkula J, Polo-Kantola P, Mankonen H, Anttila L, Jokimaa V. Multinucleation in day-two-embryos is not associated with multinucleation in sibling embryos after freezing and thawing. Manuscript.
- III Seikkula J, Oksjoki S, Hurme S, Mankonen H, Polo-Kantola P, Jokimaa V. Pregnancy and perinatal outcomes after transfer of binucleated or multinucleated frozen-thawed embryos: a case-control study. *Repro Biomed Online.* 2018;36:607-613.
- IV Seikkula J, Anttila L, Polo-Kantola P, Bloigu R, Engblom J, Tinkanen H, Jokimaa V. Effect of mid-luteal phase GnRH agonist on frozen-thawed embryo transfers during natural menstrual cycles: a randomized clinical pilot study. *Gynecol Endocrinol.* 2016;32:961-964.
- V Seikkula J, Ahinko K, Polo-Kantola P, Anttila L, Hurme S, Tinkanen H, Jokimaa V. Mid-luteal phase gonadotropin-releasing hormone agonist support in frozen-thawed embryo transfers during artificial cycles: a prospective interventional pilot study. *J Gynecol Obstet Hum Reprod.* 2018;47:391-396.

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1 INTRODUCTION

Cryopreservation of supernumerary embryos is today an integral component of assisted reproductive treatments (ART). Embryo cryopreservation has enabled an increase in the number of embryo transfers (ET) per patient and, at the same time, a decrease in the number of transferred embryos. Implementation of elective single ET (eSET) policy and freezing of supernumerary good-quality embryos has significantly reduced multiple pregnancies and improved perinatal outcomes in ART (Henningsen et al., 2015). In addition, freezing of all embryos derived from in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) cycles to perform frozen embryo transfer (FET) later on, is an option to minimize the risk for ovarian hyperstimulation syndrome (OHSS) and related maternal morbidity (Chen et al., 2012, Henningsen et al., 2015). All in all, embryo cryopreservation has promoted the cumulative success rate of IVF and ICSI cycles, cost-efficiency of ART, and patient safety. Improved FET results have encouraged clinicians to take advantage of more individualized approaches in ART to avoid pregnancy and perinatal complications.

The development of IVF culminated in the first live birth in 1978, and ICSI, developed to overcome male infertility, resulted in a live birth in 1991. The first live birth after transfer of a frozen-thawed embryo was reported in 1984 (Wang et al., 2006, Zeilmaker et al., 1984). However, the success rate for FET has remained significantly lower compared to fresh ET until the introduction of modern embryo culture and cryopreservation techniques in the first decade of the 21st century (Abdelhafez et al., 2010, Ferraretti et al., 2017, Rienzi et al., 2017). Today, FET provides comparable pregnancy outcome to fresh ET. Along with improved results, the proportion of FET cycles to all ART treatments has increased in Europe, from 12% to 21% between 1997 and 2011 (Ferraretti et al., 2017). Also, in Finland, the proportion of FET to all ART treatments have increased steadily from the beginning of the 21st century, with simultaneous improvement in FET outcomes. Altogether, 3565 FETs were performed in 2016, of which 797 resulted in a live birth, representing 1.5% of all deliveries. During the preceding 15-year period, live birth rate (LBR) of FET cycles rose from 11% to 22%. In 2016, ART treatments using own gametes, 24% of fresh ETs, and 22% of FETs resulted in live births. Today, half of the ETs are performed as FET in Finland (THL, 2018).

Although modern cryotechniques have closed the gap in pregnancy outcomes between ET and FET, failed ART cycles continue to represent a significant challenge in daily clinical practice. The probability of ET/FET resulting in pregnancy consists of a complex combination of related causes for infertility, embryo quality, and endometrial receptivity. Targeted embryo selection aims to

reduce the number of unsuccessful treatment cycles. Morphological features of an embryo and their true impact on pregnancy potential should be recognized to diminish embryo wastage. During the first cleavages, embryos may exhibit more than one nucleus within a blastomere, a phenomenon referred to as blastomere multinucleation. Multinucleation is one of the embryo quality markers and is associated with other embryo features illustrating poor embryo quality (Hardarson et al., 2001, Liu et al., 2014a, Van Royen et al., 2003, Zhan et al., 2016). Embryos carrying multinucleated (MN) blastomeres have previously been discarded from ET because of concerns for the chromosomal health of the embryo. Therefore, evidence of pregnancy and neonatal outcomes after MN ET are currently scarce. Also, data on multinucleation in frozen-thawed embryos is limited (Agerholm et al., 2008, Desai et al., 2016, Egashira et al., 2015).

Moreover, alternative approaches have been studied along with standard endometrial preparation protocol in an attempt to promote endometrial receptivity before ET/FET. Before implementing different adjuvant therapies into routine practice, safety and efficacy should be promptly analyzed. Gonadotropin releasing hormone (GnRH) agonist, combined with standard luteal support in fresh ETs, have shown promising results in increasing implantation and LBR, particularly in IVF/ICSI cycles stimulated with an antagonist protocol (Benmachiche et al., 2017, Isik et al., 2009, Kung et al., 2014, Simsek et al., 2015, Tesarik et al., 2006, Zafardoust et al., 2015). The quality of data is, however, poor and conflicting. The luteal phase in FET cycles differs profoundly from stimulated IVF/ICSI cycles, and therefore results from fresh ETs are not directly applicable to FET. Data on the effect of additional luteal phase GnRH agonist in natural cycle (NC) FETs or artificial cycle (AC) FETs is limited to only a few studies (Davar et al., 2015, Orvieto et al., 2016, Ye et al., 2019, Zarei et al., 2017).

2 REVIEW OF LITERATURE

2.1 Clinical outcomes of FET

2.1.1 Pregnancy outcomes

Embryo cryopreservation was originally designed to reduce the number of transferred embryos during fresh ETs and complications related to ART, such as OHSS, multiple pregnancies and preterm birth. Nowadays, along with improved cryotechniques, preserving supernumerary embryos for later use has become a convincing option to increase the cumulative pregnancy rate (PR) of ART, and is today perceived as a complementary, or even preferable, procedure to fresh ET (Acharya et al., 2018, Tiitinen et al., 2001). Historically, LBR has been higher among fresh ETs compared to FET. The discrepancy in outcomes is partly explained by the strategy of transferring the best-quality embryos during a fresh cycle, while cryopreserved embryos exhibited more compromised quality compared to embryos selected for ET. Advanced cryotechniques and a shift in practice patterns toward blastocyst culture and more liberal use of preimplantation genetic testing for aneuploidies (PGT-A) and freeze all strategy have nowadays equalized pregnancy outcomes between fresh ET and FET (Evans et al., 2014, Roque et al., 2019).

According to a large analysis of ART data in Europe, PR per FET increased steadily from 14% to 22% between 1997 and 2011, whereas a simultaneous rise in PR per fresh ET was from 24% to 28% (Ferraretti et al., 2017). There is growing evidence that with current cryotechniques, pregnancy outcomes of FET cycles, including clinical pregnancy rate (CPR) and LBR, yield comparable results and even better outcomes than fresh ET, especially in women with a high response to controlled ovarian hyperstimulation (COH) (Acharya et al., 2018; Roque et al., 2019, W. Zhang et al., 2018). Accordingly, in Finland, LBR in FET and fresh ET is similar (22.0% and 23.9%, respectively) (THL, 2018).

2.1.2 Neonatal outcomes

Along with a progressive increase in the use of FET, reassuring safety data regarding the impact of cryoprotectants and freezing-thawing methods on the health of the newborns have been shown. The impact of cryopreservation processes on perinatal and neonatal outcomes have been evaluated in several meta-analyses (Berntsen and Pinborg, 2018, Sha et al., 2018, Zhao et al., 2016).

According to a Finnish cohort study, a safety profile regarding the risk for preterm birth (6.5% vs. 8.8%), low birth weight (4.2% vs. 6.0%), or small for gestational age (1.5% vs. 3.1%) seems to be in favor of FET (n = 2,293) compared to fresh ET (n = 4,151), whereas course of pregnancy is the most favorable in spontaneous pregnancies (n = 31,946) (Pelkonen et al., 2010).

By contrast, current data on the increased risk of large for gestational age among neonates born from FET cycles are convincing (Berntsen and Pinborg, 2018, Kato et al., 2012, Pelkonen et al., 2010, Sha et al., 2018). In a meta-analysis of more than 100,000 FET singletons, a 1.5-fold risk for large for gestational age and a 1.7-fold risk for macrosomia was observed in neonates born from FET compared to fresh ET (Berntsen and Pinborg, 2018) Another meta-analysis of 31 studies showed results consistent with earlier evidence, pointing out a decreased risk for low birth weight, preterm birth, and perinatal mortality compared to fresh ET, whereas pregnancy-induced hypertension, postpartum hemorrhage, and increased birth weight associated with FET (Sha et al., 2018). Interestingly, two recent retrospective analyses found increased birth weight to be associated with AC-FET rather than NC-FET (Ishii et al., 2018, Tsuji et al., 2017). This finding was not, however, supported by another large retrospective analysis of different FET protocols, which instead found AC-FET to associate with hypertensive disorders during pregnancy and postpartum hemorrhage compared to NC-FET (Ginström Ernstad et al., 2019). These results suggest that the preparation of endometrium and hormonal milieu may contribute to conditions exposing maternal and perinatal complications.

Congenital anomalies (CAs) are more frequently seen in children born after ART compared to natural conception (Berntsen et al., 2019). In a meta-analysis of 112,913 ART children and 4,471,368 naturally conceived children, the risk for CAs was increased 1.4-fold in children born after ART (Zhao et al., 2018). In a large Scandinavian cohort, however, the absolute increase in risk for CAs was only 0.5% among ART children (Henningsen et al., 2018). The risk for CAs seems not to be related to the method of fertilization (IVF or ICSI) but instead, the risk for chromosomal abnormalities is reported to be associated with ICSI and male infertility compared to IVF (Berntsen et al., 2019). According to the available data, the risk for major CAs is not increased among singleton neonates born from FET cycles compared with children born after fresh ET (Aflatoonian et al., 2010, Kato et al., 2012, Pelkonen et al., 2014, J. Zhang et al., 2018). Available data suggest an overall good prognosis in the long-term health of ART children, although potential increases in blood pressure and compromised metabolic and cardiovascular profiles compared to spontaneously conceived children have been reported (Berntsen et al., 2019). No difference was detected in physical health in early childhood or academic performance at adolescence

between children born from FET or fresh ET cycles (Pelkonen et al., 2015, Spangmose et al., 2018).

The mechanism behind the increased risk for CAs in ART remains unclear. The process of IVF/ICSI, including ovarian stimulation, manipulation of the gametes and embryo, and embryo exposure to different culture media have been suggested as causative factors (Zhao et al., 2018). Some implications on the causality between infertility rather than the ART process and risk for CAs have been postulated. Sub-fertility has been found to be an independent risk factor for CAs as, for example, prolonged time to pregnancy *per se* is reported to associate with increased prevalence of CAs (Davies et al., 2012, Zhu et al., 2006). Another explanation involves epigenetic changes in gene imprinting due to the ART process. Aberrant DNA methylation may alter gene transcription that impacts embryo development negatively (Berntsen et al., 2019, Palermo et al., 2008). Further, during blastogenesis, normal interaction in nuclear mechanics and cellular organization, function, and movement are needed for subsequent normal organogenesis. Defects in blastogenesis commonly affect the formation of the midline, of which monozygotic twins represent a rare example. Hypothetically, early embryos may carry features like multinucleation, which later affect cell migration and exposure to defects in organogenesis (Halliday et al., 2010).

2.2 Factors contributing to FET results

Several factors, including clinical features of the infertile couple and the ART process, may affect FET outcomes (Figure 1). This paragraph focuses on the critical steps of FET treatment that may impact FET outcomes.

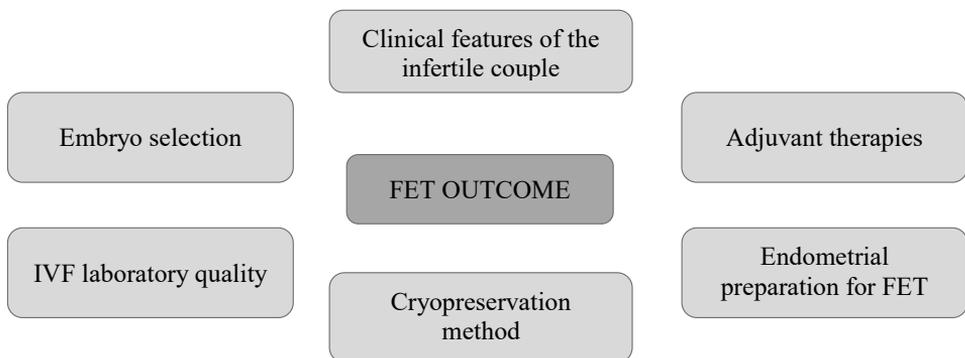


Figure 1. Factors affecting frozen embryo transfer outcome.

2.2.1 Embryo selection

Human embryos undergo sequential mitotic divisions of cells in which the entire genomic information is replicated and further reproduced by nuclear division (Fritz and Speroff, 2005, ESHRE, 2012). Visualization of female and male pronuclei within the inseminated oocyte is a sign of normal fertilization. Eventually, these two haploid complements of chromosomes merge to form a diploid chromosomal constitution and set a base for the first cell division. The first cell cycle, when the 2-cell embryo is formed, is completed approximately 20-26 hours after fertilization and after that, mitotic divisions occur within a time range of 12-16 hours. Eventually, the embryo begins compaction at the 8-cell stage, and the cleavage stage ends with the formation of morula and embryo enters to blastocyst stage generally five days after fertilization (Prados et al., 2012, ESHRE, 2012). Normally fertilized oocyte and embryo cleavage patterns are presented in Figure 2. The cleavage behavior of embryos cultured *in vitro* may be affected by the disruption of mitosis, leading to various morphological alterations.

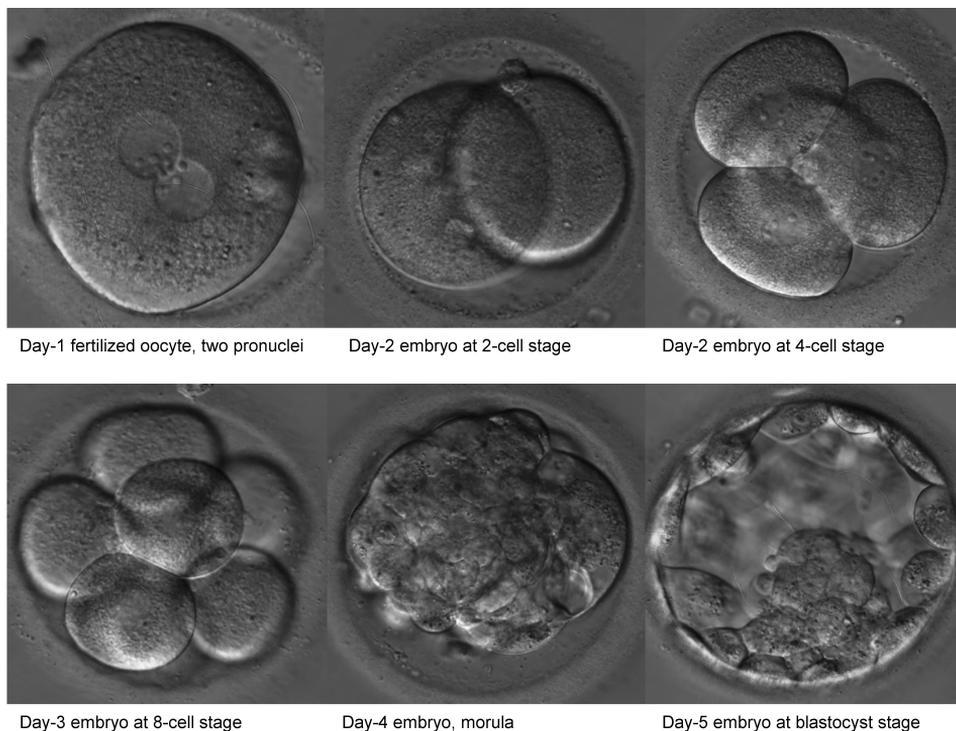


Figure 2. Embryo development from normally fertilized oocyte to day-5 blastocyst stage.

Morphological scoring of preimplantation embryos is a critical step in ART, determining embryo selection for fresh ET, cryopreservation, and subsequent FET. Sequential embryo screening includes embryo evaluation at specific time

points, whereas embryo monitoring with a time-lapse embryoscope allows continuous observation of the developmental stages of the embryo in an automated incubation system. No clear consensus regarding the superiority of these two protocols has been established (Armstrong et al., 2018). In 2011, an international consensus by the European Society of Human Reproduction and Embryology's expert panel defined the minimal criteria for embryo assessment. In the cleavage stage, the morphologic evaluation of embryos includes the evaluation of cell number and symmetry, embryo fragmentation, and blastomere nuclearity whereas blastocysts should be assessed by the developmental stage and morphology of the inner cell mass and the trophoctoderm (TE) (Alpha and ESHRE, 2011). The impact of multinucleation on FET outcome is discussed in detail in paragraph 2.3.

The embryo age at the time of ET or embryo cryopreservation generally varies from day-2 embryos to blastocysts. The use of extended embryo culture in FETs has increased significantly. In 2013, 70-80% of FETs in the United States were blastocyst transfers. A large nationwide cohort study that included more than 200,000 FETs from the United States noted significantly improved LBR, decreased miscarriage rate, and similar perinatal outcomes among blastocyst transfers compared to cleavage stage FET (Holden et al., 2017), but no benefit was found in cumulative pregnancy outcomes or LBR per FET favoring blastocyst transfers in a meta-analysis, as found by Martins et al. (2017). Extended embryo culture seems to decrease the time to pregnancy in FET cycles by enabling more detailed observation of embryo development and viability. However, prolonged exposure to *in vitro* culture conditions may adversely affect the embryo and lead to undesirable epigenetic changes in the offspring. Also, the embryo culture to the blastocyst stage seems to be associated with increased risk for monozygotic twinning (Berntsen et al., 2019).

Currently, no uniform worldwide protocol for embryo selection for cryopreservation exists, and criteria are subjected to individual IVF laboratory policies. Heterogenic protocols challenge the comparison between FET outcomes and complicate the evaluation of FET study methods and results.

2.2.2 Cryotechniques

The slow freezing technique and ultrarapid vitrification are two principal approaches used for embryo cryopreservation. During the freezing process, ice crystallization of intracellular water causes cellular damage and impairs cryosurvival of the embryos. The cells are dehydrated by a series of cryoprotectants with rising concentration to avoid ice crystal formation. Relatively low concentrations of cryoprotectants are used in the slow freezing

method, and intracellular ice formation is minimized by a controlled slow cooling rate. (Fuller and Paynter, 2004, Mazur, 1990). The slow freezing protocol was the first cryotechnique adopted in worldwide clinical practice.

In vitrification, high concentrations of cryoprotectants and ultrarapid cooling are used. The ultrarapid cooling rate is achieved by dripping the cryovials directly into liquid nitrogen. Instant cooling to -196°C results in glass-like ice without crystal formation (Vajta et al., 2015). Vitrification is gradually replacing slow freezing as the method of choice for embryo cryopreservation, although concerns regarding the impact of high concentrations of cryoprotectants during the vitrification process have emerged (Abdelhafez et al., 2010, Berntsen et al., 2019, Loutradi et al., 2008).

Blastomere survival after freezing and thawing predominantly defines the impact of cryopreservation on the pregnancy potential of thawed embryos. Fully intact embryos after thawing possess equivalent implantation rates (IR) to fresh embryos, whereas IR of embryos with 50% cell survival reach only half the rate of fully intact embryos (Edgar et al., 2000). Eluding formation of intracellular ice crystals is vital for blastomere survival. In this regard, vitrification is a more stable cryo-thaw method compared to slow freezing.

