Preimplantation Genetic Testing

Assessment and Improvement of Clinical Practice

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BY
CHRISTIAN LIEBST FRISK TOFT

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AALBORG UNIVERSITY
DENMARK

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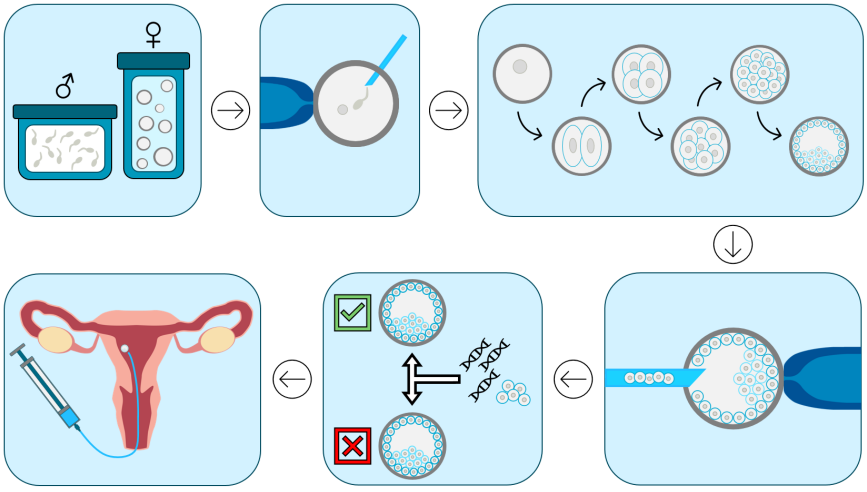

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CV

I graduated with a master's degree in Molecular Biology from Aarhus University in 2013. During my master's degree I worked with proteolytic cleavage of cell surface proteins and their role in proper nervous system development investigated by knock-down studies in Zebrafish. In May 2015, I started my employment at the Department of Molecular Diagnostics at Aalborg University, where I worked with screening of breast- and ovarian cancer predisposition genes and predictive gene testing for three years. In May 2018, I initiated my Ph.D. within the field of preimplantation genetic testing at the Department of Molecular Diagnostics at Aalborg University Hospital in close collaboration with the Fertility Unit at the Department of Obstetrics and Gynecology and the Department of Clinical Genetics at Aalborg University Hospital.

ENGLISH SUMMARY

Preimplantation genetic testing (PGT) is an option for couples at risk of passing on a hereditary disorder to their offspring. PGT entails artificial reproductive technology where human preimplantation embryos are generated *in vitro* and transferred to the uterus to establish a pregnancy. The procedure of PGT describes the process of obtaining an embryo biopsy which is subsequently tested for the genetic disorder to identify unaffected embryos for transfer.

This thesis contains five studies within the field of PGT. Study I is a systematic review of the prevalence of aneuploidy and an assessment of the clinical effect of concurrent screening for aneuploidy in the context of PGT for hereditary disorders. We found that approximately one-third of human preimplantation embryos from patients receiving PGT are aneuploid. The clinical benefit from screening for aneuploidy was challenging to assess from the currently available studies, and the lack of randomized controlled studies is concerning. Given the issues of embryonic mosaicism, intention-to-treat analyses and/or non-selection studies are warranted to properly evaluate screening for aneuploidy.

In Study II, we investigate whether fetal cells circulating in maternal blood of women achieving pregnancy following PGT-M could be isolated and tested for the hereditary disorder as an alternative to the current gold standard of chorionic villous sampling (CVS) used for prenatal testing. We published a proof-of-concept study showing that fetal cells can be isolated and that we were able to test a range of different types of mutations by a combination of short tandem repeat marker (STR) analysis and direct mutation detection. The results were verified against the result from CVS, and no disagreements were recorded. Work is currently ongoing to further evaluate the procedure aiming at introducing it into clinical practice as a non-invasive alternative to CVS.

In Study III, we set out to investigate whether biopsied trophectoderm cells could be expanded in cell culture to produce enough DNA for downstream genetic testing, including next generation sequencing (NGS), as an alternative to whole genome amplification (WGA). We succeeded in expanding biopsied trophectoderm cells in culture yielding enough DNA for downstream genetic testing by NGS without prior need for WGA. We showed that the method could be used for PGT-M by STR-marker analysis and direct mutation detection and PGT-SR by NGS. This was a proof-of-concept study, and further studies are needed to obtain more precise estimates of success rates of the procedure, as not all biopsies succeeded in expanding, and the sample size was small.

In Study IV, we assessed the clinical outcomes at our center since its establishment in 2016 and evaluated the effect of female age and the number of transferable embryos

on the chance of achieving a clinical pregnancy (fetal heartbeat in gestational week 7-8). We found that highly satisfactory clinical outcomes can be achieved at a newly established center by adhering to up-to-date methods and practice recommendations such as trophectoderm biopsy, cryopreservation by vitrification, allowing a freeze-all strategy, and single embryo transfer to be adopted without compromising clinical outcomes. Over the four years, we had a cumulative clinical pregnancy rate of 52.7 %, which increased to 87.7 % when only evaluating patients completing a full treatment offer offered by our center. Clinical pregnancy rates per oocyte retrieval and frozen embryo transfer were 28.7 and 33.2 %, respectively. We found that female age at the time of initiating PGT treatment significantly affected the chance of achieving pregnancy over the course of treatment with a reduction in the chance of 8 % per female year. Per oocyte retrieval, we found that the number of transferable embryos had a dramatic impact on the chance of achieving pregnancy. Each additional transferable embryo increased the chance of achieving clinical pregnancy two to four-fold (depending on the analysis), emphasizing that efforts to increase the number of transferable embryos will have a dramatic effect on the chances of achieving clinical pregnancy per oocyte retrieval.

In Study V, we performed a cross-sectional questionnaire survey with the aim of exploring patients' choices, opinions, and experiences with prenatal testing following PGT. This included their opinions on non-invasive testing as an alternative to CVS. We found that approximately half of the patients declined CVS, with most declining due to the risk of miscarriage associated with the procedure. Despite a desire for prenatal verification, one in four patients declined CVS. Nine out of ten patients would have accepted non-invasive prenatal testing had it been offered, emphasizing that test safety is an important aspect considered by patients. Hence, non-invasive prenatal testing is likely to cause more patients to opt for prenatal testing, which might contribute to reducing anxiety or stress experienced by patients during pregnancy. In support of this, four out of ten patients reported being concerned at some point during pregnancy that the fetus had inherited the disorder despite PGT. We found that one in four patients was not fully aware of the limitations of the nuchal translucency scan concerning its inability to determine whether the fetus has inherited the disorder for which PGT was performed. Indeed, some patients responded that they would have opted for CVS had they been aware of the limitations of the translucency scan. Additionally, approximately one-third of patients did not recall that the clinic recommended prenatal testing following PGT, although the majority recalled being informed on the option of prenatal testing. In summary, the survey indicates that improvements in clinical consultation might be required to ensure that patients can make a properly informed decision regarding prenatal testing. Additionally, non-invasive alternatives are welcomed by patients.

DANSK RESUME

Præimplantations genetisk testning (PGT), også kendt som ægsortering, er et tilbud til par som har en kendt risiko for at videregive en alvorlig arvelig sygdom til deres børn. PGT er en forlængelse af assisteret reproduktion, hvor humane præimplantationsembryoner, som er et resultat af fertilisering af oocytter med spermatozoer, bliver dyrket i laboratoriet og lagt op i livmoderen i håb om at etablere en graviditet. PGT er en procedure, hvor der tages en biopsi af embryonet, som efterfølgende testes for den arvelige sygdom. Embryoner, som ikke har arvet sygdommen bliver hermed identificeret og anvendt til oplægning, imens at de syge embryoner frasorteres.

Denne afhandling indeholder fem studier omkring PGT. Studie I er et systematisk review, hvor vi undersøgte forekomsten af kromosomantalsforandringer og evaluere den kliniske effekt ved at screene for disse samtidig med PGT for arvelige sygdomme. Vi fandt at omkring en ud af tre humane præimplantationsembryoner fra patienter, som modtog PGT for en arvelig sygdom, havde kromosomantalsforandringer. Om der var en kliniske effekt af screening for kromosomantalsforandringer, kunne ikke vurderes ud fra de nuværende tilgængelige studier, og manglen på randomiserede kontrolstudier er bekymrende. I betragtning af problematikken omkring embryonal mosaicisme, er "intention-to-treat" analyser og/eller non-selektionsstudier ønskede for at undersøge effekten af screening for kromosomantalsforandringer.

I Studie II undersøgte vi, om det var muligt at isolere fosterceller fra blodprøver fra kvinder, som havde opnået graviditet efter PGT for monogene sygdomme. Dernæst undersøgte vi, om fostercellerne kunne testes for den arvelige sygdom som et alternativ til moderkageprøve, der er den nuværende gyldne standard for prænatal testning i forbindelse med PGT. Vi publicerede et proof-of-concept studie som viste, at fosterceller kan isoleres fra moderens blod, og at vi var i stand til at teste en række forskelle typer af mutationen ved en kombination af markøranalyse og direkte mutationsdetektion. Resultaterne blev valideret ved sammenligning med resultaterne fra moderkageprøven og vidste overensstemmelse i alle tilfælde. Proceduren bliver kontinuerligt udviklet med det mål, at den kan blive introduceret i klinisk praktisk som et ikke-invasivt alternativ til modekageprøve.

Studie III havde til formål at undersøge, om biopterede trophectodermceller kunne dyrkes i cellekultur og dele sig i et omfang, så der kunne opnås en tilstrækkelig mængde DNA til at genetisk testning, herunder næste generations sekventering (NGS), uden at der var behov for helegenomamplificering. Vi viste, at det er muligt at dyrke biopterede trophectodermceller i kultur, og at der kunne oprenses tilstrækkelige mængder DNA til at udføre NGS uden forudgående helgenomamplifikation. Vi viste, at DNA oprenset fra cellekulturerne kunne anvendes til PGT for både monogene sygdomme, som blev udført med markøranalyse og

direkte mutationsdetektion, samt for større kromosomale duplikationer og deletion, som blev undersøgt med NGS. Dette studie vist, at det i princippet er muligt at undersøge, hvorvidt fosteret har arvet sygdomme ved analyse af fosterceller isoleret fra den gravides blod. Yderligere undersøgelser er nødvendige for at eftervise metodens pålidelighed og for at opnå præcise estimater for successrater.

Studie IV omhandler en evaluering af de kliniske resultater på vores Center for Præimplantations Genetisk Testning siden dets åbning i oktober 2016 og til og med udgangen af 2020. Derudover undersøgte vi effekten af kvindens alder og antallet af transferable embryoner på chancen for at opnå en klinisk graviditet (hjertelyd i graviditetsuge 7-8). Vi viste, at et nyt center kan opnå gode kliniske resultater ved at anvende moderne metoder og efterleve nuværende retningslinjer for PGT, såsom bioptering på blastocystsstadiet og cryopræservering ved vitrifikation, som tillader nedfrysning af alle embryoner og obligatorisk transferering af et enkelt embryon uden at kompromittere de kliniske resultater. Over de fire år, som blev undersøgt, havde vi en kumulativ klinisk graviditetsrate på 52,7 % for hele kohorten, hvilken steg til 87,7 % når vi kun evaluerede patienter, som havde gennemgået et komplet behandlingsforløb. Den kliniske graviditetsrate per ægudtagningscyklus og per embryo transfer var henholdsvis 28,7 og 33,2 %. Vi observerede at kvindens alder ved starten på behandlingsforløbet havde en signifikant effekt på chancen for at opnå en klinisk graviditet i løbet af behandlingstilbuddet. Chancen for at opnå graviditet blev reduceret med 8 % for hvert ekstra år, som kvinden blev ældre. Vi fandt at antallet af transferable embryoner efter en ægudtagningscyklus påvirkede chancen for at opnå graviditet i samme cyklus signifikant. Hver ekstra transferabelt embryon fordoblede eller firdoblede chancen for at opnå en klinisk graviditet (afhængigt af den udførte analyse). Dette viser, at tiltag, som kan øge antallet af transferable embryoner, vil have en dramatisk effekt på chancen for at opnå klinisk graviditet per ægudtagningscyklus.

Studie V beskriver vores resultater fra en spørgeskemaundersøgelse blandt patienter, som har opnået en graviditet efter PGT. Undersøgelse omhandler deres valg af, meninger om og erfaringer med invasiv prænatal testning (moderkageprøve) i forbindelse med deres graviditet, herunder deres meninger om et ikke-invasive prænatale alternativ. Vi erfarede, at ca. halvdelen af patienterne takkede nej til modekageprøve. De fleste takkede nej pga. den risiko for abort, som var forbundet med indgrebet. Selv hos patienter, som udtrykte et ønske om at få foretaget prænatal verificering af det oprindelige PGT-resultat, valgte ca. en ud af fire at takke nej til moderkageprøven. Ni ud af ti patienter ville have accepteret ikke-invasiv prænatal testning, hvis det var blevet tilbudt dem, hvilket indikere, at risici forbundet med prænatal testning er et vigtigt punkt for patienter. Dermed indikerer resultaterne, at man ved at indføre et ikke-invasivt alternativ til moderkageprøven, kan få flere patienter til at acceptere prænatal testning. Dette kan potentielt medvirke til at reducere stress og/eller bekymring, som nogle patienter måtte opleve i løbet af graviditeten. Dette er underbygget af, at fire ud af ti patienter rapporterede, at de havde været bekymrede på et eller andet tidspunkt i løbet af graviditeten for, om fosteret nu

også var rask på trods af PGT. Vi fandt at en ud fire patienter ikke var ordentligt klar over begrænsningerne ved nakkefoldsskanningen i forhold til dens manglende evne til at kunne sige nogen om den sygdom, som patienten fik foretaget PGT på baggrund af. Faktisk ville tre patienter ikke have fravalgt moderkageprøven, hvis de havde været klar over denne begrænsning. Vi fandt desuden, at ca. en tredjedel af patienterne ikke kunne huske, at klinikken anbefalede moderkageprøve efter PGT. Mere end ni ud af ti patienter rapporterede dog, at de var blevet informeret om muligheden for at få foretaget en moderkageprøve. Sammenlagt viser resultaterne, at man bør overveje at gennemgå den kliniske information, som gives til patienterne, for at sikre, at de er bedre klædt på til at træffe velinformerede beslutninger omkring prænatal testning efter PGT. Derudover viser studiet, at et ikke-invasivt alternativ til moderkageprøve, vil have en høj tilslutning blandt patienterne.

ACKNOWLEDGEMENTS

The work presented in this thesis would not have come to fruition were it not for contributions and support from numerous wonderful colleagues, friends, and family members.

Initially, I would like to thank my supervisor Inge Søkilde Pedersen for believing in my, supporting me, and guiding me along this journey. She was the one that initially suggested to me that I should consider a Ph.D. within the field of preimplantation genetic testing when the Center for Preimplantation Genetic Testing opened at Aalborg University Hospital in October 2016. I've since developed an enormous interest and passion for the field, so thank you, Inge, for stirring me in the right direction. I would also thank you for helping me grow and evolve into the scientist I am today and for sharing your extensive knowledge within the field of genetics with me.

I would also like to thank my co-supervisors, Hans Jakob Ingerslev and Ulrik Schiøler Kesmodel, for their support and guidance, especially within the world of assisted reproductive technology, in which I had no prior experience when I initiated my Ph.D. I would explicitly like to thank Ulrik for sharing his extensive knowledge about statistics and experience with epidemiolocal studies, from which I've learned a lot. I want to thank Jakob for his always critical eye and exhaustive corrections. Despite the often overwhelming amount of red text in returned manuscripts, they have contributed to making me a better writer. Additionally, Jakob's ability to always know someone that knows something has been helpful whenever I needed a new collaborator for a project or an expert. I've learned a tremendous amount from all three of you, for which I am grateful, and I hope that I'll be able to draw on your experience and knowledge in the future through various collaborations, as I am sure that I can still learn a lot more from you all. Hopefully, one day, I will be able to return.

I would like to express a large amount of gratitude to the various laboratories and departments that I've been in contact with during my Ph.D. All have med me and my projects with enthusiasm and opens arms.

I would like to thank my colleague at the Department of Molecular Diagnostics, where I am employed. It is a fantastic department with some wonderful people. I would especially like to thank Henrik Okkels, Anja Ernst, Birte Degn, and Marianne Nygaard Bang of the PGT team for their assistance, advice, and aid during my Ph.D. I would also like to thank Anne Grethe Sørensen and Anne Rusborg for sharing their expertise during DNA isolation and performing library construction and next generation sequencing of my samples. Whenever I ran into trouble coding in R, or

needed any technical assistance, our great team of bioinformaticians, Lasse Ringsted Mark, Mads Sønderkær, and Nina Anette Hagen Madsen, were always kind to help me solve the problem and teach me their language. For that, I am thankful, and I can assure you that your assistance will likely be needed again soon. I would also like to thank Anja Ernst, Ihab Bishara Yousef Lolas, Henrik Okkels, and Lene Rasmussen for many great moments, and I will not even begin to count the number of laughs and fun moments we have shared. I want to take Christine Flodgaard Høgsbro for our collaboration over the past few years. I enjoyed our time in the lab, which was very fun, especially considering the late hours and the at some points obscure circumstances under which we managed to perform our experiments (you know). Lastly, I would like to thank the head of the Department of Molecular Diagnostics, Henrik Krarup, for the support and guidance he has provided to me prior to and during my Ph.D. and the contacts he has put me in touch with. While his name may not be present on any of the results of this Ph.D., his part in making this Ph.D. possible is enormous. Thank you for that.

I would like to recognize all the people working at the Fertility Unit where I spent a large part of my time during my Ph.D. I can only applaud the enthusiasm and interest that they showed in my research from day one and their effort to ensure that I could perform my research at the clinic. Most importantly, they made me feel welcomed from day one. As a bonus, other departments would be jealous if they were aware of the frequency that fresh bread or delicious bakery was served, which may have statistically significantly affected the frequency or timing of my visits (Ulrik taught me how to calculate that). I owe a special thank you to Christina Hnida and especially Vibeke Bæk Christiansen, with whom I spent many educational and fun hours performing embryo biopsy or other experiments. Their expertise in embryo handling and biopsy was essential for my research. I also owe a special thank you to Janne Margrethe Roskær, for her enthusiasm and help in initiating and managing patient recruitment.

The Department of Clinical Genetics, especially Tue and Marie, whom I co-authored papers and accompanied abroad to conferences during my Ph.D., deserves a thank you. I've had many great moments with both of you, learned a lot, and produced great work, which I can only hope will continue in the future. With Tue, I have made a great friendship off of work, for which I am grateful.

Vladimir and Hiva from the Department of Stem Cell Research at Aalborg University Hospital deserve appreciation for their enthusiasm and interest when we suggested try and culture biopsied trophectoderm cells. Without their assistance, the research would not have been possible.

The collaboration that I've had with ARCEDI Biotech has been special, and I would like to thank Lotte Hatt and Palle Schelde for initially agreeing to initiate a partnership, which has now resulted in a paper and hopefully will lead to the

introduction of a non-invasive prenatal alternative into clinical practice in the near future. Their lab consists of friendly and enthusiastic people, which have been welcoming whenever I've spent time at their laboratory. Unfortunately, Corona-lockdown hindered me from staying at their lab for as long as I planned. I want to extend a special thanks to Line Dahl Jeppesen, with whom I've had many fun, exciting, and educational discussions. I hope that our regularly occurring meetings will continue in the future and hopefully spark new interesting collaborations. I would also like to extend a thank you to Peter Schelde for collecting blood samples all across the kingdom, irrespective of the day and time.

