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CALCIUM ACTIVATION OF OOCYTES FOR POOR PROGNOSIS INFERTILITY PATIENTS

Faculty of Medicine and
Health Technology
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ABSTRACT

Mirva Seppänen: Calcium Activation of Oocytes for Poor Prognosis Infertility Patients
Master's Thesis
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Examiners: Prof. Heli Skottman and M.Sc. Tiina Mikkilä
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Background & aims: Intracytoplasmic sperm injection (ICSI) is one of the most significant techniques in in vitro fertilization (IVF), especially for male factor infertility. Various studies have shown that couples may benefit more from a combination of ICSI and artificial calcium activation (AOA), than ICSI on its own. After fertilization the oocyte activation is triggered by phospholipase C zeta (PLCζ), and calcium ions are released from endoplasmic reticulum of the oocyte. A momentary rise in the concentration of the calcium ions is the first stage of oocyte activation. This study compared the patients' treatment cycles before and after the AOA. The study is centred on the quality of the transferred embryos, and the newborns' health. The aim of this study was to provide information on the quality of embryos after calcium activation by collecting and analysing the data from the treatments, and to find a possible statistical significance between the calcium activation and the better quality of the embryos. In addition, the Reproductive Endocrinology Outpatient Clinic was interested in the health of the newborns. This study defines and observes the poor prognosis patients from Fimlab IVF laboratory point of view.

Methods: This was retrospective database study of selected materials from the Babe IVF System. The data concerning infertility treatments has been gathered between years 2016 and 2020. Oocytes were collected and calcium activated between 2016 and 2019. Embryo scoring based on the modified ESHRE guidelines.

Results: Altogether in 61% of the picked up embryos the quality improved. In 24% of the cases the quality stayed the same and did not decline with the calcium activation. The fertilization rate in this study was 56%. For all the embryo transfers made, the number of clinical pregnancies was 26%, and in 18% of the pregnancies a healthy child was born.

Conclusions: Calcium activation is a promising treatment, and based on the results of this study it can be used to improve embryo quality. The results of the study were limited by several factors, such as the relatively small number of patients. Partly because of the small number of patients no clear reason was found to why some patients had no improvement in the quality of their embryos. However, the results were in many ways encouraging, most importantly the fact that the children born as a result the treatments were healthy.

Keywords: Calcium activation, artificial oocyte activation, ICSI, calcimycin, embryo quality

The originality of this thesis has been checked using the Turnit's OriginalityCheck service.

TIIVISTELMÄ

Mirva Seppänen: Munasolujen kalsiumaktivaatio heikon ennusteen hedelmöityshoitopotilailla
Pro Gradu -tutkielma
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Tutkimuksen tausta ja tavoitteet: Mikrohedelmöitys on yksi merkittävimmistä koeputkihedelmöityksen muodoista, etenkin miehestä johtuvassa hedelmättömyydessä. Useat tutkimukset ovat osoittaneet, että parit voivat hyötyä enemmän mikrohedelmöityksen yhdistämisestä keinotekoiseen munasolujen aktivaatioon kuin yksinään mikrohedelmöityksestä. Hedelmöityksen jälkeen siittiöiden fosfolipaasi C zeeta (PLCζ) aikaansaa kalsiumionien vapautumisen munasolun solulimakalvostosta eli aktivoi munasolun. Kalsiumionien konsentraation hetkellinen nousu on ensimmäinen munasolun aktivaation vaihe. Tässä tutkimuksessa verrataan saman potilaan hoitokertoja ennen sekä jälkeen keinotekoisen kalsiumaktivaation. Tutkimus keskittyy siirrettyjen alkioiden laatuun sekä hoitojen myötä syntyneiden lapsien terveyteen. Tutkimuksen tavoitteena oli kerätä ja analysoida hoidoista tallennettu tieto ja löytää mahdollinen tilastollinen merkitsevyys kalsiumaktivaation sekä paremman alkion laadun välillä. Lisäksi Hormoni- ja lapsettomuuspoliklinikka oli kiinnostunut vastasyntyneiden terveydestä. Tutkimuksessa heikon ennusteen potilaat ovat määritelty Fimlabin hedelmöityshoitolaboratorion näkökulmasta.

Menetelmät: Tutkimus on retrospektiivinen tietokantatutkimus rajatusta Babe IVF System -materiaalista. Tutkimuksen aineisto on tallennettu vuosien 2016 ja 2020 välillä. Munasolut on kerätty ja aktivoitu vuosien 2016 ja 2019 välillä. Alkioiden pisteytys on tehty ESHRE:n ohjeisiin pohjautuen.

Tutkimustulokset: Alkion laatua saatiin parannettua 61 % kaikista aktivoiduista alkioista. Lisäksi 24 % tapauksessa alkioiden laatu pysyi vähintäänkin samana eikä huonontunut tehdyn kalsiumaktivaation myötä. Tutkimuksessa hedelmöittymisprosentti oli 56 %. Kaikkien tehtyjen alkionsiirtojen jälkeen kliinisten raskauksien määrä oli 26 % ja 18 % kaikista siirtokerroista syntyi terve lapsi.

Johtopäätökset: Kalsiumaktivaatiot ovat lupaavia hoitoja ja tämän tutkimuksen tulosten perusteella alkioiden laatua voitiin parantaa kalsiumaktivaatiolla. Tutkimuksen tuloksia rajoittivat monet tekijät, kuten vielä suhteellisen pieni potilasmäärä. Pieni potilasmäärä vaikutti myös siihen ettei tutkimuksessa löydetty selkää syytä, miksi joidenkin potilaiden alkioiden laatu ei parantunut. Tulokset olivat kuitenkin monelta osin rohkaisevia ja tärkeimpänä tieto siitä, että syntyneet lapset olivat terveitä.

Avainsanat: kalsiumaktivaatio, keinotekoinen munasolujen aktivaatio, mikrohedelmöitys, kalsimysiini, alkiolaatu

Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck –ohjelmalla.

PREFACE

This study was carried out in collaboration with the Fimlab IVF laboratory, which works in cooperation with the Reproductive Endocrinology Outpatient Clinic in Pirkanmaa Hospital District. I would like to thank especially my instructor, the head embryologist Tiina Mikkilä, for the support, patience and help that she has given me during this master's thesis. Also, I would like to thank the employees in the Fimlab IVF laboratory and the Reproductive Endocrinology Outpatient Clinic for producing my study material. The grammar was checked by Anna-Tuulikki Beckers, whose help was invaluable. Last but not the least, I would like to thank my husband Jussi, for encouraging me to complete this master's thesis.

Tampere, 2nd December 2021

Mirva Seppänen

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LIST OF SYMBOLS AND ABBREVIATIONS

BABE IVF System	Database created by Cleodora Medical Ltd
ESHRE	European Society of Human Reproduction and Embryology
IBM SPSS	Data analysis tool, version 26, created by IBM Corporation
WHO	World Health Organisation
ART	Assisted reproduction technique
AOA	Artificial oocyte activation
A23187	Calcimycin, calcium ionophore
Ca^{2+}	Calcium ion
DAG	Diacylglycerol
HA	Hyaluronic acid
ICSI	Intracytoplasmic sperm injection
IP_3	Inositol 1,4,5-triphosphate
IUI	Intrauterine insemination
IVF	In vitro fertilization
NP	Non-progressive motility
PB	Polar body
PGC	Primordial germ cell
PICSI	Physiological intracytoplasmic sperm injection
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLCζ	Phospholipase C zeta
PN	Pronucleus
PR	Progressive motility
PVS	Perivitelline space
ZP	Zona pellucida

1. INTRODUCTION

On the whole, since 1900 to 2019 birth rate has been decreasing in Finland. In the early twentieth century females have had five children in average, and in 2019 1,35 children in average. (Retrieved from https://www.stat.fi/til/synt/2020/synt_2020_2021-04-23_tie_001_fi.html on 20th October 2021). Nowadays, couples are older when they birth their first child (Rotkirch et al., 2017) e.g., during the last ten years the average age of new mothers has increased by 1,4 years, thus being 29.7 years in 2019. (Retrieved from https://www.stat.fi/til/synt/2020/synt_2020_2021-04-23_tie_001_fi.html on 20th October 2021). Couples are delaying parenthood for multiple reasons, but also certain biological factors, such as the parents' age, quality of gametes and environmental factors may partly explain this negative development (Harper et al., 2017; Jørgensen et al., 2011). In 2018 the Finnish Institute for Health and Welfare reported that during the last decade the total number of assisted fertility treatments hadn't changed. However, the percentage of in vitro fertilizations (IVF) had increased, and the percentage of intrauterine inseminations (IUI) had decreased. (Finnish institute for health and welfare, 2020)

In 2016 European Society of Human Reproduction and Embryology (ESHRE) reported that in Finland 3% of all infants were born as a result of assisted reproduction technique (ART) (European Society of Human Reproduction and Embryology, 2020). In 2018 Finnish Institute for Health and Welfare reported that this number had increased to 5.4% (Finnish institute for health and welfare, 2020). These percentage values together with the change in treatment protocol underlines and supports the fact that more and more couples need IVF.

IUI, IVF and intracytoplasmic sperm injection (ICSI) are common assisted reproduction techniques (Mangoli et al., 2008). In IVF and ICSI, the gametes are extracted from the couple or a donor at an infertility clinic. In both methods the fertilization is carried out in a petri dish. In IVF method sperm cells are transferred into the same dish as the oocyte. The most vital spermatozoon fertilizes the oocyte. In ICSI method the best sperm is identified by a microscope and injected into the oocyte. Researchers have added a physiological aspect to ICSI method, where the selection of spermatozoon is carried out with hyaluronic acid (HA) (Parmegiani et al., 2010).

This master's thesis is centred on ICSI which is a significant ART. ICSI is known to help male factor infertility and poor prognosis infertility patients (Mangoli et al., 2008; Nasr-Esfahani et al., 2010). In addition, various studies show that some couples benefit more from a combination of ICSI and artificial oocyte activation (AOA), than ICSI on its own (Heindryckx et al., 2008; Montag et al., 2012; Vanden Meerschaut et al., 2012). When ICSI fails, there are often problems with the oocyte activation – this is where the ICSI-AOA combination comes in (Bonte et al., 2019; Vanden Meerschaut et al., 2012). Oocyte activation is a complex series of molecular reactions. Oocyte activation is triggered by phospholipase C zeta (PLCζ) via the inositol 1,4,5-triphosphate (IP_3) pathway (Bonte et al., 2019; Saunders et al., 2002). Ca^{2+} ions are released from endoplasmic reticulum of the oocyte as a result of the IP_3 pathway (Taylor et al., 2014). In vivo released Ca^{2+} ions trigger the formation of the fertilized egg into an embryo (Saunders et al., 2002)

This study is carried out together with Fimlab IVF laboratory which works in cooperation with the Reproductive Endocrinology Outpatient Clinic in Pirkanmaa Hospital District. Together they are responsible for approximately one third of all infertility treatments in the Finnish public sector (*Annual Report of Fimlab IVF Laboratory*2019). This master's thesis compares treatment cycles before and after the calcium activation. This study is centred on the quality of the transferred embryos and the health of the newborns. The data concerning infertility treatments has been gathered between years 2016 and 2020 in the Fimlab IVF laboratory database.

The aim of this study is to collect and analyse the data, and to find a possible significance between activated oocytes and better quality of the embryos. A few studies have found this connection between calcium activation and better embryo quality (Ebner et al., 2015; Karabulut et al., 2018), in addition to calcium activation improving e.g. fertilization rate (Bonte et al., 2019; Ebner et al., 2015; Karabulut et al., 2018; Nazarian et al., 2019). The Reproductive Endocrinology Outpatient Clinic has also been interested in the health of the newborns. This study defines and observes the poor prognosis patients from Fimlab IVF laboratory's point of view. The results indicate that calcium activation of oocytes improves the quality of embryos. 17.8% of all treatment cycles resulted in live birth, which was exactly the same rate as nationally in the public sector in 2018 (Finnish institute for health and welfare, 2020). Based on the available clinical data, each of the 38 children that were born following ICSI-AOA were healthy.

The literature review of this thesis gives an overview of the germ cell production in both females and males, as well as the fertilization of oocytes and the implantation. In addition, the review introduces the definition of a poor prognosis patient. The theory section is centred on the semen analysis and the artificial reproductive techniques, e.g., calcium activation and physiological intracytoplasmic sperm injection (PICSI). The theory section also opens up the criteria of embryo grading. The grading of cleavage stage embryos and blastocysts are represented separately. Before the results and discussion is a short section on materials and methods.