Several studies have demonstrated that vitrification is superior to the slow freezing protocol in terms of survival rate and pregnancy outcomes in all developmental stages of embryos; LBR was found to be similar or was in favor of vitrification compared to slow freezing (Balaban et al., 2008, Kaartinen et al., 2016, Z. Li et al., 2014, Peeraer et al., 2015, Rezazadeh Valojerdi et al., 2009). In a study by Kaartinen et al. (2016), LBR remained comparable, but the number of thawed embryos needed to produce a live birth was lower among vitrified than slow-frozen embryos (Kaartinen et al., 2016). In a large retrospective cohort study of 11,644 slow-frozen blastocyst transfers and 19,978 vitrified blastocyst transfers, vitrified-thawed cycles were more likely to result in FET than slow frozen-thawed cycles, and live birth occurred more frequently if the embryo was vitrified (Z. Li et al., 2014). The perinatal outcome of neonates seems comparable after vitrification or slow freezing (Kaartinen et al., 2016, Z. Li et al., 2014). It is noteworthy that current data on neonatal outcomes are predominantly analyzed from FET cycles in which cryopreservation was performed using the slow freezing method, although reassuring data on the safety of vitrification is accumulating (Berntsen et al., 2019, Pelkonen et al., 2015, Spangmose et al., 2018). Not all authors agree with the ongoing practice of substituting slow freezing of cleavage stage embryos for vitrification since equivalent LBR is still achievable with the slow freezing protocol (Edgar and Gook, 2012, Zhu et al., 2015).

2.2.3 Role of the endometrium

In the normal ovulatory cycle, the endometrium undergoes stromal cell differentiation mediated by cyclic sequential secretions of ovarian steroids, 17β -estradiol, and progesterone (Duc-Goiran et al., 1999). The regrowth of the endometrium is activated when epithelial and stromal cells, the two main cell types of the endometrium, begin proliferation as a response to rising estrogen at the beginning of the menstrual cycle. Mitotic activity and increased nuclear DNA and cytoplasmic RNA synthesis are accelerated preceding the shift from the proliferary phase to the preimplantation period of the menstrual cycle (Fritz and Speroff, 2005, Piltonen, 2016).

After ovulation, estrogen-driven endometrial proliferation is inhibited by the rising levels of progesterone. These hormonal changes induce secretory transformation of the uterine glands, called decidualization of the endometrium, and prepares the uterus for pregnancy, irrespective of the presence of a conceptus (Fritz and Speroff, 2005, Piltonen, 2016). Microarray studies have found that decidualization is a complex cascade of sequential reprogramming of functionally related families of genes involved in the extracellular matrix organization, cell adhesion, cytoskeletal organization, signal transduction, metabolism, stress responses, cell cycle progression, differentiation, and apoptosis (Gellersen et al., 2007). Upon implantation, decidua further develops to control the process of placentation.

The window of implantation (WOI) represents the period of maximal endometrial receptivity during which the uterine environment is the most favorable for nidation (Karizbodagh et al., 2017). The WOI opens approximately 6-8 days after ovulation and is maintained for four days. In humans, the time of onset and duration of WOI exhibit individual variation, and the mechanisms regulating the conversion of endometrium -first into receptive and further into refractory phase- are poorly understood (Achache and Revel, 2006, Bergh and Navot, 1992, Lessey, 1998, Piltonen, 2016).

2.2.4 Endometrial preparation in FET

Synchronization between the endometrial development and the embryo is critical for the success of implantation. Preparation of the endometrium for pregnancy requires complex cross-talk between autocrine and paracrine factors in the maternal environment and between mother and the embryo. The main mediators of stromal growth, estrogen and progesterone, also regulate the expression of several growth factors and cytokines involved in the endometrial maturation process and embryo-endometrial signaling (Karizbodagh et al., 2017). The endometrium also responds to several pregnancy-specific proteins secreted by the developing blastocyst (Duc-Goiran et al., 1999).

Table 1. FET preparation protocols.

FET –protocol	Principal selection criteria	Hormonal preparation of endometrium in follicular phase
Natural cycle FET (NC-FET)	Spontaneous ovulatory cycle	None
Modified natural cycle FET (mNC-FET)	Timing of FET in ovulatory cycle	None
Ovulation induction FET (OI-FET)	Oligo- or anovulatory cycle	Recombinant FSH, human menopausal gonadotropin, or aromatase inhibitor
Artificial cycle FET (AC-FET)	Oligo- or anovulatory cycle, flexible timing of FET	Oral/vaginal/transdermal estradiol or synthetic estrogen

FET: frozen embryo transfer, LH: luteinizing hormone, FSH: follicle stimulating hormone

In IVF/ICSI treatments, the administration of GnRH agonist or antagonist, as well as the development of multiple corpora lutei after controlled ovarian hyperstimulation, are known to induce iatrogenic luteal phase defect, which is compensated by exogenous progesterone administration. Compared to IVF/ICSI cycles, the hormonal milieu differs profoundly in the NC. Proof of the luteal phase defect is not as well described in NC-FET, in a modified natural cycle (mNC) FET, or the ovulation induction cycle (OI) FET compared to IVF/ICSI. However, the use of luteal phase support (LPS) has been adopted in routine practice in NC-FET and is also often used in mNC-FET and OI-FET (IVF worldwide, 2018, S. Li et al., 2014, Mackens et al., 2017, Yu et al., 2015).

The main issue in various FET protocols is the creation of an embryo-endometrial synchrony corresponding to the conditions that take place during natural conception and placentation. This process requires normal endometrial proliferation, the transition from the proliferative phase to the secretory phase, optimal timing of FET, and an adequate luteal phase. Receptive endometrium can be achieved both during natural and hormonally substituted cycles. Different endometrial preparation methods and basic principles for selection of FET protocol are presented in Table 1.

Vaginal micronized progesterone is the most commonly used preparation for LPS, whereas oral or intramuscular supplementation of progesterone are optional administration routes. In NC-FET, repeated dosages of human chorionic gonadotropin (hCG) have been used as an alternative to luteal phase progesterone supplementation, although recent data do not support its beneficial effect (Lee et al., 2017). In Finland, LPS is routinely continued for two weeks until the pregnancy test. In AC-FET, selection of the dominant follicle is suppressed, and endometrial proliferation is induced by administration of exogenous estrogen.

Ultrasound parameters	Intervention prior to FET	Timing of embryo transfer
Growth of the dominant follicle and thickness of endometrium	Urinary LH measurements	According to the spontaneous ovulation detected from urinary LH measurements
Growth of the dominant follicle and thickness of endometrium	Pharmacological triggering of ovulation	According to the triggered ovulation
Growth of the dominant follicle and thickness of endometrium	With or without pharmacological triggering of ovulation	According to the spontaneous ovulation detected from urinary LH measurements, or according to triggered ovulation
Thickness of endometrium and absence of the dominant follicle	Initiation of oral/intramuscular/ vaginal progesterone	According to the initiation of progesterone treatment

Once sufficient endometrial thickness is achieved, oral, intramuscular, or vaginal progesterone is combined with estrogen supplementation to initiate endometrial secretory changes (Leonard et al., 2015). Due to the absence of pituitary mediated changes in endogenous ovarian steroid production in early pregnancy, hormonal replacement therapy (HRT) must be continued until the onset of placental steroidogenesis. However, no proven concept for the optimal length of luteal support in AC-FET has been established (Mackens et al., 2017).

Miscarriages and biochemical pregnancies are described to occur more frequently in AC-FET cycles. Nonetheless, LBR remains equal compared to other FET protocols (Cerrillo et al., 2017, Tomás et al., 2012). All in all, several studies show no difference in LBR between different FET protocols (Cerrillo et al., 2017, Givens et al., 2009, Groenewoud et al., 2016, S. Li et al., 2014, Peeraer et al., 2015, Yu et al., 2015). However, the largest studies involve only the comparison of NC-FET and AC-FET, and in some cases, also extend to mNC-FET. Two meta-analyses verify comparable pregnancy outcomes of these three protocols (Groenewoud et al., 2017, Yarali et al., 2016). However, recent data give some indication of the beneficial effect of OI with letrozole for endometrial preparation compared to NC-FET or AC-FET (S. Li et al., 2014, Tatsumi et al., 2017). In a meta-analysis by Yarali et al. (2016), OI with letrozole showed a promising effect on FET pregnancy outcome, although adequately powered randomized controlled trials (RCTs) are currently lacking.

2.2.5 Adjuvant therapies in assisted reproduction treatments

A wide range of adjuvant therapies with a variety of different mechanisms of action has been used in ART in an attempt to improve ET or FET outcomes.

These interventions, several of which are experimental, are more often offered to women suffering from previous failed IVF/ICSI cycles, repeated miscarriages, or severely compromised ovarian function. Adjuvant therapies can be administered before embryo transfer, in the luteal phase or during the entire IVF/ICSI or FET cycle, although most of the studies predominantly involve fresh ET. Pretreatment prior to IVF/ICSI with different types of adjuvant therapies, such as antibiotics, dopamine agonists, intravenous immunoglobulins, intrauterine hCG, low-dose acetylsalicylic acid, heparin, uterine relaxants, Co-enzyme Q10, melatonin, acupuncture, and filgrastim have been used with little or no evidence of their positive effect on LBR (Aaleyasin et al., 2015, Gat et al., 2016, Kalampokas et al., 2017, Nardo et al., 2015, Polanski et al., 2014, Revelli et al., 2008, Seto et al., 2017, Shirlow et al., 2017, Ubaldi et al., 2002, Wang et al., 2017, Xu et al., 2018). Recent meta-analyses of the use of dehydroepiandrosterone or growth hormone as a pretreatment prior to IVF/ICSI in women suffering from poor ovarian reserve identified possible positive effects on pregnancy outcomes (Li et al., 2017; Liu et al., 2018; Pacchiarotti et al., 2016, Zhang et al., 2016). Administration of short-acting GnRH agonist as adjuvant therapy for traditional LPS has drawn attention and has been adopted widely into clinical practice, without solid evidence of its beneficial effect (Martins et al., 2016, Oliveira et al., 2010, Tesarik, 2018). The role of short-acting GnRH agonist as adjuvant therapy in fresh ET and FET is discussed in detail in paragraph 2.4.

2.3 Blastomere multinucleation, an embryo quality marker

2.3.1 Definition and classification

Blastomere multinucleation is a nuclear abnormality detected in the microscopic evaluation of the embryo. Normally only a single nucleus per blastomere is present, whereas in blastomere multinucleation at least one blastomere of the embryo exhibits more than one nucleus. Blastomere multinucleation can be further divided into two subgroups according to the number of detected nuclei: the binucleated (BN) blastomere carries two nuclei per cell, and the multinucleated (MN) blastomere more than two nuclei per cell (Figure 3). MN blastomeres are also called micronucleated, as they exhibit several small-sized nuclei. Multinucleation can be evaluated only during the early embryonic cell divisions, from 2- to 8-cell stage, while each blastomere is individually detectable (ESHRE 2012; Fritz and Speroff, 2005, Rienzi et al., 2005). Blastomere multinucleation is one of the morphologic features recommended to be evaluated in an embryo grading protocol (Alpha and ESHRE, 2011, Holte et al., 2007).

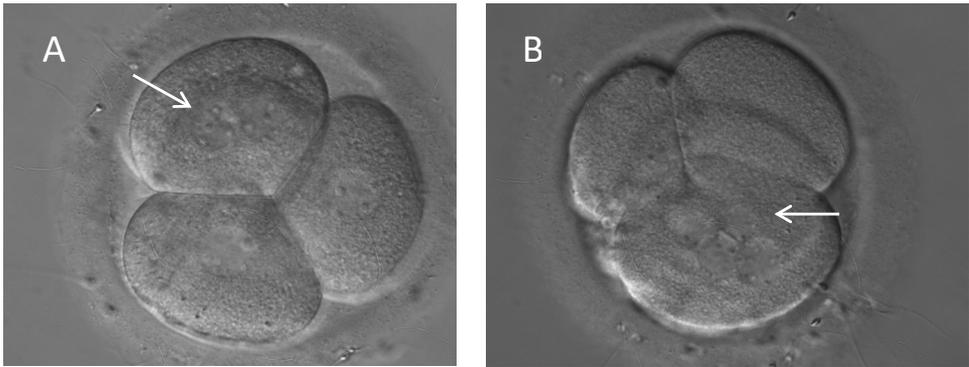


Figure 3. A. Binucleated embryo B. Multinucleated embryo.

2.3.2 Formation of multinucleation

Karyokinesis without cytokinesis, i.e., division of the nucleus without division of the cytoplasm, has been proposed as one of the mechanisms leading to the formation of multinucleation. This type of cell cycle error could be a main contributor especially for the development of BN blastomeres (Hardy et al., 1993, Pickering et al., 1995). During mitotic cell division, multiple small nuclei may arise from the abnormal organization of the mitotic spindle at prophase and defective chromosome migration at anaphase. As chromosomes move to opposite sides of the cell, lagging chromosomes may form separate micronuclei during the following interphase (Munné and Cohen, 1993, Staessen and Van Steirteghem, 1998, Winston et al., 1991). Furthermore, partial fragmentation of the nuclei may also cause blastomere multinucleation (Munné and Cohen, 1993).

Programmed cell death, called apoptosis, is strongly associated with nuclear fragmentation (Delimitreva et al., 2005, He et al., 2009). In 1987, it was discovered that MN blastomeres do not possess the typical activation of gene expression at the 8-cell stage compared to mononucleated sibling blastomeres. The authors debated that this functional inactivation could be a physiological action leading to developmental arrest and elimination of the affected blastomeres from the embryo (Tesarik et al., 1987). Therefore, certain blastomere multinucleation patterns may reflect a stage of apoptosis leading to either total developmental arrest or to embryo self-correction, in which non-viable cells are eliminated from further development (Desai et al., 2018).

Blastomere fusion, also called reverse cleavage, is a phenomenon occurring in early cleavage stage embryos. Reverse cleavage affects embryo developmental potential negatively and coincides with multinucleation (Liu et al., 2014b). Freezing and thawing of embryos have been proposed to expose the embryo to pronounced blastomere fusion, leading to numerical chromosomal changes. The

fusion of two or several blastomeres after thawing results in MN blastomeres carrying ploidy errors (Balakier et al., 2000). Theories of the formation of bi- and multinucleation are presented in Figure 4.

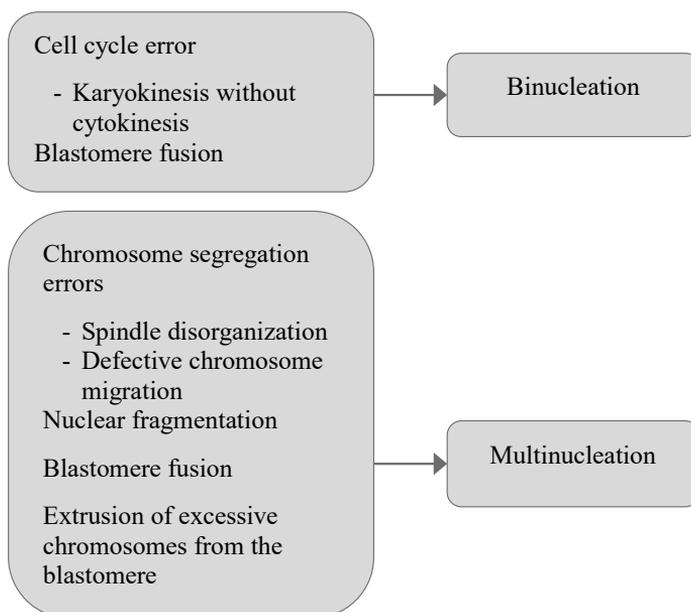


Figure 4. Theories of the formation of bi- and multinucleation.

2.3.3 Incidence of multinucleation in fresh and frozen-thawed embryos and embryo cohorts

Generally, blastomere multinucleation is highly prevalent after the first embryonic divisions, whereas it is less likely to persist during further embryo development (Balakier et al., 2016, Van Royen et al., 2003). In the earliest studies on the incidence of multinucleation, bi- or multinucleation detected in sequential embryo screening at day-2 and -3 of embryo culture were included in the reported incidence, whereupon the rate of multinucleation reached up to 80% in embryo cohorts and 30% of the embryos (De Cássia Savio Figueira et al., 2010, Jackson et al., 1998, Van Royen et al., 2003). More recently, the incidence of bi- and multinucleation has been more frequently determined in specific embryonic developmental stages, showing an incidence of 5.8% to 26.2% in 4-cell stage embryos (Egashira et al., 2015, Meriano et al., 2004, Yakin et al., 2005).

Multinucleation may be a temporary and reversible phenomenon, as some MN blastomeres exhibit an ability to restore mononuclearity (Balakier et al., 2016). A self-correction from MN to mononucleated has been demonstrated to occur in a frequency of up to 74% in 4-cell cleavage stage embryos (Aguilar et al., 2016).

Given the transient appearance of MN blastomeres within the embryo, the incidence of multinucleation may be underestimated by traditional sequential embryo screening due to the limited time interval for observation of embryo morphology. Continuous imaging of embryo development with an embryoscope enables the perception of dynamic morphological changes in the embryo without observational time restrictions. During time-lapse imaging, 43% of 2-cell stage embryos have been reported to exhibit multinucleation, and the detection rate in 4-cell stage embryos varies between 15%-25% (Aguilar et al., 2016, Balakier et al., 2016, Basile et al., 2014, Desai et al., 2014, Desch et al., 2016). *De novo* appearance of multinucleation, referring to multinucleation not detected in the earlier developmental stages of the embryo, has been reported to occur with a frequency of 7.5% in 4-cell fresh embryo cohorts and of 6.2% in fresh day-3 embryos (Aguilar et al., 2016, Van Royen et al., 2003).

2.3.4 Factors contributing to the formation of multinucleation

Multinucleation in *in vivo* fertilized human embryos was demonstrated in 1954, indicating that multinucleation is not exclusively an IVF/ICSI related phenomenon (Hertig et al., 1954, cited in Tesarik et al., 1987). However, it has been debated whether multinucleation may result from hypoxic intrafollicular conditions during COH or unfavorable embryo culture conditions (Pickering et al., 1990, Van Blerkom et al., 1997, Winston et al., 1991). In 1997, Van Blerkom et al. (1997) demonstrated that MN embryos derived predominantly from severely hypoxic follicles, and defectively oxygenated follicles were associated with an abnormal chromosomal organization within the mature oocytes. High levels of estradiol and increased number of retrieved oocytes, a result of an exaggerated response to gonadotropins during COH, have been associated with multinucleation (De Cássia Savio Figueira et al., 2010, Jackson et al., 1998, Van Royen et al., 2003, Yilmaz et al., 2014). However, results are not uniformly consistent since in a large cohort of ICSI cycles, the number of retrieved oocytes or high estradiol levels did not correlate with the appearance of multinucleation (Balakier et al., 2016), and results concerning follicle stimulating hormone (FSH) consumption and formation of multinucleation remain controversial (De Cássia Savio Figueira et al., 2010, Jackson et al., 1998, Van Royen et al., 2003).

Female age has an indisputable effect on ovarian response to gonadotropin stimulation and pregnancy outcomes in ART. Advanced female age has a strong association with an increasing number of chromosomal errors within the oocytes and preimplantation embryos. The deterioration in oocyte quality associated with increasing female age has been acknowledged to contribute to reduced fecundity and poor ART outcomes among aging women. Female age as a predisposing factor to blastomere multinucleation has been evaluated with conflicting results.

