Improving our understanding of the implications of unanticipated or unclear prenatal genetic screening results

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Thesis submitted to the University of Ottawa
in partial fulfillment of the requirements for the
Doctorate in Philosophy degree in Epidemiology

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Preface

Ethical Considerations

All analyses were conducted using secondary data from Ontario's prescribed pregnancy, birth and childhood registry, the Better Outcomes Registry & Network (BORN). As a prescribed registry, BORN Ontario reviewed the research protocols and approved for the analyses to be conducted within the BORN Ontario secure network environment to ensure privacy requirements were met.

BORN Ontario is housed within the Children's Hospital of Eastern Ontario (CHEO) and therefore, with the support of BORN Ontario, approval from the CHEO Research Ethics Board was obtained for all research projects; CHEOREB# 22/03PE for the studies presented in Chapter 2 and Chapter 3, and CHEOREB# 22/05PE for the study presented in Chapter 4. Additionally, an administrative review was also obtained from the University of Ottawa Research Ethics Board for all studies; H-06-22-8234 and H-06-22-8269, respectively.

Statement of Financial Support

I was financially supported by a doctoral grant from the Canadian Institutes of Health Research (CIHR Frederick Banting Charles Best Doctoral Award) for the first three years of my studies.

I also received funding from the University of Ottawa through an excellence scholarship throughout my doctoral studies to cover tuition costs.

I have had the opportunity to work at the Better Outcomes Registry & Network (BORN) Ontario and Prenatal Screening Ontario during my studies, which allowed me to access the datasets needed to conduct this thesis free of charge.

A Note About Language

Following current recommendations in the field of epidemiological research in pregnancy, this dissertation uses gender-inclusive language.[1,2] The wording “pregnant women and pregnant individuals” was also used in order to reflect the experiences of pregnant individuals who do not identify as female while also representing the gendered pregnancy experiences of cisgendered women.

Some terminology used to define specific screening modalities, or disciplines, remains gendered, and was therefore used as recognized in the international literature at the time the dissertation was written, although we recognize that these terms may eventually be replaced.

Contribution of Authors

With guidance from my supervisors and Thesis Advisory Committee, I, Kara Bellai-Dussault (the PhD candidate) was responsible for leading all aspects of this thesis, by developing the study protocols, conducting all analyses, interpretation of the results, and drafting the manuscripts. My supervisor Dr Beth Potter provided supervision and guidance throughout the entire process with the help of Dr Julian Little, epidemiologist, as co-supervisor. The Thesis Advisory Committee members were selected for their unique expertise and complementary skillset reflecting the multidisciplinary nature of the studies comprised in this thesis, and included Dr Mark Walker, maternal fetal medicine specialist, Dr Deshayne Fell, epidemiologist, and Shelley Dougan, director of Prenatal Screening Ontario.

The medical directors of Prenatal Screening Ontario, Dr Christine Armour and Dr Nan Okun critically reviewed the manuscripts, and provided insight into the potential clinical implications of the findings for the provincial program. Lynn Meng helped with the initial pull of the data sets from the BORN Information System as per the BORN standard procedures and provided valuable advice on coding methods. Carolina Lavin-Venegas additionally helped with administrative work including formatting of manuscripts 2 and 3, and submission to the journal. In addition, for the third manuscript, Dr Tianhua Huang provided advice on the study design and reviewed the manuscript as an epidemiologist with over 20 years of experience in multiple marker screening research in Ontario. For this study, Dr. Steven Hawken provided statistical advice and reviewed the findings, given the higher level of complexity of the methods. All authors made significant contributions to these studies through conceptualization of the study, interpretation of the results, and critical review of the manuscripts.

Abstract

Improving our understanding of the implications of unanticipated or unclear prenatal genetic screening results

Prenatal genetic screening for trisomies 21 and 18 is offered in many jurisdictions in the form of multiple marker screening, incorporating information on patient characteristics, biochemical markers, and nuchal translucency measurement, where a pocket of fluid located behind the fetal neck is measured. Although screening results are generally straightforward to interpret, unanticipated or unclear results can occur and evidence regarding their clinical implications is limited, hindering patient counselling. The aim of this doctoral thesis was to address this evidence gap by investigating associations between these unanticipated or unclear results and adverse perinatal outcomes.