The people working at the other national PGT center at Rigshospitalet, Copenhagen, also deserve a thank you. They have provided great feedback whenever I've presented ideas or results at our national meetings. The future holds some national collaborations, which I am looking forward to.

Stine from the Department of Culture and Learning deserves recognition for guiding and educating me on questionnaire surveys, which was extremely helpful during the planning and execution of my research and when interpreting the results.

I want to thank my colleagues and friends who read and gave feedback on the introduction to this thesis. Thank you, Morten Mørk, Peter Jørgensen, Tue Diemer, and Anja Ernst, for taking your time to read and contribute to the introduction.

I would like to thank all of my friends, who, in one way or another, have helped me get through this Ph.D. A special thank you has to go to Peter Jørgensen, my dear friend, and former classmate, back when we studied Molecular Biology at Aarhus University. I really appreciate the countless hours of fun conversations, computer games, and football matches we have shared over the years. It has been great to have been able to share my experiences and ups and downs with a fellow Ph.D. student and good friend, and it has helped me a lot along my journeys. I look forward to more great conversations, scientific or less so, in the future. I would also like to say thank you to my good friend Morten Kronborg and the countless hours we have spent running, weightlifting, or doing CrossFit together. I've always enjoyed having you as my training buddy. The mentally demanding process that I've been through during my Ph.D. has only caused my appreciation for our physically exhausting sessions to increased, as they have helped me stay in shape and regain energy. Lastly, I would like to thank my friends Mark and Ida, Rune and Marie, and their kids for the countless joyful gatherings that my family and theirs have spent together and the energy and love it brings into my life.

Last but most importantly, I would like to thank my nearest family for their support along the way. To my parents, Kate and Per, and my older brother Michael, I am grateful for your love and support in all aspects of life. My amazing wife, Michelle, and my two lovely kids Julie and Villum, deserve all my gratitude for always being

there to make me smile at the end of the day, no matter how things had gone at work. Thank you, Michelle, for your patience, support, understanding, and love, and for everything you do for our family and me; I love you. This work would never have materialized if not for your support and belief in me.

LIST OF ABBREVIATIONS

ART	**Assisted Reproductive Technology**
cbNIPT	**Cell-Based Non-Invasive Prenatal Testing**
CCS	**Comprehensive Chromosome Screening**
cffDNA	**Cell-free fetal DNA**
CGH	**Comparative Genomic Hybridization**
CVS	**Chorionic Villous Sampling**
ESHRE	**European Society of Human Reproduction and Embryology**
FISH	**Fluorescence *In Situ* Hybridization**
ICM	**Inner Cell Mass**
IVF	***In Vitro* Fertilization**
NGS	**Next Generation Sequencing**
NIPT	**Non-invasive prenatal testing**
PCR	**Polymerase Chain Reaction**
PGT	**Preimplantation Genetic Testing**
PGT-A	**Preimplantation Genetic Testing for Aneuploidy**
PGT-M	**Preimplantation Genetic Testing for Monogenic Disorders**

PGT-SR	**Preimplantation Genetic Testing for Structural Rearrangements**
SNP	**Single Nucleotide Polymorphism**
STR	**Short Tandem Repeat**
WGA	**Whole Genome Amplification**

LIST OF STUDIES

Study I:

A systematic review on concurrent aneuploidy screening and preimplantation genetic testing for hereditary disorders: What is the prevalence of aneuploidy and is there a clinical effect from aneuploidy screening?

Christian Liebst Frisk Toft, Hans Jakob Ingerslev, Ulrik Schiøler Kesmodel, Tue Diemer, Birte Degn, Anja Ernst, Henrik Okkels, Kristin Rós Kjartansdóttir, Inge Søkilde Pedersen.

Published in *Acta Obstetricia et Gynecologica Scandinavica, 2020;**99**:696–706.*

DOI: 10.1111/aogs.13823

Study II:

Cell-based non-invasive prenatal testing for monogenic disorders: confirmation of unaffected fetuses following preimplantation genetic testing

Christian Liebst Frisk Toft, Hans Jakob Ingerslev, Ulrik Schiøler Kesmodel, Lotte Hatt, Ripudaman Singh, Katarina Ravn, Boletta Hestbek Nicolaisen, Inga Baasch Christensen, Mathias Kølvraa, Line Dahl Jeppesen, Palle Schelde, Ida Vogel, Niels Uldbjerg, Richard Farlie, Steffen Sommer, Marianne Louise Vang Østergård, Ann Nygaard Jensen, Helle Mogensen, Kristín Rós Kjartansdóttir, Birte Degn, Henrik Okkels, Anja Ernst, Inge Søkilde Pedersen.

Published in *Journal of Assisted Reproduction and Genetics, 2021;1–12.*

DOI: 10.1007/s10815-021-02104-5

Study III:

Proof of concept: DNA amplification by culture of trophectoderm biopsies as an alternative to whole genome amplification in preimplantation genetic testing

Christian Liebst Frisk Toft*, Christina Flodgaard Høgsbro*, Hans Jakob Ingerslev, Ulrik Schiøler Kesmodel, Christina Hnida, Hiva Alipour, Vladimir Zachar, Hiroaki Okea, Takahiro Arima, Birte Degn, Tue Diemer, Inge Søkilde Pedersen.

*Shared co-first authorship

Submitted on 30th April 2021 to *Journal of Reproductive Biomedicine Online*

Study IV:

Cumulative pregnancy rates following preimplantation genetic testing in a public health care setting: a report on 330 couples

Marie Skov Hvidbjerg*, Christian Liebst Frist Toft*, Ulrik Schiøler Kesmodel, Inge Søkilde Pedersen, Birte Degn, Henrik Okkels, Anja Ernst, Tue Diemer, Hans Jakob Ingerslev

*Shared co-first authorship

Submitted on 10th June 2021 to *Fertility and Sterility*

Study V:

Patients' choices and opinions on chorionic villous sampling and non-invasive alternatives for prenatal testing following preimplantation genetic testing for hereditary disorders: A cross-sectional questionnaire study

Christian Liebst Frisk Toft, Tue Diemer, Hans Jakob Ingerslev, Inge Søkilde Pedersen, Stine W. Adrian, Ulrik Schiøler Kesmodel

Submitted on 10th June 2021 to *Prenatal Diagnosis*

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1 PROLOGUE

This thesis contains research work done within the field of preimplantation genetic testing (PGT) for hereditary disorders during the last three years which I have spent as a Ph.D. fellow at the Department of Molecular Diagnostics, Aalborg University Hospital. During my Ph.D. I had close ties to the Fertility Unit, Aalborg University Hospital, and the Department of Clinical Genetics, Aalborg University Hospital. These three departments form the Center of Preimplantation Genetic Testing at Aalborg University Hospital, established in October 2016. Our center is one of two national PGT centers in Denmark, who offers PGT for hereditary disorders.

The results span from basic research where we cultured biopsied trophectoderm cells as an alternative method of DNA amplification, over a systematic review on the prevalence of aneuploidy and clinical effect of aneuploidy screening, to clinical research involving the development of cell-based non-invasive prenatal testing, a patient questionnaire survey on prenatal testing following PGT and evaluation and analysis of clinical outcomes at our center. The diverse nature of the topics and papers that constitutes this thesis has allowed me to delve into many different aspects of the field and reflects the close link between basal and clinical research characterizing a University Hospital. As the research projects span diverse areas within the field of PGT, a joint introduction to, presentation, and discussion of the studies would not be meaningful nor constructive for the reader. Hence, the thesis contains an initial comprehensive introduction to the field of PGT followed by five separate sections, each focusing on a single study with a separate introduction, study objective, study design, results, and a discussion of the findings. The thesis will end with a joint section containing concluding remarks and future perspectives. A short introduction to the five included studies is provided below.

Study I details a systematic review of concurrent PGT for hereditary disorders and aneuploidy, assessing the extent to which aneuploidy is reported and affects clinical results during PGT (Appendix A).

Study II describes our evaluation of cell-based non-invasive prenatal testing as an alternative to invasive chorionic villous sampling following PGT for monogenic disorders (Appendix B).

Study III describes our work establishing a cell culture assay capable of supporting the growth and division of biopsied trophectoderm cells as an alternative method of whole genome DNA amplification prior to genetic testing for hereditary disorders (Appendix C).

Study IV comments on our work of assessing and evaluating clinical outcomes, including cumulative pregnancy rates, as well as factors affecting the chance of achieving pregnancy during PGT for hereditary disorders at our center (Appendix D).

Study V contains the results from a questionnaire aiming to investigate which factors govern and affect patients' decisions regarding prenatal testing following pregnancy after PGT for hereditary disorders (Appendix E).

2 INTRODUCTION

2.1 THE ORIGIN OF PGT

In 1989 at the Hammersmith Hospital in London, a few couples at risk of transmitting a recessive X-linked disorder to their offspring opted for a yet unknown treatment that would go on to revolutionize the reproductive options for couples at risk of transmitting a hereditary disorder to their offspring (Handyside *et al.*, 1990). The treatment was based upon assisted reproductive technology (ART) treatment to generate human preimplantation embryos *in vitro*, which were subsequently biopsied to obtain DNA for genetic testing. The couples in question were at risk of transmitting an X-linked disorder, which would affect 50 % of their male offspring. To identify potentially affected embryos, the sex of the embryos was determined when DNA from the embryo was amplified by Polymerase Chain Reaction (PCR) for a Y-chromosome specific sequence, a procedure published previously the same year (Handyside *et al.*, 1989). The presence of the PCR product would indicate that the embryo was a male, while absence would indicate a female embryo. Using this procedure, female embryos were identified and transferred to the uterus. The following year, in 1990, the first-ever children following what is now known as preimplantation genetic testing (PGT) were born.

While applying PCR to test for a hereditary disorder on a single cell biopsied from a human preimplantation embryo was considered an amazing achievement in 1989, the significance of this breakthrough may only be fully appreciated in hindsight. Since then, several advances, both procedural and technological, have been developed, which have greatly improved the success rate, diagnostic accuracy, and possibilities of PGT as we know it today.

2.2 THE WORKFLOW OF PGT

PGT is a risk-reduction procedure offered to couples at risk of passing on a hereditary disorder to their offspring. It entails the generation of preimplantation embryos *in vitro* from which embryonic material is obtained. The embryonic material is then tested, allowing selection of unaffected embryos for intrauterine transfer (Figure 2-1). Alternatives to PGT are spontaneous pregnancy, gamete donation, adoption, prenatal testing, or abstaining from having children. Although replacing gametes from the affected parent with those from a donor, or choosing adoption, can eliminate the risk, many couples wish to have a child to which there is a biological relation. Prenatal testing is an alternative solution, where termination of pregnancy can be performed in the case of an affected fetus, but this is not always viable as political, cultural, or religious factors might prohibit termination of pregnancy in some cases or countries. PGT provides a solution where couples can have a biological child of their own with

a significant reduction in the risk of inheriting the disorder in question. The two latest publications by the European Society of Human Reproduction and Embryology (ESHRE) PGT Consortium, which collects, and reports results from PGT centers across Europe, indicate that the risk of misdiagnosis associated with PGT is less than 1 % (De Rycke *et al.*, 2017; Coonen *et al.*, 2020).

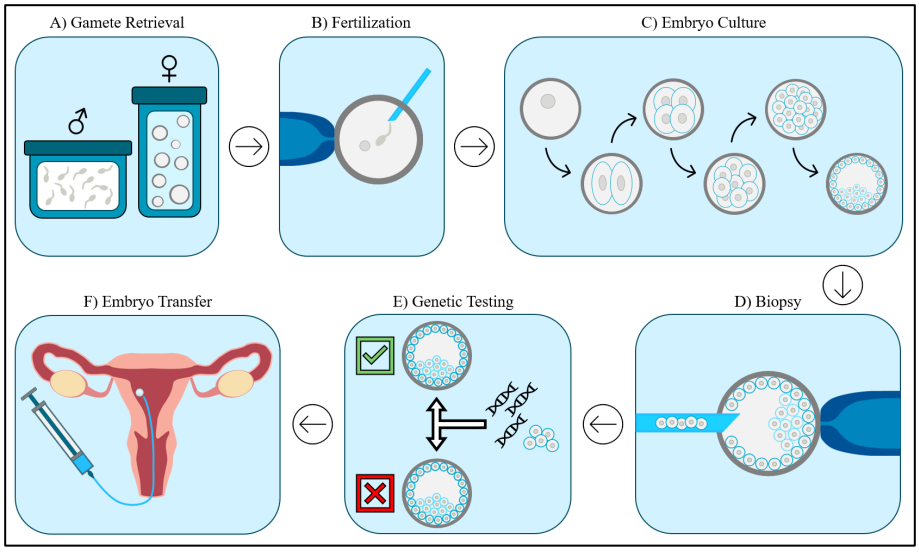


Figure 2-1: Outline of the workflow of PGT.

PGT starts with retrieval of gametes (A) followed by fertilization of oocytes in vitro (B). Successfully fertilized oocytes are then cultured in vitro, developing into preimplantation embryos (C). Embryo biopsy is performed (D) to obtain DNA for genetic testing of the hereditary disorder to differentiate affected and unaffected embryos (E). Unaffected embryos are subsequently transferred back to the uterus (F).

2.3 THE EVOLUTION OF PGT FOR INHERITABLE DISORDERS

The first-ever PGT was performed indirectly by sexing the embryos to exclude male embryos for transfer (Handyside *et al.*, 1989, 1990). While this was, without doubt, a fantastic achievement, 50 % of discarded male embryos would be expected to be unaffected using this approach. Additionally, the method was not without flaws. One misdiagnosis was reported with one fetus found to be male following prenatal testing and karyotyping, likely caused by PCR amplification failure during PGT (Lissens, 1996). Hence, there was room for improvement. At the time when the first PGT procedure was performed, DNA sequencing was still in its infancy, and knowledge about the human genome was sparse. Another year would pass from the first PGT until the human genome project was launched in October 1990, and it would take an

additional 13 years for its completion in April 2003. While the method of Sanger sequencing was introduced in the mid to late 1970s (Sanger and Coulson, 1975; Sanger *et al.*, 1977), it took another 10 years for the method to be commercialized, allowing sequencing of larger DNA sequences with the aid of computers (Smith *et al.*, 1986). The combination of sequencing techniques being in their infancy and the gaps in knowledge of the human genome meant that PGT was still only reserved for specific cases. With the completion of the human genome project and the continuous development of new methods and platforms for DNA sequencing and chromosomal analysis over the years, PGT today can be performed for a vast selection of hereditary disorders. The two major groups of hereditary disorders for which PGT is performed are monogenic disorders and structural rearrangements. PGT for monogenic disorders, which characterize disorders caused by a mutation within a single gene, has been abbreviated as PGT-M. PGT for structural rearrangements, abbreviated PGT-SR, constitutes chromosomal translocations and larger chromosomal deletions and duplications.

2.3.1 DIRECT MUTATION DETECTION

Direct testing for monogenic mutations by Sanger sequencing soon followed the first report of PGT, with the first child being born following direct testing for cystic fibrosis in 1992 (Handyside *et al.*, 1992). This showed that specific gene mutations causing monogenic disorders could be investigated directly by PCR. PCR-based genetic testing is still applied today, including at our PGT center at Aalborg University Hospital. Soon, reports followed for other monogenic disorders such as Tay-Sachs disease (Harper, 1992), hemophilia A (Snabes *et al.*, 1994), Marfan syndrome (Harton *et al.*, 1996), Rhesus typing (Avner *et al.*, 1996), Myotonic Dystrophy (Sermon *et al.*, 1997), familial adenomatous polyposis coli (Asangla *et al.*, 1998), and neurofibromatosis type 2 (Harper *et al.*, 2002). Today, with the human reference genome being available, direct mutation detection in PGT can be performed for all monogenic disorders where the mutation causing the disorder has been identified.

2.3.2 IMPROVEMENT OF DIAGNOSTIC ACCURACY

A significant challenge of PCR-based methods for the detection of monogenic disorders was the issue of allele drop-out (ADO) (Findlay *et al.*, 1995). ADO is defined as the failure to amplify and detect one of the alleles during genetic testing. Due to the low input amount of DNA used, there is a significant risk of failed or preferential amplification, where one or both alleles fail to amplify, or one allele is preferentially amplified over the other. This poses a serious issue to both the robustness and the diagnostic accuracy of PGT, in the worst case resulting in misdiagnosis and transfer of an affected embryo.

A solution to reduce the impact of ADO, and thereby reduce the risk of misdiagnosis and inconclusive test results, was suggested in 1998 by Rechitsky *et al.*, who

performed multiplex PCR for both cystic fibrosis and highly polymorphic markers within the genome known as short tandem repeats (STRs) (Rechitsky *et al.*, 1998). This procedure was later echoed by others (Dreesen *et al.*, 2000; Piyamongkol *et al.*, 2001; Harper *et al.*, 2002) and shown to increase diagnostic accuracy (Lewis *et al.*, 2001). The use of STRs markers is recommended as part of the current ESHRE PGT Consortium's good practice recommendations for the detection of monogenic disorders (Carvalho *et al.*, 2020c).

STRs are 2-6 base pair DNA sequences consecutively repeated a variable number of times distributed across the human genome (Richard *et al.*, 2008), making them useful for distinguishing alleles within and between individuals. A combination of a sufficient number of STRs serves effectively as a DNA fingerprint, which is why STR markers are used for forensic science. Their widespread presence across the human genome means that they are present in close proximity to most genes and can often be found within a few megabases upstream and downstream of a gene. This can be used in PGT to detect which alleles from each parent are inherited by the embryo to determine whether the embryos are affected or not. This is achieved by phasing the alleles of interest to the mutation in question. Phasing of alleles describes the concept of assigning STR markers to the two alleles allowing them to be tracked and differentiated from one another, e.g., when differentiating an unaffected and affected allele during PGT by tracking the inheritance of paternal and maternal STR markers. DNA from first-degree relatives is required for phasing, although this might be circumvented in the context of PGT by indirectly using a pool of embryos as "relatives". In addition to a significant increase in diagnostic accuracy and success rate, the use of STR markers simultaneously allows testing for the presence of foreign DNA.

STR markers can be used for indirect testing. Indirect testing implies that an embryo is identified as affected or unaffected without testing for the mutation itself but instead using only STR markers or other DNA markers. Simultaneously with the first report by Rechitsky *et al.* using STR markers as a means to enhance diagnostic accuracy (Rechitsky *et al.*, 1998), others reported the use of STR markers for indirect testing in a couple where the gene, but not the mutation, causing the disorder was known (Lee *et al.*, 1998). Alternatively, indirect testing can be used in cases where the patient seeking PGT does not want to know his or her carrier status, in which case it is referred to as exclusion testing. This usage was suggested in 1996 for the late-onset neurological disease Huntington's disorder (Schulman *et al.*, 1996) and first performed in 2002 (Stern *et al.*, 2002), and has later been shown to be chosen by and used for a substantial proportion of patients (approximately one third) at risk of being affected by Huntington's disease (Van Rij *et al.*, 2012). This is a useful strategy for late-onset disorders in general, where the patient seeking PGT wants to ensure that a future child does not inherit a particular disease running in the family while remaining uninformed about their own carrier status. The downside of exclusion testing is that by chance, 50 % of the embryos at risk of being affected and therefore deselected will

actually be unaffected. Additionally, 50 % of patients receiving treatment would not have needed treatment, as they will not carry the affected allele. On the contrary, deselection of potentially unaffected preimplantation embryos may seem more ethically acceptable than termination of pregnancy in the case of prenatal testing showing an affected fetus.