Moriwaki et al. (2004) found advanced female age to be associated with the formation of MN blastomeres. Later, in a study conducted by Balakier et al. (2016), blastomere multinucleation was discovered to be more prevalent among women aged over 40 years compared to women less than 35 years old. However, not all studies have confirmed these results (Ergin et al., 2014, Meriano et al., 2004, Van Royen et al., 2003, Yilmaz et al., 2014). As for the role of the male partner, no difference in the appearance of multinucleation has been detected after fertilization of oocytes with fresh sperm compared to frozen sperm or sperm collected from testicular sperm aspiration (De Cássia Savio Figueira et al., 2010).

The impact of embryo cryopreservation on multinucleation is poorly studied. Comparative studies on the frequency of multinucleation between cryo-thawed embryos and fresh embryos are lacking, but embryo cryopreservation has been postulated to increase blastomere multinucleation due to suboptimal conditions during freezing and thawing process (Agerholm et al., 2008). Factors studied previously as possible contributors to multinucleation are presented in Figure 5.

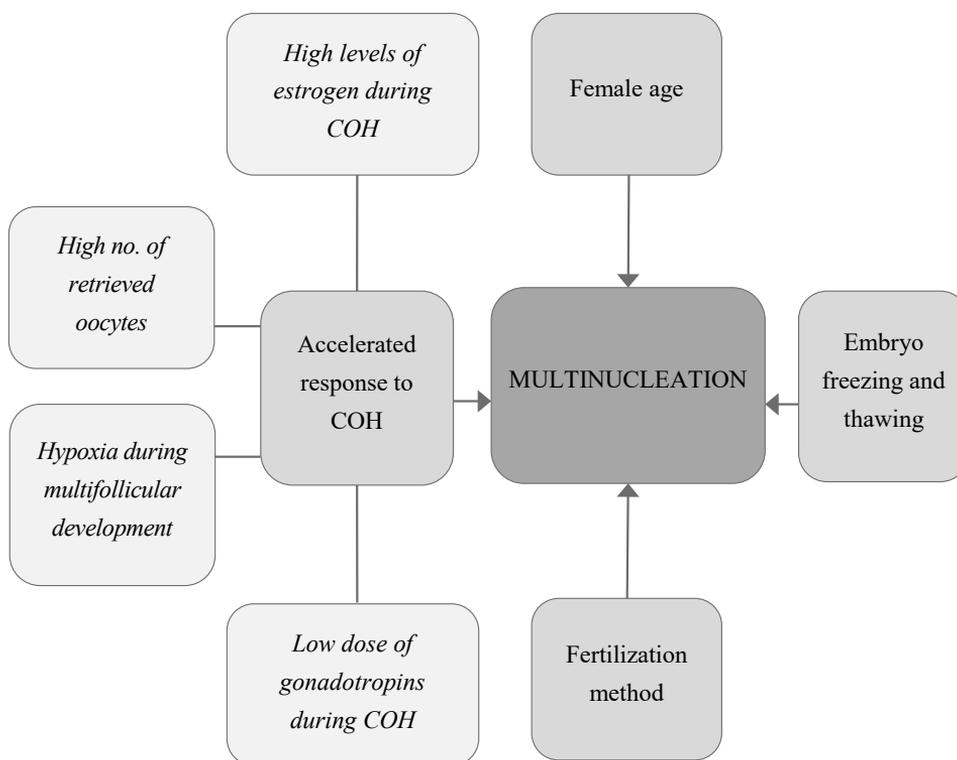


Figure 5. Factors studied as a contributors to occurrence of multinucleation.

2.3.5 Developmental capacity of MN embryos

Blastomere multinucleation appears in concordance with some morphological features generally related to diminished embryo developmental potential. Multinucleation is associated with increased embryo fragmentation and reverse cleavage. Uneven blastomere cleavage coincides with multinucleation as well as with low implantation potential and an increased rate of aneuploidy in cleavage stage embryos (Hardarson et al., 2001, Liu et al., 2014b, Van Royen et al., 2003, Zhan et al., 2016).

The rate of embryonic development most reliably reflects the overall embryo quality (Desai et al., 2016, Shapiro et al., 2001). The ideal cleavage pattern of eight cells on day-3 of embryo culture has been reported conjointly with minimal incidence of multinucleation (Van Royen et al., 2003). Some reports of reduced blastocyst formation among BN/MN embryos have been introduced, especially pointing out the detrimental effect of persisting/appearing MN blastomeres after the 2-cell stage (Desch et al., 2016, Egashira et al., 2015, Yakin et al., 2005). At the 2-cell stage, a developmental arrest occurs with similar frequency among mononucleated and BN/MN embryos if normal cytokinesis is displayed at the first cleavage. (Hashimoto et al., 2015). Furthermore, the overall comparable blastulation rate between mononucleated and BN/MN embryos have been reported (Desai et al., 2018).

Embryo cleavage time until the 4-cell stage is reported to be longer in MN embryos compared to their mononuclear counterparts, although the authors debated whether this phenomenon indicates active repair mechanisms in MN embryos rather than reflects a compromised developmental potential (Balakier et al., 2016). This finding is supported by Desch et al. (2017), who reported better LBR among MN embryos completing their second mitotic division after a longer time. Day-5 and -6 blastocysts derive from BN/MN cleavage stage embryos with similar frequency, and therefore the presence of multinucleation seems not to be associated with delayed blastulation (Balakier et al., 2016, Desai et al., 2016, Staessen and Van Steirteghem, 1998). Development to the blastocyst stage is reported to occur more frequently among BN than MN embryos (Meriano et al., 2004).

2.3.6 Developmental capacity of sibling embryos

In 2004, a difference in the pregnancy potential of mononucleated embryos from cycles containing either BN or MN sibling embryos was reported; multinucleation in an embryo cohort seemed to reduce the pregnancy potential of non-MN embryos, in opposition to BN embryos, whose presence did not decrease the odds for pregnancy among mononucleated sibling embryos

(Meriano et al., 2004). In contrast, later studies revealed that implantation, CPR, and LBR of mononucleated sibling embryos compared to embryos deriving from non-multinucleated cycles appear to be comparable, although a pronounced tendency to early miscarriage among cycles in which MN embryos were present but not transferred has been reported. (Egashira et al., 2015, Jackson et al., 1998, Yilmaz et al., 2014) Further, blastocyst formation of mononucleated embryos derived from cycles containing MN sibling embryos seems to be comparable to their counterparts from cycles exhibiting exclusively mononucleated embryos (Egashira et al., 2015).

2.3.7 Chromosomal complement of MN embryos

Since multinucleation interferes with the nuclear integrity of the blastomeres, questions on its relation with an increased incidence of chromosomal abnormalities have been asked. In general, aneuploidy at the time of conception and during preimplantation development is a very common phenomenon and is known to increase along with advanced maternal age. Chromosomal abnormalities in a fertilized oocyte originate primarily from female meiotic errors. Mitotic errors, instead, contribute to chromosomal errors like mosaicism detected at the cleavage stage. The rate of chromosomal abnormalities significantly decreases in the blastocyst stage, although the overall incidence remains as high as 60%. The difference in aneuploidy rates between women of younger and older age ranges also persists in the blastocyst stage. The decline in the aneuploidy rate is likely attributable to the developmental arrest before transitioning to the blastocyst stage and the self-correction methods modulated by the activated gene expression at the cleavage stage (Barbash-Hazan et al., 2009, Fragouli et al., 2013, Franasiak et al., 2014, Munné et al., 2005).

Complex aneuploidy at the cleavage stage is commonly accompanied by mosaicism (Gutiérrez-Mateo et al., 2011). Whether the embryo is destined to arrest or to form a blastocyst is suggested to depend on the type of aneuploidy, the way the embryo responds to aberrant gene expression, and whether the embryo and its blastomeres possess the ability to restore normal ploidy (Fragouli et al., 2013). However, it remains to be answered whether multinucleation illustrates blastomere aneuploidy or, instead, active self-correction mechanisms to eliminate the excessive chromosomal content of the blastomere.

The first studies evaluating the chromosomal constitution of cleavage stage MN embryos by the fluorescence in situ hybridization (FISH) technique were published in the 1990s, demonstrating that some of the MN blastomeres carried normal chromosomal complement, and that chromosomal content of the sibling

blastomeres did not necessarily correspond to that of BN or MN (Munné et al., 1994, Munné and Cohen, 1993, Staessen and Van Steirteghem, 1998). The chromosomal constitution was aneuploid in 80% of BN/MN embryos having all their blastomeres biopsied for FISH analysis on day-4 of embryo development. Of those embryos, half had a complex aneuploidy or mosaicism, whereas the other half had a combination of normal and abnormal nuclei (Kligman et al., 1996). In a more detailed analysis, in 3- to 8-cell embryos displaying bi- or multinucleation in both blastomeres at 2-cell stage, the chromosomal constitution was found to correspond to normal mononuclear diploid status in 30% of cases, whereas 34% presented non-diploid constitution indicative of chaotic division. Interestingly, 36% had a combination of mononuclear diploid and non-diploid blastomeres, suggesting that at least one of the initial BN/MN blastomeres possessed normal ploidy or ability to self-correction (Staessen and Van Steirteghem, 1998). In a FISH analysis including exclusively MN blastomeres, the majority of them were found to be BN after spreading. The rate for uniformly diploid nuclei varied between 22% and 50%. (Xanthopoulou et al., 2011, Yilmaz et al., 2014) The remaining nuclei consisted of the aneuploid, polyploid, or chaotic chromosomal complement. In 64% of BN/MN blastomeres containing aneuploid nuclei, genetic content was not identical, indicating unequal segregation of chromosomes into multiple nuclei (Yilmaz et al., 2014).

Aneuploidy records gained from blastocyst stage laser-assisted TE biopsies have revealed comparable aneuploidy rates between mononucleated and MN embryos. Among MN embryos, all 24 chromosomes contribute to blastocyst aneuploidy. Dominant chromosomal abnormalities included single or dual chromosomal trisomy, or monosomy of entire or segmental chromosomes (Balakier et al., 2016).

In a large cohort of embryos followed with time-lapse imaging and PGT-A, the incidence of MN decreased as the embryo cleaved from the 2- to 4-cell stage. The majority of the MN 2-cell stage developed into good-quality blastocysts, and during early cleavage, correction to mononuclear blastomeres occurred in 50% of MN embryos, regardless of the ploidy status. Half of the blastocysts exhibiting MN blastomeres at the cleavage stage carried a normal chromosomal constitution (Balakier et al., 2016), which is comparable to the overall aneuploidy rate (Fragouli et al., 2013). In concordance, in an analysis of 26 blastocysts tested using microarray-based comparative genomic hybridization to assess ploidy, half of the blastocysts developed from embryos exhibiting MN in both blastomeres at the 2-cell stage (n=9) were euploid (Hashimoto et al., 2015).

Interestingly, despite the associations reported between multinucleation and developmentally detrimental features, no strict correlation between morphological features of the embryo and ploidy status has been established.

Furthermore, chromosomal abnormalities are present in MN embryos regardless of the overall embryo morphology (Balakier et al., 2016, Kligman et al., 1996, Staessen and Van Steirteghem, 1998).

In conclusion, a high aneuploidy rate among BN/MN embryos detected in the earliest studies may be related to the timing of analysis, as only cleavage stage embryos were examined. Therefore, analysis during cleavage stage may lead to overestimation of the number of chromosomal aberrations while possible self-correction procedures might take place. Recently, more advanced genetic testing techniques with TE biopsies from blastocysts have revealed normally developing MN/BN embryos to carry normal ploidy status with similar frequency to mononuclear blastocysts, questioning the prevalent practice of excluding BN/MN embryos from ET.

2.3.8 Clinical outcomes of MN embryos

Implantation potential

Until recently, due to its assumed detrimental effect on pregnancy potential, the presence of MN/BN in embryos *per se* was regarded as a contraindication for ET selection. The first reports on pregnancy outcomes of BN/MN embryos in fresh ETs included several confounding factors, such as both BN/MN and mononucleated embryos, and up to six embryos were transferred simultaneously. However, a distinct and consistent reduction in IR compared to mononucleated counterparts was observed in early published data (De Cássia Savio Figueira et al., 2010, Jackson et al., 1998, Pelinck et al., 1998, Van Royen et al., 2003).

However, more recent data on the embryo cohorts followed with time-lapse imaging revealed a relatively high IR of 18%-28% in cleavage stage embryos, and up to 32% in blastocysts deriving from MN 2-cell embryos. According to the majority of the studies, IR is at the lowest if both blastomeres are affected at the 2-cell stage. IR for PGT-A tested euploid MN embryos remained comparable to overall IR among MN embryos. In most of the studies a significant difference in IR favoring mononucleated embryos was consistent in every subgroup, but also comparable IR has been reported regardless of the nuclear status (Aguilar et al., 2016, Balakier et al., 2016, Desch et al., 2016, Hashimoto et al., 2015). The effect of multinucleation on implantation potential seems to be more detrimental if the phenomenon persists or appears at the 4-cell stage (Aguilar et al., 2016).

Birth potential

Due to the general practice of discarding BN/MN embryos from ET, the earliest studies on the clinical relevance of multinucleation included only reports on the

development of BN/MN cleavage stage embryos but no data on the pregnancy potential. Only the most recent publications have accumulated data on the LBR of BN/MN embryos. In the cleavage stage, LBR for fresh embryos exhibiting multinucleation at the 2-cell stage has been reported to be significantly reduced compared to embryos displaying normal nuclear status. The negative impact was most prominent if MN was detected in both blastomeres (Desch et al., 2016). The pregnancy potential of MN embryos seems to increase along with normal embryo development. Egashira et al. (2015) demonstrated a reduction in LBR in fresh cleavage stage MN ETs, whereas LBR was comparable in FETs between MN and non-MN blastocysts if an MN embryo had blastulated normally. However, reports of less optimistic results postulated that vitrified-warmed blastocysts exhibiting multinucleation at the early stages of embryo development were half as likely to implant (Hur et al., 2018) or to produce a live birth (Desai et al., 2016) compared to their mononucleated counterparts.

Although the number of deliveries from either fresh or frozen-thawed MN ETs remains considerably low, growing evidence indicates that blastocysts displaying MN at the cleavage stage can produce live births at a relatively high rate. In a cohort of 61 blastocysts exhibiting multinucleation at the 2-cell stage, live birth or ongoing pregnancy was reported in 41% of fresh ET cycles. If chromosomal abnormalities were excluded with PGT-A, the corresponding percentage was as high as 64% (Balakier et al., 2016). The tendency to pregnancy loss in embryos exhibiting multinucleation at the 2-cell stage relates to the cleavage pattern of the embryos; the miscarriage rate is higher among embryos reaching the 4-cell stage with shorter cleavage time, less than 37 hours after fertilization (Desch et al., 2016).

Neonatal outcomes

Current data on pregnancy complications or neonatal outcomes after transfer of BN/MN embryos are limited to small patient series and sporadic reports. Two cases of termination of pregnancy due to trisomy 18 and a combination of trisomy 21 and 18 after the transfer of an MN embryo have been reported (Balakier et al., 2016, Balakier and Cadesky, 1997). Later, a case report of conjoined twins (CT) after the transfer of an MN embryo was published (Serapinas et al., 2016). Pregnancy was terminated in the second trimester. In full-term pregnancies, the number of reported deliveries varied from 1 to 14, and all born babies were healthy (Balakier et al., 2016, Balakier and Cadesky, 1997, Egashira et al., 2015, Hashimoto et al., 2015, Jackson et al., 1998, Yilmaz et al., 2014). To date, in the largest cohort of 61 fresh ET transfers derived from MN 2-cell embryos, 14 deliveries were documented. No obvious difference in neonatal outcomes was observed (Balakier et al., 2016). Neonatal outcomes from frozen-thawed BN/MN embryo transfer have been described in

only one study; Egashira et al. (2015) reported delivery of 9 healthy newborns from transfers of 22 MN embryos.

Multinucleation and placental development

Little is known about placental development in pregnancies originating from BN/MN embryos. A rare vascular abnormality of the placenta, placental mesenchymal dysplasia (PMD), has been related to the binucleation of cleavage embryos (Xanthopoulou et al., 2011). Histologically, PMD is characterized with enlarged edematous stem villi with dilated vessels and absent trophoblastic proliferation. Clinically, PMD is illustrated by placentomegaly and obstetrical complications, such as intrauterine growth restriction and fetal demise (Kinoshita et al., 2007). PMD is suggested to arise from abnormal fertilization or maternal disjunction during the first meiotic division (Kinoshita et al., 2007, Pawoo and Heller, 2014). However, an alternative explanation for the development of diploid cells of androgenic origin distinctive for PMD is karyokinesis of paternal haploid mononucleated blastomere without cytokinesis, and therefore, eventually diploid unipaternal cell lineage is formed (Xanthopoulou et al., 2011).

2.4 Short-acting GnRH agonist as adjuvant luteal support

2.4.1 GnRH function

GnRH, a decapeptide secreted from the hypothalamus in a pulsatile manner into the portal circulation of the hypophysis and then transported to the anterior pituitary gland, modulates the mammalian reproduction system by stimulating synthesis and secretion of gonadotropins LH and FSH. Two types of GnRH receptors are demonstrated to be expressed in multiple extra-pituitary mammalian tissues and cells, indicating that the effect of GnRH is not limited only to the secretion of pituitary gonadotropins. GnRH receptors are present in reproductive organs, such as the placenta, endometrium, myometrium, and ovary, and in peri-implantation embryos of different species. This finding has led to speculations on a potential role of GnRH as a direct regulator of endometrial cells and embryo-endometrial interaction (Casañ et al., 1999, Cheng and Leung, 2005, Maggi et al., 2016, Metallinou et al., 2007).

2.4.2 Pharmacokinetics of GnRH agonist

GnRH agonist is a synthetic decapeptide and agonist analogue of GnRH. GnRH agonist has a biphasic effect on pituitary function. Short-term use increases the

number of GnRH receptors and provokes synthesis and release of pituitary gonadotropins, a phenomenon also called a flare effect. If exposure to GnRH agonist continues for a prolonged period, the stimulatory effect of endogenous GnRH is inhibited by extended occupation and loss of GnRH receptors. As desensitization of the targeted pituitary cells occurs, pituitary gonadotropin secretory response is downregulated. In turn, in females, estrogen levels decrease to menopausal levels (Cheng and Leung, 2005, Maggi et al., 2016, Pharmaca Fennica, 2018). GnRH agonist is available as a long-acting depot and short-acting daily formulations. As adjuvant support in the luteal phase of ART cycles, only a short-acting GnRH agonist, namely triptorelin, has been investigated (Table 2).

Triptorelin

Short-acting triptorelin acetate (Gonapeptyl[®]) contains non-natural amino acid substituents, and therefore its duration of action is longer, and its affinity to bind to GnRH receptors is higher compared to endogenous GnRH. In a rat pituitary cell receptor assay, the receptor affinity of triptorelin was up to 100-fold greater compared to natural GnRH. The systemic bioavailability of triptorelin is estimated to be nearly 100%. The elimination half-life is three to five hours, and the total clearance of triptorelin occurs within 24 hours. In the liver and the kidneys, triptorelin is degraded to peptides and amino acids and thereafter eliminated renally (Triptorelin, 2015, Pharmaca Fennica, 2018).

2.4.3 Mechanism facilitating implantation

Data on the short-acting GnRH agonist supplementation as an additional LPS for progesterone supplementation has been accumulating for more than a decade. Originally GnRH agonists were investigated as a potential contraceptive and abortive agents but were proven to fail as inducers of early pregnancy loss (Skarin et al., 1982). In fact, case reports of sporadic spontaneous pregnancies under the influence of luteal phase GnRH agonist administration were reported, drawing attention toward possible mechanisms enhancing embryo implantation (Elefant et al., 1995, Gartner et al., 1997, Tesarik et al., 2004). More recently, the concept of GnRH agonist as additional luteal support to traditional LPS was extended to the sole use of repeated dosages of GnRH agonist in the luteal phase of IVF/ICSI. GnRH agonist as only LPS yielded in comparable results compared to progesterone in a pilot prospective randomized comparative study, and this result was later strengthened in a large retrospective analysis in which IR and LBR were higher in the GnRH agonist group (Bar Hava et al., 2017, Pirard et al., 2015). Theories of different mechanism of GnRH agonist postulated to enhance reproductive function are presented in Figure 6.