2. LITERATURE REVIEW

2.1 Gametogenesis to implantation

2.1.1 Primary germ cell division

Meiosis is a process which generates female and male gametes. In meiosis, cells first undergo mitosis and then meiosis I and meiosis II, whereas in somatic division cells undergo only mitotic cell division. Female and male meiosis differ from each other. The male meiosis produces four haploidic gametes, whereas the female meiosis produces one oocyte, and the remaining cells form polar bodies (PBs) in the perivitelline space (PVS). Difference between those divisions is shown in *Figure 1*. (Sainio & Sariola, 2015)

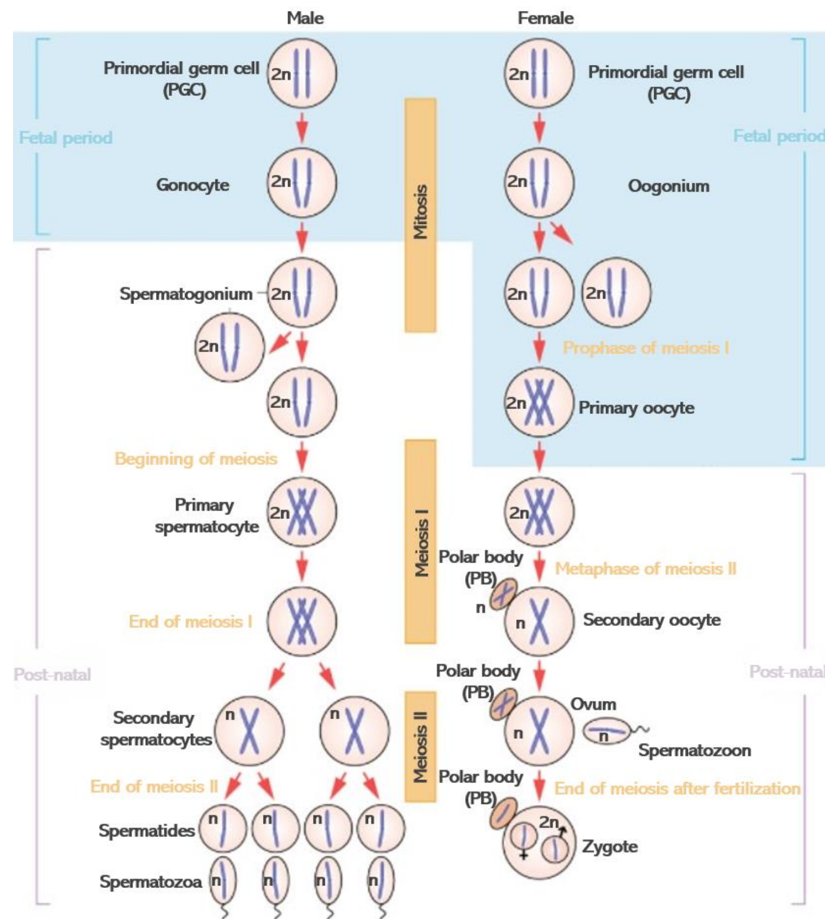

Figure 1. Gametogenesis in humans. *The female meiosis begins already during fetal stage, and then stops at meiosis I prophase. Oocytes continue to mature in puberty but proceed to meiosis II only in fertilized oocytes. In males, spermatogonia form after birth but start to mature into spermatozoa only in puberty. Picture cited and modified from (Sainio & Sariola, 2015).*

2.1.2 Oogenesis

Both male and female meiosis are prone to error, the female meiosis especially so. Female meiosis may be arrested at prophase I for decades. That way maturating oocytes can be exposed to different stress factors which may damage the DNA and cause e.g., fetal aneuploidies. The earliest form of germ cells are called primordial germ cells (PGCs). After PGCs have migrated from the wall of the yolk sac through the dorsal mesentery of the hindgut to the genital ridge, PGCs undergo proliferation and form gonads. PGCs differentiate into oogonia, that divide by mitosis in the developing ovaries. Some of the oogonia begin a meiotic division into primary oocytes, and the rest of the diplotene oogonia die by apoptosis. (Sainio & Sariola, 2015) From the 6th week to the 20th week of gestation, the number of PGCs rises exponentially (*Figure 2*). The number is highest during the 20th week of gestation. (Oktem & Urman, 2010) Thereafter, the number of cells declines. The finite number of female germ cells is reached before birth. (Sainio & Sariola, 2015) Less than 1 % of germ cells will reach the ovulatory phase before menopause and most of them undergo atresia (Oktem & Urman, 2010; Sainio & Sariola, 2015).

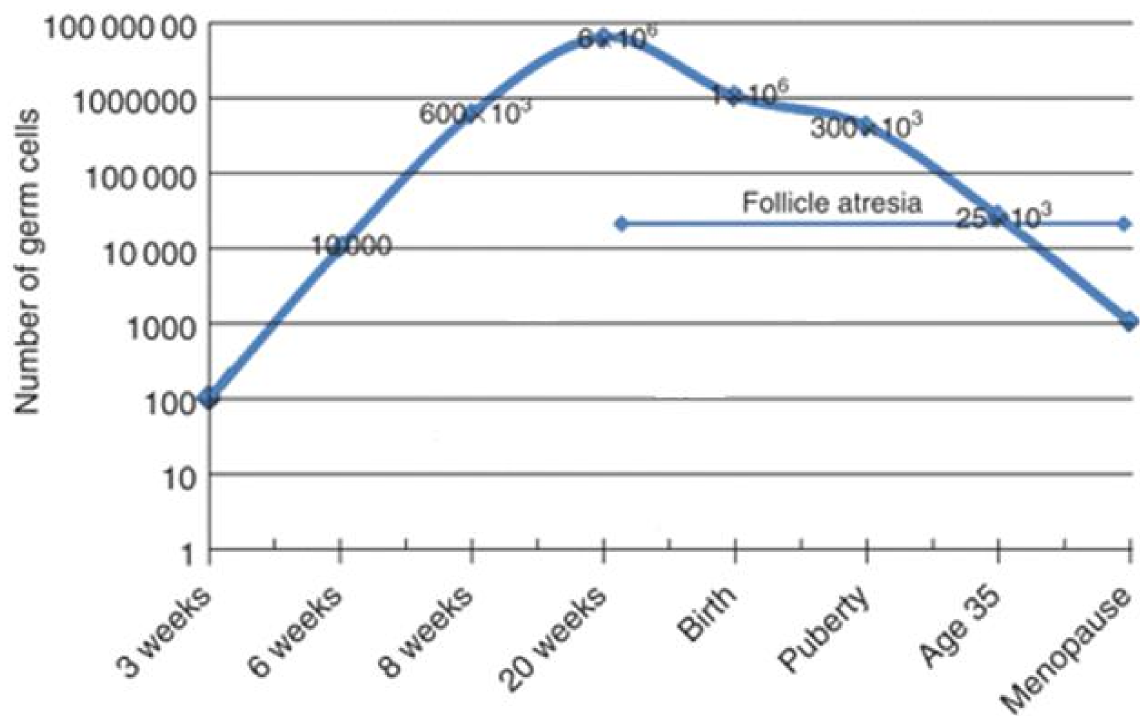

Figure 2. Number of germ cells. *The exponential increase in the germ cell count, and the atresia starting on gestation week 20. Picture cited and modified from (Oktem & Urman, 2010).*

Oocytes are arrested at prophase of meiosis I throughout the follicular growth process. Diplotene oocytes and flattened pregranulosa cells form the primordial follicles before birth. Primary oocytes in primordial follicles have finished meiotic recombination. After hormonal stimulation the cells of a sexually mature female move onto the first meiotic division. Meiotic division is arrested at metaphase of meiosis II until the oocytes become fertilized by a sperm. (Sainio & Sariola, 2015) At meiosis II stage the first PB is visible in the PVS (*Figure 3*). This indicates that the secondary oocyte's nucleus is mature. (Gardner et al., 2018)

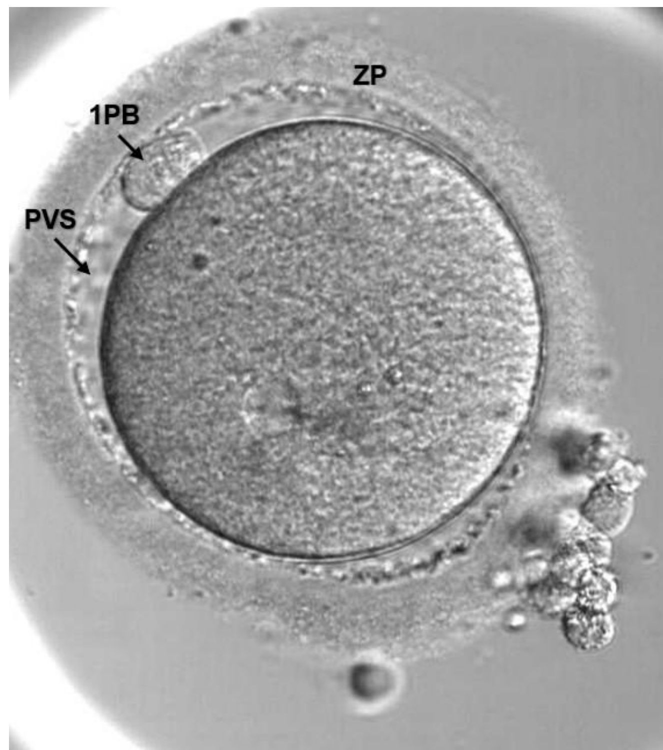

***Figure 3.* Normal metaphase, stage II oocyte.** *Mature metaphase II oocyte with normal morphology. The first polar body (1PB) is visible in the perivitelline space (PVS). The oocyte is surrounded by zona pellucida (ZP). The oocyte was cultured in EmbryoSlide® and imaged in the EmbryoScope® plus (Vitrolife) time-lapse system.*

2.1.3 Spermatogenesis

Spermatogenesis requires communication between PGCs and supporting Sertoli cells. Together those cells form seminiferous cords and later develop into seminiferous tubules. PGCs change morphologically and during this slight maturation cells become gonocytes, precursors of spermatogonia. (Sainio & Sariola, 2015; Schulz & Miura, 2002)

During embryo genesis, some of the diplotene gonocytes divide by mitosis. The differentiation of spermatogonia begins only after birth. In a sexually mature male, gonocytes resume proliferation and form spermatogonia, which begin a meiotic division into spermatozoa. (Sainio & Sariola, 2015)

During a maturation process spermatogonia go through either mitosis or two meiotic divisions. In meiotic division type B spermatogonia differentiate into primary spermatocytes. (Sainio & Sariola, 2015; Schulz & Miura, 2002) This proliferation and maturation process starts in the wall of the seminiferous tubule. Maturating spermatocytes move from the base to the lumen. After meiosis I secondary spermatocytes are formed, which then undergo meiosis II and after divisions form round spermatids. (Sainio & Sariola, 2015)

In the spermiogenesis, elongated spermatids go through morphological changes of the nucleus and cell body. During the spermiogenesis an acrosome forms on the top of the spermatozoon (Sainio & Sariola, 2015). Finally, Sertoli cells phagocytize most of cytoplasm, and spermatozoa are completely developed (Sainio & Sariola, 2015; Schulz & Miura, 2002). Spermatozoa are incapable of swimming when they are released from testis. The spermatozoa gain the ability to swim in the epididymis. (Sainio & Sariola, 2015)

2.1.4 Fertilization into implantation

During growth, eggs form extra cellular matrix (zona pellucida, ZP) by producing glycoproteins. In humans, the ZP is made of different subtype glycoproteins. There are also cumulus cells surrounding the ZP. (Jin et al., 2011; Sainio & Sariola, 2015; Tokuhiro et al., 2012) The ZP matrix has an important role in taxon-specific binding of the spermatozoa to the oocyte (Rankin et al., 2003; Sainio & Sariola, 2015). In addition, ZP enables the fertilized oocyte to proceed into the uterus and prevents it from implanting into the fallopian tube (Sainio & Sariola, 2015). Before implantation ZP begins to thin and it tears allowing the embryo to hatch. If embryos do not hatch, they cannot implant into the endometrial lining of the uterus. (Partanen et al., 2015)

Before the spermatozoa can fertilize the oocyte, they must undergo capacitation. That includes different physiological changes e.g., calcium influx and cholesterol efflux. In addition, spermatozoa undergo acrosome reaction. (Jin et al., 2011; Sainio & Sariola, 2015) In acrosome reaction, enzymes are released from the head of spermatozoa. By the help of enzymes that are released by the acrosomal reaction, spermatozoa can penetrate

inside the oocyte. During fertilization, spermatozoa bind to the surface of the ZP. Primary binding and the sperm penetration through the ZP triggers fusion of germ cells plasma membrane. In addition, the ZP of the oocyte changes in order to prevent polyspermy. Furthermore, meiosis of quiescence oocyte is resumed after the sperm penetration. (Sainio & Sariola, 2015) HA is the main component of the cumulus oophorus. *In vivo*, oocytes are surrounded by HA that may play a role in the selection of sperm. (Jin et al., 2011; Parmegiani et al., 2012; Worrilow et al., 2013)

After sperm-oocyte fusion the membrane potential of the oocyte changes. The change in the potential causes a cortical reaction which blocks polyspermia. Fertilization takes place in the fallopian tube ampulla. After fertilization, the fertilized egg goes through mitotic divisions and compaction. (Sainio & Sariola, 2015) After 6-7 days, the embryo reaches the blastocyst stage and implants into the wall of the uterus. (Partanen et al., 2015). The time when the uterus is receptive for implantation is called window of implantation. In the mid-luteal phase of the menstrual cycle, the uterus prepares implantation by production of hormones, growth factors and cytokines. (Bassil et al., 2018; Corbett & Blackburn, 2017; Dimitriadis et al., 2010; Sainio & Sariola, 2015)

2.2 Oocyte retrieval and embryo transfer in IVF

2.2.1 Ovarian stimulation and oocyte processing

The purpose of ovarian stimulation is to stimulate oocyte maturation and to prepare the uterine for the implantation. The number of collected oocytes varies depending on the response to hormonal stimulation. However, the aim of the stimulation is that several oocytes mature at same time and produce good quality embryos after fertilization by IVF. Infertility specialists can select between two types of stimulation protocols, gonadotropin-releasing hormone (GnRH) antagonists or GnRH agonist. Before the oocyte pick up protocol the woman gets an injection of human chorionic gonadotropin (hCG). The final stage before the oocyte pick up is ovulation induction with injection of hCG, which is done when the biggest ovarian follicles are 17 mm in diameter. (Morin-Papunen & Koivunen, 2012)

After the stimulation the ovarian follicles are punctured through the vaginal wall while using an ultrasound control. Follicular fluid is collected in sterile tubes, and oocytes are identified. (Morin-Papunen & Koivunen, 2012) Before fertilization the surface of the oocyte is denuded of granulosa cells mechanically using a specific media. Mature oocytes

are fertilized and activated by ICSI. After fertilization and activation, cell culture dishes will be put in the incubator under 37 ºC, 6 % CO_2, 6 % O_2. Embryos will usually be transferred at day 3-5 of culture. If necessary, embryos can be transferred or frozen also on day 2 or day 5. (Fimlab's work instruction, 2017) Two pronuclei (2PN) checks should be performed on the embryos within 16-18 hours after ICSI. (Retrieved from https://atlas.eshre.eu/ on 24th October 2021; Fimlab's work instruction, 2017)

2.2.2 Types of embryo transfer

After a few days of culture, the best embryos are chosen to be transferred into the uterus. In fresh embryo transfer, one or two embryos are transferred. In case the couple has any good quality embryos left after fresh embryo transfer, they can be frozen and stored for future use. (Morin-Papunen & Koivunen, 2012) In Fimlab IVF laboratory, either top or good quality embryos are good enough to qualify for freezing. This procedure has decreased the proportion of multiple births during the last decades. (Finnish institute for health and welfare, 2020; Morin-Papunen & Koivunen, 2012)

Extended embryo culture might also be a possibility. In that case blastocyst embryos are transferred into a uterus on day 5. This involves e.g., a higher risk of preterm delivery, monozygotic twins and large for gestational age babies. Despite the concerns regarding extended embryo culture, many IVF clinics use this strategy. (Maheshwari et al., 2016). There are also a few advantages to blastocyst stage transfers. The nutritional environment in the uterus is better for blastocysts than for cleavage stage embryos. *In vivo*, the cleavage stage embryos enter the uterus after compaction, but *in vitro* the cleavage stage embryos are transferred into the uterus before compaction. The oviduct and the uterus have different nutritional environments for embryos. This difference between nutritional environments could be one reason why blastocysts are more viable in IVF treatments than cleavage stage embryos. (Gardner et al., 1998) Blastocyst stage transfer leads to a higher pregnancy rate per embryo transfer in comparison to cleavage stage transfer (Gardner et al., 1998; Maheshwari et al., 2016) It is also possible to identify embryos that have limited developmental potential (Gardner et al., 1998). Due to circumstances Fimlab IVF laboratory is transferring mainly fresh cleavage stage embryos, and extended embryo culture is used in frozen embryo transfers.