To achieve this, three population-based retrospective cohort studies were conducted using data from Ontario's prescribed perinatal registry, Better Outcomes Registry & Network. The first study investigated the association between all levels of nuchal translucency and chromosomal abnormalities and found an increased risk of chromosomal abnormalities among pregnancies with measurements below the widely used threshold of 3.5 mm at which follow-up investigations are offered. The second study further evaluated the association between nuchal translucency and adverse perinatal outcomes, where pregnancies with increased measurements were less likely to result in a live birth, even when all identified chromosomal abnormalities were excluded. The third study examined the association between multiple marker screening results positive for both trisomies 21 and 18 at once, or 'double-positive results', and adverse perinatal outcomes. The study found an increased risk of preterm birth overall, and when all diagnosed chromosomal abnormalities were excluded.

These findings that the unanticipated or unclear results evaluated were associated with an increased risk of adverse perinatal outcomes have important implications for clinical practice and policy, for example, in decisions about thresholds for offering follow-up investigations, the types of services offered, and counselling for pregnant patients. These studies will also inform

future research to further understand the implications of all possible prenatal genetic screening results and reduce uncertainty in prenatal genetic screening.

Résumé

Améliorer notre compréhension des implications des résultats inattendus ou incertains du dépistage génétique prénatal

Le dépistage génétique prénatal des trisomies 21 et 18 est offert dans de nombreuses juridictions sous la forme de dépistage multimarqueurs, intégrant de l'information caractérisant la personne enceinte, des marqueurs biochimiques, ainsi que la mesure de la clarté nucale, qui consiste à mesurer une accumulation de liquide au niveau sous-cutané située à l'arrière de la nuque du fœtus. Bien que les résultats du dépistage soient généralement simples, il arrive qu'ils soient imprévus ou incertains et qu'il n'existe que peu de données cliniques quant à leurs implications, ce qui compromet le conseil aux patients. L'objectif de cette thèse de doctorat était de combler ces lacunes en étudiant les associations entre ces résultats inattendus ou incertains et différentes issues périnatales défavorables.

Pour ce faire, trois études populationnelles rétrospectives ont été réalisées à partir de données du registre périnatal désigné de l'Ontario, le Registre et réseau des bons résultats dès la naissance (BORN). La première étude a examiné l'association entre tous les niveaux de clarté nucale et les anomalies chromosomiques et a constaté un risque accru d'anomalies chromosomiques parmi les grossesses dont les mesures sont inférieures au seuil largement utilisé de 3,5 mm à partir duquel des examens de suivi sont proposés. La deuxième étude a approfondi sur le sujet en évaluant l'association entre la clarté nucale et les issues périnatales indésirables, et conclut que les grossesses présentant des mesures de clarté nucale plus élevées étaient moins susceptibles de mener à une naissance vivante, même lorsque toutes les anomalies chromosomiques identifiées étaient exclues. La troisième étude a examiné l'association entre des résultats de dépistage multimarqueurs positifs pour les trisomies 21 et 18 simultanément, aussi appelés "résultats doublement positifs", et les issues périnatales indésirables. L'étude a révélé un risque accru de naissance prématurée à la fois globalement et lorsque toutes les anomalies chromosomiques diagnostiquées étaient exclues.

Ces conclusions, selon lesquelles les résultats inattendus ou incertains évalués étaient associés à un risque accru d'issues périnatales indésirables, ont des implications importantes pour la

pratique clinique et les politiques de santé, par exemple pour les décisions concernant les seuils à partir desquels des examens de suivi sont offerts, les types de services offerts et les conseils aux personnes enceintes. Ces études guideront également les recherches futures visant à mieux comprendre les implications de tous les résultats possibles découlant du dépistage génétique prénatal et à réduire l'incertitude du dépistage génétique prénatal.