To ensure genetic variation, DNA is exchanged between homologous chromosomes during gametogenesis, a process known as a crossover event because DNA exchanges (crosses over) between the two homologous chromosomes. The concept of crossover means that indirect testing using only STR markers during PGT is associated with a risk of misdiagnosis in case of a crossover between the marker and the site of the mutation. Although the frequency of crossover events is not the same across the genome (Khil and Camerini-Otero, 2009), the basic mechanism is the same: the larger the distance between two loci in the genome, the larger the risk of a crossover occurring, emphasizing the need to identify STR markers in close vicinity to the gene of interest. Additionally, assessing STR markers on both sides of the mutation is strongly recommended, as this allows detection of the large majority of crossover events. Hence, utilizing at least two STR markers located on both sides of the affected gene and in close proximity (preferably within 1 Mb) is recommended in the most recent ESHRE PGT consortium good practice recommendations (Carvalho *et al.*, 2020c), as this will significantly reduce the risk of an undetected crossover event due to the extremely low risk of a double crossover event occurring within a few Mb. Due to the high accuracy associated with STR markers, direct mutation detection is nowadays often completely omitted, and testing is often based solely on STR markers or other DNA markers.

STR markers also allowed the first case of a “savior sibling”, where PGT was used as a means to obtain a HLA matching sibling to a six-year old girl suffering from Fanconi Anemia and in need of HLA-matching stem cell transplantation (Verlinsky *et al.*, 2001). While the concept of “savior siblings” is a continuing ethical debate (Ingerslev and Hindkjaer, 2012; Strong *et al.*, 2014; Rubeis and Steger, 2019), the procedure has been welcomed by patients (Zierhut *et al.*, 2013). HLA matching accounted for 5 % of PGT treatments performed in Europe in 2013-2015, which is the latest available data collection published by the ESHRE PGT Consortium (Coonen *et al.*, 2020). In Denmark, only one case of HLA matching has been published (Degn *et al.*, 2012).

One shortcoming of STR markers is that informative markers must be identified for each couple and tested to ensure that the chosen STR markers can be amplified together in a multiplex PCR reaction. This results in a significant preclinical workload and time before treatment can be initiated. Additionally, in rare cases, no relatives are available, no informative markers can be identified, or multiplexing of desired markers fails. However, these cases are considered extremely rare, at least from our experience at our center. On the contrary, PCR-based methods are cheap, especially when compared to newer and more advanced technologies.

2.3.3 DETECTING CHROMOSOMAL ABNORMALITIES

It did not take long for PGT to evolve to include larger chromosomal abnormalities such as translocations and larger deletions or duplications. This was achieved by the introduction of fluorescence in situ hybridization (FISH), which was initially used to determine the sex of embryos during PGT for X-linked conditions (Griffin *et al.*, 1993; Munné *et al.*, 1993b). FISH is performed by annealing probes coupled to a fluorophore to metaphase chromosomes spread out on a microscope slide. By inspection of the fluorescent signal(s), chromosomal rearrangements, large deletions, or large duplications can be detected. While FISH opened op a new array of disorders that could be tested by PGT, the procedure was labor intense, required case-by-base probe customization, and limitations in the number of fluorescent signals detectable affected the number of chromosomal aberrations that could be investigated. FISH was the primary method used for detection of larger chromosomal aberrations until 2002, when Malmgren *et al.* reported the use of comparative genomic hybridization (CGH) (Malmgren *et al.*, 2002). CGH is based on competitive binding of the sample of interest and a reference sample of known karyotype to a metaphase spread of chromosomes. By using fluorophores with different colors, gains and losses can be detected by visual inspection. The procedure was later automized resulting in array CGH (aCGH) where DNA microarrays are used instead of chromosome metaphase spreads. To this day, aCGH is still widely used to test for hereditary chromosomal abnormalities, while the use of FISH is declining (Coonen *et al.*, 2020).

Around the same time as CGH made its entry, a procedure utilizing single nucleotide polymorphism (SNP) in the human genome for genetic testing was introduced (Wang *et al.*, 1998). SNPs are scattered frequently across the human genome. While on each nucleotide position in the genome, there are only four possible different options (Adenosine, Guanosine, Cytosine or Thymine), the large number of SNPs present in the human genome means that a combination of SNPs can be used as a DNA fingerprint. In that regard, SNPs share many features with STR markers in that they are distributed across the entire genome and vary within and between individuals. SNPs have the additional benefit compared to STR markers in that they are much more frequent in the genome and often found in closer vicinity to a gene or position in the genome of interest compared to STRs. Utilizing SNPs for PGT was first reported in 2002 for a mutation in the *NF2* gene, where a single SNP was used in combination with direct mutation detection to select unaffected embryos (Abou-Sleiman *et al.*, 2002). SNP arrays were later developed to genotype several predefined SNPs from a DNA sample, which was then used to phase and track alleles during PGT in order to discriminate between affected and unaffected embryos in a manner similar to STR markers. The use of SNPs as a means to track alleles has been commercialized with examples such as Karyomapping (Handyside *et al.*, 2010) and Haplarithmisis (Zamani Esteki *et al.*, 2015). In addition to specific genetic disorders, genome-wide SNP analysis also allows the detection of de novo chromosomal aberrations.

While work on "second-generation sequencing" or "next generation sequencing" was initiated in the mid to late 1990s and the first next generation sequencer launched in 2000, the first PGT using NGS was not reported until 2013 (Treff *et al.*, 2013). Since then, numerous reports on the use of NGS for both PGT-M and PGT-SR have been published. Judging from the recently published literature within the field of PGT, NGS is becoming the method of choice for PGT. Part of the explanation is that NGS has the benefit of being highly scalable, so the amount of data (and thereby the cost) can be customized to each case, and highly flexible, as NGS allows both PGT-M and PGT-SR to be performed using the same platform (De Rycke and Berckmoes, 2020). Additionally, the capability of NGS to generate genome-wide data can be used for the detection of de novo chromosomal abnormalities (De Rycke and Berckmoes, 2020).

A common requirement for these more advanced methods is that the small amount of DNA obtained must be amplified by a procedure named whole genome amplification (WGA) before genetic testing. No WGA protocols are perfect, and they do not produce a true copy of the initial input DNA. Thus, issues such as ADO, genome coverage, and preferential amplification may introduce bias and artifacts (Sabina and Leamon, 2015), which must be considered when selecting WGA protocols for PGT (De Rycke and Berckmoes, 2020).

In conclusion, while PCR-based methods such as direct mutation detection, STR marker analysis, and FISH are still employed, the trend in PGT moves towards platforms and solutions that allows most if not all types of hereditary disorders (and potential other chromosomal abnormalities) to be tested on a single platform without much individual customization.

2.4 THE HUMAN PREIMPLANTATION EMBRYO

ART aims at replicating the steps of human reproduction and early embryonic development *in vitro*. Hence, to understand the procedures of ART, knowledge about human reproduction *in vivo* is essential.

2.4.1 EARLY HUMAN EMBRYONIC DEVELOPMENT *IN VIVO*

Human embryonic development initiates upon fertilization of the oocyte with the spermatozoon (Figure 2-2). Oocytes are released from the ovaries (only one ovary is shown in Figure 2-2) with an interval of approximately 28 days in women with a regular cycle. Following successful fertilization of the oocyte, a zygote is formed that will go through a series of developmental steps as it migrates through the fallopian tube towards the uterine cavity, where it will eventually implant into the endometrium and continue its development.

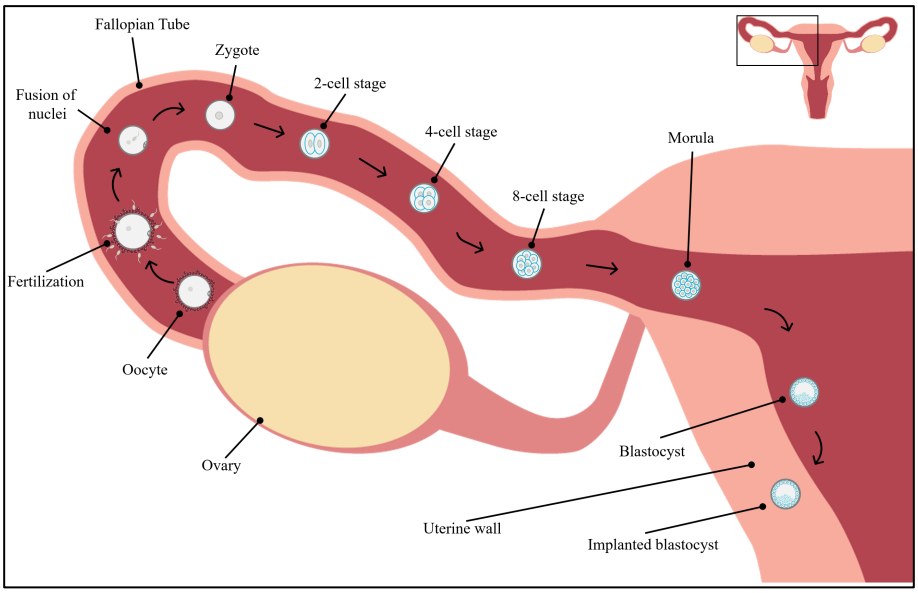


Figure 2-2: Overview of early human embryonic development in vivo.

Following fertilization of the oocyte, the two haploid nuclei fuse to form a diploid cell called a zygote. The zygote then enters the cleavage stage, where each cleavage results in a doubling of the cells within the embryo as it travels along the fallopian tube towards the uterine cavity. When the embryo consists of 16 cells, it has reached the morula-stage, at which point cell differentiation starts while the cells keep dividing. At day five to six post-fertilization, the embryo has formed a blastocyst consisting of two distinct cell lines (trophectoderm and the inner cell mass, see Figure 2-3). The blastocyst will eventually implant into the endometrium, where it will continue its development.

Following contact between the spermatozoon and the oocyte, the two haploid nuclei will fuse to form a single diploid cell, the zygote. Between day one and day two post-fertilization, the first cleavage appears forming two cells, termed blastomeres (from Greek *blastos*, to sprout), by replication and division of the DNA and partition of the existing cytoplasm. This stage of embryonic development is referred to as the cleavage stage, as the embryo divides (cleaves) into more cells without increasing its size. More cleavages occur towards day three post-fertilization where the zygote has formed a morula consisting of sixteen blastomeres. Towards day five post-fertilization the morula compacts and the blastomeres keep dividing. At the same time, the first distinct cell populations begin to form. At day five post-fertilization, the embryo consists of an outer layer of cells, referred to as the trophectoderm, that encapsulates a large fluid filled cavity called the blastocoel, and a collection of cells inside the embryo called the inner cell mass (ICM) (Figure 2-3). The presence of these two distinct cell populations and the blastocoel marks that the embryo has reached the

blastocyst stage. The trophectoderm will later give rise to the placenta while the inner cell mass will develop into the fetus, give rise to the amnion, and contribute to the placenta (Taylor *et al.*, 2014a). On day six to seven post-fertilization the blastocyst hatches from a protein-rich shell termed the zona pellucida (Figure 2-3), which has been encapsulating the embryo during the early stages of embryonic development. The zona pellucida is lost so that the embryo can initiate implantation into the endometrium at day eight to nine post-fertilization, where development will continue.

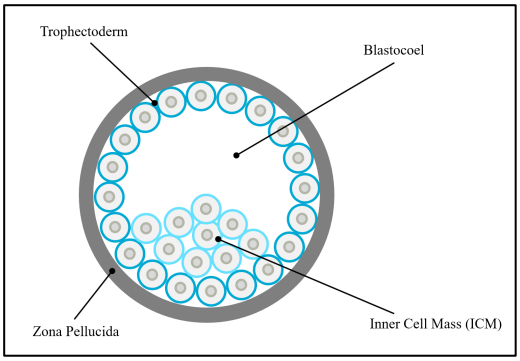


Figure 2-3: The human blastocyst.

The human blastocyst contains two distinct cell populations: the trophectoderm and the inner cell mass (ICM). The trophectoderm lines the periphery of the embryo and is surrounded by a protective protein-rich shell termed the zona pellucida. The ICM consist of a collection of cells inside the embryo. Inside the embryo is a fluid-filled cavity termed the Blastocoel.

2.4.2 ASSISTED REPRODUCTIVE TECHNOLOGY

Patients opting for PGT must go through ART procedures to generate human preimplantation embryos *in vitro*. This entails retrieval of male and female gametes (oocytes and spermatozoa) and subsequent fertilization of oocytes (Figure 2-1A and B). Female gamete retrieval entails hormone stimulation to stimulate the maturation of preovulatory follicles to allow collection of a large number of oocytes. The hormone stimulation has to be balanced to optimize the number of oocytes retrieved while simultaneously minimizing the risk of adverse side effects such as ovarian hyperstimulation syndrome (Alper and Fauser, 2017). Oocyte retrieval entails ultrasound-guided puncture of oocyte follicles and collection of oocytes. Fertilization can be performed either by in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (The method of ICSI is depicted in the illustration in Figure 2-1B). During IVF, metaphase II oocytes are attempted fertilized using numerous (several thousand) spermatozoa to each oocyte. During ICSI, the oocyte is denuded by removing cumulus and corona cells followed by the injection of a single into the cytoplasm of the oocyte (Figure 2-1B).

ICSI was initially developed to overcome issues associated with poor fertilization, primarily caused by male factor infertility (O'Neill *et al.*, 2018), and is today widely used due to its high level of standardization and efficacy (Rubino *et al.*, 2016), including PGT. For PGT, fertilization by ICSI is recommended over IVF to avoid contamination from spermatozoa and cumulus and corona cells attached to the zona pellucida, as these might disturb genetic testing by contamination with maternal and paternal DNA. According to the latest EHSRE PGT Consortium data collection, approximately 92 % of cycles between 2013 and 2015 used ICSI for fertilization (Coonen *et al.*, 2020). Following successful fertilization, the resulting zygote initiates early embryonic developments as it does *in vivo* (Figure 2-2). The presence of two pronuclei following fertilization by visual inspection has historically characterized successful fertilization *in vitro*. However, recent research has shown that fertilized oocytes with zero or one visible pronucleus can result in biparental diploid embryos if left to develop to later stages (Destouni *et al.*, 2018). This raises concern about discarding fertilized oocytes prematurely based on the pronuclei count. The latest introduction of time-lapse imaging incubators has likely reduced this problem as correct fertilization and embryo quality can now be evaluated dynamically from a time series across embryonic development instead of a snapshot in time. However, manual inspection of time-lapse images might be preferred as automatically assigned pronuclei count by the time-lapse software can be flawed (Capalbo *et al.*, 2017).

If successfully fertilized *in vitro*, the resulting zygote starts dividing, referred to as the cleavage stage of embryonic development. The cleavage stage is important in the context of PGT, as the eight-cell cleavage stage embryo has been the preferred stage for embryo biopsy since the dawn of PGT and up until recently, as detailed in a forthcoming section. Depending on the local practice, the embryo can either be transferred into the uterine cavity at the cleavage stage or cultured *in vitro* for an additional 2-3 days until it reaches the blastocyst stage and then transferred. For embryos cultured to the blastocyst stage, a morphological grading system has been developed, known as the Gardner score, where both the ICM and trophectoderm is given a score by using the letters A, B, and C, with A being the highest score (Gardner and Schoolcraft, 1999). The scoring system is a tool to allow prioritization of embryos for transfer, as there is a positive correlation between embryo morphology score and clinical outcomes (Gardner *et al.*, 2000; Ahlström *et al.*, 2011; Goto *et al.*, 2011; Van Den Abbeel *et al.*, 2013).

New research focuses on utilizing time-lapse videos to predict which embryos are most likely to result in pregnancy based on morphological and morphokinetic parameters across embryonic development. In that context, a recent deep learning model has shown promising results in predicting fetal heartbeat from embryo transfer based on time-lapse videos (Tran *et al.*, 2019). Embryonic development *in vitro* is affected by multiple aspects of cell culture conditions such as oxygen tension levels, pH, temperature, and volume of the culture medium (Wale and Gardner, 2016). Interestingly, a large national study in the UK has shown that the choice of culture

conditions can affect clinical outcomes in ART treatment (Castillo *et al.*, 2020). Thus, even though culture conditions have improved significantly over the years, no clear consensus on the optimal culture conditions exist. Future research will likely improve *in vitro* culture of human preimplantation embryos, hopefully bringing us closer to the optimal conditions.

2.5 CRYOPRESERVATION OF HUMAN EMBRYOS

As the use of ART rapidly spread across the globe following the first successful IVF treatment in 1977 and the birth of Louise Brown in 1978, the first child born as a result of ART, a need to store surplus human embryos gradually arose. Freezing of human embryos dates back to the beginning of the 1980s, with the first pregnancy following transfer of cryopreserved embryos reported in 1983 (Trounson and Mohr, 1983). The first cryopreservation protocol utilized slow freezing, where the embryos are frozen in a liquid containing cryoprotectants over several hours. The original procedures resulted in low survival rates upon thawing, which called for improvements or a less damaging alternative approach (Baust *et al.*, 2009; Konc *et al.*, 2014). Both occurred as improvements to slow freeze protocols were gradually introduced along with the introduction of a new method termed vitrification. During vitrification, embryos are rapidly frozen within a few minutes using liquid nitrogen in a high concentration of cryoprotectants (Baust *et al.*, 2009; Konc *et al.*, 2014). Despite improvements of slow freeze protocols, a recent systematic review and meta-analysis concluded that vitrification is superior to slow freezing with respect to survival rates and clinical outcomes (Rienzi *et al.*, 2017). Importantly, vitrification has demonstrated clinical outcomes comparable to fresh embryo transfer (Li *et al.*, 2014; Wong *et al.*, 2017).

Vitrification has facilitated the introduction of a “freeze-all” strategy (Wong *et al.*, 2014) in ART, where all embryos are frozen and no fresh transfers performed. The ”Freeze-all” strategy has the potential advantage of reducing the risk of ovarian hyperstimulation syndrome (Chen *et al.*, 2016). Additionally, the embryo transfer can be more easily timed with a receptive endometrium. During PGT, an additional advantage is that it allows sufficient time for genetic testing following embryo biopsy. The latest ESHRE PGT Consortium data collection reported a trend towards more PGT centers utilizing a “freeze-all” strategy (Coonen *et al.*, 2020). The “freeze-all” strategy will likely become more used as more and more PGT centers shift to biopsy at the blastocyst stage, making fresh transfer practically impossible in many cases due to the limited time available for genetic analysis.

2.6 EMBRYO BIOPSY

The first-ever PGT biopsies were performed by Handyside *et al.* at the cleavage stage by the aspiration of a single or two blastomeres (Figure 2-4B). Biopsy at the cleavage

stage allowed sufficient time for the embryo to be transferred within the so-called window of implantation, referring to the period in which the endometrium is most receptive for embryonic implantation (Harper, 1992). Fresh embryo transfer was necessary as the methods for cryopreservation were inefficient at that time, as described in the previous section.