2.2.3 Time-lapse technology

Generally, embryo selection is based on several different morphological grading systems (Gardner, D. K. & Schoolcraft, 1999), which may limit embryo assessment (Campbell et

al., 2013; Chamayou et al., 2013; Gardner & Schoolcraft, 1999; Wong et al., 2010). Time-lapse technology and a one-step culture medium allow uninterrupted embryo development and continuous control at the same time. IVF specialists get detailed information e.g., cleavages, compaction and blastulation. In addition to morphological parameters time-lapse technology offers dynamic parameters for embryo assessment (Campbell et al., 2013; Chamayou et al., 2013; Wong et al., 2010).

On this decade studies have been focused on the possibilities of detecting aneuploidies and indicating implantation potential through cleavages, compaction and blastulation. Correlations have been found. (Campbell et al., 2013; Chamayou et al., 2013; Wong et al., 2010) In addition, Zhan et al. (2016) study is based on cleavage stage embryos, especially those in the first and the second cleavage stages, where e.g., the 2-cell stage embryo has resulted in 5 or 6 blastomeres. This kind of cleavage is called direct unequal cleavage (DUC). Number after the DUC refers to the number of cleavages. After the third cleavage DUC is difficult to assess, especially due to the high number of cells and their small size. Zhan et al. (2016) noticed a decline in DUCs after the second cleavage and discussed the possible differences between DUC mechanisms in early cleavage stage embryos and later cleavage stage embryos. DUCs decrease the embryo's ability to form blastocyst and its implantation potential. (Zhan et al., 2016)

2.3 Poor prognosis infertility patients and causes of infertility

After conventional ICSI total fertilization failure is still 3-5% of all ICSI cycles (Kashir et al., 2010; Montag et al., 2012). The main reason for total fertilization failure is oocyte activation deficiencies that occur after conventional ICSI. Deficiencies can be related to both sperm and oocyte factors. (Heindryckx et al., 2008; Kashir et al., 2010; Montag et al., 2012) The report of the consensus meeting in Vienna in 2017 recommended the normal fertilization rate be more than 65% and the poor fertilization rate be less than 25% (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017). In Fimlab the IVF laboratory specialists consider the ICSI when the fertilization rate is below 50%. The tight threshold is well-founded because the couples get three treatment cycles from the public sector.

From the laboratory's point of view, both low fertilization and poor-quality embryos in previous treatment cycles decrease the prognosis. Even one of these indicators alone may decrease the prognosis. In addition, female age is a significant factor both clinically

and from the laboratory's point of view, e.g. since the oocytes are prone to error during the entire maturation.

Female age is the one the major impairing factor for couples with childlessness. Fertility decreases particularly after the age of 35 (*Figure 4*), and aneuploid oocytes increase. (Franasiak et al., 2014; Grøndahl et al., 2017; Loane et al., 2013; Nelson & Lawlor, 2011; Sainio & Sariola, 2015; Templeton et al., 1996) Especially females over 35 have higher risks for impairments, such as mitochondrial and cohesion dysfunctions, and aneuploidies (Cimadomo et al., 2018). Female age is not the only reason for infertility, but it is a complex problem, for which the reasons are difficult to study.

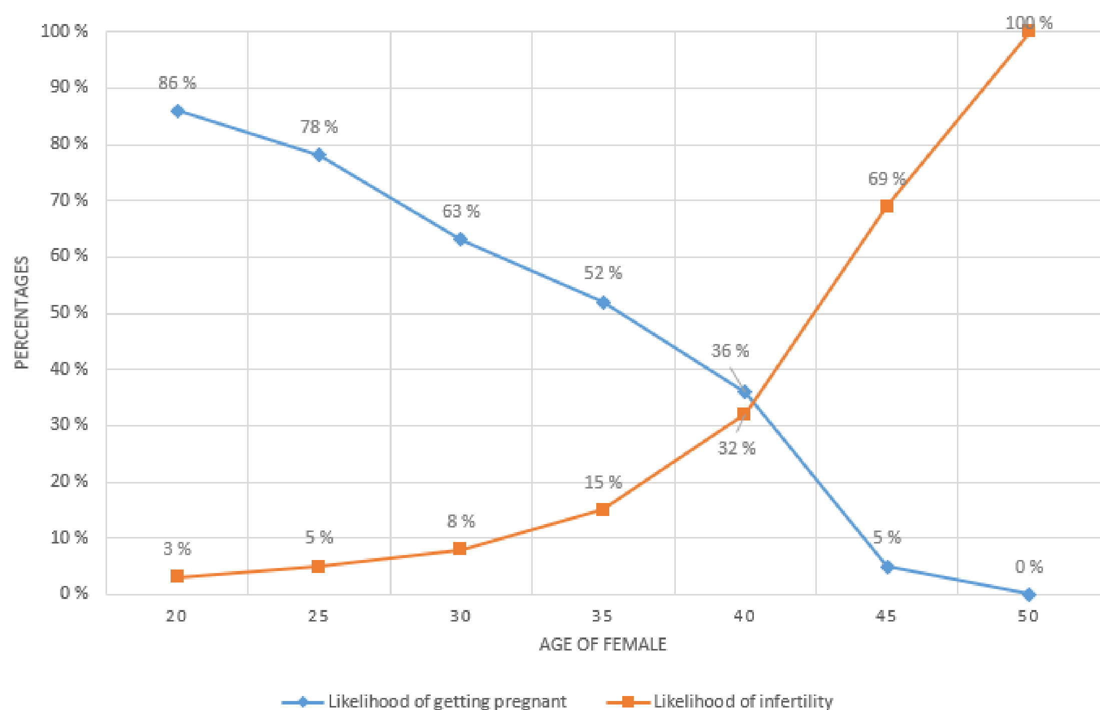

Figure 4. Fertility and age of female. *As the age of female increases, also the likelihood of infer-tility increases, and the likelihood of getting pregnant decreases. The changes start to occur faster after the age of 35. Chart made and modified based on the information by BabyCenter, L.L.C. (Retrieved from https://www.babycenter.ca/a6155/your-age-and-fertility on 25th August 2021)*

Generally, it is acknowledged that sperm-derived PLCζ is the trigger for oocyte activation after sperm-egg fusion (Kashir et al., 2010; Kouchi et al., 2005; Saunders et al., 2002). Both Yoon et al. (2008) and Heytens et al. (2009) provided the first evidence for the link between male infertility and defects in the PLCζ gene. They identified a point mutation in the PLCζ gene in infertile men (Heytens et al., 2009; Yoon et al., 2008). Furthermore, several studies have shown that both decreased protein expression and abnormal PLCζ

are causes for male infertility (Kashir et al., 2012; Nomikos et al., 2011; Nomikos et al., 2013; Yoon et al., 2008).

In addition, sperm quality is a globally significant reason for male infertility (Skakkebaek et al., 2015), and also in Finland (Jørgensen et al., 2011). Jørgensen et al. (2011) carried out their surveillance study in Turku but the results can be applied to the whole Finnish population (Jørgensen et al., 2011). According to the study, environmental factors might be the most likely reason behind the quality results. Also, testis cancer had increased during the surveillance period. Concentration, percentages of morphologically normal spermatozoa, the total number of morphologically normal spermatozoa and total sperm counts had decreased during the study period. Only the volume of ejaculate did not differ. (Jørgensen et al., 2011) Generally, semen quality has been found to decrease in several areas (Levine et al., 2017) and complex infertility problems have been noticed (Gore et al., 2015; Mascarenhas et al., 2012; Segal & Giudice, 2019).

2.4 Screening seminal fluid

Semen analysis is a common method of assessment of male fertility. Commonly, the evaluation of male infertility is based on the World Health Organization (WHO) guidelines. (Jørgensen et al., 2006) Fimlab IVF laboratory also follows this general guideline. Sperm consist of two components, the spermatozoa and the seminal fluid. Both components are defined during the semen analysis. In the evaluation volume of the ejaculate, the concentration of spermatozoa are measured. (Palermo et al., 2014) Also pH, motility, morphology and vitality are important for the sperm to function. All these four indicators are important to define during the analysis (World Health Organization, 2010).

Several studies have aided the WHO to create the concept of normal spermatozoa (Fredricsson & Bjork, 1977; Liu & Baker, 1992; Menkveld et al., 1990; Menkveld et al., 1991). Spermatozoa assessment is difficult because of variable morphology (Fredricsson & Bjork, 1977; Menkveld et al., 1990). Assessment of sperm morphology is a controversial subject (Check et al., 2002; Menkveld et al., 2011). WHO guidelines give detailed and precise instructions for morphology analysis. A normal sperm sample must contain at least 4 % sperms that have normal morphology. The reference limit is valid only if evaluation is carried out using WHO guidelines. (World Health Organization, 2010)

If the volume of a correctly given sperm sample is low, it might indicate a blockage or anatomical anomalies (Auger et al., 1995). The lower reference limit for semen volume

is 1.5 ml. The condition where semen volume is below this limit is called hypospermia. In addition to volume assessment, also pH can be assessed. The assessment should be done as soon as possible, within 30 minutes because semen pH increases with time. The consensus value of the WHO guideline book is 7.2 which is a lower limit value for pH. (World Health Organization, 2010) In Fimlab IVF laboratory pH is assessed only if semen volume is low.

Concentration is one of the most important parameter of semen analysis. The lower reference limit for sperm concentration is 15×10^6 spermatozoa per ml. The WHO recommends using this total number of spermatozoa per ejaculate. The lower reference limit for total sperm number is 39×10^6 spermatozoa per ejaculate. (World Health Organization, 2010) This threshold is based on the fact that a higher sperm concentration does not offer any benefit (Bonde et al., 1998). Low sperm count means cryptozoospermia, in which case the sperm can be detected only in a centrifuged sperm sample. Furthermore, azoospermia should be suspected if no spermatozoa are detected in the sperm analysis. (World Health Organization, 2010) It is noteworthy in the studies of Mortimer (1994) and Cooper et al. (2006) that concentration will be underestimated after centrifugation and spermatozoa motility can be lost. (Cooper et al., 2006; Mortimer, 1994).

Pregnancy rates depend on progressive sperm motility (Jouannet et al., 1988; Larsen et al., 2000; Zinaman et al., 2000). A normal sperm sample should have more than 32 % actively moving spermatozoa. Progressive motility (PR) means spermatozoa that move linearly or in a large circle. All other patterns of motility are non-progressive motility (NP). Together PR and NP equal total motility which should be more than 40 % in normospermia. In asthenozoospermia, the sperm sample contains less than 32 % linearly moving spermatozoa. The condition where the sperm sample contains less than 39×10^6 spermatozoa per ejaculate and less than 32 % linearly moving spermatozoa is called oligoasthenozoospermia. (World Health Organization, 2010)

Vitality assessment is important for sperm samples with less than 40% progressive motile spermatozoa. Both motility and vitality should be analysed as soon as possible, rather within 30 minutes. Spermatozoa viability can be assessed by using the dye exclusion method or hypotonic swelling. The dye exclusion method is based on membrane-impermeant dyes and a phenomenon where dead cells allow the entry of stains. In normospermia the sperm sample should have more than 58% membrane-intact spermatozoa. (World Health Organization, 2010)

2.5 Assisted reproduction techniques

2.5.1 Intracytoplasmic sperm injection

Originally ICSI was created to help male factor infertility (Ebner et al., 2012; Heindryckx et al., 2008; Palermo et al., 1992). The fertilization rate after ICSI is approximately 70% and the total fertilization failure is 2 to 5% of all ICSI cycles (Bonte et al., 2019; Kashir et al., 2010; Montag et al., 2012). Even if ICSI brings relief to couples suffering from male factor infertility and abnormal fertilization (Kashir et al., 2010; Montag et al., 2012), there are still couples who don’t benefit from it because they suffer from infertility caused by the sperm not being able to activate the oocytes (Mangoli et al., 2008; Nasr-Esfahani et al., 2010). Before the actual sperm injection, spermatozoon is immobilized by damaging the tail (Yanagida et al., 2001). Immobilization of the sperm is one of the tools used to assist in the activation of the oocyte.

2.5.2 Calcium activation of oocytes

Fusion of the germ cell plasma membrane enables releasing of PLCζ into the oocyte (*Figure 5*). PLCζ is the one of the key activators for oocytes. PLCζ is not expressed in the earliest stages of spermatogenesis. PLCζ is expressed at the stages from spermatids to spermatozoa. (Saunders et al., 2002) The PLCζ presence correlates with the capability to produce Ca^{2+} oscillations. Momentary increase in intracellular calcium concentration is the first signalling process in the activation of an egg. Generally, Ca^{2+} oscillations are a result of IP_3 mediated Ca^{2+} release. Phosphatidylinositol 4,5-bisphosphate (PIP_2) from plasma membrane hydrolyses into IP_3 and diacylglycerol (DAG) (Nomikos et al., 2013; Taylor et al., 2014; Yoon et al., 2008). IP_3 binds to IP_3 receptors within the endoplasmic reticulum and releases Ca^{2+} ions from the storage (Nomikos et al., 2005; Taylor et al., 2014). *In vivo*, Ca^{2+} oscillations activate the oocyte (Martínez et al., 2021) and trigger the formation of the fertilized egg into an embryo (Saunders et al., 2002). Depending on the species Ca^{2+} activation in vivo is either single transient or oscillatory (Vanden Meerschaut et al., 2014).