Acknowledgements

Foremost, I must thank my PhD supervisor, Beth Potter. I am extremely grateful for your continued guidance throughout my doctoral studies. You were incredibly generous in sharing your expertise, resourceful, patient, and kind. I sincerely could not have imagined a better supervisor.

I am also incredibly grateful for the support and guidance provided by my thesis advisory committee. Thank you to Julian Little for being my PhD co-supervisor. I appreciated your enthusiasm for the projects, as well as your meticulousness and expertise. To Deshayne Fell and Mark Walker, I cannot express how fortunate I feel to have had you alongside me throughout this process. I am proud to collaborate on research projects with you. I also wish to express my appreciation to Shelley Dougan for supporting me from the very first moment I showed interest in pursuing this degree. I still remember sitting in your office in the first years of our screening program, trying to absorb your wisdom and learning immensely from you. I still do and hope to continue working with you for a very long time.

A special thought for Andrea Lanes, for inspiring me to pursue this doctoral degree in the first place. I feel incredibly lucky to have had the privilege to work with you and admire you greatly.

To my husband Anson, thank you for your unwavering support, your interest in my research, and for always believing in my abilities to achieve this goal.

Finalement, un remerciement tout particulier à mes parents, qui m'ont inculqué les valeurs de persévérance, d'éthique de travail et l'importance de l'éducation, sans lesquelles je ne serais pas là où j'en suis aujourd'hui. Merci infiniment pour votre soutien et tous vos encouragements dans cette étape importante. Vi voglio tanto bene.

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Chapter 1. Introduction and Literature Review

Chapter 2. Manuscript 1: Cytogenetic outcomes following an increased nuchal translucency measurement

List of Acronyms and Abbreviations

AFP	Alpha fetoprotein
ART	Assisted reproductive technologies
BORN Ontario	Better Outcomes Registry & Network Ontario
βhCG	Beta human chorionic gonadotropin
cfDNA screening	cell-free DNA screening
CIHI	Canadian Institute for Health Information
CI	Confidence interval
CNV	Copy number variant
CRL	Crown-rump length
DAD	Discharge Abstract Database
DAG	Directed acyclic graph
EDD	Estimated Date of Delivery
eFTS	enhanced First Trimester Screening
FISH	Fluorescent In Situ Hybridization
GA	Gestational age
IVF	In vitro fertilization
ICSI	Intracytoplasmic sperm injection
MMS	Multiple marker screening
MSS-Quad	Maternal Serum Screening

NICU	Neonatal intensive care unit
NIPT	Noninvasive prenatal testing
NPV	Negative predictive value
NT	Nuchal translucency
PAPP-A	Pregnancy-associated plasma protein A
PlGF	Placental growth factor
PPV	Positive predictive value
QF-PCR	Quantitative Fluorescent Polymerase Chain Reaction
RR	Risk ratio
RD	Risk difference
SCA	Sex chromosome aneuploidy
SD	Standard deviation
UE3	Unconjugated estriol

Chapter 1. Introduction and Literature Review

1.1 Organization of dissertation

This manuscript-based dissertation is presented in accordance with the School of Epidemiology and Public Health (SEPH), University of Ottawa, Guidance for Thesis by Article. The dissertation begins with an introductory chapter (Chapter 1) providing context for the studies presented, including literature reviews and rationales for the study questions.

The dissertation comprises three manuscripts related to outcomes associated with prenatal genetic screening results that are unanticipated or unclear. The first two manuscripts focus on the association of fetal nuchal translucency measurements with 1) chromosomal outcomes (Chapter 2) and 2) pregnancy and perinatal outcomes (Chapter 3), while the third manuscript focuses on the outcomes of pregnancies with multiple marker screening results that are 'double-positive' for trisomies 21 and 18 (Chapter 4). Each manuscript is presented in a chapter and includes a preface, as well as the manuscript that was published or submitted to the peer-reviewed journal.