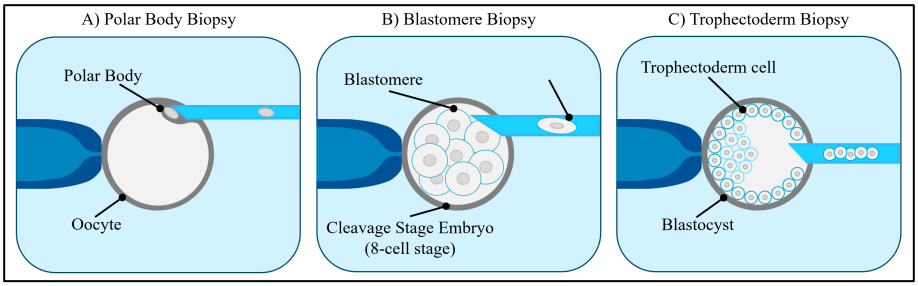


Figure 2-4: The three different embryonic stages of embryo biopsy.

DNA for genetic testing can be obtained by either A) Polar body biopsy performed on the oocyte, B) Blastomere biopsy (also known as cleavage stage biopsy) of one or two blastomeres performed at the 8-cell cleavage stage, and C) Trophectoderm biopsy of 5-10 trophectoderm cells at the blastocyst stage.

Immediately following the first PGT report, another group reported genetic testing on polar bodies (Verlinsky *et al.*, 1990). Polar bodies are diploid or haploid genomes generated as a byproduct during female meiosis I and II. They can be removed either simultaneously or sequentially from the oocytes using a biopsy pipette preceded by access through the zona pellucida, preferable generated mechanically or by laser (Kokkali *et al.*, 2020) (Figure 2-4A). While genetic testing of the polar body might have applications in ART and research into female meiosis and aneuploidy (Fragouli *et al.*, 2011; Wei *et al.*, 2015), its use in PGT for hereditary disorders has the disadvantage that it only allows testing for maternal disorders. In theory, the procedure is less invasive, and in countries where manipulation of human embryos is not legal, testing polar bodies can be the only way to perform PGT. Nonetheless, the cumulative ESHRE PGT Consortium data reveals that polar body biopsy never reached widespread application, with only 6 % of all biopsies in Europe performed by this method in 2015 (Coonen *et al.*, 2020).

Instead, blastomere biopsy at the cleavage stage has been the preferred biopsy technique since the dawn of PGT up until recently. Today, cleavage stage biopsy is performed using a biopsy pipette, and access through the zona pellucida is made either mechanically or by using a laser (Kokkali *et al.*, 2020) (Figure 2-4C). Previously, the zona pellucida at the biopsy site was dissolved by adding an acidic solution, or the zona thinned by applying a digestive enzyme, but those practices are no longer common. Although traditionally, one or two blastomeres have been biopsied at the

cleavage stage, biopsying only one cell is currently recommended (Kokkali *et al.*, 2020). This recommendation serves to avoid removing too large a fraction of the cell population, as biopsy of two versus one blastomere may negatively affect the chance of live birth (De Vos *et al.*, 2009). However, a tradeoff exists between biopsy of one versus two blastomeres as the latter might affect embryonic development and implantation potential but might simultaneously help increase diagnostic accuracy (Lewis *et al.*, 2001; Combelles, 2008). The most recent ESHRE PGT Consortium data collection presenting data from 2013-2015 reported that cleavage stage biopsy was still the most widely used method (Coonen *et al.*, 2020). Since then, judging on the literature published, a dramatic uptake of biopsy of trophectoderm cells at the blastocyst stage has happened. There are numerous good reasons for a transition from biopsy at the cleavage stage to the blastocyst stage, as will be detailed below.

Perhaps the most significant argument for biopsy at the blastocyst stage is that biopsy at this stage has less of an effect on embryonic implantation potential than biopsy at the cleavage stage (Scott *et al.*, 2013). Notably, the implantation potential of biopsied blastocysts may not be significantly different from non-biopsied blastocysts (Scott *et al.*, 2013; Tiegs *et al.*, 2020). A study on live-birth rates reported similar outcomes from transfers of biopsied and non-biopsied blastocysts (He *et al.*, 2019). In addition, the procedure is technically less challenging as a larger embryo is handled and a larger number of cells biopsied. Typically, 5-10 trophectoderm cells are biopsied at the blastocyst stage, compared to one or two blastomeres at the cleavage stage. As detailed previously, biopsy at the blastocyst stage necessitates cryopreservation in most cases to allow sufficient time for genetic testing of the biopsied cells. Thus, one of the main factors facilitating the transition to biopsy at the blastocyst stage has been improvements to cryopreservation previously detailed.

Technically, biopsy at the blastocyst stage entails aspiration of 5-10 trophectoderm cells at the opposite side of the embryo to which the inner cell mass is located (Figure 2-4C). Biopsying the blastocyst at the opposite side of the ICM ensures minimal disturbance of the ICM. Different methods are available to access the cells. A hole in the zona pellucida can be made immediately before biopsy using laser pulses followed by aspiration of 5-10 trophectoderm cells. The biopsied cells are then detached from the embryo either mechanically or assisted by laser pulses. Alternatively, the hole in the zona pellucida can be made at the cleavage stage, which causes a small herniation of trophectoderm cells as the embryo develops to the blastocyst stage. The herniating cells can then be detached mechanically or assisted by laser pulses and biopsied. (Kokkali *et al.*, 2020). The larger number of cells biopsied provides advantages to downstream genetic testing, as more DNA will be available.

Recently, a proposed alternative way of obtaining DNA is to collect cell-free DNA from the blastocoel fluid by extracting it with a fine needle or in the spent culture media. Especially the non-invasive alternative of using spent culture media is interesting as biopsy may then be avoided. The use of cell-free DNA for PGT is still

at the research stage. Issues such as karyotype and genotype concordance, diagnostic efficiency, and contamination from maternal or foreign DNA still need to be improved or addressed (Brouillet *et al.*, 2020; Leaver and Wells, 2020).

2.7 EMBRYONIC MOSAICISM

As detailed in the previous section, genetic testing of preimplantation human embryos entails analyzing biopsied embryonic material to infer a conclusion with respect to the ICM. When screening embryos for de novo chromosomal abnormalities, as will be discussed in the next section, the ability to infer the genetic status of the ICM from an embryo biopsy is complicated by a concept called embryonic mosaicism.

The concept of embryonic mosaicism describes the existence within the embryo of two or more cell lines with different chromosomal constitutions. Mosaicism predominantly originates from errors during mitotic division as the embryo develops (Taylor et al., 2014a; Popovic et al., 2020; Levy et al., 2021) (Figure 2-5). As these errors propagate through cell division to daughter cells, the resulting blastocyst will be a mosaic of cells with abnormal and normal chromosomal constitutions (Figure 2-5).

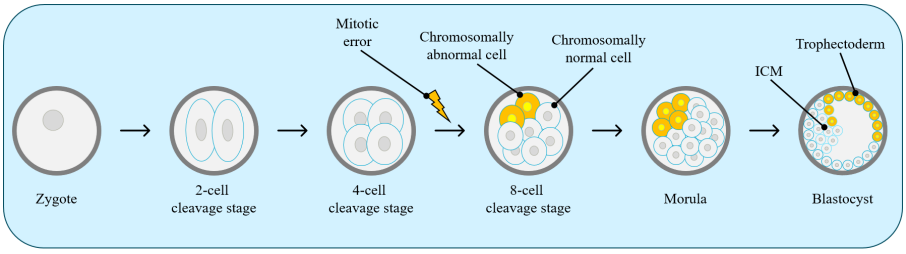


Figure 2-5: Embryonic mosaicism during embryonic development.

In the illustrated example, an error occurs during mitosis at the 4-cell in one of the blastomeres, causing the resulting daughter cells (orange) at the 8-cell stage to be have a chromosomally abnormal constitution. As embryonic development continues, the resulting chromosomal abnormality is propagated to daughter cells resulting in a blastocyst containing a mosaic of chromosomally normal (grey) and abnormal (orange) cells.

2.7.1 MOSAICISM AND EMBRYONIC DEVELOPMENT

Current data on embryonic mosaicism stems from analyses of in vitro generated embryos, and the prevalence of mosaicism in human preimplantation embryos reported in the literature varies dramatically from 4 to 90 % (Capalbo *et al.*, 2016; Sachdev *et al.*, 2017; Popovic *et al.*, 2020). In striking contrast, mosaicism is only

detected in 1-2 % of prenatal samples of placental tissue (Malvestiti *et al.*, 2015) and 1-6 % of products of conception from miscarriages (Martínez *et al.*, 2010; Kroon *et al.*, 2011; Li *et al.*, 2013; Segawa *et al.*, 2017). In general, there appear to be a reduction in the prevalence of aneuploidy as embryonic development advances, and mosaicism even seems to decrease during the early steps of embryonic development from the cleavage stage to the blastocyst stage (Capalbo *et al.*, 2016; Vera-Rodriguez and Rubio, 2017; Popovic *et al.*, 2020). One explanation for this observed decrease as embryonic development advances might simply be that most mosaic embryos are not viable. Alternatively, the embryo may be able to correct chromosomal errors to some extent, supported by sequential analysis showing that aneuploidies initially detected at the cleavage stage could not be detected at the blastocyst stage (Barbash-Hazan *et al.*, 2009; Capalbo *et al.*, 2013). Indeed, embryonic self-correction have previously been suggested (Bazrgar *et al.*, 2013), later supported by evidence of fetal cell lineage-specific depletion of chromosomally abnormal cells in mice embryos (Bolton *et al.*, 2016) and very recent evidence of elimination of aneuploidy in human embryos (Orvieto *et al.*, 2020; Yang *et al.*, 2021). It is likely, that the observed reduction in mosaicism as embryonic development advances is a combination of self-correction mechanism and differences in viability of chromosomally normal and mosaic embryos.

2.7.2 ASSESSING THE TRUE INCIDENCE OF MOSAICISM

The true incidence of mosaicism in human preimplantation embryos is extremely difficult to assess for multiple reasons. First, the method of choice for genetic testing can affect the degree of mosaicism observed due to differences in detection limits and types of chromosomal abnormalities detectable (Capalbo *et al.*, 2016; Vera-Rodriguez and Rubio, 2017; Popovic *et al.*, 2020). Second, embryo culture conditions may affect the prevalence of mosaicism, although hard evidence is still lacking (Swain, 2019). Third, how mosaicism is accessed can affect results. Initial attempts at assessing mosaicism were based on analysis of the isolated ICM and the trophectoderm as a whole or partitioned into smaller portions (Capalbo and Rienzi, 2017). Bias in assessing mosaicism in this way arises from two situations. First, when analyzing a collection of cells such as the entire ICM or larger partitions of the trophectoderm, mosaicism may be undetectable if only a few cells are chromosomally abnormal within the cell population. Second, in studies where multiple biopsies are analyzed, there is a risk that sites of mosaicism are not biopsied, and therefore not detected, simply by chance. Interestingly, different biopsy protocols (zona pellucida opening at the cleavage stage versus at the blastocyst stage) may affect the rate of mosaicism (Xiong *et al.*, 2021).

Methods such as single-cell sequencing will significantly enhance the resolution when investigating mosaicism. Interestingly, a recent study found at least one aneuploid cell in 59 out of 74 (80 %) preimplantation embryos following single-cell analysis (Starostik *et al.*, 2020). More data over the coming years, especially from single-cell

studies of disaggregated blastocysts, will aid in examining the true prevalence of embryonic mosaicism, as suggested by Popovic *et al.* (Popovic *et al.*, 2020).

During PGT for a hereditary disorder, where the mutation investigated is segregated during meiosis, embryonic mosaicism does not pose an issue, as mosaicism results from errors occurring during mitosis in the developing embryo. On the contrary, mosaicism is a challenge when screening human preimplantation embryos for chromosomal abnormalities arising during embryonic development, which is the topic of section 2.8.

Importantly, it should be addressed and emphasized that the term mosaicism is used in the literature to describe two distinct phenomena. Mosaicism may describe the presence of two or more genetically different cell lines within the embryo; hence, the embryo as an entity is mosaic. But, the term mosaicism may also describe the presence of mosaicism within cells of a trophectoderm biopsy (the biopsy is mosaic) (Nakhuda *et al.*, 2018). The detection of mosaicism within a biopsy might represent actual mosaicism in the embryo as a whole, be confined to the trophectoderm, or be an artifact from the biopsy procedure, but deciding which is the case can be complicated (Capalbo *et al.*, 2016; Capalbo and Rienzi, 2017). Hence, a mosaic biopsy does not necessarily equal a mosaic embryo. This differentiation is critical to keep in mind when discussing mosaicism, as it might impact interpretation. For clarification, unless explicitly stated otherwise, mosaicism in this thesis refers to the state of the embryo being mosaic as an entity.

2.8 PGT FOR ANEUPLOIDY

The presence of the normal number of chromosomes within a human cell is termed euploidy (Greek *eu,* for "true or "even"). The normal number of chromosomes is 22 pairs of autosomes and one pair of sex chromosomes, each pair consisting of a paternal and maternal component. Numerical deviations from the normal number of chromosomes, or part of a chromosome, are termed aneuploidy (Greek *an,* for "not" or "without"). Aneuploidy further divides into whole chromosomal aneuploidies (the loss or gain of an entire chromosome), segmental aneuploidies (loss or gain of subchromosomal segments), or polyploidy (additional copies of all chromosomes).

2.8.1 FEMALE AGE, REPRODUCTION, AND ANEUPLOIDY

A clear correlation exists between female age and female fertility rate and rate of miscarriage (Heffner, 2004). As female age increases, the chance of achieving pregnancy decreases while the risk of experiencing miscarriage increases in an almost exponential manner (Figure 2-6). Combined, this leads to an age-dependent reduction of the chance of achieving a live birth.

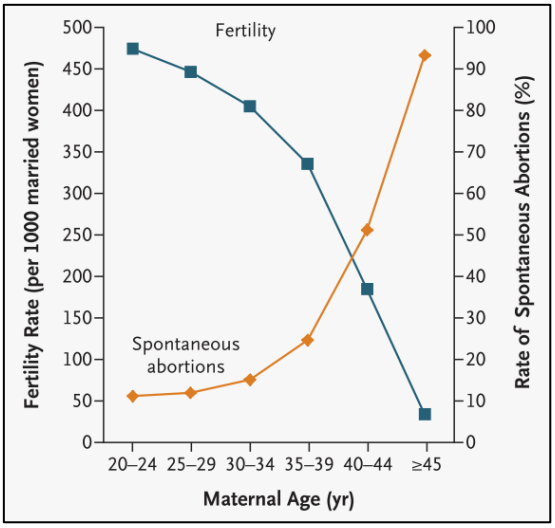


Figure 2-6: Relation between female age, fertility rate, and rate of miscarriage

Note: the synonym "spontaneous abortion" is used instead of miscarriage in the figure.

Reproduced with permission from (Heffner, 2004), Copyright Massachusetts Medical Society.

The prevalence of aneuploidy in male and female gametes varies considerably. Aneuploidy affects less than 10 % of spermatozoa, while 20-25 % of oocytes are aneuploid in young women (age 25-30) (Hassold and Hunt, 2001; Martin, 2008; Wartosch *et al.*, 2021). In addition, aneuploidy in female oocytes increases with female age due to an increase in errors during meiosis (McCoy, 2017; Webster and Schuh, 2017; Wartosch *et al.*, 2021). As a result, aneuploidy in human preimplantation embryos increases with female age (Franasiak *et al.*, 2014a; Demko *et al.*, 2016) (Figure 2-7).

Aneuploidy negatively affects embryonic implantation potential (Rubio *et al.*, 2017; Tiegs *et al.*, 2020). Additionally, aneuploidy is prevalent in miscarriage products of conception, ranging from 40 to 80 % (Martínez *et al.*, 2010; Kroon *et al.*, 2011; Li *et al.*, 2013; Segawa *et al.*, 2017). Combined, this suggests that aneuploidy originating from female oocytes is a primary explanation for both the observed reduction in fertility rates and the increasing rate of spontaneous miscarriages as female age increases. Consequently, female age is a good predictor of the chance of achieving pregnancy both spontaneously and following ART (Demko *et al.*, 2016).

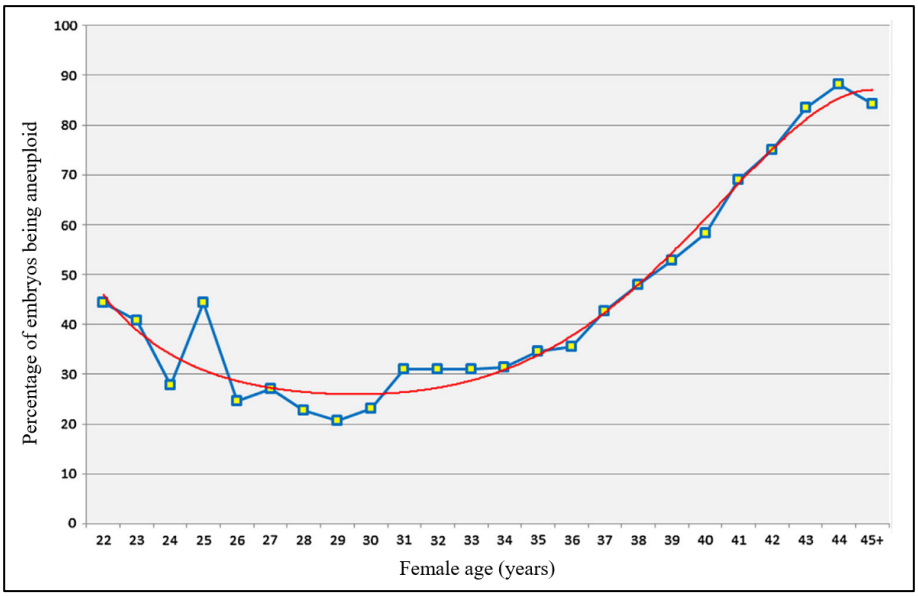


Figure 2-7: The relationship between female age and prevalence of aneuploidy in human preimplantation embryos.

Reproduced with permission from (Franasiak et al., 2014a), Copyright Elsevier.

2.8.2 INITIAL SCREENING FOR ANEUPLOIDY USING FISH

The age-related decrease in the chance of achieving live birth caused the suggestion in the early 1990s of screening human preimplantation embryos during ART for their ploidy status. The idea was that selection of euploid embryos for transfer would improve clinical outcomes, as euploid embryos should have a higher chance of implanting and a lower chance of miscarrying.

The introduction of FISH provided a method to test for chromosomal aberrations, including aneuploidy. Improvements to FISH allowed analysis of more than two chromosomes with screening performed for chromosome X, Y, 13, 18, and 21 (Munné *et al.*, 1993a); the primary chromosomes which may cause birth of children with physical and/or mental disabilities when affected by aneuploidy. The first deliveries following screening for aneuploidies using FISH were reported in 1995 (Verlinsky *et al.*, 1995). Slowly but steadily, the use of FISH as a tool for embryo selection began, which at the time was referred to as preimplantation genetic screening (PGS). The term PGS was used to distinguish it from PGT for hereditary disorders (known back then as PGD, preimplantation genetic diagnosis) (Geraedts *et al.*, 1999). Based on the data collections published by the ESHRE PGT Consortium, the use of FISH for embryo selection to enhance clinical outcomes grew through the 2000s until around

2010-2012 when array technologies started to replace it (De Rycke *et al.*, 2017). Unknown at the time, this procedure was flawed and was implemented into clinical practice without proper clinical validation but based solely on the theoretical assumption that the selection of euploid embryos would enhance clinical outcomes. Initially, smaller studies failed to show a significant clinical benefit from PGS using FISH (Staessen *et al.*, 2004; Platteau *et al.*, 2005a, 2005b). Then followed a large randomized controlled trial in 2007 showing that PGS using FISH not only failed to improve clinical outcomes but significantly reduced live birth rates in women of advanced maternal age (Mastenbroek *et al.*, 2007). These findings were echoed in another study a year later (Hardarson *et al.*, 2008). A later systematic review and meta-analysis of the use of FISH concluded that it reduced birth rates in women of advanced maternal age while also showing that there was no evidence to support that it improved birth rates in general (Mastenbroek *et al.*, 2011). Serious critique ensued, primarily focusing on the fact that the method was never adequately evaluated before being implemented into clinical practice, and emphasis was put on not to repeat this same mistake with new methods (Ankum *et al.*, 2008; Mastenbroek *et al.*, 2011; Gleicher *et al.*, 2014). The failure of PGS using FISH can be attributed to multiple factors.