Table 2. Previous studies on the effect of the gonadotropin releasing hormone agonist (GnRH_a) administered on the day six (d6) of the luteal phase of IVF/ICSI and FET cycles.

Author, year, and country	Study design	N:o of transferred embryos mean (range or [SD])		Implantation rate %			Clinical pregnancy rate %		
		GnRH _a d 6	Control	GnRH _a d6	Control	p-value	GnRH _a d6	Control	p-value
IVF/ICSI stimulation with agonist protocol									
Fujii et al. 2001, Japan ⁴⁶	RCT	2.1 (0.1)	2.0 (0.1)	28.5	19.6	<0.05	44.5 (65/146)	34.3 (47/137)	<0.05
Tesarik et al 2006, Spain ¹⁵⁹	RCT	2.3 (0.5)	2.3 (0.5)	29.8	18.2	<0.05	51.1 (72/141)	41.5 (59/142)	NS
Ata et al 2008, Turkey ¹⁴	RCT	2.65 (2.58-2.72) ^b	2.51 (2.42-2.60) ^b	21.1	20.1	NS	42.8 (122/285)	42.1 (120/285)	NS
Razieh et al 2009, Taiwan ¹³⁷	RCT	2.3 (0.8)	2.4 (0.8)	12.3	7.3	0.04	25.6 (23/90)	10.0 (9/90)	0.015
Yldiz et al 2014, Turkey ¹⁸¹	RCT	2.71 (0.7)	2.68 (0.7)	20.7	1.3	NS	40.0 (40/100)	31.6 (30/95)	NS
Inamdar et al 2012, India ⁷¹	RCT	2.4 (2.31-2.53) ^b	2.54 (2.44-2.64) ^b	17.6	17.1	NS	NA	NA	
Kung et al 2014, Taiwan ⁹⁴	Retrospective cohort	NA	NA	NA	NA		48.1 (37/77)	37.5 (21/56)	NS
Simsek et al 2015, Turkey ¹⁵²	Retrospective cohort	3 (1-6)	3 (1-5)	NA	NA		NA	NA	
Aboulghar et al. 2015, Egypt ³	RCT	2.6 ± 0.6	2.7 ± 0.6	NA	NA		36.2 (81/224)	30.6 (68/222)	NS
IVF/ICSI stimulation with antagonist protocol									
Isik et al 2009, Turkey ⁸²	RCT cross-over	2.3 (1.2)	2.2 (1.2)	26.5	9.3	<0.0001	40.5 (30/74)	20.0 (16/80)	0.0055
Zafardoust et al 2015, Iran ¹⁸³	RCT	3.2 (0.9)	2.9 (1.02)	NA	NA		14.0 (6/43)	7.5 (3/40)	NS
Tesarik et al 2006, Spain ¹⁵⁹	RCT	2.2 (0.4)	2.3 (0.5)	27.1	17.4	>0.05	47.6 (69/145)	37.5 (54/144)	NS
Kung et al 2014, Taiwan ⁹⁴	Retrospective cohort	NA	NA	NA	NA		50.0 (35/70)	27.0 (10/37)	0.025
Check et al 2015, United States ³¹	Prospective cohort	1.9	2.0	37.9	33.1	NS	51.5 (69/134)	49.2 (97/197)	NS
Simsek et al 2015, Turkey ¹⁵²	Retrospective cohort	2 (1-5)	1 (1-5)	NA	NA		NA	NA	
Benmachiche et al 2017, Denmark ²³	RCT	2.3 (0.5)	2.4 (0.8)	27.3	23.8	NS	38.2 (63/165)	30.9 (51/165)	NS
Natural cycle FET									
Orvieto et al. 2016, Israel ¹²²	Retrospective cohort	1.8 (0.5)	1.8 (0.5)	31.1	17.3	<0.012	50.8 (30/59)	25.7 (19/74)	0.002
Artificial cycle FET									
Davar et al. 2015, Iran ³⁵	RCT	2.4 (0.62)	2.5 (0.63)	14.7	13.1	NS	26	21	NS
Ye et al. 2019, China ¹⁷⁹	RCT	2.0 (0.1)	2.0 (0.1)	41.0	39.2	NS	57.7 (246/426)	58.7 (212/361)	NS

^aLBR per transferred embryo, ^bmean (95% CI), ^cof positive pregnancy test

FET= frozen embryo transfer, hCG= human chorionic gonadotrophin, ICSI= intracytoplasmic sperm injection, IVF= in vitro fertilization, NA= not available, NS= not significant, RCT= randomized controlled trial

Ongoing or live birth rate			Miscarriage rate			Notification
GnRHa d6	% Control	p-value	GnRHa d6	% Control	p-value	
IVF/ICSI stimulation with agonist protocol						
23.6 (73/309) ^a	15.7 (44/280) ^a	<0.05	15.4 (10/65)	27.7 (13/47)	NS	Daily administration of 600 µg buserelin until pregnancy test
46.8 (66/141)	38.0 (54/142)	NS	NA	NA		0.1 mg triptorelin
31.2 (89/285)	29.5 (84/285)	NS	NA	NA		0.1 mg triptorelin
NA	NA		NA	NA		0.1 mg triptorelin
36.0 (36/100)	27.4 (26/95)	NS	4.0 (4/100)	4.2 (4/95)	NS	1 mg leuprolide
27.7 (59/213)	26.3 (56/213)	NS	24.3	30.7	NS	Three repeated dosages of 1 mg leuprolide
40.3 (31/77)	32.1 (18/56)	NS	NA	NA		Cause for administration of 0.1 mg triptorelin not available
40.8 (469/1149)	41.2 (230/558)	NS	16.8 (193/1149)	10.9 (61/558)	0.001	Up to six embryos transferred at ET. Cause for administration of 0.1 mg triptorelin not available
30.4 (68/224)	25.7 (57/222)	NS	NA	NA		Daily 0.1 mg triptorelin until pregnancy test
IVF/ICSI stimulation with antagonist protocol						
35.1 (26/74)	16.3 (13/80)	0.007	NA	NA		Cross-over study, combination of 0.5 mg leuprolide and hCG
NA	NA		NA	NA		0.1 mg triptorelin
44.8 (65/145)	31.9 (46/144)	<0.05	NA	NA		0.1 mg triptorelin
42.9 (30/70)	21.6 (8/37)	0.035	NA	NA		Cause for administration of 0.1 mg triptorelin not available
47.8 (64/134)	38.6 (76/197)	NS	NA	NA		1 mg leuprolide administered according to the patient's preference
32.8 (230/701)	34.4 (114/331)	NS	12.1 (85/701)	14.5 (48/331)	NS	0.1 mg triptorelin
36.4 (60/165)	30.7 (50/163)	NS	17.1 (13/76) ^f	27.5 (19/69) ^f	NS	0.1 mg triptorelin
Natural cycle FET						
45.8 (27/59)	20.3 (15/74)	<0.001	NA	NA		Intervention and control group from two consecutive time periods, combination of 0.1 mg triptorelin and hCG
Artificial cycle FET						
NA	NA		5	8	NS	0.1 mg triptorelin and repeated dosages of acetylsalicylic acid
46.7 (199/426)	49.3 (178/361)	NS	11.0 (27/246)	9.9 (21/212)	NS	0.1 mg triptorelin

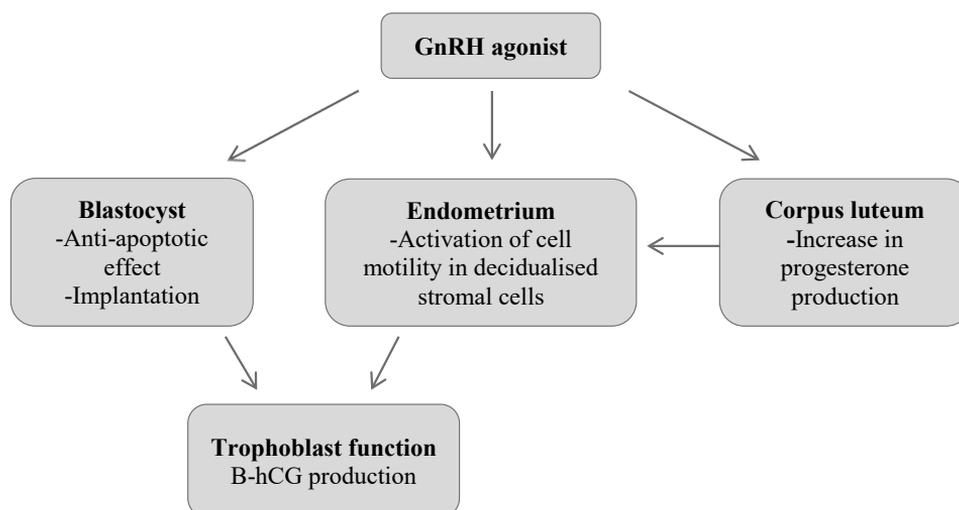


Figure 6. Theories of GnRH agonist action on reproductive function.

GnRH agonist in embryo-endometrial interaction

The expression of GnRH and its receptors is up-regulated in mouse embryo after the early blastocyst stage and in pregnant mouse uteri. Treatment with GnRH agonist has been shown to promote blastocyst development and inhibit apoptosis under *in vitro* culture conditions. Also, GnRH agonist antagonized the apoptotic effect of GnRH antagonist on blastocyst development. In all, these findings suggested that GnRH acts as an important growth factor in preimplantation mouse embryos. (Kawamura et al., 2005). Similarly, GnRH agonist added to the culture medium enhanced *in vitro* blastocyst formation of porcine embryos in a dose-dependent fashion, whereas the embryotrophic effect was reversed by supplementation of GnRH antagonist (Nam et al., 2005).

In human *in vitro* models, the results of the beneficial effect of GnRH agonist on embryo-endometrial interaction are inconsistent. In 1999 the presence of both GnRH and GnRH receptor in human blastocysts was demonstrated for the first time (Casañ et al., 1999). In a study by Klemmt et al. (2009), the *in vitro* decidualization capacity of human endometrial stromal cells or the level of hCG secreted by the implanting blastocysts were not affected by the presence of GnRH agonist administered in the culture medium. By contrast, Wu et al. (2015) demonstrated that GnRH agonist activated cell invasion, migration, and motility of human decidual endometrial stromal cells, suggesting an active role in the regulation of embryo-endometrial interactions. The difference in study protocols may explain this discrepancy; in a study by Klemmt et al. (2009), an endometrial sample was collected from non-pregnant uteri during the assumed WOI, whereas

Wu et al. (2015) investigated decidual tissue from women undergoing elective pregnancy termination in the 6th-12th weeks of gestation.

In vivo and *in vitro* β -hCG production has been demonstrated to be stimulated in the human placenta by GnRH agonist (Barnea et al., 1991, Siler-Khodr et al., 1997). Therefore, in clinical studies, β -hCG levels have been interpreted as a marker of a potential positive effect of GnRH agonist on early pregnancy development in IVF/ICSI cycles. Higher levels of β -hCG and increased LBR after administration of mid-luteal GnRH agonist in IVF/ICSI cycles were first reported by Tesarik et al. (2006). Furthermore, a retrospective analysis of an IVF/ICSI cohort with repeated GnRH agonist as sole luteal support revealed higher β -hCG levels among women older than 35 years compared to traditional progesterone support (Bar Hava et al., 2017). However, another study (Check et al., 2015) reported no difference in the β -hCG production in a non-randomized prospective study.

GnRH agonist and corpus luteum function

In addition to a possible direct effect on the endometrial receptivity and early embryo, mid-luteal GnRH agonist supplementation may facilitate corpus luteum function by increasing luteinizing hormone levels and thus prevent luteolysis. Luteal phase deficiency is evident in IVF/ICSI cycles, whereas in FET cycles, the endometrial milieu is either under normal cyclic hormonal regulation or endometrial receptivity is being controlled by exogenous hormonal substitution.

A stimulatory effect of GnRH agonist on corpus luteum in antagonist IVF/ICSI cycles is supported by the finding of increased levels of luteal phase progesterone and estradiol in cycles in which single dose GnRH agonist additional to progesterone or repeated dosages of GnRH agonist only were used (Bar Hava et al., 2017, Benmachiche et al., 2017). Interestingly, this effect was not evident in IVF/ICSI cycles stimulated with agonist protocol (Razieh et al., 2009, Fujii et al., 2001). Understandably no difference in steroid production was noted in hormonally substituted oocyte donation recipients due to the absence of corpus luteum (Tesarik et al., 2004).

Clinical studies demonstrate either a neutral or a stimulatory effect of luteal phase GnRH agonist on steroidogenesis in IVF/ICSI cycles. These findings conflict with *in vitro* studies in the human ovary, reporting that endogenous GnRH acts as a luteolytic factor in luteinized granulosa cells and thus decreases progesterone production. Treatments with GnRH agonist has been postulated to mimic the effect of endogenous GnRH, although results are not consistent (Metallinou et al., 2007).

2.4.4 Pregnancy outcomes

A summary of the study outcomes of luteal phase GnRH agonist in IVF/ICSI, NC- and AC-FET cycles are presented in Table 2.

Fresh embryo transfer

The first prospective controlled trial in 2004 demonstrated that IR and LBR after fresh ET in donated oocyte recipients under HRT were higher if a single dose of triptorelin was additionally administered in the luteal phase (Tesarik et al., 2004). Few studies have been conducted with findings of the positive effect of additional luteal phase GnRH agonist in IVF/ICSI, both in GnRH agonist and antagonist cycles (Razieh et al., 2009, Isik et al., 2009, Kung et al., 2014).

The use of GnRH agonist as adjuvant luteal support seems to increase IR in IVF/ICSI cycles when using the agonist protocol. Two RCTs reported a difference in IR, favoring a mid-luteal single dose GnRH agonist; Razieh et al. (2009) reported an increased CPR, and Tesarik et al. (2006) found an increased LBR per transferred embryo. Better IR, CPR, and LBR per transferred embryo were reported in an RCT in which repeated dosages of GnRH agonist were used in the luteal phase (Fujii et al., 2001). In two RCTs a clinically relevant, although statistically insignificant, difference in CPR and LBR favoring GnRH agonist use was observed (Aboulghar et al., 2015, Yıldız et al., 2014). In contrast, the largest RCT so far published failed to demonstrate a difference between traditional progesterone supplementation and additional single dose GnRH agonist in terms of ongoing pregnancy (Ata et al., 2008).

In IVF/ICSI cycles stimulated with antagonist protocol, three RCTs have shown an increase of IR if additional GnRH agonist was used. (Isik et al., 2009, Tesarik et al., 2006, Zafardoust et al., 2015). However, in an RCT by Benmachiche et al. (2017), no difference in IR was demonstrated. Most of those RCTs were underpowered to reveal statistical significance in CPR, although a clinically relevant difference was systematically noted (Benmachiche et al., 2017, Tesarik et al., 2006, Zafardoust et al., 2015). LBR was found to be increased in an RCT by Tesarik et al. (2006), both CPR and LBR were higher in GnRH agonist added cycles compared to traditional progesterone supplementation in RCT by Isik et al. (2009). In a retrospective analysis by Kung et al. (2014) CPR and LBR were higher in IVF/ICSI cycles stimulated with antagonist protocol when additional luteal phase GnRH agonist was administered, whereas in cycles stimulated with agonist protocol, no difference was observed. Notably, the effect of mid-luteal GnRH supplementation in antagonist IVF/ICSI cycles was most prominent in a subgroup of women with higher basal FSH and lower number of mature oocytes.

Natural cycle FET

The effect of a single dose GnRH agonist on pregnancy outcomes in NC-FET has been evaluated in only one retrospective study in which the outcomes of two different NC-FET LPS protocols from consecutive periods were compared. During the first period, 74 women used progesterone for luteal support, whereas during the second period, a modified luteal support protocol with two additional injections, hCG and GnRH agonist on the day of transfer and four days later, were administered in 46 women. Improvement in IR and ongoing PR were observed in the group using a modified luteal support protocol (Haas et al., 2015). Later, the same research group extended their study to include pregnancy outcomes of AC-FETs from the corresponding periods in the analysis. In this analysis, the 59 women using modified NC-FET LPS protocol were included. The beneficial effect of modified NC-FET luteal support protocol on pregnancy outcomes also sustained in the re-analysis of the data (Orvieto et al., 2016).

Artificial cycle FET

The positive effect of a single dose mid-luteal GnRH agonist supplementation to hormonally substituted cycles was first demonstrated by Tesarik et al. (2004) on oocyte recipients. Recipients were prepared according to the AC-FET protocol, whereas embryo was attained from the oocyte donor after COH performed according to the agonist protocol. Oocytes from one donor were equally shared by two recipients, and these recipients were randomized to receive either placebo or GnRH agonist in addition to progesterone. Fresh ET of cleavage stage embryo was performed into the hormonally prepared uterus of the recipient. IR was found to be increased in the intervention group, whereas the difference of 12.3 percentage points in LBR pointing to the benefit of GnRH agonist use remained but was statistically insignificant.

To date, three RCTs on mid-luteal GnRH agonist supplementation additional to progesterone in AC-FET cycles have been conducted. Davar et al. (2015) used repeated dosages of ASA along with estradiol supplementation, and 200 women were randomized to receive either progesterone only or an additional single dose of GnRH agonist three days after FET. The IRs were similar between the groups, and the difference of 5 percentage points in ongoing PR favoring GnRH agonist supplementation remained insignificant. The results were in line with the most recent RCT by Ye et al. (2019), in which a total of 868 AC-FET cycles were randomized to receive a single dose of mid-luteal GnRH agonist or standard HRT. The difference in IR favoring GnRH agonist administration was only seen in the subgroup of women over 35 years old (Ye et al., 2019). In an RCT by Zarei et al. (2017), women undergoing AC-FET were randomized into four groups to receive luteal support with vaginal progesterone (n=100), oral

dydrogesterone (n=100), vaginal progesterone combined with a single dose of GnRH agonist (n=100), and oral dydrogesterone combined with hCG (n=100). Ongoing PR was compromised in the group using oral dydrogesterone, whereas no difference was observed among the other groups. However, there were inconsistencies in the reported results, and the quality of the study was questionable.

2.4.5 Safety of GnRH agonist administration in the luteal phase

Safety data of GnRH agonist on neonatal outcomes is based on previous reports of normal pregnancies and healthy neonates born after intended or inadvertent exposure to luteal phase GnRH agonist (Abu-Heija et al., 1995, Cahill et al., 1994, Chardonens et al., 1998, Gartner et al., 1997, Marcus and Ledger, 2001). None of the studies evaluating single or continuous use of short-acting GnRH agonist as LPS in addition to progesterone in fresh ET or FET cycles have included neonatal data in the study outcomes.

2.5 Quality of evidence and open questions

Earlier data on the developmental capacity of MN embryos suggested very poor pregnancy potential but has been contradicted by newer studies. All in all, data on the pregnancy and obstetric outcomes of the BN/MN embryos are still scarce. Since the BN/MN embryos are often deselected from ET, evidence regarding the significance of blastomere multinucleation has remained limited. Therefore, it is still an open question of whether BN/MN embryos are safe to transfer or suitable for cryopreservation.