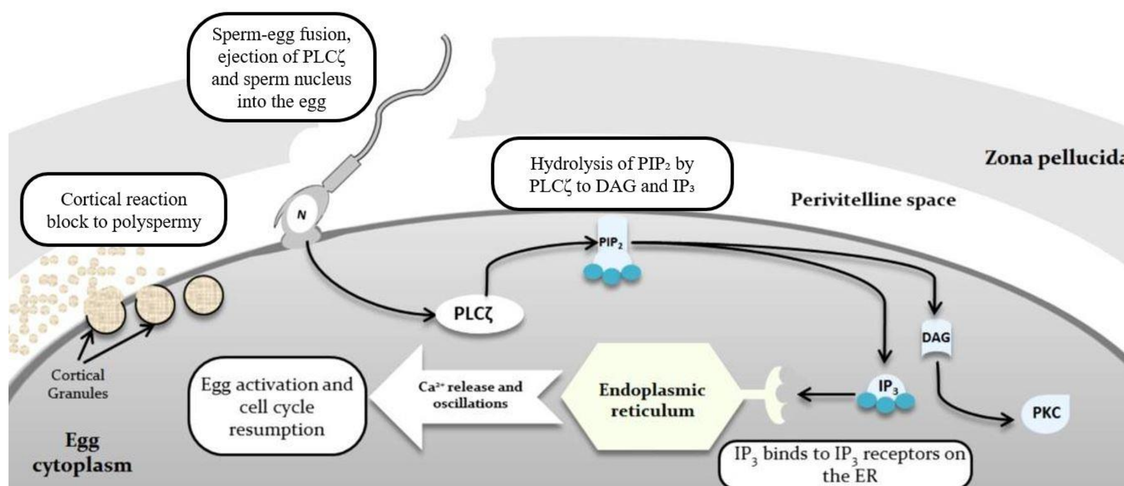

Figure 5. Fusion of germ cell plasma membrane, and releasing of PLCζ. *As a result of the sperm-egg fusion, PLCζ is released from the sperm cell. PLCζ hydrolyses PIP2 into IP3 and DAG. DAG activates protein kinase C. IP3 binds to IP3 receptors on the ER. The egg gets activated after the release of calcium. The cortical reaction taking place after the sperm-egg fusion blocks the polyspermy. Picture cited and modified from (Ramadan et al., 2012).*

After ICSI, Ca^{2+} oscillation can be mimicked by the artificial calcium influx from the med ium (Heindryckx et al., 2008; Martínez et al., 2021). Artificially produced Ca^{2+} oscillations do not exactly mimic natural oocyte activation and natural Ca^{2+} oscillations (Heindryckx et al., 2008). In addition to chemical activation methods, mechanical or electrical methods are also feasible. (Heindryckx et al., 2008) In vitro, Ca^{2+} activation with ionophores is like a one top curve (Nikiforaki et al., 2016). Various reports show that couples benefit from ICSI together with AOA (Bonte et al., 2019; Heindryckx et al., 2008; Montag et al., 2012; Murase et al., 2004; Vanden Meerschaut et al., 2012). Van Meerschaut et al. (2012) have shown that ICSI together with AOA is effective for couples with an oocyte-related activation problem after previous ICSI failure (Vanden Meerschaut et al., 2012).

Generally used activating substances for human oocytes are calcium ionophores (Bonte et al., 2019; Ebner et al., 2012). Ionophores make the plasma membrane of the oocyte more permeable, and also have an effect on e.g., endoplasmic reticulum. The most popular calcium ionophores are calcimycin (A23187) and ionomycin (Ebner et al., 2012; Kashir et al., 2010; Nikiforaki et al., 2016) Nikiforaki et al. (2016) have proved that ionomycin is more effective than calcimycin, by activating both mouse and human oocytes (Nikiforaki et al., 2016). For human oocytes, the difference between the two ionophores did not reach the limit of statistical difference. However, a smaller difference between those ionophores was observed. In addition, ionomycin can produce one top Ca^{2+} curve with higher amplitude than calcimycin. (Nikiforaki et al., 2016) The difference might be

based on the observations that embryonic development could be mediated by total summation of Ca^{2+} (Ozil et al., 2005; Tóth et al., 2006). For Ca^{2+} activations, Fimlab IVF laboratory has followed, the instructions of the commercial calcimycin producer, as is appropriate.

In addition to assisting with oocyte activation, calcium can improve embryo quality. Ebner et al. (2015) considered the calcium activation for patients whose embryos were suffering from developmental issues (Ebner et al., 2015) Karabulut et al. (2018) have also found a connection between better embryo quality and AOA. In their study patients had different indications such as total fertilization failure or severe sperm quality impairment. Fertilization rate and pregnancy rate also increased in their study. (Karabulut et al., 2018) Lack of calcium causes e.g. cleavage anomalies, and arrests embryo development at mitotic division (Ebner et al., 2015) is one of the reasons why calcium can improve embryo quality. Apart from these studies, also Bonte et al. (2019) and Nazarian et al. (2019) have found a connection between better fertilization rate and AOA, but not an exact connection between embryo quality and AOA. (Bonte et al., 2019; Nazarian et al., 2019) Many studies can prove the improvement of fertilization rate in connection to AOA but not so many the improvement of embryo quality.

2.5.3 Physiological intracytoplasmic sperm injection

Several small clinal studies have shown that ICSI with HA improves both embryo quality and birth rates. This physiological ICSI (PICSI) is based on the fact that HA is a physiological selector of sperm *in vivo*. (Mokánszki et al., 2014; Parmegiani et al., 2010; Parmegiani et al., 2012; Worrilow et al., 2013) Miscarriage rate was decreased compared to normal ICSI. Miller et al. (2019) do not recommend the use of PICSI extensively at present because PICSI does not substantially promote livebirth rate. (Miller et al., 2019) DNA breaks in a spermatozoon have many negative influences on fertility. *In vitro*, sperm binding to HA indicates only slight DNA fragmentation and normal shape. (Parmegiani et al., 2010; Yagci et al., 2010) This selection might be critical in ICSI cases where limited number of oocytes are available. However, male infertility and causes behind it are studied significantly less than female infertility, even though sperm assessments are common.

2.6 Embryo grading

2.6.1 Cleavage stage embryo

In post-insemination assessments of cleavage stage embryos, Fimlab IVF laboratory follows the guidelines of the Atlas of Human Embryology by ESHRE, as is appropriate (retrieved from https://atlas.eshre.eu/ on 7th April 2021). The Atlas of Human Embryology has general instructions for scoring and grading human oocytes and preimplantation embryos. Mainly, the instructions for cleavage stage embryos are created in-house, and they are based on morphology. Assessments of morphology are performed in special time points.

After the fertilization, normal fertilized oocytes should have 2PN and 2PB (*Figure 6*) (retrieved from https://atlas.eshre.eu/ on 7th April 2021). More accurately, after the fertilization the first PB undergoes cleavage and the second is normally extruded (Partanen et al., 2015). In addition, cytoplasm, oolemma, ZP and PVS should be typical. Early cleavage happens on day 1 (26±1 h, post-ICSI). The embryo is typically at 2-cell stage and PNs have disappeared (retrieved from https://atlas.eshre.eu/ on 7th April 2021).

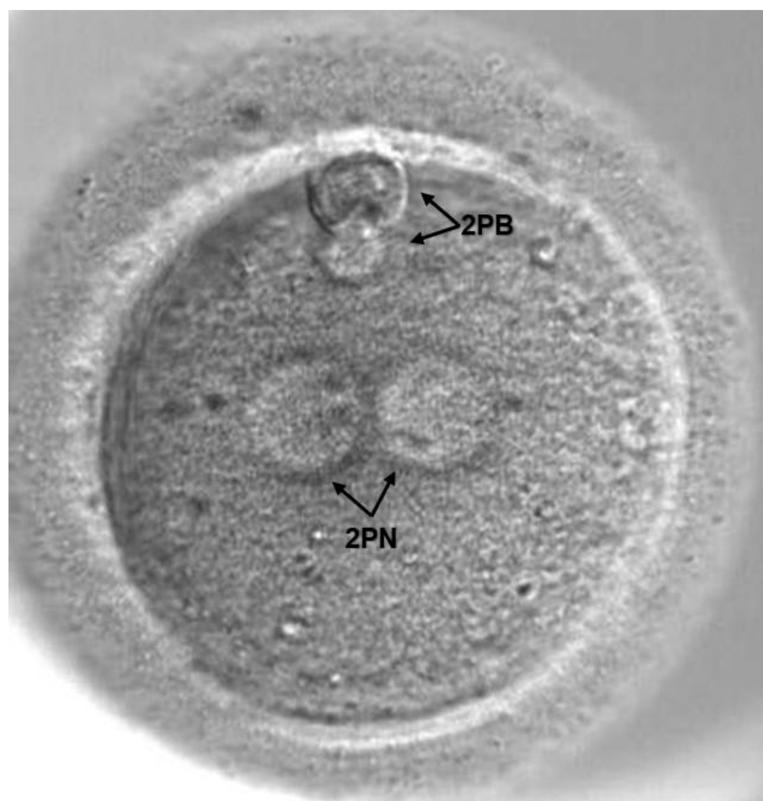

Figure 6. Normal fertilized oocyte. *Two polar bodies (2PB) are visible in the upper region of the oocyte, and two pronuclei (PN) in the middle. The zygote was cultured in EmbryoSlide® and imaged in the EmbryoScope® plus (Vitrolife) time-lapse system.*

On day 2 (44±1 h, post-ICSI), the embryo is typically at 4-cell stage. The blastomeres' sizes are compared to each other. Regularity in blastomere sizes has been noticed to increase pregnancy outcome (Giorgetti et al., 1995; Hardarson et al., 2001; Holte et al., 2007; Ziebe et al., 1997). Blastomeres should be equal in all 2-, 4- and 8-cell stages (*Figure 7*). Bigger than a 20 % difference in size is significant (*Figure 8*). Also fragmentation, nucleation, cytoplasm and ZP are assessed and rated. (Retrieved from https://atlas.eshre.eu/ on 7th April 2021) On day 3 (68±1 h, post-ICSI), when the embryo is at 8-cell stage, assessment and scoring are performed.

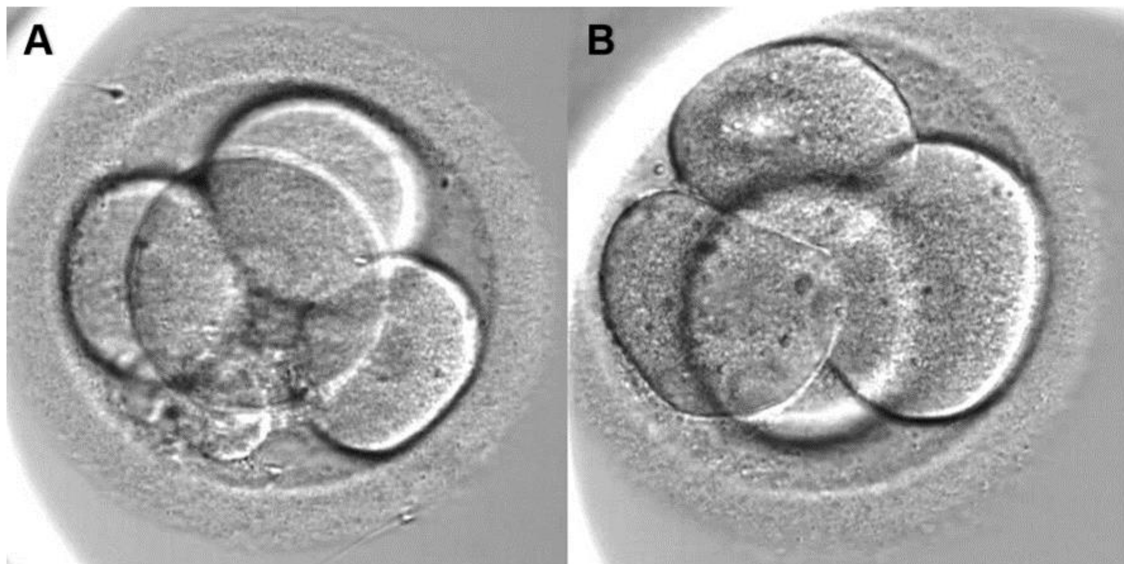

Figure 7. 4-cell stage embryos on day 2. *A. 4-cell stage embryo with over 20% size difference in blastomeres. B. 4-cell stage embryo with maximum score. Equal sized blastomeres, and no anomalies in morphology. Embryos were cultured in EmbryoSlides® and imaged in the EmbryoScope® plus (Vitrolife) time-lapse system*

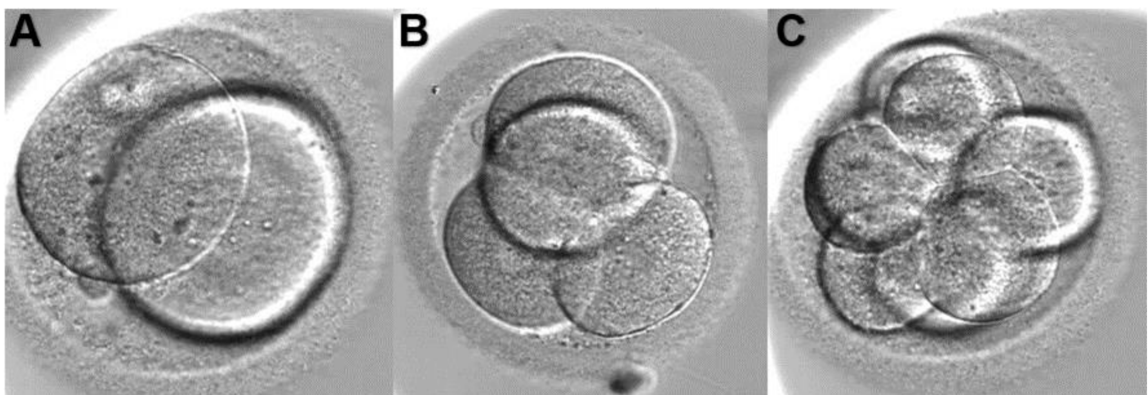

Figure 8. The size difference of the blastomeres can be maximum 20%. *A. 2-cell stage embryo without significant size difference in blastomeres. B. 4-cell stage embryo without significant size difference in blastomeres. C. 8-cell stage embryo without significant size difference in blastomeres. Embryos were cultured in EmbryoSlides® and imaged in the EmbryoScope® plus (Vitrolife) time-lapse system.*

Fragmentation is reported as the percentage of the total cytoplasmic volume. The desirable degree of fragmentation is less than 10 % (Retrieved from https://atlas.eshre.eu/ on 7th April 2021). The implantation potential of the embryo is weaker if e.g., multinucleation is detected (Jackson et al., 1998; Moriwaki et al., 2004; Pelinck et al., 1998; Van Royen et al., 2003). Anomalies in cytoplasm can be granularity, vacuolization and cytoplasmic pitting. A possible predictive value of anomalies in e.g., embryo quality is unclear. (Retrieved from https://atlas.eshre.eu/ on 7th April 2021) For example, wide vacuolization might be harmful for the embryo, however the presence of small vacuoles in not significant (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). In the Fimlab IVF laboratory's scoring system, maximum score is 3.5, and minimum score 2.0, in which range the embryo is usable. Each morphology difference cuts half a point from the maximum score, and multinucleation cuts a whole point. For example, a 4-cell stage embryo, on day 2, that consists of equal size blastomeres, and in which morphology anomalies are not observed, will get a score 3.5 (*Figure 8*).