First, due to technical limitations associated with FISH, only a limited fraction of all chromosomes is investigated: often only a quarter or less. Since then, chromosomal aberrations have been found to affect all 24 chromosome pairs (Franasiak *et al.*, 2014b; McCoy, 2017; Escribà *et al.*, 2019). Hence, FISH falls short as a method to efficiently discriminate between euploid and aneuploid embryos, as it does not assess the ploidy status of a significant fraction of the chromosomes. Therefore, the limited number of chromosomes tested contributed to FISH failing in systematically improving clinical outcomes.

Second, at that time, the gold standard of embryo biopsy was to biopsy a single or two blastomeres at the cleavage stage (Figure 2-4B). As detailed previously, biopsy at the cleavage stage affects the implantation potential of the embryo (Scott *et al.*, 2013). Hence, it is not unlikely that the biopsy procedure contributed to the failure of FISH to systematically improve clinical outcomes. Additionally, aneuploidy (Fragouli *et al.*, 2013, 2014; Babariya *et al.*, 2017; Liñán *et al.*, 2018) and mosaicism (Capalbo *et al.*, 2016; Vera-Rodriguez and Rubio, 2017; Popovic *et al.*, 2020) appears more prevalent at the cleavage stage than the blastocyst stage. Sequential analysis of embryos showed that aneuploidies initially detected at the cleavage stage were not detectable at the blastocyst stage (Barbash-Hazan *et al.*, 2009; Capalbo *et al.*, 2013). This supports current suggestions of self-correction mechanisms within the embryo, as previously discussed. These findings raise the question if some of the embryos discarded due to aneuploidy at the cleavage stage might have developed into euploid blastocysts, causing an unnecessary reduction in the number of embryos available for transfer.

Combined, the stage of embryo biopsy commonly used at the time and the limited number of chromosomes analyzed by FISH are probably the primary reason that FISH-based screening for aneuploidy failed as a tool to enhance clinical outcomes during ART treatment.

2.8.3 CURRENT SCREENING FOR ANEUPLOIDY AND THE ISSUE OF EMBRYONIC MOSAICISM

The introduction of new methodologies that allowed screening of all 24 chromosome pairs gave birth to a new and ongoing era of aneuploidy testing. This era was originally named PGS 2.0 in reference to FISH-based PGS (named PGS 1.0 retrospectively) but is today officially referred to as PGT-A (Zegers-Hochschild *et al.*, 2017). The use of methods that allow testing of all 24 chromosome pairs is also called comprehensive chromosome screening (CCS), but to adhere to current terminology, the term PGT-A will be used in this thesis to refer to aneuploidy testing for all 24 chromosomes unless stated otherwise.

The ability to test all 24 chromosome pairs combined with the introduction of trophectoderm biopsy has removed the major pitfalls associated with FISH performed at the cleavage stage. Currently, PGT-A is used globally, and its use is increasing in both Europe (Coonen *et al.*, 2020) and the United States (Roche *et al.*, 2021). Nevertheless, the benefit of the procedure, and the possible harm associated with it, are still highly debated. The theory behind PGT-A is that euploid embryos can be identified and selected for transfer, avoiding unnecessary transfer of aneuploid embryos, which would improve implantations rates and reduce miscarriage rates, resulting in reduced time to live birth. This theory assumes that the ploidy status of biopsied cells represents the ploidy status of the ICM, which is currently heavily debated due to the concept of mosaicism.

The concept of embryonic mosaicism, detailed in section 2.7, challenges the interpretation of the ploidy status of the ICM from a trophectoderm biopsy, as mosaicism can impact diagnostic accuracy depending on its presence and distribution within the embryo (Figure 2-8). Mosaicism may cause false-negative and positive ploidy calls (Figure 2-8). Discarding euploid embryos due to a false-positive result will cause an unnecessary reduction of the number of embryos available for transfer and thereby reduce the cumulative chance of achieving pregnancy. Transferring an aneuploid embryo due to a false-negative result decreases the likelihood of implantation and increases the risk of miscarriage and birth of an affected child.

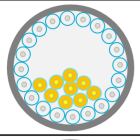
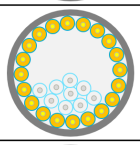
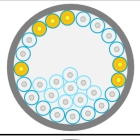
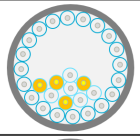
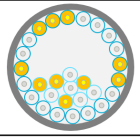
Type of embryonic mosaicism Euploid cell Aneuploid cell		Possible types of trophectoderm biopsies	Test results
Euploid trophectoderm Aneuploid ICM		Euploid	Falsely classified as euploid
Aneuploid trophectoderm Euploid ICM		Aneuploid	Falsely classified as aneuploid
Mosaic trophectoderm Euploid ICM		Euploid	Correctly classified as euploid
		Aneuploid	Falsely classified as aneuploid
		Mosaic	Falsely classified as mosaic
Euploid trophectoderm Mosaic ICM		Euploid	Falsely classified as euploid
Mosaic trophectoderm Mosaic ICM		Euploid	Falsely classified as euploid
		Aneuploid	Falsely classified as aneuploid
		Mosaic	Correctly classified as mosaic

Figure 2-8: How embryonic mosaicisms may affect diagnostic accuracy.

Abbreviations: ICM; Inner Cell Mass

The challenge posed by mosaicism has resulted in some authors claiming that PGT-A should not be performed as the ploidy status cannot be determined from a trophectoderm biopsy of 5-10 cells with enough reliability to justify its use in clinical practice (Gleicher and Orvieto, 2017). In line, others have suggested that uncertainties related to the method only qualifies it as a tool for research and not for clinical practice (Sciorio and Dattilo, 2020). A study assessing experts' opinions within the field showed a great variety in if, when, and how to use PGT-A (Sermon *et al.*, 2016). Others have questioned the lack of proper regulatory review and restrictions for the advertisement of new reproductive add-ons, such as PGT-A, emphasizing the challenge of adequately informing patients so they are allowed to make their own informed decision (Wilkinson *et al.*, 2019). Caution has recently also been raised against the use of PGT-A in a recent opinion statement from the American Society of Reproductive Medicine (Penzias *et al.*, 2018).

Non-selection studies are currently likely to be the best ways of accessing the impact of mosaicism on the ability of PGT-A to identify the embryos that are most likely to

result in live birth of an unaffected child. In a non-selection study, embryos are tested for aneuploidy and transferred without knowledge of the result. Subsequently, assessment of ploidy calls and corresponding clinical outcomes allows estimation of the positive and negative predictive values of PGT-A. Current data from non-selection studies show that transfer of euploid embryos compared to embryos of unknown ploidy and aneuploid embryos have a significantly higher chance of resulting in live birth (Scott *et al.*, 2012; Tiegs *et al.*, 2020). This supports the notion that PGT-A can be used to predict which embryos are most likely to result in live birth. Interestingly, one of the studies showed that 4 of 99 embryos expected not to result in pregnancy based on PGT-A, resulted in healthy live births (Scott *et al.*, 2012), while the other study reported 0 live births from transfer of 102 aneuploid embryos (Tiegs *et al.*, 2020). This shows that although the negative predictive value of PGT-A may be high, PGT-A is not flawless, and there is a risk of discarding embryos capable of resulting in a healthy live birth along the way. The acceptable threshold percentage of falsely discarded embryos is highly subjective, and an ethical debate needs to be had on whether the benefits of PGT-A outweigh the risk of discarding reproductive competent embryos. The risk of falsely discarding euploid embryos will likely differ from center to center, as will the clinical benefit of PGT-A, further complicating the evaluation of PGT-A.

Indeed, the currently available clinical data on PGT-A reflects a very diverse effect. An initial systematic review (Lee *et al.*, 2015) and meta-analysis (Dahdouh *et al.*, 2015) on the effect of PGT-A concluded that it could improve clinical outcomes in good prognosis patients. A large multicenter study performing an intention-to-treat analysis, the so-called STAR-trial, concluded that PGT-A did not improve overall pregnancy outcomes (Munné *et al.*, 2019). However, they observed increased ongoing pregnancy rates per transfer in patients age 35-40 but not in younger patients (Munné *et al.*, 2019). A later single-center study reported improved live birth rates from PGT-A following intention-to-treat analysis across all age categories (Anderson *et al.*, 2020). Quite remarkably, they showed that PGT-A was capable of eliminating the age-related decrease in implantation and live birth rates (Anderson *et al.*, 2020). However, this was not a randomized controlled trial, meaning that confounding of the results cannot be excluded. A very recent systematic review and meta-analysis of randomized controlled trials reported that PGT-A improves cumulative live birth rates (Simopoulou *et al.*, 2021). However, the "quality of evidence" was reported as "very low" using the "Grading of Recommendations Assessment, Development, and Evaluation (GRADE)" approach. In general, the study reported that the available evidence to evaluate the effect of PGT-A was "very low" or "low" for most outcome parameters evaluated, highlighting previous reports that more research and thorough evaluation of PGT-A is needed.

Different factors may explain the diverse reports on the effect of PGT. Obviously, the age of the patient cohort must be considered, and the effect of PGT-A should be evaluated in age-matched cohorts. While trophectoderm biopsy and vitrification have

a minimal effect on implantation potential, as previously detailed, this might not always be the case. Therefore, biopsy and cryopreservation are at risk of confounding the results of PGT-A compared to a control group of non-biopsied embryos and/or fresh transfer. Especially biopsy at the cleavage stage is likely to negatively affect clinical outcomes as previously detailed. Obviously, if PGT-A is performed concurrently with PGT-M/SR where biopsy and often cryopreservation are already being performed, their impact can be ignored. The choice of platform, bioinformatic pipeline, and experience in classifying and evaluating embryo ploidy status may all affect which aneuploidies are called and when they are called from center to center. Combined, these factors contribute to explaining the divergent reports of the effect of PGT-A, and why the lack of a standard procedure for PGT-A complicates comparison between studies.

Given this, careful consideration and validation are required when evaluating whether to implement PGT-A into clinical practice or offer it to patients as an add-on. While PGT-A may improve pregnancy and live births rates per transfer, the method may simultaneously be at risk of reducing cumulative pregnancy or live birth rates, which all in all will only reduce the patient's overall chance of success.

Currently, the best-proposed way to evaluate PGT-A in a given clinical setting appears to be in the form of a prospective non-selection study, as suggested by Scott *et al.* (Scott *et al.*, 2012; Tiegs *et al.*, 2020). Such a study allows the determination of positive and negative predictive values of PGT-A. Those predictive values are valuable when evaluating whether to implement aneuploidy screening. It would be interesting to see more non-selection studies from different centers to assess how predictive values fluctuates. Alternatively, instead of discarding aneuploid embryos, they may simply be prioritized last for transfer. However, in such cases, care must be taken to ensure proper counseling of patients on the associated risk and recommendations for prenatal testing, especially in cases of known liveborn abnormalities. Additionally, more randomized controlled trials evaluating PGT-A on an intention-to-treat bias are warranted.

2.8.4 TRANSFER OF MOSAIC EMBRYOS

When detecting mosaicism in a trophectoderm biopsy, the interpretation becomes more complex (Figure 2-8). Transfer of mosaic embryos may result in unaffected live births (Greco *et al.*, 2015; Victor *et al.*, 2019), although the chance of achieving ongoing pregnancy is lower and the risk of miscarriage higher compared to transfer of euploid embryo (Viotti *et al.*, 2021). This has fostered opinion statements from the major stakeholders within the field on how to interpret and prioritize embryos with a mosaic result (CoGEN, 2016; Cram *et al.*, 2019; ASRM, 2020), placing mosaic embryos as an intermedia group between embryos with fully euploid and aneuploid test results. Prioritization includes evaluating the degree of mosaicism, the number of chromosomes affected, and the chromosome(s) involved.

2.9 PRENATAL TESTING FOLLOWING PGT

The risk of misdiagnosis associated with PGT for hereditary disorders is considered extremely low (< 0.1 %), and the two latest ESHRE PGT Consortium data collections reported no cases of misdiagnosis (De Rycke *et al.*, 2017; Coonen *et al.*, 2020). Despite the low risk, when misdiagnosis occurs, the consequences are severe, as the child will be affected by a severe disorder from birth or later in life. Hence, at our center, we recommend prenatal testing to confirm the test result from PGT performed for hereditary disorders in line with the ESHRE PGT good practice recommendations (Carvalho *et al.*, 2020a).

2.9.1 PRENATAL TESTING

The current gold standard for prenatal testing is chorionic villus sampling (CVS) and amniocentesis. A biopsy of cells from the chorionic villi or amniotic fluid serves as a source of DNA for genetic testing. As CVS can be performed earlier in pregnancy compared to amniocentesis, this method is preferred. CVS entails a small increased risk of miscarriage, estimated to be less than < 0.2 % (Salomon *et al.*, 2019). Despite recommendations to have prenatal testing performed, approximately half of all patients at our center decline. There may be many reasons for declining CVS, such as the unwillingness to terminate the pregnancy in case of an affected fetus or fear of the risk of miscarriage associated with the procedure. The latter reason indicates that a less invasive alternative to CVS may sway some patients into opting for prenatal testing, backed up by a recent multinational survey among patients in the context of prenatal testing for Down syndrome (Hill *et al.*, 2016). In fact, questionnaire surveys among pregnant women in England (Hill *et al.*, 2012) and Denmark (Lund *et al.*, 2018) have shown that patients are willing to compromise with test accuracy and amount of genetic information in exchange for a non-invasive test. Interestingly, this was in contrast to preferences of health professionals, who put most emphasis on accuracy, highlighting the need to investigate patient preferences as they may differ significantly from health professionals (Hill *et al.*, 2012). In a study of patients at risk of transmitting cystic fibrosis to their offspring, 43.5 % were willing to have invasive testing for cystic fibrosis, which increased to 94.4 % if presented with a non-invasive alternative (Hill *et al.*, 2015). In conclusion, the available data suggest that a non-invasive alternative to CVS will increase the number of patients opting for prenatal testing, which will increase the chance of identifying the rare cases of misdiagnosis with the added benefit of providing assurance to patients during pregnancy.

2.9.2 NON-INVASIVE PRENATAL TESTING

Today, non-invasive prenatal testing can be performed by either analyzing cell-free fetal DNA (cffDNA) or circulating fetal cells (Figure 2-9). The idea of non-invasive

prenatal testing has been around since the presence of fetal cells in maternal blood was documented in 1959 (Zipursky *et al.*, 1959), and its potential in non-invasive diagnosis was proposed a decade later (Walknowska *et al.*, 1969). The first report describing a successful enrichment of fetal cells was published in 1979 (Herzenberg *et al.*, 1979) and later followed by what has been named the "golden era" of circulating fetal cell research spanning from 1993 until 2003 (Singh *et al.*, 2017). Despite this golden era, research has not yet led to the introduction of cell-based non-invasive prenatal testing (cbNIPT) into routine clinical practice. Just before the beginning of the golden era of circulating fetal cell research, cell-free fetal DNA (cffDNA) was identified in maternal blood (Lo *et al.*, 1989).

CffDNA is fragmented DNA circulating in maternal blood with a size of approximately 150-200 base pairs (Chan *et al.*, 2004; Lo *et al.*, 2010; Yu *et al.*, 2014) originating from apoptotic trophoblastic cells (Alberry *et al.*, 2007; Faas *et al.*, 2012). The use of cffDNA to test for the most common fetal aneuploidies causing liveborn disorders (chromosome 13, 18, and 21) was reported in 2008 (Fan *et al.*, 2008). A rapid introduction into clinical practice followed in many countries (Minear *et al.*, 2015). Currently, it serves as a screening tool for the above-mentioned aneuploidies while it can be used as a diagnostic test for Rhesus D and fetal sex (Mackie *et al.*, 2017). The use of cffDNA in prenatal testing for monogenic disorders in clinical practice was reported in 2012, and the method is currently part of the public health care service in England (Scotchman *et al.*, 2020). The fragmented state of cffDNA can complicate the analysis of certain types of hereditary disorders, such as repeat expansion disorders, which might often extend beyond the fragment length of cffDNA. An additional limitation of cffDNA is that the fraction of fetal to maternal DNA depends on maternal weight (Ashoor *et al.*, 2013; Wang *et al.*, 2013; Yared *et al.*, 2016) and gestational age (Lo *et al.*, 1998; Lun *et al.*, 2008; Wang *et al.*, 2013), which can complicate the use of cffDNA for non-invasive testing in overweight women or early pregnancy.

An alternative to cffDNA as a source of fetal DNA for prenatal testing is the isolation of fetal cells from maternal blood, which in principle might provide intact fetal genomes allowing genetic testing of all sort of genetic disorders (Figure 2-9). Two additional potential benefits of using fetal cell isolation compared to cffDNA are that cells can be isolated from maternal blood earlier in pregnancy and that the isolation is not affected by female weight (Kruckow *et al.*, 2018). Fetal cells have been isolated as early as gestational week 5 (Mouawia *et al.*, 2012) and 6 (Ravn *et al.*, 2020)

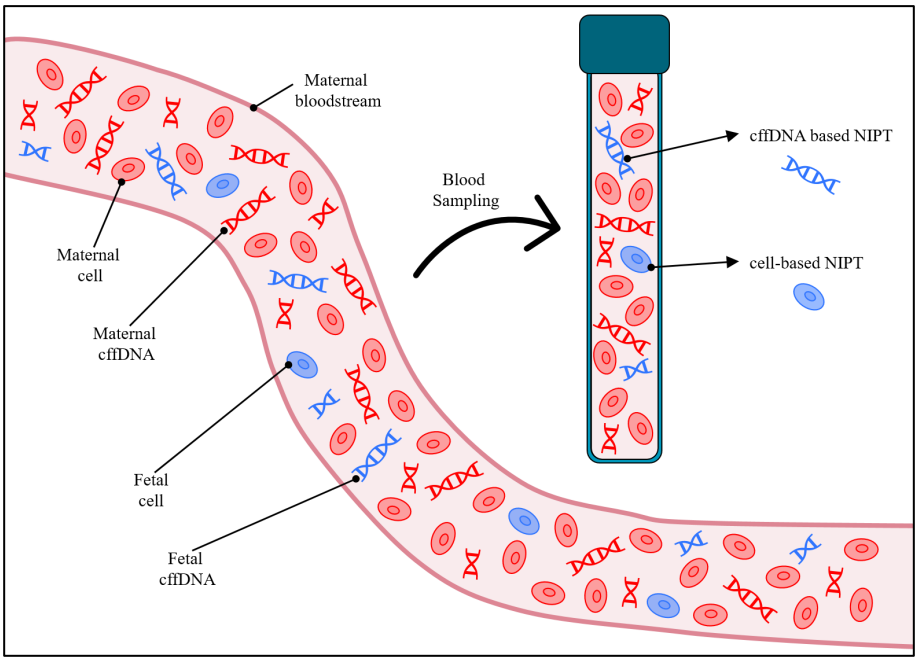


Figure 2-9: Cell-free fetal DNA and fetal cell in maternal blood.