The heterogeneity in the LPS regimens in different studies generates limitations on the interpretation of the results. In three meta-analyses conducted on the subject, a mid-luteal single dose of GnRH agonist improved IR, CPR, ongoing pregnancies, and LBR, especially in the antagonist ICF/ICSI cycles. However, the overall quality of evidence is too low to draw clear conclusions on the potential effect of mid-luteal GnRH agonist supplementation (Kyrou et al., 2011, Martins et al., 2016, Oliveira et al., 2010). Evidence on the effect on mid-luteal use of GnRH agonist in FET cycles is even more limited. However, the policy of using GnRH agonist as luteal support is adopted widely in clinical use. Further studies are warranted to provide information about the clinical relevance of GnRH agonist as luteal support.

3 AIMS OF THE STUDY

This thesis was designed to study the significance of embryo quality and modified luteal support regarding the results of FET cycles. Regarding embryo quality, multinucleation was the focus of interest. More specifically, the occurrence and contributing factors of multinucleation in frozen-thawed embryo cohorts and the effect of multinucleation on the course of pregnancy and neonatal health were explored. Furthermore, in the present study, the effect of the short-acting GnRH agonist, triptorelin, as additional adjuvant therapy in the mid-luteal phase of FET cycles was evaluated.

The specific aims were as follows:

- 1 To evaluate the *de novo* occurrence of embryo multinucleation before and after embryo cryopreservation and assess whether the presence of BN/MN embryos in an embryo cohort before cryopreservation was associated with a higher occurrence of multinucleation after thawing (**Study II**).
- 2 To assess whether female age or ovarian response to COH was associated with *de novo* occurrence of multinucleation before and after embryo cryopreservation (**Study II**).
- 3 To study pregnancy results, obstetric outcomes, and health of the newborns in FET cycles using BN/MN embryos (**Studies I and III**).
- 4 To study whether a single subcutaneous dose of GnRH agonist (triptorelin) administered at the mid-luteal phase of NC- or AC-FET cycles could improve pregnancy outcomes (**Studies IV and V**).

4 MATERIALS AND METHODS

4.1 Study populations and study designs

This thesis consists of five publications. **Study I:** A case report of CT after the transfer of an MN frozen-thawed embryo. **Studies II-III:** Two historical cohorts representing the occurrence of multinucleation in frozen-thawed embryo cohorts, clinical factors related to the formation of multinucleation as well as pregnancy and obstetric outcomes of BN and MN FETs. **Studies IV-V:** A prospective randomized clinical pilot study (**Study IV**) and a prospective interventional study (**Study V**) evaluating the effect of a single dose of triptorelin as an additional LPS in NC- and AC-FET cycles. The information of data collection times and locations, as well as inclusion and exclusion criteria, are described in Table 3.

Table 3. Study period, location, and inclusion and exclusion criteria.

Study I
Year 2013 Turku University Hospital, Turku, Finland
Inclusion criteria: A case of conjoined twins after transfer of frozen-thawed MN embryo
Exclusion criteria: None
Study II
January 2010 – December 2012 Turku University Hospital, Turku, Finland
Inclusion criteria: 415 consecutive IVF/ICSI cycles and embryo cryopreservation and frozen embryo transfers until 4/2014 Subanalysis: Multinucleation in embryo cohorts with or without embryo cryopreservation (2012)
Exclusion criteria: None
Study III
January 2009 – December 2015 Turku University Hospital, Turku, Finland
Inclusion criteria: Case group: Single or double BN or MN FET Control group: Single or double mononucleated FET Subanalysis: Pregnancy outcomes of BN vs. MN FET
Exclusion criteria: Indistinct documentation on blastomere multinucleation Subanalysis: double FETs including BN and MN embryos
Study IV-V
May 2013 – December 2014 Turku University Hospital, Turku, Finland and Väestöliitto Fertility Clinic ¹ , Turku, Finland (Study IV) Turku University Hospital, Turku, Finland and Tampere University Hospital, Tampere, Finland (Study V)
Inclusion criteria: Scheduled NC FET (Study IV) or AC-FET (Study V) Duration of infertility ≥ 12 months
Exclusion criteria: Female age > 42 years at the time of embryo freezing Female or male chromosomal abnormality, use of testicular or donated sperm without female cause for infertility, use of donated oocytes Congenital uterine anomalies, intramural myomas (> 4 cm) Pathologies distorting the uterine cavity Endometrium thickness < 6 mm prior to FET Untreated thyroid dysfunction or hyperprolactinemia Allergy to triptorelin

IVF = in vitro fertilization, ICSI = intracytoplasmic sperm injection,
FET = frozen embryo transfer, BN = binucleated, MN = multinucleated,
NC = natural cycle, AC = artificial cycle

¹Now Mehiläinen Felicitas

4.1.1 Study I

Study I was a case report of CT pregnancy after a transfer of MN frozen-thawed embryo. A 33-year-old healthy woman and a 37-year-old man suffered from infertility due to azoospermia after stem cell transplantation for hematologic malignancy. Sperm was frozen before the malignancy treatment and used for ICSI. An antagonist protocol (Gonal-F 175 IU for 9 days and Cetrotide 0.25 mg for 6 days) was followed for COH and out of 10 obtained oocytes, eight fertilized normally. At the 2-cell stage, all embryos contained MN blastomeres, and after cleavage at the 4-cell stage, each embryo eligible for freezing carried either one BN or MN blastomere. The slow freezing method was used for embryo cryopreservation. After an unsuccessful transfer of a fresh mononucleated 4-cell stage embryo, FET was performed during NC. An 8-cell embryo containing three MN blastomeres was selected for transfer after overnight culture of three thawed embryos.

4.1.2 Studies II and III

Studies II - III involved a retrospective analysis of prospectively collected data from Turku University Hospital medical records and IVF laboratory documents. Data included detailed information on the embryo classification, freezing, and thawing methods, as well as the demographics of the women and characteristics of the IVF/ICSI and FET cycles.

Study II consisted of 415 consecutive IVF/ICSI cycles with embryo cryopreservation by slow freezing. Data on embryo thawing and post-thaw culture was tracked until the end of April 2014. Drop out in sample data included cryopreserved but not thawed embryos (n=2227) as well as embryos that had degenerated during the thawing process (n=143) (Figure 7). Data on objectively measurable parameters known to reflect ovarian competence during gonadotropin treatment were retrieved from medical records and included female age at the time of oocyte pick up, IVF/ICSI stimulation protocol, number of retrieved oocytes, consumption of total recombinant FSH or human menopausal gonadotropin, and the proportional dose of FSH or human menopausal gonadotropin per obtained oocyte. Due to the difference in pharmacokinetic profiles, cycles stimulated with corifollitropin alfa (n=25) were not included in the analysis of FSH consumption. Two additional approaches were used to further analyze associations between clinical factors and the occurrence of multinucleation: IVF/ICSI cycles were divided into three groups according to the female age (20–29 years, 30–35 years and 36–40 years) and assessed in relation to number of harvested oocytes (1–8; 9–17; ≥18) and proportional FSH used per harvested oocyte (1– 84 IU, 85–237 IU and ≥ 238 IU). Division to the categories for oocyte number and FSH consumption per collected oocyte was defined from upper and lower quartiles of the data distribution.

Flowchart of the study II		≥ one MN embryo/ embryo cohort	MN embryos/ all embryos
D2	No of 415 IVF/ICSI cycles: 415 No of embryos: 3430	62.4% (259/415)	16.7% (572/3430)
D2t	No of embryo cohorts with thawing: 330 No of thawed embryos: 1203		
	No of embryo cohorts with survived embryos: 320 No of survived embryos: 1060	16.3% (52/320)	5.8% (61/1060)
D3t	No of embryo cohorts with overnight culture: 265 No of cultivated embryos after thawing: 771	31.7% (84/265)	14.9% (108/771)

D2: day of embryo culture
D2t: day of thawing
D3t: day after thawing and an overnight culture

Figure 7. Number of IVF/ICSI cycles and *de novo* occurrence of multinucleation in embryo cohorts and embryos at each time point of evaluation.

Compared to 2010 and 2011, the database from 2012 was more comprehensive and included information about IVF/ICSI cycles not eligible for cryopreservation. These data provided an opportunity to evaluate whether the incidence of MN was different in IVF/ICSI cycles with cryopreservation (n=137) compared to cycles without freezing (n=58).

For **Study III**, 1,335 frozen-thawed ETs were evaluated from the IVF laboratory documents, and single and double ETs comprised exclusively of BN/MN embryos (n=136) were included in the Case group. The Control group (n=136) consisted of randomly selected ETs of mononucleated embryos among women matched for age at the time of oocyte pick up. Also, a sub-analysis of pregnancy results of FETs containing either BN or MN embryos exclusively were conducted. FET protocols included NC, AC, and OI cycles with clomifene citrate or low dose gonadotropin. Demographics of the women and cycle characteristics are presented in Table 4. Standard LPS with vaginal micronized progesterone 200mg twice daily was administered in natural and OI cycles. In AC-FET, standard HRT included oral and transdermal estrogen combined to 600 mg vaginal micronized progesterone when the endometrial thickness was at least 6 mm and the absence of a dominant follicle was determined with vaginal ultrasound. Data on the course of pregnancy and health of the newborns were obtained from the medical records.

4.1.3 Studies IV and V

Study IV was a prospective randomized clinical pilot study and **Study V**, a prospective interventional pilot study. Demographics of the women and cycle characteristics are presented in Table 4.

Table 4. Demographics of the women and cycle characteristics.

	Study III		Study IV		Study V	
	Case	Control	Intervention	Control	Intervention	Control
N	136	136	65	62	72	72
	Mean (SD)					
Female age at OPU	32.3 (4.2)	32.5 (4.3)	33.0 (3.8)	34.0 (5.7)	31.9 (3.6)	32.5 (3.7)
BMI (kg/m ²)	24.1 (3.9)	24.0 (3.7)	23.2 (3.3)	23.8 (3.4)	24.0 (4.0)	24.3 (4.5)
No of previous pregnancies	0.8 (1.2)	0.6 (1.0)	—	—	—	—
No of previous deliveries	0.3 (0.6)	0.3 (0.6)	—	—	—	—
Antral follicle count	21 (10.4)	19 (9.2)	—	—	—	—
Number of transferred embryos	1.1 (0.3)	1.1 (0.3)	1.1 (0.4)	1.1 (0.3)	1.1 (0.3)	1.1 (0.2)
Endometrial thickness (mm)	—	—	9.7 ± 2.0	9.1 ± 1.8	9.7 (2.0)	9.1 (1.8)
Duration of infertility at OPU	47.7 (23.9)	47.4 (30.9)	42.0 (27.6)	42.1 (31.1)	41.0 (22.4)	43.1 (24.6)
	n (%)					
Primary infertility	71 (52.2)	89 (65.4)	36 (55.4)	35 (56.5)	32 (44.4)	34 (47.2)
Etiology for Infertility						
Female only	58 (42.7)	64 (47.1)	22 (33.4)	31 (50.0)	39 (54.2)	39 (54.2)
Male	42 (30.9)	32 (23.5)	16 (24.6)	17 (27.4)	10 (13.9)	9 (12.5)
Combined	23 (16.9)	15 (11.0)	10 (15.4)	3 (4.8)	11 (15.3)	12 (16.7)
Unknown	12 (8.8)	24 (17.7)	17 (26.2)	11 (17.7)	12 (16.7)	10 (13.9)
Not available	1 (0.7)	1 (0.7)	0	0	0	2 (2.8)
Long stimulation protocol	76 (55.9)	75 (55.1)	35 (53.8)	35 (56.5)	27 (37.5)	27 (37.5)
Live birth from fresh ET	30/125 (24.0)	24/124 (19.4)	—	—	—	—
All embryos frozen (n)	11	12	—	—	—	—
FET during natural cycle	79 (58.0)	95 (69.9)	65 (100)	62 (100)	—	—
FET during artificial cycle	55 (40.4)	41 (30.1)	—	—	72 (100)	72 (100)
FET during ovulation induction	2 (1.5)	0	—	—	—	—
Single embryo transfer	121/136 (89.0)	119/136 (87.5)	56/65 (86.2)	54/62 (87.1)	65/72 (90.3)	68/72 (94.4)
FET at cleavage stage	130 (95.6)	126 (92.6)	65 (100)	62 (100)	40 (55.6)	42 (58.3)
Completely survived embryos	116/151 (76.8)	118/153 (77.1)	58/74 (78.4)	58/70 (82.9)	68/80 (85.0)	73/76 (96.0)
Normally cleaved embryos ^a	77/122 (63.1)	74/109 (67.9)	33/57 (57.9)	34/53 (64.2)	54/59 (91.5)	51/58 (87.9)
Detection of BN/MN						
2 – cell stage	1/151 (0.7)					
4 – cell stage	63/151 (41.7)					
8 – cell stage	87/151 (57.6)					

Values are mean (SD) unless otherwise indicated

^aNormal cleavage defined as doubled blastomere number after an overnight culture

BMI= body mass index, ET= embryo transfer, FET= frozen embryo transfer, OPU= oocyte pick up, BN= binucleation, MN= multinucleation

Study IV included 98 women scheduled for NC-FET and **Study V** 107 women scheduled for AC-FET. Selection of the intervention treatment was carried out using sealed opaque envelopes. Women randomized to NC-FET Control group received standard LPS and women randomized to the AC-FET Control group underwent standard HRT as noted above in the description of **Study III**. In both intervention arms, an additional single dose of triptolerin 0.1 mg was administered subcutaneously when the age of the transferred embryos reached six days.

In NC-FETs, vaginal ultrasound assessment was performed in the preovulatory phase to identify the dominant follicle growth, and in AC-FET, the absence of a dominant follicle was determined. Endometrial thickness of at least 6 mm was confirmed before proceeding to NC-FET or progesterone supplementation in AC-FET. In NC-FET, ovulation was determined using commercial urine-based ovulation kits, and standard LPS administration was started when the embryonic age reached three days.

Standard LPS was continued until the pregnancy test in NC-FET, whereas in AC-FET, HRT was continued until the ninth week of gestation or a negative pregnancy test. When no ongoing pregnancy occurred, and surplus embryos were available, a second attempt with crossover design was scheduled (Figure 8).

	Study III		Study IV	
	98		107	
Participants				
Randomisation	48	50	51	56
Exclusion	6	2	0	2
	Control	Intervention	Control	Intervention
N	42	48	51	54
Exclusion, <i>pregnant or no surplus embryos</i>	25	28	33	33
	Crossover		Crossover	
	Control	Intervention	Control	Intervention
N	20	17	21	18
Total no of FET cycles	62	65	72	72

Figure 8. Flowchart of the study population, **Studies III and IV.**

In **Study IV**, an analysis of β -hCG- concentrations from plasma samples by electrochemiluminescence immunoassay (Cobas 8000, Roche Diagnostics GmbH, Mannheim, Germany) was performed when the embryonic age reached 16 days.

4.2 Laboratory methods

4.2.1 Embryo assessment (Studies I-III)

Blastomere number and evenness, fragmentation, and the nuclei of the embryos were systematically evaluated before cryopreservation (D2), within one hour after thawing (D2t) and 23-25 hours after thawing (D3t). Embryo assessment was performed simultaneously by two experienced observers, one via inverted microscope (Nikon Diaphot 300) with 400 \times magnification and the other via a connected PC monitor, and in case of discrepancy in embryo grading repeated for consensus. In **Study II**, embryos were graded as either mononucleated or MN (two or more nuclei detected in at least one blastomere). Because the objective was to assess the *de novo* occurrence of multinucleation at each time point of evaluation, all MN embryos detected on D2 or D2t were excluded from further analysis.

In **Study III**, embryos were classified as mononucleated, BN, or MN according to the nuclear status of the embryos (Figure 9). Embryos exhibiting BN/MN before or after cryopreservation were grouped together due to the limited number of cases. If there was no blastomere loss after thawing, embryos were rated as “completely survived”. Resumption of mitosis was classified as normal if the number of blastomeres had doubled during an overnight culture following thawing. BN/MN embryos were transferred if normal embryos of good quality were not available. A maximum of two embryos was transferred per FET cycle.

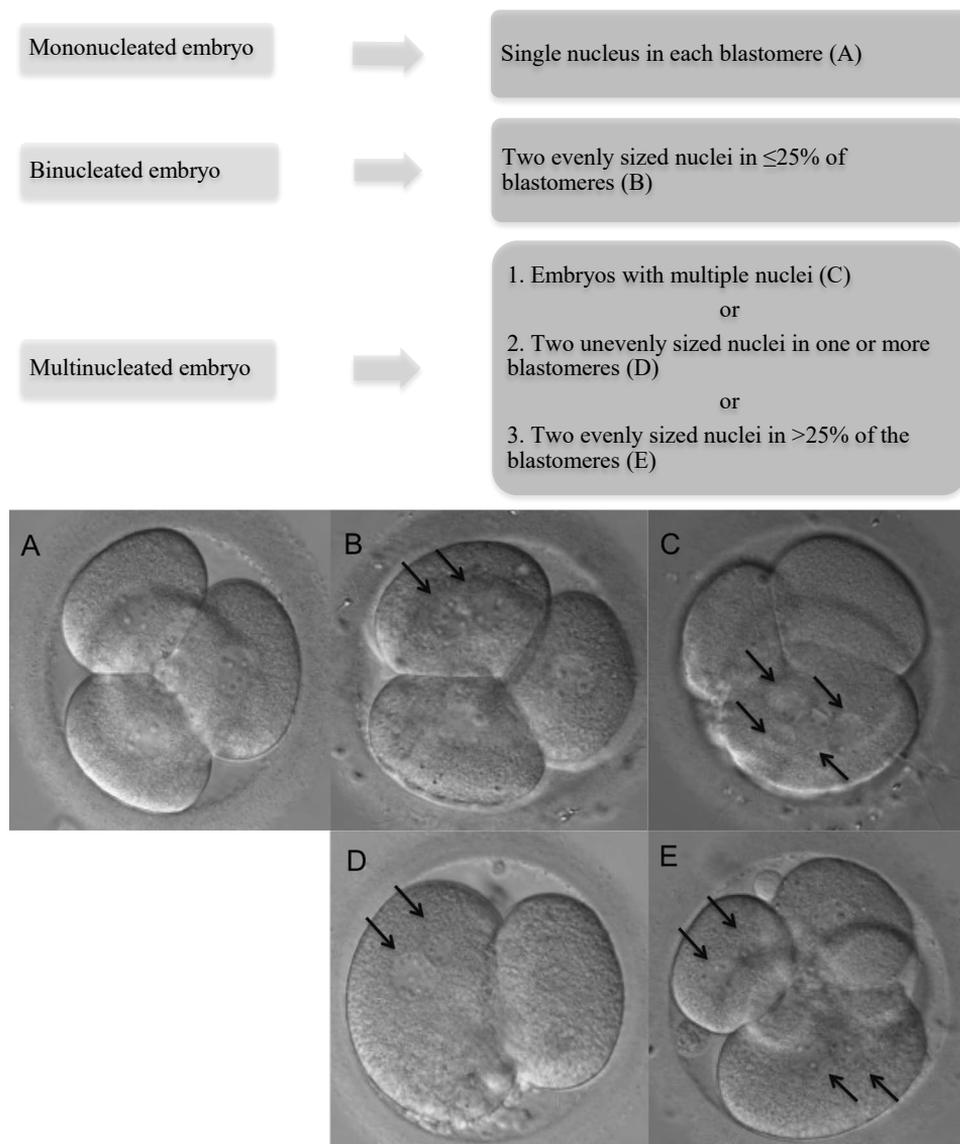


Figure 9. Definition of nuclear abnormalities (**Study III**).

Eligibility criteria for embryo cryopreservation included less than 25% fragmentation or difference in blastomere size. BN/MN embryos were not automatically excluded from cryopreservation if general criteria were met.