On day 4, assessment focuses on the compaction process of blastomeres. What is evaluated is the proportion of the compaction rate (in percentages) to the cells involved in the compaction (in percentages). Tao et al. (2002) have proved that implantation and pregnancy rates are better if all blastomeres are included in the compaction process (Tao et al., 2002). Furthermore, fragmentation and other anomalies are observed. In *Figure 9* is a top-quality morula in which the compaction stage is ≥ 75 %. At the 4- to 8-cell stage assessments are important because before the embryonic genome is activated, the evaluation of the embryo and of the growth potential may be erroneous (Tao et al., 2002).

2.6.2 Blastocyst

In vitro, between day 4 and 5, the volume of fluid increases in the cavity (the blastocoel). By day 5 embryos develop into blastocysts. Cells start to specialise, becoming inner cell mass (ICM) and trophectoderm (TE). The trophoblast cells' Na^+/K^+-ATPase membrane channels raise Na^+ concentration within the embryo. Salt concentration increases because osmosis and water enlarge the cavity. (Partanen et al., 2015; Watson et al., 2004) TE has an important role in embryo implantation, e.g., those cells form the placenta, and ICM differentiates in stages into the fetus. Fgf4 signalling between ICM cells and TE cells stimulates trophoblast cell division and differentiation. The inner layer of trophoblast cells differentiates into cytotrophoblast cells which can attach to the uterus. Then cytotrophoblast cells differentiate into syncytiotrophoblast cells which penetrate into the wall of the uterus. (Partanen et al., 2015)

In the Fimlab IVF laboratory, the grading of blastocyst is based on the Gardner and Schoolcraft system (Gardner & Schoolcraft, 1999). The system incorporates the rate of the blastocoel enlargement and both the quality and number of two different cell lines. The rate of the blastocoel is assessed on a six-step scale, where number one is an early blastocyst and number six is a hatched blastocyst. ICM and TE quality analysis are performed for blastocysts that score ≥ 3 (full blastocyst). ICM and TE quality points are given in three classes, where A is the highest and C is the lowest. For example, top-quality blastocyst will get the grade 5AA on day 5 (*Figure 10*). This is a completely expanded blastocyst with a tightened ICM and a multicellular epithelium. (Gardner & Schoolcraft, 1999)

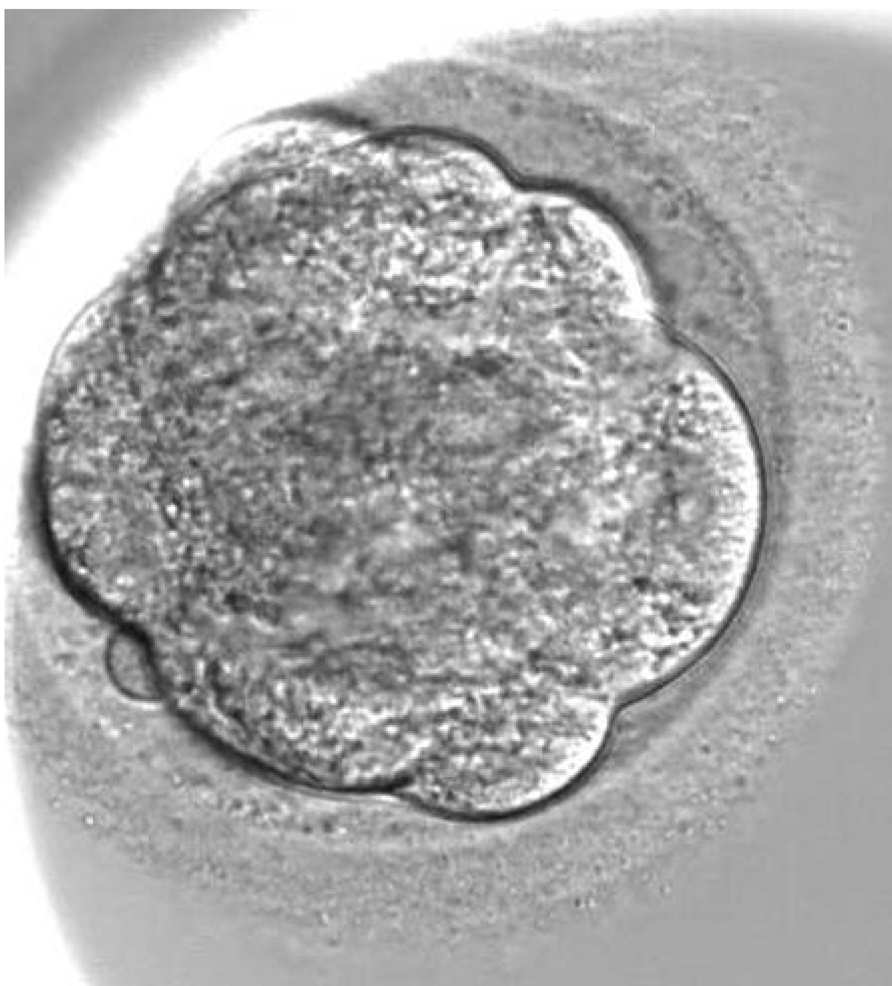

Figure 9. Morula on day 4. *This morula is top quality. Assessment focuses on the compaction process of blastomeres. The embryo was cultured in EmbryoSlide® and imaged in the EmbryoScope® plus (Vitrolife) time-lapse system.*

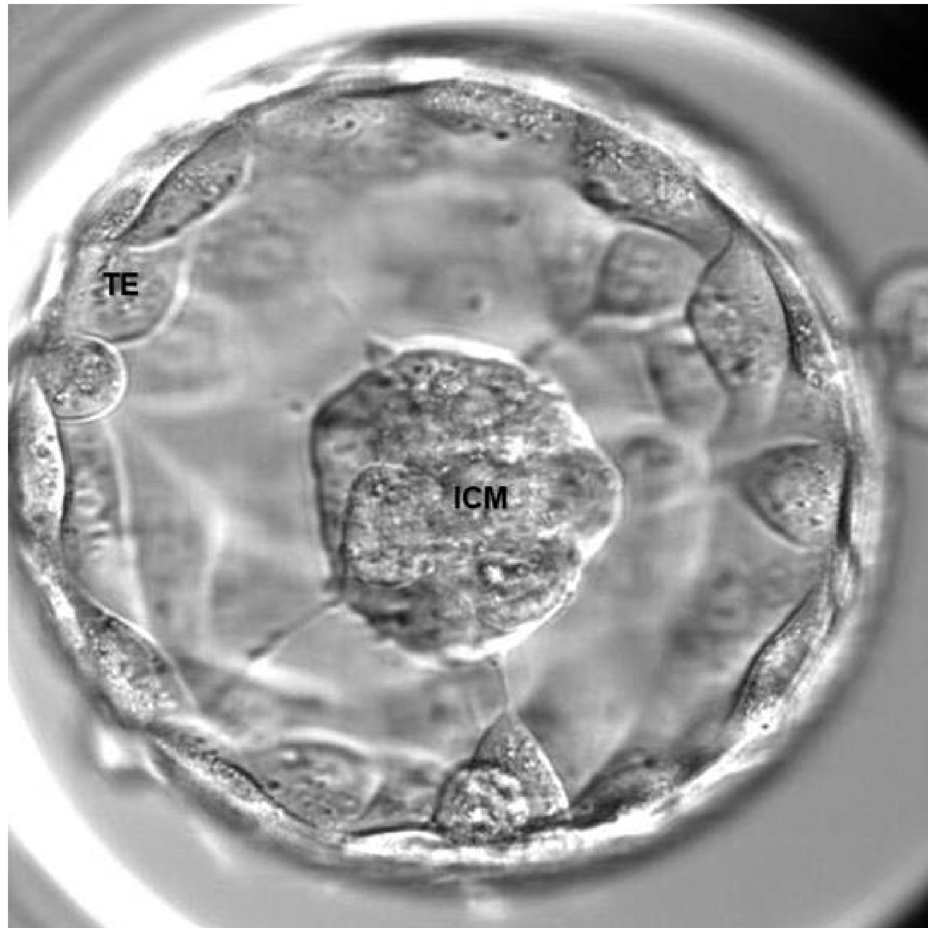

Figure 10. Blastocyst on day 5. *5AA blastocyst – a hatching blastocyst with a tightly packed inner cell mass (ICM) and cohesive trophectoderm (TE). The embryo was cultured in EmbryoSlide® and imaged in the EmbryoScope® plus (Vitrolife) time-lapse system.*

3. OBJECTIVES

The aim of this study is to provide information on the quality of embryos after ICSI-AOA. Cases are quite rare, so the timeline is somewhat long. Fimlab IVF laboratory was interested in the quality differences between oocytes without calcium exposure and oocytes within calcium exposure. Oocytes were collected by Fimlab IVF laboratory employees. In addition, both fresh embryo transfers and frozen embryo transfers were carried out, by IVF laboratory employees.

- Hypothesis one was that cycles with calcimycin would produce better quality embryos than earlier treatment cycles without calcium.
- Hypothesis two was that treatment cycles with both calcium activation and PICSI would produce better quality embryos than earlier treatment cycles.
- Hypothesis three was that the quality and age of the transferred embryo with calcium would not affect the birthweight of a newborn or the total pregnancy weeks. Both fresh and frozen embryos were analysed separately.
- Hypothesis four was that the quality and age of the transferred embryo with calcium would have a significance in the result of the pregnancy test. Furthermore, the clinical pregnancy rate and how many clinical pregnancies ended in childbirth were observed. Hypothesis four is the most important hypothesis because a healthy newborn is the most significant indicator of a successful treatment in Fimlab IVF laboratory. Both fresh and frozen embryos were analysed separately.
- Hypothesis five was that maternal age would not affect the birthweight of a newborn or the total pregnancy weeks.

4. MATERIALS & METHODS

4.1 The basis of database study

Data used in this study was saved in Babe IVF System (Cleodora Medical Ltd) between 2016-2020 by the Reproductive Endocrinology Outpatient Clinic employees. Oocytes were picked up and calcium activated between 2016-2019. However, both fresh and frozen embryo transfers were carried out between 2016-2020. Oocyte activation was performed immediately after ICSI, as Gynemed GmbH & Co. KG, the producer of A23187 has recommended.

For the purposes of this study, the quality of all embryos was re-assessed by one person only, so they are intercomparable. Embryo scoring has been assessed according to the Fimlab's in-house instructions which are based on the ESHRE guideline. Embryos have mainly been assessed without the benefits of time-lapse technology. Only eleven embryos have been in the EmbryoScope time-lapse system. Sperm volume, concentration and motility have been assessed according to the recommendations of the WHO. The sperm evaluations have been assessed by several persons.

A variable called *pregnancy test* is used in the analysis. Pregnancy test result is not typically used as an indicator of the treatments' success in studies. However, for an IVF laboratory pregnancy test result is a useful preliminary indicator since it demonstrates that the laboratory is operating as it should. This study defines and observes calcium activation from Fimlab IVF laboratory's point of view, and thus it is well-founded to use this variable in this analysis. In addition, the Reproductive Endocrinology Outpatient Clinic gets clinical patient records after a long delay, and this delay sets challenges for using other variables that better represent the treatments' successfulness. The typically used variables clinical pregnancy rate and live birth rate could be limiting reliable analyses in this study, because those cases were so small in number. Because of this, the use of the variable pregnancy test result is well-founded.

4.2 The part of data collection and grouping

This was a retrospective database study of selected materials from the Babe IVF System. The linkage between database and privacy policy was carried out by using personal

treatment IDs. A Microsoft office Excel worksheet which consists of basic information from treatment cycles was exported from the BABE IVF System. This basic information was for example, treatment ID, maternal age, the main diagnose, number of mature oocytes, number of frozen embryos, and outcome of treatment. In addition, variables such as paternal age, quality of sperm, knowledge of PICSI and number of activations, knowledge of previous fertilization and embryo quality, knowledge of embryo quality and embryo age within calcium activation were selected from the BABE IVF System. Also, information such as the weight of the newborns and the total pregnancy weeks was included. The variables were chosen based on their importance to Fimlab IVF laboratory. The choosing of the variables is also based on the Finnish national criteria of a full-term delivery and a healthy newborn.