Illustration of cell-free fetal DNA (cffDNA) and fetal cells circulating in maternal blood. Both cffDNA and fetal cells can be obtained by blood sampling and used for prenatal testing.

Abbreviations: cffDNA, cell-free fetal DNA; NIPT, non-invasive prenatal testing

2.9.3 CELL-BASED NON-INVASIVE PRENATAL TESTING

Initially, the main obstacles for routine clinical implementation of cbNIPT were the inability to identify fetal cells in all pregnancies and a high false-positive rate of aneuploidy and sex detection (Bianchi *et al.*, 2002). One of the main reasons for the inability to identify fetal cells in every pregnancy was the inability to characterize the type of fetal cells present in the circulation and subsequently to identify specific markers allowing them to be separated from the maternal cell population (Beaudet, 2016; Singh *et al.*, 2017). Another obstacle was the scarceness of fetal cells in maternal blood and the need for methods capable of enriching these cells (Beaudet, 2016; Singh *et al.*, 2017). Recently, a Danish Biotech company published a protocol for enrichment of fetal cells, which are believed to be extravillous trophoblasts (EVTs) based on microarray profiling (Hatt *et al.*, 2014), capable of yielding an average output of 12.8 fetal cells from 30 ml of blood (0,43 fetal cells/ml) (Kølvraa *et al.*, 2016). Notably, the authors identified fetal cells in all samples from 111 pregnancies while also showing that the isolated trophoblastic cells were applicable for downstream

analysis by both aCGH and NGS. Later, Vossaert *et al.* reported a similar number of trophoblastic cells (0,18 per ml) isolated from maternal blood. (Vossaert *et al.*, 2018). However, they identified fetal cells in only 102 of 125 blood samples (82 %) from pregnant women. Numerous commercial solutions for single-cell collection and analysis have become available in the last few years (Valihrach *et al.*, 2018). Still, peer-reviewed published proof of their ability and efficacy in identifying fetal cells from maternal blood is lacking. To date, cbNIPT has been used to test for carrier status of monogenic disorders, such as cystic fibrosis (Saker *et al.*, 2006; Mouawia *et al.*, 2012; Pfeifer *et al.*, 2016; Dahl Jeppesen *et al.*, 2020) and spinal muscular atrophy (Mouawia *et al.*, 2012; Pfeifer *et al.*, 2016), for various monogenic disorders following PGT-M (Toft *et al.*, 2021), and for aneuploidy, unbalanced structural translocations and smaller deletions (Vestergaard *et al.*, 2017; Hatt *et al.*, 2020).

Further assessment of cbNIPT is still needed, such as large-scale studies on the reliability of fetal cell isolation, risk of not having an informative test result, sensitivities, and specificities. When non-invasive solutions are ready, the current evidence suggests that they will increase patient adherence to prenatal testing in addition to the apparent benefits obtained from transitioning from an invasive to a non-invasive procedure.

3 STUDY I

3.1 INTRODUCTION

As female age increases, the chance of achieving pregnancy decreases while the risk of experiencing a miscarriage increases (Heffner, 2004), reducing the likelihood of achieving a live birth. Aneuploidy has been established as one of the primary explanatory factors behind this relationship. First, aneuploidy in human preimplantation embryos increases with increasing female age (Franasiak *et al.*, 2014a; Demko *et al.*, 2016). Second, aneuploidy has been shown to decrease implantation rates (Rubio *et al.*, 2017; Tiegs *et al.*, 2020). Third, aneuploidy is prevalent in miscarriage products of conception (Martínez *et al.*, 2010; Kroon *et al.*, 2011; Li *et al.*, 2013; Segawa *et al.*, 2017). Combined, this establishes a link between aneuploidy and reduced chance of achieving pregnancy and increased risk of miscarriage as female age increases.

This led to the suggestion that screening for aneuploidy during ART might improve pregnancy or live birth rates on a per transfer basis, reducing time to pregnancy and cost of ART. As discussed in the introduction, the concept and use of PGT-A is a subject of debate and intense research. Obviously, as the initial attempts to improve clinical outcomes using FISH and cleavage stage biopsy (PGS 1.0) failed and were prematurely brought into clinical practice, caution is taken not to make the same mistake again. Some of the primary issues have been addressed by switching to blastocyst stage biopsy and comprehensive chromosome screening. However, and despite widespread clinical implementation, the use of PGT-A is still heavily debated, primarily due to the issue of embryonic mosaicism and the risk of discarding reproductive competent embryos, as thoroughly discussed in the introduction.

The majority of studies assessing the prevalence of aneuploidy and the clinical effect of PGT-A have been performed in cohorts receiving ART treatment for infertility. PGT-A performed concurrently with PGT for hereditary disorders has also been reported, but a systematic review of the published literature has not previously been performed. Two significant differences exist between patients receiving PGT-A concurrently with PGT for hereditary disorders and patients receiving ART for infertility. During PGT for hereditary disorders, embryo biopsy is already being performed, while in ART, biopsy is performed solely for the purpose of PGT-A. Hence, in the latter, the biopsy procedure, if not performed properly, could affect the embryo's implantation potential, thus confounding the effect of PGT-A. This is not an issue during PGT for hereditary disorders, as PGT-A is performed on the biopsied material obtained for PGT. Additionally, and most importantly, patients referred to PGT for hereditary disorders are mostly considered fertile. Could it be that there is a difference in the prevalence of aneuploidy between infertile and fertile couples?

3.2 STUDY OBJECTIVE

We performed a systematic review of the available literature to determine the prevalence of aneuploidy and investigate the effect of screening for aneuploidy concurrently with PGT for hereditary disorders. Additionally, we investigated to what extend PGT-A reduced the number of transferable embryos.

3.3 STUDY DESIGN

A systematic review in accordance with the Preferred Reporting Items for Systematic Reviews and MetaAnalyses (PRISMA) was performed. As biopsy at the blastocyst stage has been shown to be less detrimental to embryonic implantation potential compared to biopsy at the cleavage stage, only studies performing biopsy at the blastocyst stage were included to reduce confounding. Additionally, only studies performing CCS were included. Studies performing PGT-M and/or PGT-SR with concurrent screening for aneuploidy reporting either aneuploidy rates and/or clinical outcomes were included. The search was last updated in July 2019.

The prevalence of aneuploidy and the percentage of transferable embryos prior to and post PGT-A were evaluated from all the studies by weighted analysis. The effect of PGT-A was evaluated in studies including a control group.

3.4 STUDY RESULTS

Twenty-six studies were identified that met inclusion criteria, all of which reported aneuploidy rates (Figure 1 and Table 1). Of the 26 studies, 17 reported clinical outcomes. Of those, three were historical cohort studies comparing a group receiving PGT-A to one not receiving PGT-A. No randomized trials were identified.

Aneuploidy rates varied considerably between studies, ranging from 17.2 % to 83.3 % (Figure 2). When evaluating studies having analyzed 100 or more embryos, aneuploidy ranged from 21.5 % to 56.5 %. Not all studies reported the mean female age of the cohort. In the studies where mean female age was reported, it ranged from 29.2 to 38.0 (Table 1). A weighted aneuploidy rate of 34.1 % (CI_{95} 33.1 % - 35.2 %) was calculated (Figure 2). PGT-A significantly reduced the percentage of embryos suitable for transfer from 57.5 % (CI_{95}: 56.3 % - 58.6 %) to 37.2 % (CI_{95}: 36.1 % - 38.4 %) (Figure 3A). Only three studies included a control group not receiving PGT-A. Of those, two reported a statistically significant difference between the cohort receiving PGT-A and the one that did not (Figure 4).

3.5 STUDY DISCUSSION

The calculated weighted aneuploidy rate of 34.1 % does not seem to differ from those reported for women receiving ART of approximately the same age (Franasiak *et al.*, 2014a; Demko *et al.*, 2016). However, direct comparison is not possible as the average age of the cohort from which the weighted aneuploidy rate was calculated could not be determined. Aneuploidy varied significantly, even when trying to reduce bias by excluding smaller studies from the analysis. The large span in reported aneuploidy rates is likely a reflection of differences that might directly affect aneuploidy rates and/or detection. Examples are female age, culture conditions, and the method of genetic testing. Various methods of genetic testing might have different lower limits of resolution where aneuploidy can be reliably detected. Additionally, procedures on how mosaic results were interpreted and handled were often not reported, causing speculation that mosaics may have been classified as either euploid or aneuploid embryos in some studies. Additionally, differences in experience might also affect the calling of aneuploidy, possibly adding to the observed differences. Unfortunately, the findings provided in our paper cannot definitively answer the question of whether aneuploidy is more or less prevalent in patients receiving PGT-M/SR compared to ART, but they suggest that they are comparable. Thus, aneuploidy appears to affect one out of three embryos during PGT.

As expected from the prevalence of aneuploidy, PGT-A significantly reduced the number of embryos suitable for transfers in the accessed studies. Assuming that aneuploid embryos either do not implant or result in a miscarriage, reducing the number of transferable embryos is beneficial as unnecessary transfers can be avoided. On the contrary, if euploid embryos are falsely discarded as aneuploid, PGT-A might end up reducing the overall chance of achieving pregnancy.

In summary, these findings indicate that aneuploidy is prevalent in human preimplantation blastocyst from patients undergoing PGT for hereditary disorders, and that the prevalence of aneuploidy is comparable to those reported in patients receiving ART for infertility.

Whether or not PGT-A entails a clinical benefit could not be determined from the included studies. The lack of randomized controlled trials is a problem as unknown factors might confound the three identified historical cohort studies. An updated search as of May 2021 on PubMed revealed that no randomized controlled trial had been published since we conducted our study. In general, the lack of randomized controlled trials PGT-A in general and concurrently with PGT-M/SR is worrying. Investigating PGT-A by an intention-to-treat analysis in randomized controlled trials will provide a better measure of its effect. Additionally, non-selection studies will aid in evaluating the risk of discarding reproductive competent embryos in the process.

We are currently planning a national non-selection study between the two PGT centers in Denmark located at Aalborg University Hospital and Rigshospitalet in Copenhagen. PGT-A is currently performed at both centers in connection with PGT-SR by NGS. For PGT-M, both centers are currently performing STR marker analysis with no aneuploidy screening. At the time of writing, we are in the process of planning a non-selection study on the PGT-M cohorts to evaluate positive and negative prediction rates for PGT-A at the two centers. This will be an exciting study allowing us to access the predictive values of PGT-A at our centers while contributing to the continuous assessment of and discussion on PGT-A.

4 STUDY II

4.1 INTRODUCTION

PGT for hereditary disorders entails a very low risk of misdiagnosis, expected to primarily stem from failure to correctly assign the results following genetic testing to the correct embryos or transferring a wrong embryo. For obvious reasons, centers should take measures according to published guidelines (Carvalho *et al.*, 2020b) to track the steps from biopsy to genetic testing to transfer to reduce the risk that biopsies, test results, and embryos are unintentionally mixed up. Currently used genetic tests are designed to deliver unambiguous results so that when embryos are labeled as affected or unaffected, the risk of misdiagnosis is extremely low. Otherwise, they are labeled as inconclusive. Diagnostic accuracy is enhanced using STR markers and SNPs, as discussed in the introduction. Hence, when misdiagnosis occurs, it must be expected to be due to human error in most cases. While the risk of misdiagnosis is low, the consequence is severe, as it might result in the birth of a child affected by a hereditary disorder. For these reasons, prenatal testing to confirm the original test result from PGT is recommended at our center at Aalborg University Hospital.

The current gold standards for prenatal testing are the invasive procedures CVS and amniocentesis, which are associated with discomfort to the patient and a small risk of miscarriage (Salomon *et al.*, 2019). For obvious reasons, non-invasive alternatives eliminating the risk of miscarriage would be preferred. The risk of miscarriage affects patient's decision-making of prenatal testing (Hill *et al.*, 2012, 2015, 2016; Lund *et al.*, 2018), something we also experience at our center. Patients referred to PGT might already have tried to achieved pregnancy on multiple occasions or experienced one or more miscarriages prior to PGT. Additionally, achieving pregnancy by PGT might also take some time, maybe even years, with failed implantations or miscarriages along the way. It is then, perhaps not surprising, that those couples refuse invasive prenatal testing upon achieving pregnancy. A non-invasive solution for prenatal testing will likely be more acceptable for those patients, increasing the chance of detecting the few cases of misdiagnosis that may occur while it may additionally provide comfort and assurance to the patients during the pregnancy.

As detailed in the introduction, fetal cells stemming from the trophoblast can now be identified and isolated from maternal blood and used as a source of DNA for prenatal testing. At least some of those cells will contain an intact fetal genome that can be isolated and tested for the genetic disorder in question. This procedure is known as cell-based non-invasive prenatal testing (cbNIPT). The Danish biotech company ARCEDI Biotech has recently developed and validated an efficient method for isolation of fetal trophoblast from maternal blood and shown that DNA isolated from the cells can be used for genetic testing (Hatt *et al.*, 2014; Kølvraa *et al.*, 2016;

Vestergaard *et al.*, 2017; Dahl Jeppesen *et al.*, 2020). This study aimed to evaluate this procedure as a tool for prenatal testing following PGT-M.

4.2 STUDY OBJECTIVE

The objective of this study was to investigate the use of cbNIPT following PGT-M. The study was intended as a proof-of-concept study. We wanted to examine the efficiency of fetal cell isolation and the ability to test various types of genetic mutations on isolated trophoblast cells using STR marker analysis combined with direct mutation detection when possible.

4.3 STUDY DESIGN

Patients achieving pregnancy following PGT-M opting for CVS had blood sampled on the day of the CVS. Blood samples were collected, and potential fetal cells were isolated within 48 hours by ARCEDI Biotech . Potential fetal cells were tested by STR marker analysis and direct mutation detection when possible, using the same setup that was designed and used for PGT of the embryos. STR marker analysis and direct mutation detection allowed us to determine the origin of the isolated cells (fetal/maternal) and their mutational status (affected/unaffected). Additionally, we calculated the ADO rate from the STR marker analysis.

4.4 STUDY RESULTS

The study included eight patients from which 33 potential fetal cell samples were identified and isolated (Table 1 and 2). At least one fetal cell sample was isolated from each patient (range 1-6) (Table 2). Of those, 27 (82 %) were successfully tested, allowing determination of origin and mutational status (Figure 2). Of the 27 cell samples successfully tested, 24 were verified to be of fetal origin based on the STR-marker analysis showing paternal markers (Figure 2). All 24 fetal cells were shown to be unaffected, although four were designated as “conditionally unaffected” as the test result was based on only one STR marker (Figure 2). All test results were in concordance with the results from CVS.

4.5 STUDY DISCUSSION

In the presented proof-of-concept study, we showed that fetal cells can be successfully isolated from maternal blood and tested for various genetic mutations spanning from point mutation over repeat expansions to larger deletions and duplications. This was accomplished using STR-marker analysis and direct mutation detection. Fetal cells were identified in all cases, although the number varied significantly.

Unfortunately, we were only able to include eight patients in the study. While a large fraction of patients referred to PGT at our center enters the study, many of them do

not opt for CVS, which disqualified them from participating in the study. Hence, while this method shows the potential of cbNIPT, a larger sample size is needed to further evaluate the assay. It is essential to obtain a more precise estimate of the median number of fetal cells isolated and, more importantly, how often the test does not yield a conclusive result per pregnancy. CbNIPT may be performed in early pregnancy so that in case of an inconclusive test result, invasive testing by CVS is still possible. Hence, if cbNIPT eventually shows a lower test success rate than CVS, it may still be considered a first-line non-invasive solution, followed by CVS in case of an inconclusive test result.

We choose to evaluate the procedure using the direct mutation detection and STR marker setup used during PGT because this was a fast and economical solution that did not require additional testing of additional markers. Our experience from PGT is that a single STR marker on each side of the gene is sufficient to obtain an informative test result reliably. When moving from testing 5-10 trophectoderm cells to a single fetal cell, the PCR reaction becomes more prone to errors such as allele dropout and failed amplification. Hence, optimization of the procedure might include using more markers, although adding additional markers might also cause the PCR conditions to be suboptimal for the markers already included.

Since the publication of this study, ARCEDI Biotech has developed a new method to isolate more fetal cells per blood sample. The method is currently being used in an ongoing project, and the result are promising. Although the new procedure has a lower specificity for fetal cell isolation than the previous one used in the published study (28.8 % [CI_{95} 21.4 % - 37.1] versus 69.7 % [CI_{95} 51.3 % - 84.4 %], exact 95 % confidence intervals), the efficiency of fetal cells isolated has increased from a median of 2.5 a median of 3.5, which will increase the chance of obtaining an informative test result per blood sample. A downside is that the larger number of cells isolated increases the cost for genetic testing as more cells must be tested (A median of 16 cells per sample with the new method compared to 4.5 with the old method). Therefore, improvement of the specificity is an obvious goal and one that ARCEDI Biotech is continuously working on. So forth, only ten blood samples have been assessed with the new methods, so recruitment is ongoing to assemble a large dataset for proper evaluation.

The large number of maternal cells isolated with the new method has enabled estimation of the sensitivity of cbNIPT. In cases where the female is known to carry the mutation, the sensitivity can is estimated by how often a maternal cell is correctly tested as affected. The specificity of cbNIPT is calculated using the number of fetal cells correctly tested as being unaffected by comparison with the CVS results. With the increasing number of cells analyzed with the new method plus those reported in the study presented here (63 fetal cells and 37 maternal cells), sensitivity has been estimated to 100 % (CI_{95} 90.5 % - 100.0 %, exact 95 % confidence interval) and specificity to 100 % (CI_{95} 94.3 % - 100.0 %, exact 95 % confidence interval). Based

on this accuracy, we have applied to the regional ethics committee to expand cbNIPT to all patients irrespective of whether they opt for CVS. Hopefully, this will allow us to include more patients in a shorter time, which will aid in the continuous evaluation of cbNIPT. Hopefully, its implementation into clinical practice is not far away, which will likely enhance patient adherence to prenatal testing and provide patients with a non-invasive procedure with no associated risk of miscarriage.

The current study was limited to patients achieving pregnancy following PGT-M as the use of STR markers allowed us to determine the origin of the isolated cells. At our center, NGS is used for PGT-SR, which does not allow the origin of the sample to be determined due to the low depth of sequencing. As part of the new setup at ARCEDI Biotech, all potential fetal cells from patients having received PGT-SR are whole genome amplified, and the origin is determined by STR marker analysis. information about the origin of each cell will allow us to evaluate cbNIPT in the context of PGT-SR in a similar manner as has been done for PGT-M, hopefully bringing cbNIPT closer to clinical practice irrespective of the type of hereditary disorder.

5 STUDY III

5.1 INTRODUCTION

While PCR-based methods allow genetic testing directly on DNA from a single or few cells, methods such as array CGH, SNP arrays, and NGS require a larger input DNA than is obtained from a regular trophectoderm biopsy. Hence, prior to genetic testing, the DNA must be artificially amplified, which is accomplished by WGA. WGA is sometimes also performed prior to PCR-based methods to allow for reanalysis in case of failed diagnosis. Without WGA, all the DNA obtained from the biopsy is used for genetic testing. In case of a failed test or inconclusive test results, thawing, re-biopsy, and an additional round of cryopreservation of the embryos is required to obtain new DNA for testing, potentially negatively affecting the survival (Taylor *et al.*, 2014b) and implantation rates (Bradley *et al.*, 2017).