4.2.2 Embryo freezing and thawing

During the period covering **Studies I-V** (2009-2015) embryo cryopreservation in the Turku University Hospital was performed using a slow freezing protocol. A slow freezing cryopreservation kit (Sydney IVF Cryopreservation kit, K-SICS-5000, Cook Australia 2009-2013 and Freeze Kit Cleave, 10166, Vitrolife Sweden, 2014-2015) was used according to the manufacturer's protocol and the cooling rate was controlled with a freezer (Planer Kryo 10-MRV, Planer PLC, Sunbury on Thames, UK). The thawing was performed by rapid warming in a 30°C water bath and rehydration in a series of media with decreasing cryoprotectant concentrations (Sydney IVF Thawing Kit, K-SITS-5000, Cook Australia years 2009-2013 and Thaw Kit Cleave, 10167, Vitrolife Sweden years 2014-2015). Afterwards, the embryos were incubated for 5 minutes in a cryopreservation buffer at RT, and subsequently, the dish was placed for 5 minutes on a heated stage at 37 °C. If feasible, the embryos were cultured overnight (Sydney IVF, K-SICM-50, Cook Australia years 2009-2012 and G-1-Plus medium, 10128, Vitrolife Sweden years 2013-2015) in 7% CO₂, 8% O₂, and 85% N₂ at 37±0.1°C.

In the Väestöliitto Fertility Clinic (now Mehiläinen Felicitas), Turku embryos were cryopreserved using the slow freezing protocol (**Study IV**). Cryopreservation and thawing kits were used according to the manufacturer's protocol (Freeze Kit Cleave and Thaw Kit Cleave, Vitrolife Sweden) and cooling rate was controlled using Planer Kryo 360-1.7. Embryo cryopreservation method in the Tampere University Hospital (**Study V**) included vitrification of embryos using Vitri Freeze Kit ES, Fertipro, Belgium, and thawed using Vitri Thaw Kit ES, Fertipro, Belgium.

4.3 Statistical analyses

The main aspects of the statistical analyses in sub-studies are described in Figure 10. The statistical significances of the differences between frequency distributions were tested with Pearson's chi-squared or Fisher's exact test for categorical variables and with Student's t-test for continuous variables with a normal distribution. Normality of the distributions was evaluated visually and with Shapiro-Wilk's test. Odds ratios and 95% confidence intervals (CIs) were calculated using univariable and multivariable binary logistic regression analysis.

Statistical analyses

STUDY II

Variables:

Dependent variable: Blastomere multinucleation in an embryo cohort

Independent variables: Female and male age, total and proportional FSH consumption, number of retrieved oocytes

Statistical analyses:

SAS version 9.4 (SAS Institute, Cary, NC, USA)

1. Differences in frequency distributions: **Pearson's Chi Square**
2. **Univariable binary logistic regression analysis** was used to assess the associations between the dependent and the independent variables
3. **Multivariable binary logistic regression analysis** included the independent variables showing significance in the univariate analysis

STUDY III

Outcome measures:

1. Pregnancy and obstetric outcomes, obstetric complications and health of the newborns after transfer of mononucleated or bi/multinucleated frozen-thawed embryo
2. Reproductive potential of bi- vs. multinucleated embryos
3. Subanalysis: Pregnancy outcomes of bi- vs. multinucleated embryos

Statistical analyses:

JMP Pro, Version 12 and SAS System for Windows, version 9.4

1. Categorical variables: **Pearson's chi-squared** or **Fisher's exact test**
2. Continuous variables: **Student's t-test**
3. **Multivariable logistic regression** was used to adjust for duration of infertility, BMI, degree of blastomere survival, resumption of mitosis, and number of transferred embryos
4. Birth weight was analyzed using the **linear mixed model**, adjusted for BMI

STUDIES IV-V

Outcome measures:

Pregnancy outcomes in natural and artificial frozen embryos transfer cycles after administration of luteal phase single-dose triptolerin

Statistical analyses:

SPSS software system for Windows, versions 22 and 23

1. Carryover effect: **Logistic regression analysis**
2. **Logistic regression analysis with generalized estimation equation extension** was applied. Results are adjusted for maternal age at COH and duration of infertility (**Studies IV-V**) and cryopreservation method and embryonic age at the time of FET (**Study V**)
3. β -hCG- concentrations: **Student's t-test**

Figure 10. Statistical analyses.

In **Study III**, stratified logistic regression analysis and linear mixed models were used in the analyses to take account the matching of cases and controls.

For **Studies IV-V**, analysis was conducted on the FET cycle level for all cycles, and the carryover effect was first tested using a logistic regression model. A logistic regression model was applied with generalized estimation equations to account for dependence among repeated treatments. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using SAS System for Windows, Version 9.4 (SAS Institute Inc, Cary, NC, USA); (**Study II-III**), JMP Pro, Version 12-(**Study III**); SPSS software (IBM SPSS Statistics for Windows, Versions 22.0 (**Study IV**) and 23.0 (**Study V**)).

4.4 Ethics

Written informed consent for publication of the case report (**Study I**) was given by the patient. **Study II** had approval from the Joint Ethics Committee of Turku University Hospital (117/1801/2013), and **Studies III-V** from the Ethical Committee of the Hospital District of Southwest Finland (**Study III** 34/1801/2014 and **Studies IV-V**: 36/1800/2013). For **Studies IV-V**, each participant signed a written informed consent (Clinical Trials study number NCT02620124).

5 RESULTS

5.1 *De novo* occurrence of MN embryos before freezing, after thawing and after overnight culture (Study II)

De novo occurrence of multinucleation was evaluated among embryos and embryo cohorts before freezing, after thawing and after an overnight culture after thawing (Figure 7). Embryo multinucleation was most common before freezing. Accordingly, the occurrence of multinucleation in embryo cohorts was more prominent before freezing than after an overnight culture after thawing ($p < 0.001$). The presence of MN embryos in an embryo cohort before freezing (D2) was not associated with *de novo* multinucleation in sibling embryos after thawing (D2t) ($p = 0.113$) or after an overnight culture after thawing (D3t) ($p = 0.845$).

In the sub-analysis of the IVF/ICSI cycles (2012), the overall incidence of multinucleation before freezing (D2) was similar between the cycles with ($n = 137$) or without ($n = 58$) cryopreservation (66.4% vs. 70.7%, $p = 0.560$).

5.2 Multinucleation in embryo cohorts, female age and ovarian response (Study II)

The mean age of the women at the time of ovum pickup was 33 ± 4 years (range 23–40). The frequency of MN embryos decreased steadily along with increasing female age (OR for four years increase in female age: 0.69 [95% CI 0.56–0.85, $p < 0.001$]).

To further explore the effect of female age and ovarian response, embryo cohorts were divided into three groups according to the female age (20–29 years, 30–35 years and 36–40 years). An interaction between the presence of MN embryos, female age, and the number of harvested oocytes ($p < 0.001$) was detected on day two of embryo culture before freezing (D2): the frequency of MN embryos was the lowest in older women with a low number of collected oocytes. For a more detailed analysis, the three female age categories were divided into subgroups based on the number of retrieved oocytes (1–8; 9–17; ≥ 18). MN embryos were most frequently observed in the age group of 20–29 years, and in this age group, the occurrence was not associated with the number of retrieved oocytes. On the contrary, in the age groups of 30–35 years and 36–40 years, an association between high ovarian response and blastomere multinucleation in embryo cohorts was seen (Table 5). Notably, these associations were only seen before cryopreservation (D2) and disappeared after thawing (D2t) ($p = 0.964$ and $p = 0.551$, respectively) and after an overnight culture after thawing (D3t) ($p = 0.806$ and $p = 0.189$, respectively). Furthermore, at

these points of evaluation, no interaction effect between multinucleation and number of collected oocytes ($p=0.926$ and $p=0.405$) was detected in any age group.

Table 5. Multinucleation on day-2 of embryo culture in relation to female age and number of obtained oocytes per ovum pick up (**Study II**).

Female age (years)	No. of IVF/ICSI cycles	No of oocytes 1–8 % (n/n) ^a n= 95	No of oocytes 9–17 % (n/n) ^a n=214	No of oocytes > 17 % (n/n) ^a n=106	<i>p</i> -value ^b
20 – 29	87	69.2 (9/13)	72.5 (29/40)	79.4 (27/34)	NS
30 – 35	207	38.5 (15/39)	69.2 (81/117)	78.4 (40/51)	<0.001
36 – 40	121	20.9 (9/43)	59.7 (34/57)	71.4 (15/21)	<0.001

^aNo. of embryo cohorts with multinucleation/all cohorts in a subgroup in parentheses

^bChi Square test

IVF = in vitro fertilization, ICSI = intracytoplasmic sperm injection, FSH = follicle stimulating hormone

A similar pattern in the occurrence of MN embryos in embryo cohorts was detected when FSH consumption was taken into account. Before cryopreservation (D2), the occurrence of multinucleation decreased with an increase in the total and proportional FSH consumption, but the association vanished after thawing (D2t) and after overnight culture after thawing (D3t) (Table 6). An interaction between female age, proportional FSH consumption, and the presence of MN embryos were also seen when the study group was further divided into age groups ($p=0.003$). In line with the associations detected between female age and number of retrieved oocytes, no difference on the occurrence of MN embryos was detected in the age group of 20–29 years, whereas in women aged 30–35 years and 36–40 years MN embryos were more common when the proportional dose of FSH was low. In the most advanced age group of 36–40 years, a similar association was also detected after thawing (D2t) and after an overnight culture after thawing (D3t) (Table 7).

Table 6. Multinucleation in relation to total and proportional FSH consumption in IVF/ICSI cycles on day-2 of embryo culture (D2), after thawing (D2t) and on day-3 of embryo culture after thawing (D3t) (**Study II**).

Time point of embryo evaluation		Embryo cohorts without multinucleation	Embryo cohorts with multinucleation	<i>p</i> -value ^a	OR (95% CI)
D2	No. of IVF/ICSI cycles	146	244		
	FSH dose (IU)	2167.4 ± 899.1	1815.5 ± 721.4	<0.001	0.65 (0.52-0.80)
	Prop. FSH ^b	275.4 ± 237.2	144.6 ± 116.9	<0.001	0.36 (0.36-0.50)
D2t	No. of IVF/ICSI cycles	255	46		
	FSH dose (IU)	1980.7 ± 815.5	1882.3 ± 761.0	NS	0.89 (0.63-1.23)
	Prop. FSH ^b	199.5 ± 190.5	167.3 ± 193.9	NS	0.81 (0.55-1.20)
D3t	No. of IVF/ICSI cycles	177	81		
	FSH dose (IU)	1987.7 ± 815.5	1893.2 ± 763.0	NS	0.88 (0.67-1.17)
	Prop. FSH ^b	201.9 ± 179.7	158.3 ± 144.4	NS	0.71 (0.49-1.02)

Numbers are mean ± SD

^aLogistic regression analysis

^bProportional FSH (IU/oocyte)

IVF/ICSI cycles stimulated with corifollitropin excluded from the analysis (n=25)

FSH= follicle stimulating hormone, IVF=, in vitro fertilization, ICSI, intracytoplasmic sperm injection

Table 7. Multinucleation in relation to proportional FSH consumption and female age in IVF/ICSI cycles on day-2 of embryo culture (D2), after thawing (D2t), and on day-3 of embryo culture after thawing (D3t) (**Study II**).

Time point of embryo evaluation	Female age (years)	No. of IVF/ICSI cycles	Categories of proportional FSH consumption, % (number of embryo cohorts with multinucleation/ all cohorts in the subgroup)			p-value ^a	OR (95% CI)
			1 – 84 IU/ oocyte	85 – 237 IU/ oocyte	≥ 238 IU/ oocyte		
D2	20 – 29	86	81.1 (30/37)	71.1 (27/38)	72.7 (8/11)	NS	0.95 (0.53-1.69)
	30 – 35	195	88.2 (45/51)	63.6 (68/107)	40.5 (15/37)	<0.001	0.25 (0.13-0.47)
	36 – 40	109	75.0 (6/8)	59.6 (31/52)	28.6 (14/49)	<0.001	0.35 (0.20-0.63)
D2t	20 – 29	73	25.8 (8/31)	9.4 (3/32)	20.0 (2/10)	NS	1.33 (0.75-2.35)
	30 – 35	142	12.8 (5/39)	13.2 (10/76)	11.1 (3/27)	NS	0.87 (0.44-1.71)
	36 – 40	86	37.5 (3/8)	23.1 (9/39)	7.7 (3/39)	0.033	0.33 (0.12-0.91)
D3t	20 – 29	60	46.4 (13/28)	16.0 (4/25)	57.1 (4/7)	NS	0.89 (0.30-2.67)
	30 – 35	120	28.1 (9/32)	29.2 (19/65)	21.7 (5/23)	NS	0.83 (0.49-1.42)
	36 – 40	78	75.0 (6/8)	34.3 (12/35)	25.7 (9/35)	0.028	0.47 (0.20-0.90)

^aLogistic regression analysis

IVF/ICSI cycles stimulated with corifollitropin excluded from analysis (n=25)

IVF = in vitro fertilization, ICSI = intracytoplasmic sperm injection, FSH = follicle stimulating hormone

5.3 Pregnancy and perinatal outcomes after transfer of BN or MN frozen-thawed embryos (Study III)

Univariate analysis was first carried out to test differences in pregnancy outcomes between the Case and the Control groups. IR, CPR, ongoing PR at gestational week 12 and LBR were lower in the Case group than in the Control group (28.0% and 39.3%, $p = 0.036$, 29.4% and 44.1%, $p = 0.012$, 22.8% and 36.0%, $p = 0.017$, and 22.1% and 36.0%, $p = 0.011$, respectively). However, no difference in miscarriage rate was detected (20.0% vs. 15% in the Case and the Control group, respectively). Multivariable logistic regression analysis confirmed the differences in CPR and LBR and similar miscarriage rate between the Case and the Control groups after adjusting for confounding factors. The birth weights in all newborns in both the Case and the Control groups were appropriate for gestational age. No difference in perinatal outcomes (gender distribution, placental weight, CAs, and the occurrence of obstetric complications) was observed. Clinical outcome data are presented in Table 8, and detailed information on the CAs detected in the Case and the Control group are shown in Figure 11.

Table 8. Pregnancy, obstetric and perinatal outcomes (Studies III, IV and V).

	Study III				Study IV				Study V			
	Case (n = 136)	Control (n = 136)	<i>p</i> - value	OR (95% CI)	Intervention group (n = 65)	Control group (n = 62)	<i>p</i> - value	OR (95% CI)	Intervention group (n = 72)	Control group (n = 72)	<i>p</i> - value	OR (95% CI)
	n (%)											
Live birth	30 (22.1)	49 (36.0)	0.038 ^j	0.48 (0.24 – 0.96)	20 (30.8)	15 (24.2)	NS ^{k,l}	1.37 (0.27 – 3.28)	21 (29.2)	14 (19.4)	NS ^{k,m}	1.76 (0.87–3.56)
Implantation rate ^a	42/151 (27.8)	61/153 (39.9)	0.011 ^j	0.39 (0.19 – 0.81)	—	—			—	—		
Clinical pregnancy	40 (29.4)	60 (44.1)	0.012 ^j	0.40 (0.19 – 0.82)	25 (38.5)	17 (27.4)	NS ^{k,l}	1.65 (0.77 – 3.55)	29 (40.3)	26 (36.1)	NS ^{k,m}	1.13 (0.63–2.03)
Miscarriage ^b	8 (20.0)	9 (15.0)	NS ^j	1.39 (0.44 – 4.35)	3 (12.0)	2 (11.8)	NS ^k	1.13 (0.20 – 6.52)	8 (27.6)	11 (42.3)	NS ^k	2.15 (0.20–22.78)
Ongoing pregnancy at gwk 12	31 (22.8)	49 (36.0)	0.017 ^{fi}	0.52 (0.31 – 0.89)	—	—			—	—		
Extrauterine pregnancy ^b	1 (2.5)	2 (3.3) ^e			2 (8.0)	0 (0.0) ^e			0 (0.0)	1 (3.8) ^e		
Induced abortion ^a	1 (2.5)	0 (0.0) ^e			0 (0.0)	0 (0.0) ^e			0 (0.0)	0 (0.0) ^e		
Congenital malformations ^c	3 (9.7)	2 (4.1)	NS ^{g,i}	2.52 (0.40 – 16.00)	—	—			—	—		
Sex of the infant												
Male	16 (51.6)	23 (46.0)			—	—			—	—		
Female	15 (48.4)	27 (54.0)	NS ^{fi}	0.80 (0.33 – 1.96)	—	—			—	—		
Obstetric complications ^c	6 (20.0)	10 (20.4)	NS ^{g,i}									
Pre-eclampsia	0 (0.0)	2 (4.1)	NS ^{g,i}		—	—			—	—		
GDM	6 (20.0)	8 (16.3)	NS ^{g,i}		—	—			—	—		
	mean ± SD											
Birth weight ^d	3526 ± 689	3632 ± 420	NS ^{h,i}		3519 ± 551	3807 ± 341	NS ^{h,i}		3600 ± 580	3666 ± 373	NS ^{h,i}	
Placental weight ^d	610 ± 139	603 ± 132	NS ^{h,i}		—	—			—	—		

^aNo. of gestational sacs per total no. of transferred embryos, ^bPercentage from clinical pregnancies, ^cCalculated from ongoing pregnancies, ^dOnly singleton pregnancies included in the analysis, ^eInsufficient data for statistical analysis, ^fPearson's Chi-square, ^gFisher's exact test, ^hStudent's t-test, ⁱUnivariate analysis, ^jMultivariable analysis adjusted for body mass index, duration of ^fPearson's Chi-square, ^gFisher's exact test, ^hStudent's t-test, ⁱUnivariate analysis, ^jMultivariable analysis adjusted for body mass index, duration of infertility, number of transferred embryos infertility, blastomere loss and resumption of mitosis, ^kLogistic regression analysis, generalized estimating equalities, ^lAdjusted for maternal age at oocyte pick up and duration of infertility ^mAdjusted for maternal age at oocyte pick up, duration of infertility, cryopreservation method and embryonic age at the time of frozen embryo transfer
Gwk= gestational week, GDM= gestational diabetes mellitus

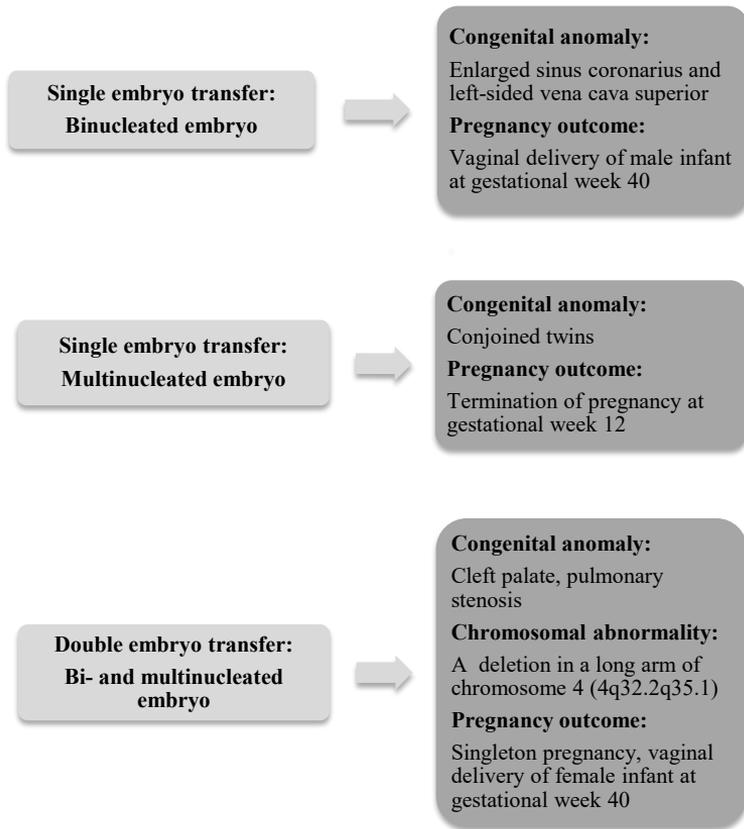
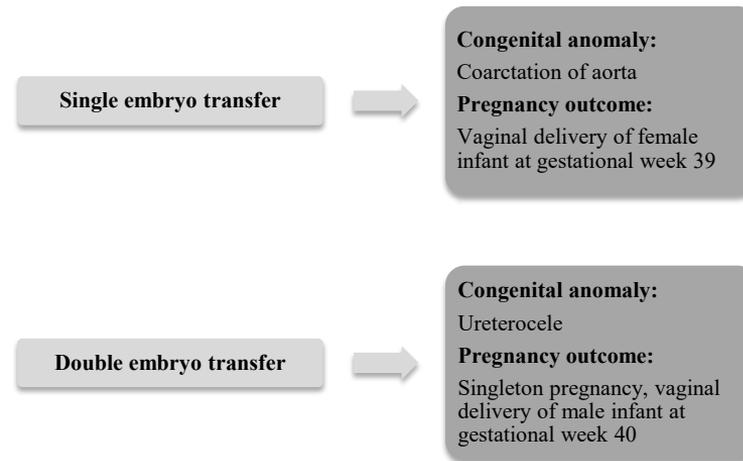


Figure 11 a) Nuclear status and pregnancy outcome of frozen embryo transfers leading to congenital anomaly in the Case group (**Study III**).



b) Pregnancy outcome of frozen embryo transfers leading to congenital anomaly in the Control group (**Study III**).