Quality of sperm was categorized into three classes, normozoospermia, oligoasthenozoospermia and other quality abnormalities. The categorization was based on the knowledge that oligoasthenozoospermia was the main abnormality for males. Percentages of other abnormalities: hypospermia, cryptozoospermia, azoospermia and asthenozoospermia, were significantly lower, so these were combined into one category. Furthermore, the information of oligoasthenozoospermia on its own was significant because in such cases the sperm had less spermatozoa than the recommended reference limit, and the spermatozoa do not move correctly. These factors may indicate e.g., that the spermatozoa are not mature. (World Health Organization, 2010)

Also, Age of women was categorized. There were three classes, 30 years old or under, 31-36 years old and 37 years old or over. This categorization is modified from the categorization of Finnish Institute of Health and Welfare. From the age of 31 to 36 birth rate (a newborn baby per embryo transfer) was at least 27 %, and after the age of 36 it decreased to less than 19 %. This steep decline is why the middle age group is from 31 to 36. In frozen embryo transfers, maternal age in this study is set as the age when the oocytes are picked up. This is validated by the timeline of the study being as long as four years.

4.3 Statistical analysis

The achieved quantitative results were analysed with IBM SPSS® Statistics version 26 (IBM Corp., Armonk, NY, USA) to establish the statistical significance of the results. The quantitative data was analysed by using crosstabs with Fisher’s exact test; one-way

ANOVA (three or more samples) with Fisher's exact test; or the Kruskal-Wallis test. The level of significance was set at $p<0.05$.

5. RESULTS

Between 2016 and 2020, 52 clinical pregnancies were achieved in the Reproductive Endocrinology Outpatient Clinic following an ICSI-AOA combination. 35 of those ended in childbirth. Total number of different couples in this study data was 108. Overall, they had N=197 embryo transfers (both fresh and frozen). Clinical pregnancy rate was 26.4% and fertilization rate was 55.5% (data not shown). Three out of those 52 clinical pregnancies were twin pregnancies (5.8%), and two out of those 197 transfers were ongoing pregnancies (1.0%). 15 spontaneous miscarriages (28.8%) and 12 biochemical pregnancies (6.1%) occurred. Any clinical pregnancies which ended in childbirth were not interrupted by fetal aneuploidies. For all 38 children born following AOA, neonatal outcomes were normal as no malformations were observed. The key rates in ICSI-AOA cycles are summarized in *Table 1*. The numbers and rates are total figures containing both fresh and frozen embryo transfers.

Table 1. ICSI-AOA cycles at Fimlab in 2016-2020. *Values indicating the outcome of the treatment cycles, fresh and frozen embryo transfers together.*

	AOA Cycles (N=197)
Positive Pregnancy test	64 (32.5%)
Biochemical Pregnancy	12 (6.1%)
Clinical Pregnancy	52 (26.4%)
Live Birth	*35 (17.8%)*
Ongoing Pregnancy	*2 (1.0%)*
Twin Pregnancy	*3 (5.8%)*

Fresh embryo transfers were n=112 (57.1%), and frozen embryo transfers were n=76 (38.4%). N=9 embryos (4.5%) were abnormally fertilized, or the transferred embryos were not calcium activated, because in those cases activated embryos had inferior quality compared to embryos without activation. 173 treatment cycles were onefold A23187 activations (87.8%) and 24 were twofold A23187 activations (12.2%). PICSI was used in 93 cycles (46.3%).

In the Reproductive Endocrinology Outpatient Clinic between 2016 and 2019, the total number of ICSI cycles with fresh embryo transfers was N=920, and in frozen embryo

transfers N=1066. Values of 2020 were not available yet. Clinical pregnancy rate was 32.2% in fresh embryo transfers and 34.4% in frozen embryo transfers. Ten (3.4%) out of the 296 clinical pregnancies with fresh embryo transfers and eleven (3.0%) out of those 367 clinical pregnancies with frozen embryo transfers were twin pregnancies. In fresh embryo transfers 18.6% and in frozen embryo transfers 20.2.% of clinical pregnancies resulted in a spontaneous miscarriage. Fimlab IVF laboratory's own key reference rates for ICSI-AOA cycles are summarized in *Table 2*. Fresh and frozen embryo transfers are listed separately.

Table 2.Reference values from ICSI transfers at Fimlab in 2016-2019. *Reference values indicating the outcome of the treatments, fresh and frozen embryo transfers separately.*

	Fresh embryo transfers (N=920)	Frozen embryo transfers (N=1066)
Positive Pregnancy test	333 (36.2%)	439 (41.2%)
Biochemical Pregnancy	37 (4.0%)	72 (6.8%)
Clinical Pregnancy	296 (32.2%)	367 (34.4%)
Live Birth	*241 (26.2%)*	*293 (27.5%)*
Twin Pregnancy	*10 (3.4%)*	*11 (3.0%)*

The mean ± standard deviation was 32.96 ± 4.21 years for maternal and 34.97 ± 5.44 years for paternal age. For 96 couples (45.3%), the main cause of assisted reproductive treatments was *male factor only*. For 110 couples, quality of sperm was oligoasthenozoospermia (52.1%), for 59 couples normozoospermia (28.0%) and for 40 couples other quality abnormalities (19.0%). In rest of the cases quality of sperm was undefinable, and reasons for that were e.g., one couple had donor sperm or sperm was testicular sperm instead of ejaculated sperm.

5.1 The effect of AOA on quality of embryos

The difference between previous embryo quality and AOA embryo quality was significant in all three embryo quality classes, and in cases where embryos were abnormally fertilized or there were no embryos. In *Figure 11* the number of top-quality embryos increased by 28.5% after the AOA, but on the other hand the number of poor-quality embryos decreased by 26.4%. In the other two classes changes were smaller. The number of good-quality embryos increased by 11.3%. The cycles where oocytes did not fertilize at all, or fertilization was abnormal, decreased by 13.2% (*Figure 11*). Before calcium

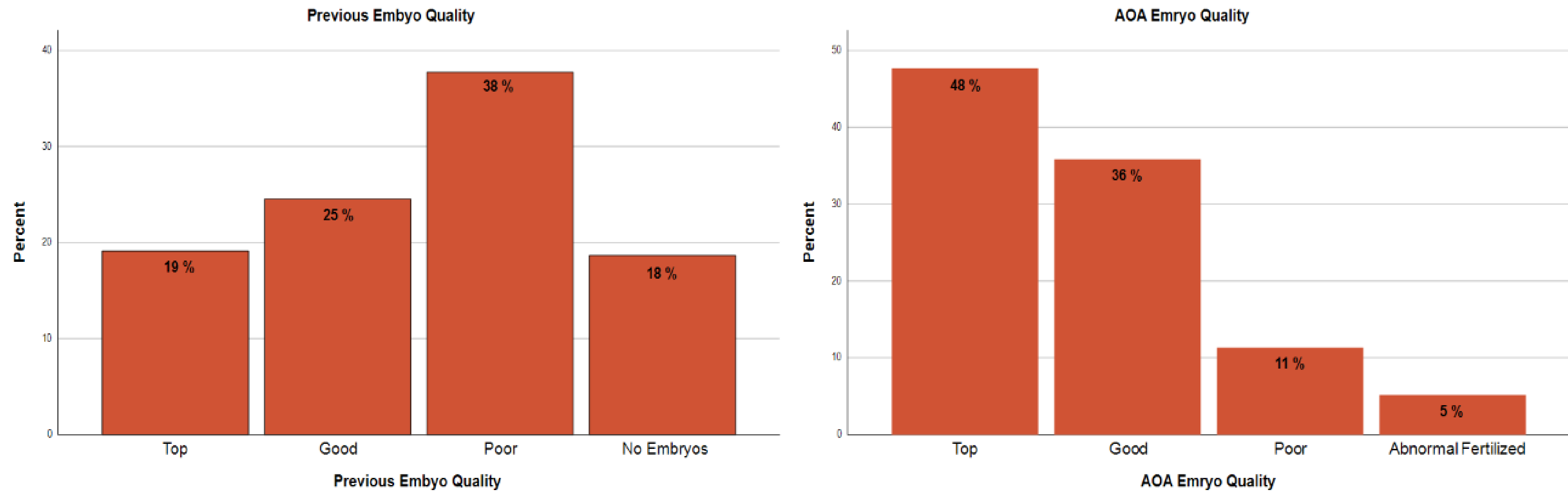

Figure 11. ***Changes in fertilized embryo quality.*** *On the left the quality of embryos before calcium activation, and on the right after calcium activation. The number of top-quality embryos has increased after calcium activation by 28.5%, and the number of good-quality embryos by 13.2%. The number of poor-quality embryos decreased after calcium activation by 26.4%., and the cycles where oocytes did not fertilize at all or fertilization was abnormal decreased by 13.2%.*

activation only 44% of embryos were top- or good-quality, whereas after the calcium activation the number was as much as 84%.

When the differences were observed in more detail, patient specifically, cycle-by-cycle, embryo quality improvement was 61.3%. In 24.3% of cycles no change for embryo quality was observed, and in 14.4% of cycles an impairment of quality was observed. Also, PICSI was not statistically significant for poor prognosis patients in this study case. PICSI had no positive effect but on the other hand, neither a negative effect.

5.2 The effect of AOA on pregnancy and the newborns

Because the data was so small, it was not meaningful to analyse statistically the effect of AOA quality and age of embryos on pregnancy weeks and birthweight of newborns. According to the original hypothesis, fresh and frozen transfers would have been studied separately. The analysis wasn't meaningful, even if this distinction was ignored. The mean ± standard deviation was 38.61 ± 1.93 weeks for pregnancy weeks and 3253 ± 578 g for birth weight of the newborns. Means are analysed without splitting the data.

The prevalence of missing data was negligible by 8% (n=15) in the next two analyses. The effect of AOA embryos' quality on pregnancy test results was analysed with cross tabulation. The quality of the transferred AOA embryos was relevant to the pregnancy test results in fresh transfers (p=0.001), but the quality of the transferred AOA embryos was not relevant to the pregnancy test results in frozen transfers (p=0.053). However, top-quality embryos achieved the same level of percentages on both positive and negative pregnancy tests in both fresh and frozen transfers (*Figure 12* and *Figure 13*).

For positive pregnancy tests after fresh transfers, percentages in three quality classes were: top-quality 49%, good-quality 24% and poor-quality 6% (*Figure 12*). After AOA 27.4% of all fresh embryo transfers resulted in clinical pregnancies, and 82.8% of clinical pregnancies ended in childbirth (*Table 3*). For positive pregnancy tests after frozen embryo transfers, percentages in three quality classes were: top-quality 48%, good-quality 22% and poor-quality 20% (*Figure 13*). After AOA 30.3% of all frozen embryo transfers resulted in clinical pregnancies, and 47.8% of clinical pregnancies ended in childbirth (*Table 3*).

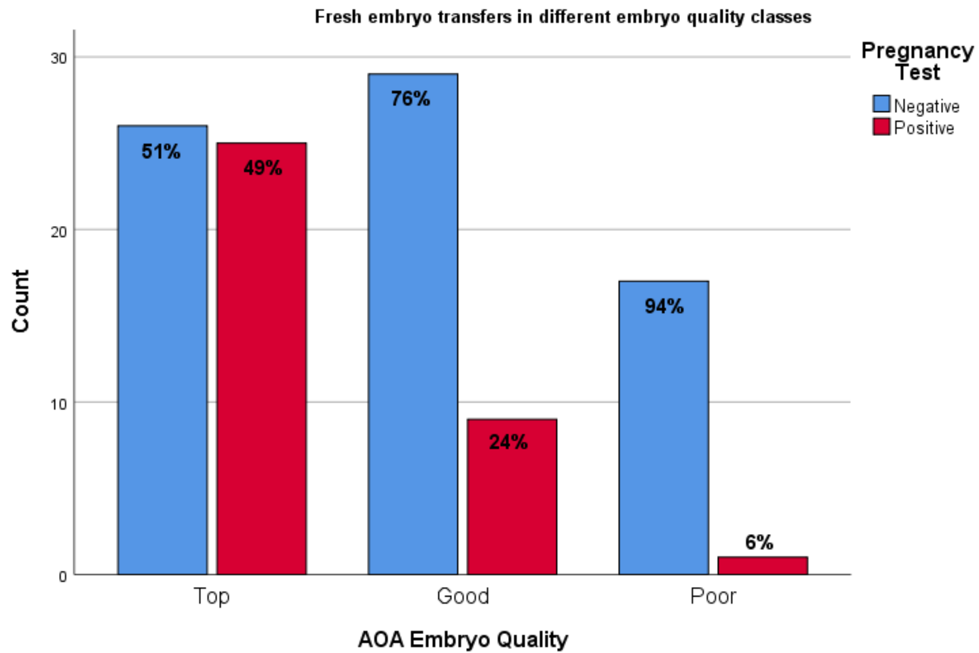

Figure 12. ***Pregnancy test results after calcium activation for fresh embryo transfers in different embryo quality classes.*** *After calcium activation the percentage of positive pregnancy test results in top-quality embryos was 49%. In good and poor-quality em-bryos the likelihood of getting a positive pregnancy test result was much smaller than the likelihood of a negative test result.*