An integrated discussion of the findings (Chapter 5) follows the three manuscripts, summarizing the findings with a discussion of the clinical and research implications.

Finally, an appendix is included to provide additional information including ethics certificates, confirmation of submission of manuscripts, and the published version of the first manuscript.

1.2 Background

1.2.1 Prenatal genetic screening and diagnosis

Prenatal genetic screening aims to provide information to expecting parents to help guide reproductive choice or prepare to welcome a child with a genetic condition. The screening results can also inform pregnancy management, including further monitoring and testing; and can inform delivery and postnatal management, such as ensuring the appropriate level of care for the pregnant individual and the newborn.

Prenatal genetic screening programs are designed to identify individuals for which there is a higher probability that one or more fetuses in the index pregnancy has one of the conditions screened and to offer follow-up investigations including prenatal diagnosis. In jurisdictions in which prenatal genetic screening programs are implemented, it is intended that prenatal screening should be offered to all pregnant women and pregnant individuals, but uptake is optional, and individuals should be supported to make an informed decision as to whether screening is right for them.

1.2.2 Multiple marker screening

Traditional multiple marker screening has been offered as the primary approach to prenatal genetic screening since the 1980s.[1–3] This approach initially included only biomarkers measured in the blood of the pregnant individual; starting in the 1990s a fetal nuchal translucency ultrasound was added in many programs.[1,2] The nuchal translucency is a collection of fluid behind the fetal neck that is measured by ultrasound in the first trimester of pregnancy.[4] Most screening programs focus on the detection of trisomy 21, and some also include a risk assessment for trisomies 18 and 13.[1]

The different modalities of multiple marker screening have varied over the years and have included screening in the first trimester of pregnancy, the second trimester, or both.[5,6] Many jurisdictions now offer a first trimester screen, often referred to as First Trimester Screening or Combined First Trimester Screening, which commonly includes an ultrasound measuring the nuchal translucency as well as serum analytes from the pregnant individual's blood such as

beta-human chorionic gonadotrophin (bhCG) and pregnancy-associated plasma protein A (PAPP-A).[7–12] The level of each marker (ultrasound or serum analytes) is calculated and expressed as a multiple of the median based on normative data accounting for the gestational age at measurement, and allows screening programs to determine if each marker is increased or decreased compared to what would be expected for the corresponding gestational age.[11,12] This information is incorporated into an estimate of the risk of trisomy based on age of the pregnant individual, and further adjusted for factors that can affect the analytes, such as cigarette smoking.[13] The information is then analyzed by the laboratory software to generate a final screening risk estimate for the pregnancy to have the aneuploidy.[12] Screen positive cutoffs are predefined and will vary depending on the screening modality and the screening algorithm of the program.[1] The choice of the screen positive cutoff is a balancing act to maximize accuracy with consideration of both sensitivity and specificity.

The performance of the multiple marker screen will depend on: the modality (e.g., first trimester screening or established alternatives in place historically or currently and that differ in analytes and timing of the screening, such as integrated prenatal screening, second trimester Quad screen, etc.); the specific analytes; the quality of the nuchal translucency ultrasound if included; and the population screened.[6,14] Regardless of modality, multiple marker screening is known to have a higher sensitivity and lower specificity with increasing age of the pregnant individual.[15] Although the positive predictive value will depend on the specific population, given the rarity of the conditions targeted by the screen, it is generally very low, at around 3-4%.[16–18]

1.2.3 cell-free DNA screening

cell-free DNA (cfDNA) screening, also commonly referred to as Noninvasive Prenatal Testing (NIPT) has higher accuracy (sensitivity and specificity for trisomy 21 >99%)[11] and much higher positive predictive value relative to multiple marker screening. Thus, to improve their prenatal genetic screening algorithm, many jurisdictions have incorporated cfDNA screening in a contingent manner following a positive multiple marker screen to reduce false positives. A small number of jurisdictions have implemented a universal cfDNA screening approach, such as Belgium and the Netherlands.[19]

cfDNA screening measures placental DNA fragments circulating in the blood stream of the pregnant individual, and most platforms estimate the probability that the pregnancy has trisomies 21, 18, or 13 and potentially sex chromosome aneuploidies, and fetal sex, while some also extend to large deletions and duplications in the form of genome-wide cfDNA screening.[11,20–23] The screen requires collection of a blood sample and can be performed as early as 9 or 10 weeks gestation depending on the screening platform, and throughout the pregnancy up to delivery.[18,24]