5.4 Pregnancy outcomes of BN and MN embryos: a sub-analysis (Study III)

Pregnancy results of the transferred frozen-thawed embryos including exclusively either BN or MN embryos were compared ($n = 97$ vs. 42 , respectively) in a sub-analysis. IR was comparable between the subgroups and difference in LBR was not different. The number of born infants per transferred BN and MN embryos approached but did not meet, statistical significance (24.7% vs. 11.9% , $p = 0.065$). P-value was also calculated for the number of born infants per implanted embryos in BN and MN groups, with only a tendency of significance (82.7% vs. 50.0% $p = 0.056$). The miscarriage rate was 30% among MN embryos and 17.9% among BN embryos, but the difference was not statistically significant (Table 9).

Table 9. Pregnancy results after transfer of BN or MN frozen-thawed embryos (Study III).

	BN n (%)	MN n (%)	p- value ^a	OR (95% CI)
Number of FETs	93	38		
Total number of transferred embryos	97	42		
Implantation rate ^b	29 (29.9)	10 (23.8)	NS	0.73 (0.32 – 1.68)
Clinical pregnancy ^c	28 (30.1)	10 (26.3)	NS	0.83 (0.36 – 1.93)
Live birth ^c	23 (24.7)	5 (13.2)	NS	0.46 (0.16 – 1.32)
No of infants ^b	24/97 (24.7)	5/42 (11.9)	NS	0.41 (0.15 – 1.16)
No of infants ^d	24/29 (82.7)	5/10 (50.0)	NS	4.80 (0.10 – 23.07)
Miscarriage ^e	5 (17.9)	3 (30.0)	NS	1.97 (0.37 – 10.40)

Values are numbers (percentages)

^aFisher's exact test, ^bCalculated per transferred embryo, ^cCalculated per FET, ^dCalculated per implanted embryo, ^ePercentage from clinical pregnancies

Double FETs including both BN and MN embryos were excluded from the analysis

FET = frozen embryo transfer, BN = binucleated, MN = multinucleated

5.5 Pregnancy and perinatal outcomes of NC-FET cycles after administration of single dose luteal phase triptorelin (Study IV)

An additional luteal phase triptorelin was given in 65 NC-FET cycles (Intervention group); the Control group was comprised of 62 NC-FET cycles with standard luteal support (Figure 8). In the Intervention group, LBR was 30.8% , and the control group was 24.2% , but all differences in pregnancy outcomes were statistically insignificant.

Mean maternal β -hCG concentration measured from ongoing singleton pregnancies when the embryo age reached 16 days was lower in the Intervention group ($n=18$) than in the Control group ($n=13$) (282.6 ± 179.1 IU/l vs. 416.6 ± 177.6 IU/l, $p=0.048$, respectively). Two single FETs in the Intervention group produced twin pregnancies; one resulted in a vanishing twin pregnancy, and the

other was a result of simultaneous implantation of frozen-thawed and spontaneously fertilized embryos. There was no difference in birth weight of the singleton newborns between the groups. Pregnancy and perinatal outcomes are presented in Table 8.

5.6 Pregnancy and perinatal outcomes of AC-FET cycles after administration of single dose luteal phase triptorelin (Study V)

Altogether, 72 AC-FET cycles were enrolled in both the Intervention and the Control groups (Figure 8). The rates for implantation, positive pregnancy test, and clinical pregnancy were similar between the groups. A difference of 9.8 percentage points in LBR and 14.7 percentage points in miscarriage rate between the Intervention group and the Control group (29.2% vs. 19.4%, and 27.6% and 42.3%, respectively) were not statistically significant.

Newborns in the Intervention (n=22) and the Control group (n=15) were healthy, and no malformations were detected. Mean birth weights from singleton deliveries were comparable between the groups. Pregnancy and perinatal outcomes are presented in Table 8.

The aims and the main results of this thesis are summarized in Table 10.

Table 10. Aims, study design, and main results of the studies.

Study details	Study II	Study III	Study IV	Study V
Aim of the study	<i>De novo</i> occurrence of MN after embryo freezing and thawing Association between multinucleation, MN sibling embryos and clinical factors affecting ovarian sensitivity to gonadotropin treatment	Transfer of mononucleated or BN/MN frozen-thawed embryo: - pregnancy outcomes - obstetric and neonatal outcome	Administration of luteal phase single dose triptorelin in NC-FET cycles: - pregnancy outcomes - β -hCG- concentrations at embryo age of 16 days - Birth weight	Administration of luteal phase single dose triptorelin in AC-FET cycles: - pregnancy and neonatal outcomes
Study design	Retrospective cohort study	Retrospective case-control study	Prospective randomized clinical pilot study	Prospective interventional pilot study
Main results	The presence of MN embryos prior to freezing was not associated with formation of MN sibling embryos after thawing Multinucleation associated with factors related to good prognosis in ART, such as young age and ovarian sensitivity to controlled ovarian hyperstimulation among women with advanced age The presence of multinucleation was similar in embryo cohorts with and without cryopreservation	Frozen-thawed BN and MN embryos transferred at the cleavage stage had lower than normal but still acceptable implantation potential and ability to produce healthy pregnancies and newborns Implantation potential of MN embryos tended to be lower compared to BN embryos	Live birth rate was 30.8% in the intervention group and 24.2% in the control group, but all differences in pregnancy outcomes were statistically insignificant β -hCG- concentration was lower in the Intervention group	Live birth rate was 29.2% in the intervention group and 19.4% in the control group, but all differences in pregnancy outcomes were statistically insignificant No difference in neonatal outcome was recorded

ART = assisted reproductive treatment, β -hCG = human chorionic gonadotropin, FET = frozen embryo transfer, BN = binucleated, MN = multinucleated, NC = natural cycle, AC = artificial cycle

6 DISCUSSION

6.1 Methodological considerations

Studying prognostic factors related to FET outcome is challenging, especially when ethical and safety aspects are in question. Several study protocols were utilized in this thesis, including a case report, a retrospective cohort study, a retrospective case-control study, and two prospective interventional studies with a crossover design. All these methods have strengths and limitations, which are summarized in Table 11.

Regarding case reports, the endpoint of a single case cannot be generalized, nor can conclusions on causal effects be drawn, but case reports enable the presentation of novelties and generation of hypotheses for further research (Nissen and Wynn, 2014). Cohort studies and case-control studies, two types of observational studies, offer the possibility to explore the prevalence of events and associations between exposure and endpoints, especially when RCTs are ethically questionable. Because subject selection is defined by outcome status, case-control studies require a fewer number of subjects compared to cohort studies and are suitable for evaluating rare outcomes (Song and Chung, 2010). Furthermore, cohorts derived from the unselected patient population illustrate real world data. RCTs demonstrate a reference standard for exploration of the efficacy of medical interventions but compared to real world data, the study population in RCTs is highly selected. Crossover design in prospective studies provide a higher number of events in a limited study population and additionally increase the homogeneity of the study groups.

Using the above mentioned study methods, the present study provided novel, albeit preliminary, information on *de novo* multinucleation in embryo cohorts after slow freezing and on the clinical significance of blastomere multinucleation on pregnancy outcomes after BN/MN FET. Further, this was the first study to evaluate the role of triptorelin as adjuvant therapy in NC-FET cycles in a prospective setting. Concerning studies involving the evaluation of pregnancy outcomes of FET, LBR, and the neonatal outcome was also included. However, this study has certain limitations that are discussed below.

Table 11. Strengths and limitations.

Study I
<p>Merits: The first report of conjoined twins after transfer of frozen-thawed MN embryo</p> <p>Limitations: Case report study of a single case</p>
Study II
<p>Merits: Data was enrolled from clinical patient sample (representing real world data) Embryo screening was performed simultaneously by two experienced observers Adjustment was performed for several factors (female age, male age, number of oocytes, total and proportional FSH consumption)</p> <p>Limitations: Retrospective analysis of the data Lack of fresh day-3 control group Sequential embryo screening Results are not directly applicable to vitrification</p>
Study III
<p>Merits: Data was enrolled from clinical patient sample (representing real world data) Embryo screening was performed simultaneously by two experienced observers Adjustment was performed for several factors (duration of infertility, BMI, degree of blastomere survival, resumption of mitosis, number of transferred embryos) Detailed and reliable information on the health of the newborns drawn from medical records Double FETs of both mononucleated and BN/MN embryos were excluded</p> <p>Limitations: Retrospective analysis of the data Low number of Cases and Controls Sequential embryo screening Cleavage stage of embryos in which bi- or multinucleation was observed was not included in the multivariable analysis Matching was based only on female age at the time of oocyte retrieval</p>
Study IV
<p>Merits: Randomized prospective study design Strict inclusion criteria Logistic regression model was used to exclude carryover effect Logistic regression analysis was used for binary variables with generalized estimation equation extension to account for dependence among repeated treatments Pregnancy outcomes were adjusted for maternal age at COH and duration of infertility</p> <p>Limitations: Low number of participants Lack of placebo Drop-out of participants</p>
Study V
<p>Merits: Prospective interventional study design Logistic regression model was used to exclude carryover effect Logistic regression analysis was used for binary variables with generalized estimation equation extension to account for dependence among repeated treatments Pregnancy outcomes were adjusted for several confounding factors (see Figure 10)</p> <p>Limitations: Low number of participants Lack of placebo Drop-out of participants after first FET due to cross-over design Heterogeneity in developmental age of transferred embryos Heterogeneity in the endometrial preparation methods</p>
<p>BMI = body mass index, BN = binucleated, COH = controlled ovarian hyperstimulation, FET = frozen embryo transfer, FSH = follicle stimulating hormone, MN = mononucleated</p>

Study I was the first published case report of CT pregnancy derived from the transfer of frozen-thawed MN embryo. Albeit limited in scientific value, this documented case inspired us to explore the occurrence of multinucleation in frozen-thawed embryos and the relation between nuclear alterations and possible complications manifesting later in pregnancy.

Embryo cohorts in **Study II** included all consecutive IVF/ICSI cycles during the study period, illustrating a cross-section of the IVF/ ICSI cycles performed in the public health care IVF Unit of Turku University Hospital without selection bias. Although data on the presence of bi- or multinucleation in **Studies II** and **III** were collected online during ART treatments, analysis of the results was performed retrospectively. Some inconsistency in embryo grading records led to drop-outs during the data sampling. Several drop-outs also occurred because some of the embryos were destroyed when requested by the couple or had remained unfrozen. Due to the lack of fresh day-3 embryos as controls and the natural reduction in multinucleation rate after second and third embryonic cleavages, no firm conclusions on the role of cryopreservation on the post-thaw occurrence of multinucleation could be made. As for cryopreservation techniques, slow freezing was generally used during data collection. More recently, a large proportion of IVF clinics have shifted to embryo vitrification. Nevertheless, **Study II** provided novel data on clinical factors associated with embryo multinucleation.

In **Studies II and III**, embryo evaluation was performed by sequential embryo screening, a method used in normal clinical practice. Embryo screening was performed simultaneously by two experienced observers to minimize inter-observer variability. Multinucleation is known to occur with the highest frequency after the first embryonic division. The first evaluation of the cleavage in sequential screening takes place at day-2 when the normal cleavage stage ranges from two to six blastomeres. Therefore, the precise order of mitotic divisions by which multinucleation occurred could not be defined. Also, some alterations in the nuclear structure may have been underestimated because of the transient nature of multinucleation and consequently, some of the embryos included in the Control group in **Study III** may have exhibit multinucleation between the screening checkpoints.

Study III reports the largest number of newborns from BN/MN embryos so far. Nevertheless, the study size is still limited. Reliable data on the incidence of CAs in newborns conceived from BN/MN FETs require large follow-up studies that are not currently available. Thus, our results should be considered preliminary. Further, the decision to transfer frozen-thawed BN/MN embryos was based on clinical judgment, mainly due to the lack of optional mononucleated embryos. However, double FETs of mononucleated and BN or MN embryos were

systematically excluded, and only distinctly evaluated BN or MN FETs formed the Case group. Moreover, matching was based only on female age at the time of oocyte retrieval, and the Control group included a similar number of women as in the Case group. In any case, multivariable analysis for confounding factors was conducted to minimize bias (Figure 10).

In our prospective **Studies IV** and **V**, a major limitation was the low number of participants. Due to limited resources, both studies were designed as pilot studies to gain tentative data on the subject. An adequately powered prospective randomized study with a difference of 9.8 percentage points in LBR, as detected in **Study V**, would have required a sample size of 299 women per group to reach statistical significance with 80% power. Further, no placebo was available, and therefore, both participants and clinicians were aware of the allocated arm. Strict inclusion criteria were set to verify the study's validity (Table 3). Participants had undergone IVF/ICSI for diagnosed female or male infertility and were recruited during their clinical visits at the infertility unit without financial compensation. Use of donor gametes was an exclusion criterion. Therefore, the study population was representative of an ordinary infertility population.

Study IV was conducted in co-operation with the Väestöliitto Fertility Clinic (now Mehiläinen Felicitas) in Turku, and **Study V** with Tampere University Hospital's Infertility Unit to ensure the target number of participants in the study period. For the same reason, a crossover setting was adopted after excluding the carryover effect. In both study protocols, a short-acting mid-luteal single dose of triptorelin was added to the standard FET protocol. Although no difference in clinical outcomes regarding the route of estrogen administration have been demonstrated (Glujovsky et al., 2010), use of both transdermal and oral estradiol for AC-FET created heterogeneity in the randomization protocol. Furthermore, both cleavage stage embryos and blastocysts were transferred. Blastocyst cultures would minimize bias caused by embryonic factors, and thus, blastocyst transfers would be optimal in studying the effects of different endometrial preparation methods, luteal support, and adjuvant therapies.

As noted above, this study has its limitations. Nevertheless, it provided novel data that applies to clinical practice. This study displayed new perspective when weighting the significance of embryo multinucleation, and pointed out the need for further studies on factors contributing to outcomes of FET, whether related to embryos, pharmacological interventions, or patient characteristics. Below, the study findings are discussed in detail.

6.2 Occurrence of multinucleation in embryo cohorts and embryos before freezing and after thawing

The multinucleation rate was the highest in day-2 embryo cohorts before freezing. At least one BN/MN embryo was detected in more than half of the embryo cohorts, a finding within the range of previously reported multinucleation rate detected both with sequential and continuous embryo screening (Aguilar et al., 2016, Balakier et al., 2016, Basile et al., 2014, Van Royen et al., 2003). Immediately after thawing, *de novo* multinucleation was seldom detected, and the rate of *de novo* multinucleation was also lower in day-3 frozen-thawed embryo cohorts compared to day-2 pre-freeze embryo cohorts. Likewise, the proportion of MN embryos to all embryos did not increase in the freezing-thawing process but instead showed a slight decline. Even with modern cryotechnology, some embryos do not survive freezing and thawing due to injuries of the cell membranes or intracellular components. The impact of freezing on nuclear reconstruction should become evident after the resumption of mitosis (Guerif et al., 2002). As the decline in multinucleation rate from day-2 to day-3 in frozen-thawed embryos and *de novo* multinucleation on day-3 after thawing resembles that described in fresh embryo cohorts (Van Royen et al., 2003, Yakin et al., 2005), it may be that slow freezing does not cause a major risk for formation of blastomere multinucleation during post-thaw mitosis. However, due to the lack of a control group of fresh day-3 embryo cohort in the present study, a firm conclusion cannot be made.

Considering the relatively high frequency of the multinucleation among embryos, it was of interest to investigate whether multinucleation *per se* reflects the quality of the entire embryo cohort. Embryo eligibility for freezing is coordinated by specific selection criteria and therefore, cryopreservation of supernumerary embryos can be considered as a sign of the adequate or good quality of embryos in a given IVF/ICSI cycle. Although the main focus of the study was on the post-thaw occurrence of multinucleation, a sub-analysis of IVF/ICSI cycles from year 2012 allowed comparison of the occurrence of multinucleation in fresh day-2 embryo cohorts having no suitable embryos for cryopreservation. Multinucleation occurred with the same frequency in cycles with and without further cryopreservation. Thus, multinucleation of blastomeres was not overrepresented in cycles that did not meet the criteria for cryopreservation. Interestingly, the presence of MN embryos in good quality embryo cohorts before cryopreservation was not associated with the occurrence of multinucleation after thawing. In contrast to Meriano et al. (2004), who suggested impaired developmental quality of mononucleated sibling embryos, the present study supports studies suggesting that multinucleation is an intrinsic feature of an embryo rather than a reflection of compromised quality of an entire embryo cohort (Egashira et al., 2015, Jackson et al., 1998, Yilmaz et al., 2014).

6.3 Factors affecting formation of multinucleation

Previous studies have evaluated the impact of female age on multinucleation independently of other factors affecting ovarian response to COH. In general, no association has been found apart from two studies by Moriwaki et al. (2004) and Balakier et al. (2016), showing that multinucleation was more common in older women. Likewise, the association of multinucleation with findings illustrating high response to gonadotropins, such as high estradiol levels, low FSH consumption, and an increased number of collected oocytes have been reported with conflicting results (Balakier et al., 2016, De Cássia Savio Figueira et al., 2010, Jackson et al., 1998, Van Royen et al., 2003, Yilmaz et al., 2014).

The **Study II** was the first to explore multinucleation and its interaction between female age and ovarian response to gonadotropins. In opposite to previous studies, multinucleation in embryo cohorts before freezing was found to be the most frequent among women less than 30 years. In that age category, highly prevalent multinucleation rate in embryo cohorts was seen independently of the number of harvested oocytes or FSH consumption per collected oocyte. In contrast, in women aged over 30 years, the multinucleation rate varied according to the number of harvested oocytes and FSH consumption. Ovarian sensitivity to gonadotropins seemed to be a risk for blastomere multinucleation in this age group. These results add to the evidence that ovarian responsiveness to COH associates with the occurrence of multinucleation (De Cássia Savio Figueira et al., 2010, Jackson et al., 1998, Van Royen et al., 2003, Yilmaz et al., 2014).