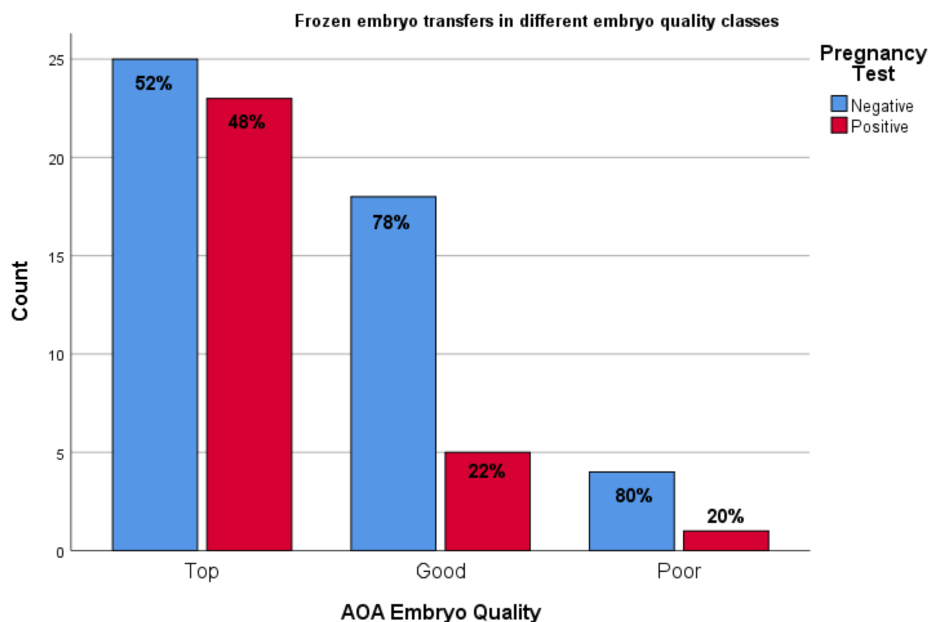

Figure 13. ***Pregnancy test results after calcium activation for frozen embryo transfers in different embryo quality classes.*** *After calcium activation the percentage of positive pregnancy test results in top-quality embryos was 49%. In good-quality embryos the likelihood of getting a positive pregnancy test result was much smaller than the likelihood of a negative test result. In poor-quality embryos as many as every fifth transfer resulted in a positive pregnancy test, which is much better than the corresponding result for fresh quality embryos.*

Table 3. Clinical results after fresh and frozen embryo transfers in different quality classes. *The chart shows negative test results in both numbers and percentages. Positive results are divided into biochemical and clinical pregnancies. The clinical pregnancies are divided further into three groups: spontaneous abortion (SAB) after less than 22 pregnancy weeks; ongoing pregnancy; and live birth. Notice that the chart shows the number of live births, and the number of children born may differ. The total number of transfers differs in this table because the prevalence of missing data was 8% (n=15).*

		Fresh Embryo Transfers, n (%)	Frozen Embryo Transfers, n (%)	Total
Top	Negative Pregnancy test	26 (51.0%)	25 (52.1%)	
	Biochemical Pregnancy	3 (5.9%)	4 (8.3%)	
	Clinical Pregnancy	22 (43.1%)	19 (39.6%)	
	SAB Less Than 22 Weeks	*3*	*9*	
	Ongoing Pregnancy	*0*	*1*	
	Live Birth	*19*	*9*	
	Total	51	48	99
Good	Negative Pregnancy test	28 (75.7%)	18 (78.3%)	
	Biochemical Pregnancy	3 (8.1%)	1 (4.3%)	
	Clinical Pregnancy	6 (16.2%)	4 (17.4%)	
	SAB Less Than 22 Weeks	*2*	*1*	
	Ongoing Pregnancy	*0*	*1*	
	Live Birth	*4*	*2*	
	Total	37	23	60
Poor	Negative Pregnancy test	17 (94.4%)	4 (80.0%)	
	Biochemical Pregnancy	0	1 (20.0%)	
	Clinical Pregnancy	1 (5.6%)	0	
	SAB Less Than 22 Weeks	*0*	*0*	
	Live Birth	*1*	*0*	
	Total	18	5	23
		106	76	182

There was no statistical significance in fresh embryo transfers (p=0.257) between AOA embryo age and the result of pregnancy test. However, in frozen embryo transfers there was a statistical significance (p=0.006). The effect of AOA embryos' age on pregnancy test result was analysed with cross tabulation. Percentages of positive pregnancy test after fresh transfers at four time points were: 21% on day 2, 36% on day 3, 30% on day 4 and 56% on day 5, as illustrated in *Figure 14*. Day 5 or later embryo transfers gave the best result in positive pregnancy tests, but only 8.4% of all fresh embryo transfers were carried out on day 5 or later (*Table 4*).

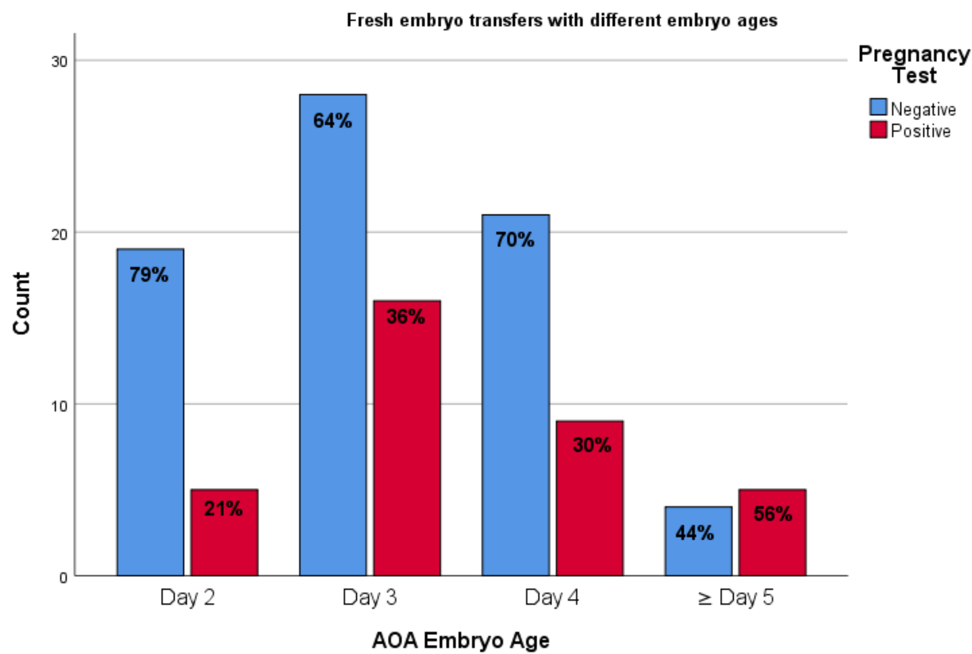

Figure 14. Pregnancy test results for fresh embryo transfers with different embryo ages. *After calcium activation, day 5 transfers had the largest number of positive pregnancy test results. The number of day 5 or later transfers was small compared to the number of day 2, 3, or 4 transfers. Day 2, 3, or 4 transfers had a smaller number of positive pregnancy test results than day 5 transfers.*

Table 4. Clinical results after calcium activation for fresh and frozen embryo transfers with different transfer days. *The chart shows in numbers and percentages the amount of negative pregnancy test results. Positive results are divided into biochemical and clinical pregnancies. The clinical pregnancies are divided further into three groups: spontaneous abortion (SAB) after less than 22 pregnancy weeks; ongoing pregnancy; live birth. Notice that the chart shows the number of live births, and the number of children born may differ. The total number of transfers differs in this table because the prevalence of missing data was 8% (n=15).*

		Fresh Embryo Transfers, n (%)	Frozen Embryo Transfers, n (%)	Total
Day 2	Negative Pregnancy test	19 (79.2%)	2 (100%)	
	Biochemical Pregnancy	1 (4.2%)	0	
	Clinical Pregnancy	4 (16.6%)	0	
	SAB Less Than 22 Weeks	*1*	*0*	
	Live Birth	*3*	*0*	
	Total	24	2	26
Day 3	Negative Pregnancy test	28 (63.6%)	10 (100%)	
	Biochemical Pregnancy	3 (6.8%)	0	
	Clinical Pregnancy	13 (29.6%)	0	
	SAB Less Than 22 Weeks	*1*	*0*	
	Live Birth	*12*	*0*	
	Total	44	10	54
Day 4	Negative Pregnancy test	20 (69.0%)	11 (44.0%)	
	Biochemical Pregnancy	2 (6.9%)	3 (12.0%)	
	Clinical Pregnancy	7 (24.1%)	11 (44.0%)	
	SAB Less Than 22 Weeks	*1*	*3*	
	Live Birth	*6*	*8*	
	Total	29	25	54
Day ≥5	Negative Pregnancy test	4 (44.4%)	24 (61.5%)	
	Biochemical Pregnancy	0	3 (7.7%)	
	Clinical Pregnancy	5 (55.6%)	12 (30.8%)	
	SAB Less Than 22 Weeks	*2*	*7*	
	Ongoing Pregnancy	*0*	*2*	
	Live Birth	*3*	*3*	
	Total	9	39	48
		106	76	182

Percentages of positive pregnancy test after frozen transfers at different embryo ages were: 56% on day 4, and 39% on day 5 or later as illustrated in *Figure 15*. On both day 2 and day 3 transfers, there were no positive pregnancy tests. In contrast to fresh transfers, 51% of all frozen embryo transfers were carried out on day 5 or later (*Table 4*). However, 56% of day 4 embryo transfers gave the best result in positive pregnancy tests (*Figure 15* and *Table 4*).

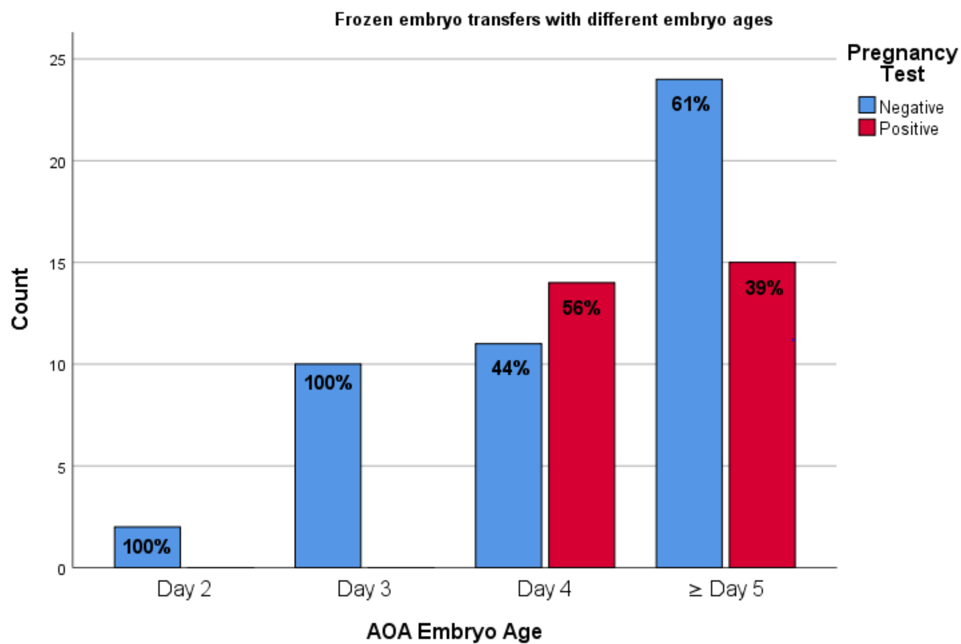

Figure 15. ***Pregnancy test results for frozen embryo transfers with different embryo ages.*** *After calcium activation, day 4 transfers had the largest number of positive pregnancy test results. In frozen embryo transfers the difference between the number of day 4 transfers and day 5 transfers was smaller than in fresh embryo transfers. There was a significantly smaller number of day 2 or 3 transfers than day 4 or 5 transfers. In addition, none of the day 2 or 3 transfers resulted in a positive pregnancy test.*

5.3 The effect of maternal age on childbirth

In this study case there was no statistical significance between categorized maternal age and birthweight of newborn by one-way ANOVA. In *Figure 16*, birthweight is categorized in three groups and presented in boxplots. In three groups (maternal age ≤30 years, 31-36 years, and ≥37 years) birthweight mean ± standard deviation was 3522 ± 461 g, 3053 ± 609 g and 3404 ± 485 g, respectively.

There was no statistical significance between categorized maternal age and pregnancy weeks by Kruskal-Wallis test. In *Figure 17* pregnancy weeks are categorized in three groups and presented in boxplots. In three groups (maternal age ≤30 years, 31-36 years and ≥37 years) pregnancy weeks mean ± standard deviation was 39.18 ± 1.78 weeks, 38.25 ± 2.10 weeks and 38.71 ± 1.60 weeks, respectively.

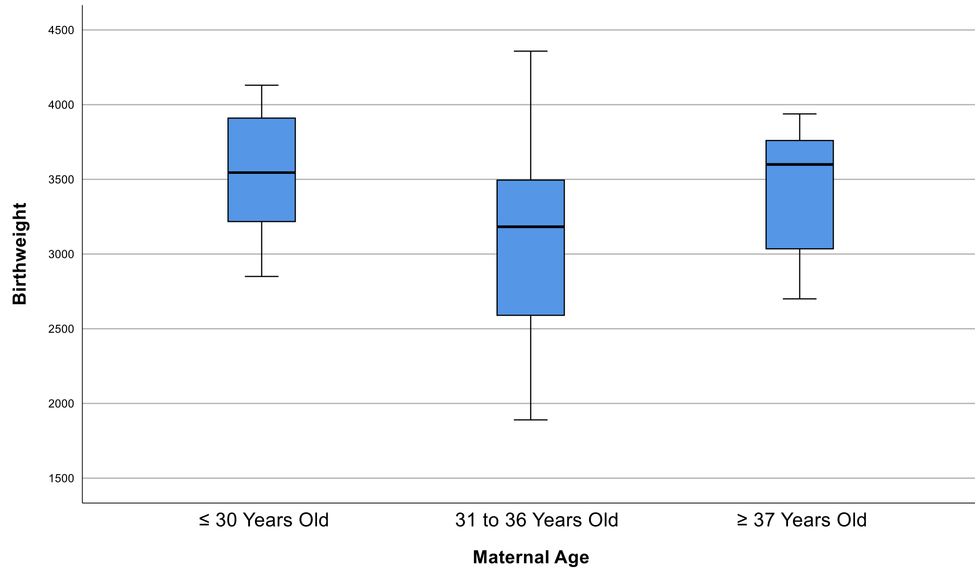

Figure 16. Boxplot chart showing maternal age and birth weight of newborn. *There was no statistical significance between categorized maternal age and birthweight of newborn. Birthweight mean ± standard deviation was largest in maternal age group 31-36 years.*