While WGA can successfully produce large amounts of DNA, the artificial nature of the amplification procedure may affect the degree to which the amplified products represent the original input DNA. WGA can cause uneven amplification of the genome, selection or drift bias distorting the representation of the amplicons between the original and final product, and polymerase artifacts (Sabina and Leamon, 2015). Uneven amplification of the genome might cause "blind spots" complicating genetic testing in those regions. Distorted amplification of amplicons might also affect genetic testing, as it might cause heterozygous loci to appear homozygous. Polymerase artifacts might directly affect direct mutation detection if the site of mutation is not correctly replicated.

Hence, while WGA allows genetic testing to be successfully performed in most cases, the method does not provide a 1:1 replicate of the original DNA template. The extent to which WGA affects the interpretation of genetic analyses may depend on multiple factors: the choice of WGA protocol, the method and platform used for genetic testing, the genetic aberration under investigation (size, type, and location in the genome), and the bioinformatic pipeline used for data processing and interpretation.

The survival of an organism depends on the ability of cells to replicate their DNA during cell division efficiently. Hence, the DNA replication machinery of human cells contains safety mechanisms to ensure proper replication of the genome, including proofreading and mismatch repair mechanisms (Bębenek and Ziuzia-Graczyk, 2018). Although *in vivo* DNA replication is not perfect (nor is it meant to be), it must be expected to be superior to artificial *in vitro* procedures such as WGA in producing a product representing the original template DNA.

Since blastocyst biopsy during PGT entails aspiration of approximately 5-10 cells, we speculated whether these cells could be expanded in cell culture as an alternative way

of DNA amplification, thereby utilizing the cellular DNA replication machinery as an alternative to WGA. Thus, we designed a study where we sought to test this concept.

5.2 STUDY OBJECTIVE

This study aimed to explore whether trophectoderm cells biopsied from human preimplantation blastocyst could be expanded in culture, producing enough DNA for genetic analysis without prior need of WGA.

5.3 STUDY DESIGN

We established a collaboration with the authors behind a recent paper describing successful culture of intact human blastocysts beyond the implantation stage (Okae *et al.*, 2018), thinking that those culture conditions might support expansion of biopsied trophectoderm cells. In the first experiment, donated blastocyst affected by a monogenic disorder were biopsied twice, and subsequently, biopsies and the remains of the embryo were cultured. The use of blastocysts affected by a monogenic disorder allowed us to verify the origin of the DNA from expanded cultures by STR marker analysis. In the second experiment, we used blastocysts affected by chromosomal deletions and duplications to investigate whether DNA purified from potential expanded cultures could be tested by NGS without prior WGA. Repeating the procedure from the first experiment, we performed two biopsies, and subsequently, biopsies and the remains of the embryo were cultured. In both experiments, we cultured intact blastocyst as done originally by Okae *et al.*, as a positive control.

5.4 STUDY RESULTS

We successfully showed that biopsied trophectoderm cells could be amplified in culture (Figure 2 and Supplementary Figure S2 and S11), although with variable success (Table 1-3). In the initial experiment, only 25 % (2/8, CI_{95} 3.2 % - 65.1 %) of primary biopsies formed colonies, which increased to 87.5 % (7/8, CI_{95} 47.4 % - 99.7 %) in the second experiment (Table 3), where the biopsies were performed in a manner more resembling normal PGT conditions (biopsy of non-hatched blastocysts). The size of the cultures (Figure 2 and Supplementary Figures S2 and S11) and the amount of purified DNA varied substantially (Table 1 and 2). However, PGT could be performed on DNA purified from expanded cultures for monogenic disorders using STR marker analysis and direct mutation detection (Figure 1 and Supplementary Figure S7-S9) and for chromosomal abnormalities by NGS without prior WGA (Figure 3 and Supplementary Figure S16-S22). We observed an ADO rate of 4.7 % (CI_{95}:1.7 % - 9.9 %) following STR marker analysis. We observed complete concordance with respect to ploidy status (Table 2).

5.5 STUDY DISCUSSION

The presented paper provides proof of concept that trophectoderm cells biopsied from human blastocyst can be expanded *in vitro,* producing enough DNA for downstream genetic analysis by both STR-marker analysis and NGS. A precise estimate of the success rate is impossible due to the low number of samples in the presented paper. Although we observed an increase in the number of biopsies that successfully expanded in culture when biopsy was performed on primarily un-hatched blastocyst in the second experiment, drawing any conclusion on whether this makes a significant difference is not possible from the limited sample size. Nonetheless, as the prerequisite for establishing a cell culture is viable cells, it makes sense that a less invasive or stressful biopsy technique, as used in the second experiment, will increase the chance of establishing a culture by increasing the likelihood of obtaining viable cells. The larger the number of viable cells present in the biopsy, the higher the chance of success. Hence, measures that will reduce the stress and tear on cells during biopsy are likely to increase the likelihood of success. One such solution might be zona breaching at the cleavage stage followed by biopsy of herniating trophectoderm cells at the blastocyst stage (McArthur *et al.*, 2005), as detailed in section 2.6. This procedure might require less suction and laser pulses than zona breaching and subsequent aspiration of trophectoderm cells at the blastocyst stage. Unfortunately, we cannot test this hypothesis as all embryos at our center are biopsied and frozen at the blastocyst stage and subsequently donated for research. Another way to obtain more viable cells would be to perform larger biopsies. There is obviously a limit to the size of the biopsy that can be performed without affecting embryonic development. However, data from our center shows that biopsy of 10-15 cells does not negatively impact embryonic implantation potential compared to biopsies containing less than ten cells, suggesting that larger biopsies can be performed within reasonable limits. Contrary to our findings, others have shown that larger biopsies (average ten cells) might affect clinical outcomes compared to smaller biopsies (average five cells) (Guzman *et al.*, 2019). Hence decisions on whether to increase biopsy size should be carefully considered and evaluated.

While we showed complete concordance for ploidy status, the size of the study does not allow a precise evaluation of the concordance between the original biopsy tested during PGT and the cultured biopsies. The issues associated with mosaicism, discussed in sections 2.7 and 2.8, further complicate such comparison, as any potential discrepancies might simply represent actual mosaicism of the embryo between the two biopsy sites. Nonetheless, the issues associated with detecting aneuploidy, such as mosaicism, following cell culture remains the same as during traditional PGT-A and would require preclinical validation.

While the proposed method is an intriguing alternative to WGA, it is not without limitations that might complicate its introduction into clinical practice. The first hurdle is that a special laboratory setting for cell culture and personnel with experience

in cell culture is required, which could hinder implementation in many PGT centers. Next, while WGA can be performed within a few hours, cell culture requires days or weeks, 8-12 days in our study, which will increase the time from biopsy until a test result is available. However, this should be a minor issue, at least in clinics utilizing the freeze-all strategy.

DNA amplification by expansion of biopsied trophectoderm cells in culture may allow investigation of epigenetic modifications, which may be complicated following WGA (Bundo *et al.*, 2012). While Epigenetic markers are preserved through cell division, whether this is also the case when trophectoderm cells are cultured *in vitro* remains to be investigated. Additionally, epigenetic markers of the trophectoderm cell population might be different from the ICM.

Alternatively, the proposed approach might be used as a tool to investigate the type and degree of bias introduced by WGA, which might help identify shortcomings of the WGA in the context of PGT and where it might need to be improved.

In conclusion, the current study provides proof of concept that biopsied trophectoderm cells can be expanded in culture, producing enough DNA for downstream genetic testing. Still, more studies are required to determine the actual success rate and practical applicability of the assays. Whether the suggested approach can be used for PGT-A remains to be investigated.

6 STUDY IV

6.1 INTRODUCTION

Assessment of clinical success rates and factors affecting these are important not only for proper quality management but also for proper patient counseling at any given clinic as discussed in the ESHRE PGT Consortium good practice recommendations for the organization of PGT (Carvalho *et al.*, 2020b).

Continuous evaluation of clinical success rates helps ensure that fluctuations in clinical success rates are detected in due time. Tracking of changes to clinical procedures might aid in identifying factors causing those changes. This is important as numerous non-patient-related factors can affect clinical outcomes, as discussed in the introduction, such as embryo culture, cryopreservation, embryo biopsy, and the type and extent of genetic testing.

Clinical outcomes can be monitored at different stages, including positive hCG, biochemical pregnancy, fetal heartbeat (clinical pregnancy), and live birth and miscarriage. Additionally, they can be evaluated in three ways: per completed treatment (cumulative over multiple oocyte retrievals), per oocyte retrieval (and subsequent transfer of resulting embryo), and per embryo transfer. The clinical or scientific context will dictate how which and how clinical outcomes are measured. As an example, when evaluating the effect of PGT-A, clinical pregnancy and live birth rates are likely to be improved if assessed on a per transfer basis. In contrast, a smaller or no effect may be observed if they are evaluated cumulatively.

Providing patients with information on the cumulative chance of achieving clinical pregnancy or live birth will aid them in deciding whether to initiate PGT or possibly choose other alternatives. Additionally, information on the chance of achieving pregnancy per oocyte retrieval and per transfer will help patients better adjust expectations during treatment.

Estimated clinical success rates drawn for a cohort are not necessarily representative of the individual patient. Numerous patient-related factors can affect clinical success rates from PGT, such as female age, ovarian reserve, and type of genetic disorder, as detailed in the introduction. Thus, knowing how such factors influence success rates might better allow patient-specific counseling on the chances of achieving clinical pregnancy or live births, which may aid patients in managing expectations.

Following the four-year anniversary of our Center for Preimplantation Genetic Testing at Aalborg University Hospital, we wanted to assess our performance by evaluating clinical pregnancy rates cumulatively, per oocyte retrieval, and per frozen embryo transfer. Additionally, we sought to investigate the effect of female age and

the number of transferable embryos on the chance of achieving clinical pregnancy cumulatively and per oocyte retrieval.

6.2 STUDY OBJECTIVE

This study aimed to assess the clinical outcomes of our newly established Center for Preimplantation Genetic Testing from October 2016 to December 2020 reported as clinical pregnancy rates per treatment (cumulative clinical pregnancy rate from all oocyte retrievals and subsequent transfers), per oocyte retrieval, and per embryo transfer. Additionally, we investigated the effect of female age at the time of initiating PGT on the chance of achieving a clinical pregnancy during PGT, and the effect of female age and the number of transferable embryos on the chance of achieving clinical pregnancy per oocyte retrieval.

6.3 STUDY DESIGN

All patients who had initiated PGT for hereditary disorders (defined as having received at least one oocyte retrieval) between October 2016 and December 2020 were included. Cumulative clinical pregnancy rate per treatment was defined as the number of patients initiating PGT who achieved a clinical pregnancy. Clinical pregnancy rate per oocyte retrieval was defined as the fraction of oocyte retrievals in which a clinical pregnancy was achieved following transfer of the resulting embryos. Clinical pregnancy rate per frozen embryo transfer was defined as the fraction of embryo transfers resulting in a clinical pregnancy. A clinical pregnancy was defined as the presence of a fetal heartbeat determined by ultrasound monitoring at gestational week 7-8. Clinical pregnancy rates were evaluated combined for both PGT-indications and separately for PGT-M and PGT-SR.

Since not all patients in the cohort had completed their treatment (not all patients had received the total number of oocytes retrievals and transfer possible) at the time of data acquisition and analysis, some patients had not yet achieved a clinical pregnancy while still having the possibility of doing so. A sensitivity analysis was performed to investigate the potential bias caused by those patients. The sensitivity analysis assessed the clinical pregnancy rate as described above but only for the subgroup of patients who had completed a full treatment offer. A full treatment offer was defined as the patient having accepted, received, and completed the maximum number of oocyte retrievals and subsequent embryo transfers offered to them by the clinic.

The effect of female age at the time of initiating PGT on the chance of achieving clinical pregnancy during PGT was evaluated by logistic regression as a categorical and continuous variable. Adjustment was made for the PGT indication and odds ratios reported as unadjusted and adjusted odds ratios with 95 % confidence intervals.

The effects of female age at the time of oocyte retrieval (continuous variable) and the number of transferable embryos resulting following genetic testing (continuous variable) on the chance of achieving clinical pregnancy per oocyte retrieval were evaluated by multilevel logistic regression separately for PGT-M and PGT-SR. Separate analyses were performed, as the variable "transferable embryos" potentially differs between the two groups with respect to implantation potential due to aneuploidy screening being performed in the PGT-SR group and not in the PGT-M group. Multilevel logistic regression was used to account for each patient contributing with multiple cycles. Each variable was adjusted for the other, and odds ratios reported as unadjusted and adjusted odds ratios with 95 % confidence intervals. Some patients in the dataset who had not yet achieved clinical pregnancy in a given cycle still had embryos available for transfer. Hence, those patients still had a chance of achieving clinical pregnancy from transfer of the remaining embryos. To address the potential bias caused by those patients, a sensitivity analysis was performed. In the sensitivity analysis, only cycles where all embryos had been transferred, or clinical pregnancy achieved, were included. The same logistic regression analyses as for the entire cohort were performed.

6.4 STUDY RESULTS

Assessment of clinical outcomes from the 330 couples who initiated PGT in the four-year period showed a cumulative clinical pregnancy rate of 52.7 % (CI_{95} 47.2 % - 58.2 %) (Table 2 and Figure 1). Following sensitivity analysis, which included 187 couples who had completed a full treatment offer, the cumulative clinical pregnancy rate increased significantly to 87.7 % (CI_{95} 82.1 % - 92.0 %) (Supplementary Table 3 and Supplementary Figure 2). Clinical pregnancy rates per oocyte retrieval was 28.7 % (CI_{95} 25.2 % - 32.4 %), which increased significantly to 48.4 % (CI_{95} 42.9 % - 53.8 %) following the sensitivity analysis (Table 2 and Supplementary Table 3). Per frozen embryo transfer, the clinical pregnancy rate was 33.2 (CI_{95} 29.4 % - 37.2 %), which also increased significantly to 47.4 (CI_{95} 42.0 % - 52.8 %) following the sensitivity analysis (Table 2 and Supplementary Table 3).

Logistic regression analysis of the effect of female age at the time of initiating PGT revealed that each additional year caused an 8 % (CI_{95} 3 % - 13 %) reduction in the chance of achieving a clinical pregnancy over the course of a PGT-treatment (Table 3). Female patients initiating PGT after turning 35 years old had a 65 % (CI_{95} 36 % - 81 %) reduction in the chance of achieving clinical pregnancy compared to patients less than 30 years of age at the time of initiating treatment. Patients initiating PGT between the age of 30 and 35 performed equally to patients initiating prior to turning 30 (Table 3 and Supplementary Figure 1).

Multilevel logistic regression showed that the number of transferable embryos significantly affected the chance of achieving clinical pregnancy per oocyte retrieval for both PGT-M and PGT-SR with an adjusted odds ratio of 2.01 (CI_{95} 1.73-2.38) and

2.64 (CI_{95} 1.69-5.07), respectively, for each additional embryo available for transfer (Table 3). Female age significantly affected the chance of achieving clinical pregnancy per oocyte retrieval for PGT-M with the chance decreasing by 8 % (CI_{95} 2 % - 13 %) per female year prior to adjustment; an effect that was no longer statistically significant following adjustment for the number of transferable embryos (Table 3). Similar estimates were obtained for PGT-SR but with larger confidence intervals due to the smaller sample size of the PGT-SR cohort, causing the effect of female age prior to adjustment to not be statistically significant (Table 3). Sensitivity analysis showed an odds ratio of 3.67 (CI_{95} 2.76-5.48) and 4.45 (CI_{95} 2.57-8.91) for PGT-M and PGT-SR, respectively (Supplementary Table 4).

6.5 STUDY DISCUSSION

We showed a cumulative clinical pregnancy rate following PGT of 52.7 % for the entire cohort and 87.7 % for the cohort having completed a full treatment offer (sensitivity analysis). The sensitivity analysis revealed that a large fraction (43.3 %) of the couples included in the original analysis had not yet completed treatment, which is not surprising since our center was founded in October 2016. The increased cumulative clinical pregnancy rate observed in the sensitivity analysis leads to two interpretations. First, it could indicate that the actual cumulative clinical pregnancy rate for the entire cohort once all included couples have been allowed to complete a full treatment offer will be significantly higher than the observed 52.7 %. Second, it is not unlikely that the cohort used in the sensitivity analysis consists of patients who become pregnant more easily than those still undergoing treatment. In that case, the cumulative clinical pregnancy rate for the remaining couples in the cohort will be significantly lower once they have finished their treatment. Hence, the cumulative clinical pregnancy rate for the entire cohort will not increase much from the currently observed 52.7 % once all patients have completed a full treatment offer. Reassessment of the data once all couples in the present cohort have concluded their treatment will provide answers to these questions.

Compared to other studies evaluating cumulative clinical pregnancy rates following PGT (Verpoest *et al.*, 2009; Bay *et al.*, 2016; Girardet *et al.*, 2018), the cumulative clinical pregnancy rate reported by us is either similar or higher. Importantly, this was achieved by obligatory frozen single embryo transfer, which eliminates the pre- and perinatal complications associated with twin-pregnancies (McLernon *et al.*, 2011), compared to the other studies performing a mix of single and double embryo transfer. The adherence of our center to up-to-date laboratory procedures such as trophectoderm biopsy and cryopreservation by vitrification are likely explanatory factors to the observed cumulative clinical pregnancy rate. Those methods have been shown to improve clinical outcomes compared to previously used methods such as cleavage stage biopsy (Scott *et al.*, 2013) and slow-freeze protocols (Li *et al.*, 2014), as detailed in section 2.5 and 2.6. Besides laboratory procedures, differences in the examined cohorts might have contributed to the observed differences in clinical

outcomes. Especially factors such as average female age and PGT-indication of the examined cohort are important. Age can directly affect clinical outcomes as older cohorts will have fewer euploid embryos. The PGT-indication can directly affect outcomes by affecting the number of unaffected embryos available for transfer according to the mode of inheritance (e.g. recessive versus dominant monogenic disorders). When comparing our clinical outcomes with the most recent ESHRE PGT Consortium data collection (Coonen *et al.*, 2020), we report comparable or better clinical outcomes, which we believe likely is a result of the use of more updated methods and practices as described previously.

In summary, our results indicate that a newly established center can achieve highly satisfactory clinical outcomes by applying up-to-date methods and practices. Additionally, procedures such as trophectoderm biopsy and vitrification allow freeze-all strategies and obligatory single embryo transfer to be adopted without seemingly compromising outcomes.

As detailed in the introduction, a close link exists between female age and fertility (Heffner, 2004). Hence, we would expect clinical pregnancy rates to decrease as female age increases. Indeed, logistic regression analysis showed that age at the time of initiating PGT treatment significantly affects the cumulative chance of achieving a clinical pregnancy. Each additional female year causes an 8 % reduction in the chance of achieving a clinical pregnancy. The observed decrease in clinical pregnancy chance was significant for the PGT-M cohort and not for the PGT-SR cohort. This difference is likely due to the low sample size of the PGT-SR cohort, as the two estimates were not statistically significantly different. These findings show that female age is an important factor to consider when evaluating chances of success during PGT for hereditary disorders, in agreement with previous reports from ART treatment for infertility (Franasiak *et al.*, 2014a; Demko *et al.*, 2016). Despite being interesting from a clinical perspective, this information might be a valuable tool during patient counseling and contribute to patients being able to make a more informed decision on whether or when to initiate PGT treatment.