The number of collected oocytes and the gonadotropin dose during COH in normal responders have not been related to increased aneuploidy rate (Sekhon et al., 2017). Meiotic errors in oocytes increase with age but interestingly, in this study, multinucleation was the most common in the youngest age category, and, in the older age categories, multinucleation was associated with a higher response to COH. These patient groups show a good prognosis in ART. Therefore, findings of the present study could be explained by the hypothesis that multinucleation reflects active reparation of abnormal chromosome content (Tesarik et al., 1987), especially among women with normal ovarian reserve and overall good quality embryo cohort.

This study provided novel information on the interaction of age and ovarian sensitivity to gonadotropins as a modulator of the multinucleation rate. Based on this, it can be hypothesized that the origin of blastomere multinucleation can be explained, at least partly, by the characteristics of the patient. Further, multinucleation may reflect self-correction events in an embryo.

6.4 Pregnancy and obstetric outcomes of BN/MN FETs

The causes and physiological significance of multinucleation have remained unclear. In addition to the most dominant hypothesis for the origin of multinucleation, cell cycle and chromosome segregation errors (Hardy et al., 1993, Munné and Cohen, 1993, Pickering et al., 1995, Winston et al., 1991), defective reassembly of nuclear envelope could also be one mechanism leading to multinucleation. Also, multinucleation has been postulated to represent a form of self-correction mechanism, by which embryo is restoring its viability (Aguilar et al., 2016, Balakier et al., 2016, Tesarik et al., 1987). Indeed, few reports of healthy newborns from ET or FET of MN embryos have been documented (Balakier et al., 2016, Balakier and Cadesky, 1997, Egashira et al., 2015, Hashimoto et al., 2015, Jackson et al., 1998, Yilmaz et al., 2014). However, due to a limited number of live births, a final conclusion for safety regarding neonatal health cannot be drawn.

In practice, MN embryos are mostly rejected from fresh ET but secondarily used for FET if other embryo parameters met the criteria for cryopreservation (Desai et al., 2016, Egashira et al., 2015). In this regard, it was natural to choose a cohort of BN/MN FETs for our study object. In all parameters evaluating the ability to achieve clinical pregnancy, the Control group yielded significantly better results. However, in the Case group, an IR of nearly 28%, within the range of latest reports from fresh cleavage stage MN ETs, was achieved (Aguilar et al., 2016, Ergin et al., 2014). In line with the study by Desai et al. (2016) investigating LBR of vitrified-warmed blastocysts, the odds for live birth among BN/MN frozen-thawed embryos was half of that in Control group. Intriguingly, the LBR of 22% observed in the Case group was identical to the LBR of FETs reported in National Institute for Health and Welfare records from 2016 in Finland (THL, 2018). Thus, although a reduction in pregnancy potential among BN/MN embryos was evident, the pregnancy outcome could still be interpreted as acceptable.

Most of the studies report a clear reduction of IR in fresh blastocysts exhibiting multinucleation at 2-cell stage (Balakier et al., 2016, Desch et al., 2016, Ergin et al., 2014). However, Aguilar et al. (2016) postulated that the implantation potential appears to correlate with the developmental stage of the embryo in which MN blastomeres are present; that is, reduction in IR seemed to be less evident if multinucleation was present only after the first embryonic cleavage. Although questioned by Aguilar et al. (2016), an effect on implantation potential was

further reported to be most obvious if, at the 2-cell stage, multinucleation affects both blastomeres (Desch et al., 2016). In the present study, multinucleation was

detected mostly at the 4-cell or 8-cell stage. Unfortunately, the analyses of the pregnancy results according to the developmental stage at the time of multinucleation could not be conducted due to a limited number of FET cycles and sequential screening of embryos.

The type of multinucleation pattern has been postulated to relate to different chromosomal contents. Meriano et al. (2007) found that fresh MN embryos carry a higher number of chromosomal abnormalities compared to BN embryos and correspondingly, reported higher IR among BN embryos. Aguilar et al. (2016) did not find any relationship between IR and the type of multinucleation detected at the 2-cell stage, but interestingly, IR was lower if multinucleation was observed at 4-cell stage compared to binucleation. To further explore the subject, pregnancy outcomes of frozen-thawed BN and MN embryos were compared in a sub-analysis of the present study. However, the number of FETs containing exclusively MN embryos remained considerably low, and although the difference tended to favor BN FETs, it did not reach statistical significance. Nevertheless, such differences in LBR would be clinically relevant, and therefore, further studies are warranted. Similarly, statistically insignificant differences in LBR (24.7% vs. 13.2%) and miscarriage rate (17.9% vs. 30.0%) in the sub-analysis of BN and MN embryos require further studies.

A tendency to pregnancy loss in MN embryos has earlier been related to short cleavage time (Desch et al., 2016). Overall, delayed cleavage during early development of MN embryos have been debated to indicate active repair mechanisms of the affected blastomeres and therefore, better prognosis. Furthermore, if detectable after the 2-cell stage, multinucleation have been linked to a more compromised pregnancy outcome (Aguilar et al., 2016, Desai et al., 2018). In the present study, bi- and multinucleation was detected at a rather late cleavage stage. At this stage of embryo development, multinucleation compared to binucleation could, therefore, illustrate a more severe form of a nuclear/chromosomal abnormality or compromised ability to self-correct, and a more pronounced tendency to early pregnancy loss. In light of the reduced potential of live birth, cultivation of MN embryos to the blastocyst stage is recommended or, secondarily, the transfer of two frozen-thawed MN embryos could be justified if mononucleated embryos are not available.

If not eliminated from the developing blastocyst, the significance of MN blastomeres for the viability of pregnancy may depend on whether the BN/MN blastomeres are destined to create the inner cell mass or the TE, which later forms the placenta. It is unknown whether BN/MN cell lineages in the placenta affect placental growth and function and thereby, on obstetrical complications (Xanthopoulou et al., 2011). This study closely evaluated the obstetric outcomes

of the pregnancies deriving from BN/MN embryos in consideration of possible consequences to the pregnancy.

Study III provided novel data on obstetric outcome, placental weight, and neonatal health of pregnancies deriving from frozen- thawed BN/MN embryos that had not been evaluated in detail earlier. The results of the study were reassuring, while no signs of disturbed trophoblast function or increased obstetric complications were detected. In both the Case and the Control groups, full-term pregnancies with similar gender distribution were attained, and placental weight was comparable between the groups. There was no difference in birth weights of the neonates or occurrence of pre-eclampsia or gestational diabetes mellitus, obstetric complications related to placental dysfunction and intrauterine growth of the fetus. However, detection of obstetric and neonatal problems requires large data and results of this study should, therefore, be considered preliminary.

6.5 Congenital anomalies of neonates conceived from BN/MN FETs

Neonatal health, including structural deviations of the developing fetus deriving from BN or MN embryos, was investigated. CAs are increased in ART pregnancies, but the reasons for this increase are obscure. Inspiration to explore further the perinatal outcome of MN FET originated from a case of CT after the transfer of an 8-cell frozen-thawed embryo exhibiting three MN blastomeres at the time of transfer. Thoragopagus twins with two heads, four limbs, and a common heart were detected at gestational week 12, and the pregnancy was terminated (**Study I**).

The prevalence of monozygotic twins is increased after ART compared to natural conception, presumably due to *in vitro* conditions during embryo culture (Aston et al., 2008). CT, a complication of monozygotic twins, is an extremely rare condition estimated to occur in one per 100,000 to 200,000 spontaneous pregnancies (Rees et al., 1993). Among children born after ART treatments, the described case of CT was the first in Finland to be reported since the beginning of the National Data Collection on infertility treatments (1992-2011) (Mika Gissler, personal communication). Remarkably, an identical CT case was presented three years later in pregnancy after fresh ET (Serapinas et al., 2016). For example, monozygotic twins and also CT are suggested to represent a form of blastogenesis defect, which frequently leads to defects in midline formation and is typically without genetic origin (Halliday et al., 2010). Considering the rarity of the condition, two reported cases of CT after the transfer of an MN embryo raises the question: does blastomere multinucleation disturb nuclear and

cellular coordination by exposing the embryo to defective cell migration in the inner cell mass and failures during organogenesis?

Present knowledge on the perinatal outcome of fresh MN ETs is limited to case reports and small patient series up to 14 deliveries, including predominantly healthy newborns. In addition to the case of CT reported by Serapinas et al. (2016), a trisomy 18 and combination of trisomy 21 and 18 after the transfer of MN embryo have been documented (Balakier et al., 2016, Balakier and Cadesky, 1997). Previously, the health of newborns conceived from MN FETs has been evaluated only in one study presenting nine healthy babies from the FETs of altogether 22 MN embryos (Egashira et al., 2015).

In this study, the rate for CAs did not differ from that detected in the control group, but still, the number of children was too low in both groups to make conclusions. Thus, the present study does not give any answer to the question of whether there is a causal relationship between multinucleation and CAs. Concerns regarding the effect of multinucleation on embryonic development have been related to possible deviations in the chromosomal complement, affecting the nuclear integrity of the blastomere. The effect of multinucleation on the chromosomal constitution would be most likely be seen as segregation errors of chromosomes that clinically manifest either as aneuploidy of all blastomeres, chromosomal mosaicism, or segmental aneuploidy (Balakier et al., 2016, Kligman et al., 1996). However, it is noteworthy that in the Case group of the present study, only one neonate with CA had an abnormality in the chromosomal constitution that corresponded to segmental aneuploidy.

Because most authors recommend either to reject MN embryos from transfer or to use them as a secondary option (Aguilar et al., 2016, Desch et al., 2016, Fauque et al., 2013, Meseguer et al., 2011), adequately powered large scale studies on the incidence of CAs among MN embryos remain currently beyond the bounds of possible. In light of new evidence that multinucleation may represent active repair mechanism to restore euploidy of a mosaic embryo rather than to be a definite sign of compromised embryo quality, attitude toward more liberal use of MN embryos is taking place. This change in clinical practice emphasizes the need for more research on the perinatal consequences of the phenomenon. The present study was the first to shed light on the subject systematically. The study population is the largest so far to explore the occurrence of CAs among neonates born from the transfer of frozen-thawed BN/MN embryos, but it is still limited in number. Considering the preliminary nature of the results, counseling the couple before the transfer of the BN/MN embryos is advisable, and, in the case of ongoing pregnancy, thorough antenatal screening is recommended.

6.6 Effect of luteal phase GnRH agonist on pregnancy and perinatal outcomes

Study IV was the first prospective randomized study on the effect of short-acting luteal phase GnRH agonist on pregnancy outcomes in NC-FET cycles compared to standard LPS. The study was planned as a pilot, and, consequently, it was not powered enough to show statistical significance. The differences in CPR and LBR (11.1 percentage points and 6.6 percentage points, respectively) point to a potential benefit of additional luteal phase GnRH agonist and indicate a need for larger studies. The effect of luteal phase GnRH agonist substitution in NC-FET has previously been evaluated only in one retrospective analysis of different NC-FET LPS protocols that were used during two consecutive time periods; standard LPS was used during the first period and the following period, additional two dosages of recombinant hCG and GnRH agonist on day of FET and four days later was administered (Orvieto et al., 2016). The ongoing PRs were twice as high when the modified LPS protocol was used. It should be questioned whether the difference in pregnancy outcomes could be explained by other factors, such as a change in laboratory techniques, rather than by the modified FET protocol only.

One possible mechanism in which luteal phase GnRH agonist could affect the pregnancy outcomes in NC-FET is the enhanced corpus luteum function and therefore, higher levels of endogenous progesterone. As a true agonist, GnRH agonist first produces a flare effect, an outburst in gonadotropin secretion, before subsequent receptor downregulation. As a consequence, activation of corpus luteum function is likely to increase progesterone secretion. Because of its short half-life, a single dose of short-acting triptorelin administered during WOI does not cause downregulation after a flare. Even if administered daily, suppression of gonadotropin secretion would take place only after a few days, when hCG production from the implanted embryo would secure the endogenous progesterone production.

A direct effect of GnRH agonist on embryo and embryo-endometrial interaction to facilitate implantation has also been proposed. Such an effect could influence hCG production by trophoblasts. Tesarik et al. (2006) demonstrated higher levels of β -hCG after administration of mid-luteal GnRH agonist. This finding was not confirmed by Check et al. (2015) or by the present study. By contrast, in the present study, hCG levels were found to be lower among the GnRH receivers than in the control group. It has been demonstrated that GnRH and its receptor are expressed in human blastocysts (Casañ et al., 1999).

Furthermore, *in vitro* experiments as well as *in vivo* analyses on the relationship between GnRH and hCG secretion indicate that GnRH and GnRH agonist indeed

induce placental hCG secretion. However, no difference in hCG production was recognized in human *in vitro* model when GnRH agonist was administered in the culture media of blastocysts incubated with endometrial cells collected from non-pregnant uteri during assumed WOI (Klemmt et al., 2009). Taken together, this study is in line with data suggesting that a single dose of GnRH agonist administered during WOI does not have such an effect on embryo that would subsequently modulate trophoblast function and increase hCG secretion. However, the absolute hCG level does not as such reflect trophoblast function, for example, their invasiveness (Alfthan et al., 1993, Fritz and Speroff, 2005). Thus, no conclusion can be made on the hypothetical effect of GnRH on embryos, trophoectoderm, and trophoblasts.

Study V was the first to report live birth and perinatal outcomes after luteal phase single dose GnRH agonist in AC-FET cycles. Like **Study IV**, this study was a pilot study and lacked the power to show statistical significance for the 9.8 percentage points difference in LBR favoring intervention. Due to the lack of corpus luteum in AC-FET, the positive effect of luteal phase GnRH agonist administered during WOI would be transmitted solely through a direct effect on the embryo or the endometrium. In hormonally substituted cycles of oocyte recipients, GnRH agonist in the luteal phase increased both IR and LBR in a prospective RCT by Tesarik et al. (2004). However, no difference in IR, CPR, or ongoing PR was detected in the prospective randomized trials on GnRH support in AC-FET (Davar et al., 2015; Ye et al., 2019). One explanation for these discrepancies may be due to the lack of power, but different immunological adaptations to invading trophoblasts and the possible modulatory effect of GnRH agonist may explain the difference in pregnancy outcomes between oocyte recipients and women utilizing autologous oocytes (Savasi et al., 2016).

Interestingly, the difference in LBR in this study was based on a higher number of miscarriages in the group receiving standard LPS. Previously, few studies have documented early pregnancy wastage to be more prominent in AC-FET cycles compared to NC- or OI-FET cycles (Hatoum et al., 2018, Jouan et al., 2016, Tomás et al., 2012). Whether the GnRH agonist at the time of implantation in AC-FETs could modulate endometrial receptivity and decrease the tendency to early pregnancy loss merits further attention.

In Studies IV and V, the birth weight of the newborns was recorded. In both studies, no statistical differences in birth weights of newborns from singleton deliveries were detected, although in **Study IV** newborns from the Intervention group weighed 300g less than in the Control group. In **Study V**, the occurrence of CAs was documented, and no congenital anomalies were detected after administration of luteal phase GnRH agonist. These preliminary results on the health of newborns are the first reported after mid-luteal administration of GnRH

agonist in NC- or AC-FET. According to the available data, the utilization of GnRH

agonist during pregnancy seems safe (Abu-Heija et al., 1995, Cahill et al., 1994, Chardonnens et al., 1998, Gartner et al., 1997, Marcus and Ledger, 2001). However, before implementing adjuvant therapy into standard ART protocol, the neonatal outcome should be comprehensively evaluated in larger, adequately powered studies.

6.7 Future aspects

As shown in the present study, MN embryos are relatively common in embryo cohorts subjected to slow freezing. The presence of MN embryos in an embryo cohort before freezing was not associated with *de novo* multinucleation rate after thawing. In line with the previously reported decline in multinucleation rate in fresh embryos from day-2 to day-3 of culture, multinucleation rate was higher in day-2 embryos than in day-3 embryos, after thawing and overnight culture. However, to verify any influence of cryopreservation on multinucleation, further studies with fresh embryos as controls are warranted.

The present study was carried out using the slow freezing technique, which was widely utilized at the time of data collection. Since then, the cryopreservation techniques have shifted toward vitrification. Therefore, studies comparing multinucleation in frozen-thawed and vitrified-warmed cleavage stage embryos would bring new information on the safety of these cryopreservation methods, especially regarding nuclear dynamics.

The clinical relevance of blastomere multinucleation is yet to be determined. A particularly intriguing question is whether blastomere multinucleation is a consequence rather than a cause of aneuploidy, as suggested by Tesarik (2018) in his comment to **Study III**. Therefore, further studies are needed to clarify whether multinucleation demonstrates a physiological repair mechanism during early embryonic development, which is, however, challenging as the origin of multinucleation is probably multifactorial. For example, the hypothesis that defective reassembly of the nuclear envelope may be one mechanism leading to multinucleation needs to be tested.

Prognostic value of multinucleation for FET outcomes is not thoroughly clarified. Larger studies are needed to detect whether the significance of blastomere multinucleation after the first mitotic cleavage differs from that after the second or third cleavage. Continuous embryo monitoring should be the preferred evaluation method due to the transient nature of the phenomenon. Further, the inclusion of only blastocyst stage embryos into the analysis of

pregnancy outcomes would reduce bias caused by heterogeneity in the developmental potential of cleavage stage embryos. Also, further effort should focus on potential differences in clinical outcomes between BN and MN FET. In all, follow-up studies with larger sample sizes are necessary to verify the impact of blastomere multinucleation on pregnancy, obstetric, and neonatal outcomes.

The literature on the efficacy of mid-luteal GnRH agonist as adjuvant therapy in fresh IVF/ICSI cycles is conflicted. In FET cycles, only limited preliminary data are available, indicating a possible beneficial effect of luteal phase GnRH agonist in FET cycles. However, adequately powered large multicenter randomized prospective studies are warranted to evaluate the effect of mid-luteal phase GnRH agonist adjuvant therapy in clinical use, both in NC- and AC-FET cycles. In addition to single dose administration, exploration of the effect of repeated dosages would also be of interest. Future research on the mechanisms behind GnRH action in the luteal phase is awaited. Furthermore, the obstetric and neonatal outcomes should always be included as outcomes in future studies to obtain data on the safety of modified ART treatments.

7 CONCLUSIONS

The main conclusions were as follows:

1. Multinucleation is commonly seen in embryos and good quality day-2 embryo cohorts before freezing, but *de novo* multinucleation is less likely to occur in day-3 frozen-thawed embryo cohorts. The presence of BN/MN embryos in day-2 fresh embryo cohorts does not illustrate the propensity of sibling embryos to develop multinucleation after freezing and thawing.
2. Multinucleation is highly prevalent in fresh day-2 embryo cohorts in women less than 30 years old, regardless of ovarian response during COH. In older women, the occurrence of multinucleation is associated with ovarian sensitivity to COH, and this association is sustained in frozen-thawed embryos. Thus, female characteristics and conditions during COH are possibly more probable contributors to occurrence of multinucleation than slow freezing process.
3. Pregnancy potential of frozen-thawed BN/MN embryos is compromised compared to mononucleated embryos, but nevertheless, acceptable CPR and LBR are achieved. Compared to BN embryos, MN embryos may be more prone to early pregnancy loss in FET cycles. According to the preliminary data, the risk for obstetric complications or fetal malformations is similar in pregnancies deriving from mononucleated and BN/MN embryos. The present results speak against the policy of discarding BN/MN embryos from cryopreservation and transfer.
4. CPR and LBR in NC-FET cycles and CPR in AC-FET tended to be higher when a single dose of triptorelin was given during WOI as an adjuvant to standard luteal support. As the prospective studies were designed as pilots, they were underpowered to show statistical significance. As such, the results imply a need for larger multicenter studies.

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A handwritten signature in black ink, consisting of a large, stylized loop followed by a horizontal line that ends in a small hook.

Jaana Seikkula

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