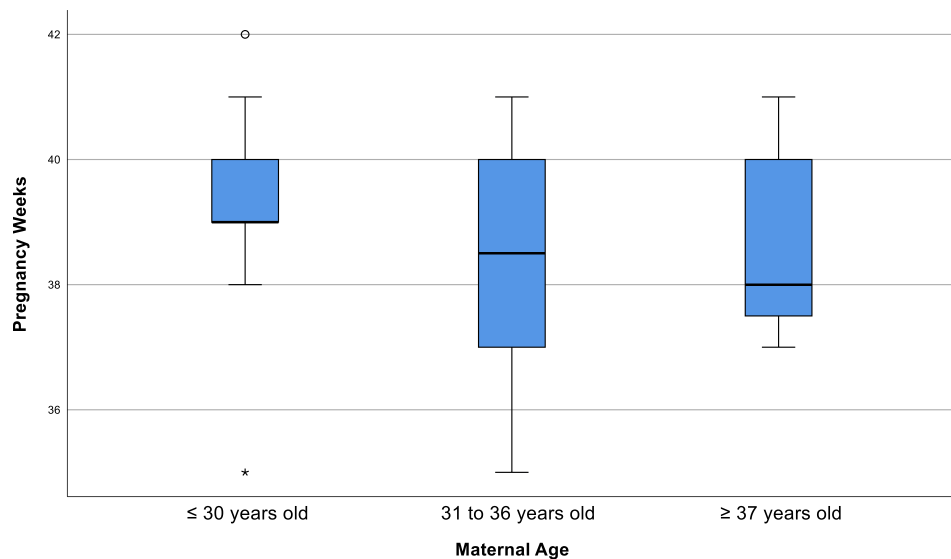

Figure 17. Boxplot chart showing maternal age and total pregnancy weeks. *There was no statistical significance between categorized maternal age and pregnancy weeks. Pregnancy weeks mean ± standard deviation was largest in maternal age group 31-36 years.*

6. DISCUSSION

All 38 children born following AOA were healthy, and that is the most important information for the whole Reproductive Endocrinology Outpatient Clinic, not only the IVF laboratory. It is globally recognised that babies from medically assisted reproduction have a higher risk of adverse birth outcomes than babies that are conceived naturally. That risk is also recognised nationally in Finland. Still the large dataset study concluded that the higher risk of adverse birth outcomes relates to other factors than the infertility treatment itself. (Goisis et al., 2019) In the future, researchers and reproductive clinics need more surveillance studies which are centred on the development of ICSI-AOA children, both globally and nationally. Vanden Meerschaut et al. (2014) got encouraging results in their study but the size of the study was too small to reach a distinct conclusion. The study dealt with the neurodevelopmental and behavioural outcomes of children born following AOA (Vanden Meerschaut et al., 2014). In addition, Miller et al. (2016) concluded that their retrospective study did not indicate an increased rate of fetal defects after ICSI-AOA (Miller et al., 2016).

Based on current knowledge maternal age is one of reasons for childlessness. The mean age for females was 32.96 ± 4.21 years in this study. Still maternal age alone did not explain poor prognosis. In addition, the quality of sperm might be an underlying cause for couples' infertility in this study, because less than one third of males had normozoospermia. Anomalous expression of PLCζ has been noticed to cause ICSI failure in infertile men. In the future research might focus more on increased male subfertility and especially on more precise sperm examinations. These days most of research is centred only on female subfertility. Even if the male has normozoospermia, there might be complications with the DNA of a spermatozoon. For example, recombinant human PLCζ might be a potential treatment (Kashir et al., 2012; Yoon et al., 2008) together with calcium activation. There is very strict regulation in Finland, so this kind of treatment might not be used here in the near future.

Fertilization rate was nearly 56% which was good, since poor prognosis patients had an earlier fertilization rate of under 50%, and in 18% of the previous treatment cycles there were no embryos. The result that the quality of AOA embryos was better than the quality of embryos without AOA was also encouraging. In all of the three embryo quality classes, improvement was at least 10%, and in the top-quality class improvement was nearly

30%. In 86% of cycles the quality of embryos either improved or was not impaired, and only in 14% of cycles quality of embryos was impaired when the difference was observed in more detail, cycle-by-cycle. These results indicate that calcium activation had a positive effect on the prognosis. Based on literature, Fimlab IVF laboratory had special criteria for the couples who got the possibility for the calcium activation, (Bonte et al., 2019; Heindryckx et al., 2008; Montag et al., 2012; Vanden Meerschaut et al., 2012). Now they also have knowledge of having the right direction with poor prognosis patients by improving the embryo's quality. However, this study did not reveal any clear common factor for couples whose embryo quality did not improve with calcium activation. Only in 14% of the treatment cycles the quality of the embryo was impaired, which contributes to why no clear cause consequence relationship could be found.

PICSI did not offer any additional value to the quality of embryos in this study. However, it was promising that PICSI had no negative effect on embryos. There is no clear indication in literature that PICSI would have a positive effect on livebirth rate, although it apparently has a lowering effect on the miscarriage rate (Miller et al., 2019).

In the future, it would be a good thought to analyse the effect of PICSI on the quality of embryos again. The timeline was four years which is quite a long time for a master's thesis. Still, the data was so small that it limited the reliability of the results. In future, when the IVF laboratory has more calcium cycles combined to the PICSI analysis, it will be possible to get more reliable results. Nowadays, and in this data, the IVF laboratory uses A23187 for the activations of oocytes, but e.g., Nikiforaki et al. have proved that ionomycin is more effective than calcimycin (Nikiforaki et al., 2016). However, the unavailability of ionophores can limit the IVF laboratory's possibilities to change the activation medium in the future.

Because the data was so small, analysing the effect of AOA quality or the effect of embryo age on pregnancy weeks and birthweight of newborns was not statistically meaningful. That was unlucky because the main aim of the IVF laboratory is to ensure the birth of a healthy baby. It was promising that the mean and standard deviations of weight and pregnancy point out that babies were full-term. A birth before the 38th (37+0) pregnancy week is considered a preterm birth and a preterm baby. Earlier a preterm baby was a baby whose birth weight was under 2500 g. Nowadays weight has been discarded as criteria, because pregnancy weeks are clinically more important. (Retrieved from https://www.kaypahoito.fi/hoi50089 on 7th April 2021) There was no statistical significance between categorized maternal age and birthweight of newborn or pregnancy

weeks. The means of those variables were normal when compared to national reference values.

The most important reference values for the ICSI-AOA cycles in this study are the patients' previous treatment results. However, the results of this study have also been compared to both the Fimlab IVF laboratory's own reference values and the national reference values to determine whether the poor prognosis patients' results improve to that same level.

Quality of the transferred AOA embryos was relevant to the pregnancy test results in fresh transfers, but not in frozen transfers. In both types of embryo transfers percentages of positive pregnancy test decreased when the quality of the embryo decreased. *Figure 12* and *Figure 13* point out that the quality of the transferred AOA embryos was relevant in both types of transfer in the end, even if the analysis got a border line result in frozen transfers. The P value was suggestive of a trend that might have been revealed with a greater sample size.

Quality of the transferred AOA embryos being relevant to the pregnancy test results speaks for the importance of embryo grading, and also time-lapse monitoring in the future. After AOA clinical pregnancy rate was at the same level in both fresh and frozen embryo transfers. When both the 26% clinical pregnancy rate and the 18% live birth rate in this study were compared to national reference values, those rates after AOA were excellent. In 2018 the Finnish Institute for Health and Welfare reported that nationally clinical pregnancy rate was 23% and live birth rate was 18% (Finnish institute for health and welfare, 2020) It is important to notice that the reference values include also IUI treatments and treatments which have been carried out by using donor germ cells. Generally, IUI treatments may decrease the national reference values.

The results of this study are in line with the reference values because Reproductive Endocrinology Outpatient Clinic had good results from IVF and ICSI treatments compared to the national results. It is encouraging, that when results after AOA are compared to Fimlab IVF laboratory's own reference values, clinical pregnancy rate 26 % and live birth rate 18 %, they are quite close to those reference values in fresh embryo transfers. The reference rates after ICSI treatment combined to fresh embryo transfer are 32 % and 26 %, respectively in Fimlab IVF laboratory between 2016-2019. After frozen embryo transfers the reference rates are even higher, 34% and 28%, respectively. Couples with poor prognosis benefitted from calcium activation treatment. It is important to notice that

the reference values separate fresh and frozen embryo transfers. This is the reason why these results are not completely intercomparable, but indicative.

In this study, in Fimlab IVF laboratory between 2016-2019, the proportion of twin pregnancies in ICSI-AOA treatments was 5.8%, and in ICSI treatments combined to fresh embryo transfers it was 3.4%. In frozen embryo transfers the proportion of twin pregnancies was 3.0%. This higher rate in ICSI-AOA treatments might be a consequence of the infertility patients' poor prognosis. When the prognosis is poor, it is more likely that more than one embryo is transferred. In 2018 the Finnish Institute for Health and Welfare reported that nationally the multiple birth rate was 1.85 in IVF treatments, and 5.9% in IUI treatments (Finnish institute for health and welfare, 2020). As expected, the twin pregnancy rate in this study was higher than Fimlab's reference values or the national reference value for IVF treatments. In addition, in Fimlab IVF laboratory between 2016-2019, the 29% spontaneous miscarriage rate in ICSI-AOA treatments is higher than the miscarriage rate in ICSI treatments (both fresh and frozen). In ICSI treatments in 2016-2019, the spontaneous miscarriage rate was 19% in fresh embryo transfers and 20% in frozen embryo transfers. At least 84% of all transferred AOA embryos were good quality, however 29% of clinical pregnancies resulted in a spontaneous miscarriage. A successful infertility treatment is a sum of many factors: besides embryo quality, e.g., endometrial receptivity has an effect on the pregnancy.

There was no statistical significance in fresh embryo transfers between AOA embryo age and pregnancy test result, but there was one in frozen embryo transfers. Nearly 70% of fresh embryo transfers were carried out on day 3 or day 4. However, extended embryo culture produced the smallest difference between percentages on both positive and negative pregnancy tests. The limitation of this observation is that only 8% of fresh embryo transfers were carried out on day 5. In contrast, 51% of frozen embryo transfers were carried out on day 5/6.

Altogether, there is no clear conclusion about the significance between AOA embryo age and pregnancy test results. If we look at the proportion of live births in total embryo transfers, blastocyst will give the best opportunity in fresh transfers for having a baby. In frozen transfers, day 4 embryos will give the best opportunity. These results are similar as Gardner et al. (1998) and Wei et al. (2019) pointed out (Gardner et al., 1998; Wei et al., 2019). In their studies they suggested that frozen blastocyst transfer is better than fresh blastocyst transfer. It is important to notice that Wei et al. (2019) carried out their study on women with good prognosis. (Wei et al., 2019) This is the biggest reason why these

results are not completely intercomparable, but indicative. In the future the IVF laboratory needs more treatment cycles with more reliable results. In addition, the knowledge that human embryonic genome does not occur until day 2-3 supports the theory that the transfers would benefit from extended embryo culture (Tao et al., 2002).

In fresh embryo transfers, in every embryo age group, at least 60% of clinical pregnancies resulted in birth. In frozen embryo transfers, in embryos transferred after day 5/6 25% of clinical pregnancies resulted in birth. The difference between fresh and frozen embryo transfers is conspicuous. This does not support the theory that the clinical pregnancy rate per transfer is grater in frozen embryo transfers than in fresh embryo transfers. One contributing factor might be the use of an antagonist protocol, which is reported to result in lower success rates. (Shapiro et al., 2011) However, this study was not designed to evaluate differences between exogenous gonadotropins, hence information about the protocol type is not included. The difference might also be partly explained by the small number of transfers or that calcium activation might reduce the embryo's freezability. However, there is yet no literature supporting this latter hypothesis.

Based on the earlier global knowledge (Campbell et al., 2013; Chamayou et al., 2013; Wong et al., 2010) and the result of this master's thesis, it is important that assessments and grading of embryos are done accurately. Assessment and embryo selection provide the best base for implantation and further development of the fetus (Campbell et al., 2013; Chamayou et al., 2013; Wong et al., 2010). Only eleven embryos have been in the EmbryoScope time-lapse system, and that is why a morphokinetic aspect cannot be taken into account in this study. However, morphokinetics might become more common in the future because the time-lapse system enables assessment of embryo morphology almost continuously, which gives more precise information. Time-lapse will give additional value to embryo grading, especially for poor prognosis patients in the future. Getting more precise information is important e.g. because in the public sector the treatment cycles have been limited to three.

7. CONCLUSIONS

The present study has some notable limitations. In the end the data was quite small and this master's thesis did not consider e.g., the effect of time-lapse system. A23187 was used during the activation cycles in the IVF laboratory, which is not as effective as ionomycin. Generally, calcium activations are experimental treatments and for that reason this method requires validation and evaluation so that results of different IVF laboratories are more comparable. In Fimlab IVF laboratory calcium activation is a clinical treatment and a so-called last chance treatment when previous embryo transfers have failed.

Despite many limitations the present study offers encouraging results. The quality of embryos was better after the calcium activation than without it, and thus the quality of the transferred embryos was a significant factor in the pregnancy test results taken during the treatment. In addition, after calcium activation the clinical pregnancy rate and the live birth rate were at same level as national reference values in IVF. Also, the fertilization rate improved after calcium activation. This study did not reveal any clear common factor for couples whose embryo quality did not improve with calcium activation. In the end, the most important result was that every single baby born after AOA was healthy, and most were of full term.

In the future, when there are more couples to study, Fimlab IVF laboratory can analyse more results and will get more informative results. Especially, it would be recommended that calcimycin would be switched to ionomycin, which is more effective. In the future, PICSI analysis needs a grater sampling, so that Fimlab IVF laboratory could get a clearer result on whether PICSI has a positive effect on embryo quality. It could also give a result on whether AOA embryo quality and AOA embryo age have an effect on the pregnancy being full term, to which this study offered no result. Live birth rate in this study was at a good level, when compared to the national reference value. This value could further improve in the future when Fimlab IVF laboratory gets to use constant evaluation of embryos more through the time-lapse system. It would also be beneficial to study male factor infertility more in the future. A greater sampling would also make it possible to analyse the treatment results with the help of the different semen parameters.

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