Upon having initiated treatment, couples often wish to know their chances of achieving clinical pregnancy following oocyte retrieval. The chance of achieving clinical pregnancy in an oocyte retrieval cycle depends on factors such as the number of transferable embryos and female age at the time of oocyte retrieval. The number of transferable embryos directly dictates the number of transfers while female age affects the quantity and quality of the retrieved oocyte, and thereby the resulting embryos transferred.

We showed that the number of transferable embryos significantly affected the chance of achieving clinical pregnancy following PGT-M and PGT-SR. Although not statistically significant, each additional transferable embryo had a greater estimated impact on the chance of achieving clinical pregnancy following PGT-SR compared to

PGT-M. This difference was not unexpected, as transferable embryos had been screened for aneuploidy concurrently with PGT-SR. Hence, a transferable embryo following PGT-SR is expected to have a higher chance of implanting than a transferable embryo following PGT-M, which has not been screened for aneuploidy (Tiegs *et al.*, 2020).

The sensitivity analysis showed the number of transferable embryos to have a significantly higher impact on the chance of achieving clinical pregnancy compared to the primary analysis of the entire cohort. Each additional transferable embryo caused an approximately fourfold increase in the chance of achieving a clinical pregnancy. As for the primary analysis, a larger impact of each additional transferable embryo was observed for PGT-SR compared to PGT-M in the sensitivity analysis, although the difference was still not statistically significant. The cycles excluded from the sensitivity analysis had, on average, a larger number of transferable embryos and a larger number of not yet transferred embryos compared to the cycles included in the sensitivity analysis. Therefore, cycles excluded from the sensitivity analysis are expected to have a higher chance of resulting in a clinical pregnancy than cycles included in the sensitivity analysis. For this reason, we expect the odds ratios from the sensitivity analysis to be more representative of the actual effect of each additional transferable embryos on the chance of achieving a clinical pregnancy per OR compared to the analysis performed on the entire cohort.

While it might seem evident that additional embryos affect the chance of achieving clinical pregnancy per OR, the magnitude of the effect is interesting. The magnitude suggests that efforts made to increase the number of transferable embryos will dramatically affect the chance of achieving clinical pregnancy per OR. One example that might affect the number of transferable embryos is stimulation regiments, which can be modified to increase the number of retrieved oocytes. However, increasing hormone stimulation might not necessarily lead to an increase in the number of good-quality blastocysts (Arce *et al.*, 2014). Additionally, attempts to increase the number of oocytes must be weighed against the potential side effects (Alper and Fauser, 2017). Another option is to utilize time-lapse imaging to assess pronuclei count better and avoid discarding correctly fertilized oocytes (Destouni *et al.*, 2018). Lastly, optimizing embryo culture conditions might increase the number and quality of embryos developing to the blastocyst stage (Wale and Gardner, 2016), and culture conditions have indeed been shown to impact live birth rates (Castillo *et al.*, 2020).

Age was observed to significantly affect the chance of achieving a clinical pregnancy per OR prior to adjustment for the number of transferable embryos. However, this effect was no longer statistically significant following adjustment. This lack of significant difference might result from the link between age and the number of transferable embryos, where age affects the quantity and quality of oocytes and the resulting number of transferable embryos. Hence, by adjusting for the number of transferable embryos, a sufficient proportion of the effect that age has on the chance

of achieving clinical pregnancy is removed to render the effect no longer statistically significant.

The primary way in which age affects fertility rates is via aneuploidy, as discussed in the introduction. Hence, we would expect the effect of age to be neglectable following adjustment for the number of the transferred embryos in the PGT-SR group. In other words, two women with the same number of transferable embryos would be expected to fare equally with respect to their chance of achieving clinical pregnancy per OR irrespective of their ages. On the contrary, in the PGT-M group, where no aneuploidy screening is performed, older women would be expected to have a reduced chance of achieving clinical pregnancy per OR compared to younger women with the same number of transferable embryos. The reason being that by chance, the older woman would have fewer euploid embryos. Contrary to expectations, we observed age to have no significant impact in neither the PGT-SR nor PGT-M group. Two likely explanations present themselves. First, the average female age of our cohort is relatively low, which will reduce the impact of aneuploidy. Second, the point in time in which we measure the clinical outcome (clinical pregnancy in gestational week 7-8) might remove some of the effects of age. In addition to failed implantation or early miscarriage, aneuploidy may also cause miscarriage later in pregnancy, which is not detected in our study. We would, although, only expect a limited number of miscarriages, as fetal heartbeat in gestational week 6-8 has been established as a good predictor of live birth for fertile women (Hyer *et al.*, 2004). Hence, in the case of an older cohort and/or live births as the clinical outcome measured, we may have observed an effect of age despite adjustment for the number of transferable embryos in the PGT-M cohort.

In conclusion, while these findings indicate that age does not significantly affect the chance of achieving clinical pregnancy per OR following PGT-M when adjusted for the number of transferable embryos, one should be careful about extrapolating these findings to other cohorts. In the context of PGT-SR with concurrent aneuploidy screening, these results indicate that more emphasis should be put on the number of transferable embryos obtained in a given cycle than female age when counseling patients on their chances of achieving clinical pregnancy. This might help reduce stress or concerns experienced by patients of advanced maternal age who have a sufficient number of embryos available for transfer.

An obvious limitation to this study is the short inclusion period and the fact that many patients have not used the full treatment offer available to them at the time of data analysis. A strength of the study is that the treatment procedures and techniques used during the inclusion period have been uniform, which reduces possible confounding. Continuous assessment of clinical outcomes and factors affecting clinical outcomes should be performed to provide up-to-date information relevant for quality management and proper patient counseling.

7 STUDY V

7.1 INTRODUCTION

While PGT can significantly reduce the risk of achieving a clinical pregnancy with an affected fetus, a small risk of misdiagnosis cannot be excluded. Methods used today for PGT are designed for optimal diagnostic accuracy, such as the use of DNA markers. However, errors in interpretation and swapping of embryos, biopsies, or test results pose a risk despite attempts to avoid such situations by adhering to currently published guidelines (Carvalho *et al.*, 2020a). If undetected and allowed to go to term, cases of misdiagnosis obviously have severe consequences for both the future child and the parents to be. Hence, patients should be informed of the risk of misdiagnosis and the possibility of having prenatal testing to confirm the result from PGT (Carvalho *et al.*, 2020a). Communication between health care professionals and patients is difficult (Ha and Longnecker, 2010), with reports of patients not understanding the delivered information despite the health care professional being convinced that they had (Berger *et al.*, 2017). Inadequate communication skills have been suggested as an issue (Leithner *et al.*, 2006). Reproductive behaviors may be affected by health literacy, thereby influencing patient decision-making, emphasizing the need for proper counseling of patients, especially when considering a subject as complex as genetics.

Prenatal testing in the context of PGT has historically been performed by CVS, which entails a small risk of miscarriage estimated to be less than 0.2 % (Salomon *et al.*, 2019). The risk of miscarriage associated with the procedure has previously been reported as a reason for patients not to choose CVS (Çakar *et al.*, 2016; Ternby *et al.*, 2016). The fact that patients do not accept the offer of CVS due to the associated risk of miscarriage is also reported by health care professionals at our center for Preimplantation Genetic Testing based on their experience when discussing prenatal testing with patients. However, it has never been qualified or quantified. Studies have found that patients prioritize test safety to a degree, where they are willing to compromise with the type and amount of genetic information obtained to have a risk-free prenatal test (Hill *et al.*, 2012, 2015, 2016; Lund *et al.*, 2018). Given these findings, non-invasive alternatives are likely to cause more patients to opt for prenatal testing following PGT, increasing the chance of identifying the rare cases of misdiagnosis. At the same time, it may help alleviate anxiety and stress experienced by patients during pregnancy. Non-invasive prenatal testing might differ from CVS in many aspects, such as the time in pregnancy at which the procedure can be performed, diagnostic accuracy, and the type of genetic information obtainable. All of these aspects should be thoroughly discussed with patients to ensure that they look beyond the issue of safety and are provided with all the relevant information to make an informed decision.

Evaluation of patients' thoughts, experiences, and preferences concerning prenatal testing, different alternatives, and the counseling they received might aid health care professionals in understanding the decision making of patients with respect to prenatal testing and how counseling might be altered or improved to allow the patients to make a properly informed decision concerning prenatal testing following PGT.

7.2 STUDY OBJECTIVE

This study aimed to investigate the extent to which patients opted for prenatal testing by CVS after achieving clinical pregnancy following PGT, their reasons for accepting or declining PGT, their experience with counseling about prenatal testing, and their opinions about non-invasive alternatives to CVS.

7.3 STUDY DESIGN

A questionnaire was designed to answer the aims mentioned above and was distributed electronically to men and women who had achieved a clinical pregnancy following PGT at the PGT center at Aalborg University Hospital between January 2016 and December 2020.

7.4 STUDY RESULTS

We found that 43.6 % (CI_{95} 36.1-51.4 %) of respondents opted for prenatal testing following PGT (Table 5). The primary reason for declining CVS was the associated fear of miscarriage associated with the procedure (69.5 %, [CI_{95} 59.2-78.5%]), while a significant part of respondents also declined CVS as they were not willing to terminate the pregnancy no matter the result (32.6 %, [CI_{95} 23.4-43.0 %]) (Table 5). Nineteen percent (CI_{95} 11.6-28.3 %) of respondents responded that a normal result from the nuchal translucency scan was sufficient to decline CVS (Table 5). Almost one in five respondents (18.2 %, [CI_{95} 13.2-24.2 %]) was not aware that the nuchal translucency scan only rarely provides information on the genetic disorder for which PGT was performed (Table 6). In fact, three respondents reported that they would have chosen CVS had they been aware. The primary reasons for accepting CVS were to allow for termination of pregnancy in case of an affected fetus (69.3%, [CI_{95} 57.6-79.5 %]) and due to recommendations from the clinic (56.0 %, [CI_{95} 44.1-67.5%]) (Table 5). Of all respondents, 54.2 % (CI_{95} 46.9-61.4 %) responded that the clinic recommended CVS, while 35.3 % (CI_{95} 28.5-21.5 %) reported that the clinic did not have any recommendation with respect to CVS (Table 8).

Almost one in ten respondents reported having a child affected by the hereditary disorder prior to initiating PGT (17.7 % %), and nearly one third (31.5 %) of respondents reported that they were not aware that they were at risk of passing on a hereditary disorder to their offspring when attempting to achieve pregnancy prior to PGT (Table 1). We found that when respondents were aware of their risk of passing

on a hereditary disorder, significantly fewer reported having a child affected by the hereditary disorder prior to initiating PGT (Table 2).

Approximately 52.7 (CI_{95} 45.6-59.7 %) and 38.4 % (CI_{95} 31.7-45.5) of respondents reported having a desire for verification that the fetus was unaffected or a concern that the fetus may have inherited the disorder despite PGT, respectively (Table 4). Although the concern and desire for verification significantly affected the choice of CVS, 25.8% (CI_{95} 17.1-36.2) of respondents not opting for CVS (n = 89) responded that they had a desire for prenatal verification that the fetus was unaffected (Table 5). There was a significant difference in choice of CVS between female respondents below and above the age of 30, with a larger fraction of the older respondents accepting CVS compared to the younger respondents (Table 7). No statistically significant difference was observed for male respondents.

We found that nine out of ten respondents (89.2 %, [CI_{95} 84.1-93.1 %]) would have accepted non-invasive prenatal testing had it been offered to them (Table 9). They reported the absence of an associated risk of miscarriage as the primary reason (93.4 %, [CI_{95} 88.7-96.5 %]) followed by the procedure being more pleasant than invasive testing (51.4 %, [CI_{95} 43.9-58.9 %]) (Table 9). The primary reason for declining non-invasive prenatal testing was that termination of pregnancy would not be an option no matter the test result (76.9 % [CI^{95} 46.2-95.0 %]) (Table 9).

7.5 STUDY DISCUSSION

In line with our experiences from clinical practice, we observed that a significant fraction of respondents declined CVS after achieving pregnancy by PGT. In line with previous studies (Çakar *et al.*, 2016; Ternby *et al.*, 2016), the risk of miscarriage associated with CVS caused patients to decline CVS; in our case, it was the primary reason reported. Approximately 25 % of respondents declining CVS reported having a desire for prenatal testing to ensure that the fetus was unaffected. These findings suggest that a significant fraction of patients wishes to have prenatal verification of the original PGT result but that the risk of miscarriage discourages them from accepting CVS. This is supported by the finding that approximately 90 % of respondents would opt for a non-invasive alternative if it was offered to them. The primary reason being that risk of miscarriage is not associated with this procedure. Hence, non-invasive alternatives to CVS will likely result in more patients accepting prenatal testing. While this increases the chance of identifying the rare cases of misdiagnosis, it seems that it might also contribute to reduce anxiety, stress, or worry experienced by patients during pregnancy with respect to whether the fetus is unaffected.

Proper counseling of patients is important to allow them to make properly informed choices with respect to prenatal testing. While almost all respondents reported that they had been informed about the option of prenatal testing, whether the clinic recommended prenatal testing following PGT was less clear. Either the recommendation has not been adequately communicated during counseling, or the respondents have simply forgotten the information. This may indicate that more effort needs to be made to ensure that patients understand and recall the provided recommendations. Different prenatal tests or screenings exist, each capable of providing different information about the fetus. Emphasis should be made to ensure that patients understand the differences and limitations between the different types of prenatal tests and screenings available. We found that approximately 25 % of respondents were unaware that the nuchal translucency scan can only rarely provide information on whether the fetus has inherited a particular disorder. This suggests that some patients might have misinterpreted a normal result from the scan as an indication that the fetus was unaffected by the specific hereditary disorder for which PGT was performed. Others have previously reported the misinterpretation of the purpose or capability of the translucency scan (Lalor and Devane, 2007; Gourounti *et al.*, 2008; Dahl *et al.*, 2011). Indeed, three respondents reported that they would have opted for CVS had they been aware of this fact, supporting this theory. Adding non-invasive prenatal testing to the array of tests available will further complicate counseling and patient decision-making. Test safety has been reported as one of the primary considerations when patients evaluate prenatal tests (Hill *et al.*, 2012, 2015, 2016; Lund *et al.*, 2018). This highlights the importance of properly informing patients about the different alternatives of prenatal testing and the differences, such as at in which gestational week they can be performed, test accuracy, and the amount and type of genetic information obtained, to ensure that patients not only focus on the issue of safety.

Our results suggest that being aware of the risk of passing on a hereditary disorder reduces the risk of couples having an affected child prior to initiating PGT. This suggests that efforts made to spread awareness of hereditary disorders to couples during family planning may reduce the number of affected children born. Importantly, awareness of the risk will allow couples to consider alternative solutions to spontaneous pregnancy, such as PGT or adoption.

During the questionnaire survey, respondents were informed to provide their name and date of birth. It is possible or even likely that some of the answers provided would have been different had the questionnaire survey been anonymous or performed as an interview-based survey. The former might have made the respondents report more honestly to some questions. The latter might have allowed the respondents to have the interviewer elaborate on the questions, potentially resulting in fewer incorrect responses from patients due to them not understanding the questions. However, it is also likely that an interviewer may unintentionally affect the answers given.

We recently published a proof-of-concept paper on cbNIPT (Study II) following PGT-M for a variety of different types of genetic disorders. The findings from the presented study suggest that the method would be welcomed by patients if implemented into clinical practice. As detailed in Study II, work is currently ongoing to validate cbNIPT, hoping that it may be implemented into clinical practice in the near future.

8 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The work detailed in this thesis spans multiple different aspects of PGT from assessment and evaluation of clinical outcomes, a systematic review of concurrent aneuploidy screening and PGT for hereditary disorders, investigation of trophectoderm cell culture as a method of DNA amplification, proof-of-concept of cbNIPT, and a questionnaire survey on patients' choices, opinions, and experiences with prenatal testing and non-invasive alternatives.

Despite the challenge associated with working with five studies so diverse in nature, I am grateful for having had the opportunity to explore so many different aspects of PGT and diverse fields of research. Not to mention the incredible people and connections that I have been so fortunate to have made on the way.

The work presented in this thesis and my experiences within the last three years has made me conclude that while PGT has evolved significantly since its introduction in 1989, the field is still in its infancy in many aspects and are undergoing rapid changes and likely will continue to do so in the near future. I would not be surprised if the concept of PGT looked quite different from how we know it today in ten years. Additionally, it appears that we are beginning to learn that the human preimplantation embryo is more intelligent than we may have originally thought, given the recent evidence of self-correction mechanisms. It will be exciting to follow this topic in the future.

We contributed to the development of new methods by proposing that biopsied trophectoderm cells could be expanded in cell culture as an alternative to artificial DNA amplification by WGA (Study III). Alternatively, the method might be used as a tool to explore bias introduced by WGA. The study provided proof of concept, but the method needs to be properly evaluated, and we acknowledge that logistic, personnel, and economic issues associated with cell culture might complicate the use of the procedure in clinical practice.

The lack of standardized methods for PGT and the continuous rapid introduction of new technologies and methods has been interesting. The high demand for PGT, not only for hereditary disorders but also as an add-on to ART, has contributed to the rapid advancement of the field and introduction of new technologies – sometimes too fast as was experienced with the premature implementation of FISH-based aneuploidy screening. While consensuses appear to be reached in certain areas such as the preferred stage of biopsy and method of cryopreservation, embryo culture conditions and the use of PGT-A especially is far from standardized across the world. The latter is especially debated due to embryonic mosaicism and the emerging evidence of

embryo self-correction mechanisms, which is a subject of intense research. Despite the initial warning from FISH-based aneuploidy screening, PGT-A is already used in many clinics worldwide while properly designed randomized controlled trials with intention-to-treat analyses and non-selection studies remain few, as discussed in the introduction and Study I. As shown in Study I, aneuploidy is prevalent in preimplantation embryos from patients receiving PGT, affecting approximately one-third of embryos. Hence, selection of euploid embryos could, in theory, improve clinical pregnancy and live birth rates and decrease miscarriage rates resulting in reduced time to pregnancy. However, proper evaluation of the extent to which reproductively competent embryos are discarded is warranted. We are currently planning a national non-selection study executed in collaboration between Denmark's two national PGT centers, which will aid us in evaluating PGT-A in Denmark.

In my opinion, as health professionals, it is our responsibility to the patients that new treatments are properly evaluated prior to clinical implementation or, as a minimum, that we can provide patients with comprehensive information on new procedures allowing them to make an informed decision about treatment options. As we found out in Study V and as has been echoed by others, communicating between patients and health care professionals is challenging. The complex concepts of genetics associated with PGT, and subsequent prenatal testing, only contributes further to the challenge of ensuring that patients receive sufficient and comprehensive information to allow them to make their own informed decision. In general, the literature and our findings indicate that focus should be put on proper communication with patients to facilitate them in making properly informed decisions. Assessment of clinical success rates and factors aiding in predicting chance of success, as discussed in Study IV, and assessment of patients experiences and opinions, as discussed in Study V, might aid health care professionals in achieving this aim by allowing them to provide patients with better information on which to base their choices or their expectations.

Part of clinical counseling in the context of PGT also entails prenatal testing. We found that a significant fraction of patients would accept non-invasive prenatal testing if it was offered to them (Study V). In Study II, we provided proof-on-concept of cbNIPT as a non-invasive alternative to CVS. The study is ongoing, and cbNIPT is hopefully not far from clinical implementation where it will provide patients with a risk-free and less invasive prenatal alternative.

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APPENDICES

Appendix A Study I

Appendix B Study II

Appendix C Study III

Appendix D Study IV

Appendix E Study V

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