The discovery of placental cfDNA in the bloodstream of pregnant individuals during pregnancy was made in 1997.[25] It soon became a promising method to screen for fetal aneuploidies and screening for clinical purposes was first offered in 2011.[26] Different platforms have been created to screen for fetal aneuploidies with placental cfDNA. Some platforms use massively parallel sequencing, which can either analyze all chromosomes or be restricted to specific chromosomes of interest, to detect unbalanced quantities of these chromosomes.[27] The excess or deficit of chromosomal material is assumed to represent the fetal genetic material, as the pregnant individual is presumed to be euploid.[27] Platforms using the Single-Nucleotide Polymorphism (SNP) based approach can distinguish between DNA fragments from the pregnant individual or from the placenta and incorporate this information in their analysis.[27]

A limitation of cfDNA screening is that the genetic material analyzed originates from the placenta, not from the fetus. This aspect of the screen is often forgotten, and this misunderstanding is exacerbated by the fact that cfDNA screening is often referred to as cell-free **fetal** DNA screening, and that laboratories calculate and report the **fetal** fraction (the proportion of DNA fragments that are placental in origin in reference to the DNA fragments of the pregnant individual). This can be problematic in cases of mosaicism, where for instance the cell line that gave rise to the placenta (originating from the trophoblasts) is euploid, but the cell line that gave rise to the fetus (inner cell mass) is aneuploid.[11]

1.2.4 Diagnostic testing

Although cfDNA screening performs very well and is even perceived by some as diagnostic, it does not constitute a diagnosis. Pregnant individuals seeking definitive answers can have

prenatal diagnostic testing in the form of chorionic villus sampling or amniocentesis or may choose to get this information postnatally through a blood sample.

Amniocentesis is generally performed starting at 15 weeks of gestation.[28] This procedure is guided by ultrasound and consists of collecting amniotic fluid, which contains fetal cells.[11] There is a risk of pregnancy loss following this procedure, which is estimated at 0.3% by a recent meta-analysis.[29]

Chorionic villus sampling is usually performed from 11 weeks to 13 weeks of gestation.[28] In approximately 1% of samples, ambiguous results are obtained, and further testing is required.[11] The procedure-related risk of pregnancy loss is estimated at 0.2%.[29] For chorionic villus sampling, cells of the trophoblast are collected, and therefore extraembryonic tissue is analyzed.[11] Although this is a diagnostic procedure, this sample constitutes a limitation in cases of mosaicism.

Cytogenetic testing is performed on the sample obtained by amniocentesis or chorionic villus sampling, and generally includes a rapid aneuploidy detection in the form of Quantitative Fluorescent Polymerase Chain Reaction (QF-PCR) or Fluorescent In Situ Hybridization (FISH) to provide results for specific chromosomal conditions (most often for common viable aneuploidies such as trisomies 21, 18, 13 and sex chromosome aneuploidies) in a timely manner if reproductive decisions need to be made; a karyotype to determine if chromosomal rearrangements or rare aneuploidies are present; and potentially a microarray analysis to detect copy number variants. The cytogenetic tests offered have varied over time, are dependent on the clinical indication that led to testing and are also dependent on the ordering provider or the centre where they practice. For instance, some centres offer microarray testing to all individuals undergoing a prenatal diagnosis procedure, whereas others will only offer microarray for specific clinical indications.[30]

Some individuals decline prenatal diagnosis in the form of chorionic villus sampling or amniocentesis, in which case cytogenetic testing can be performed on a sample obtained postnatally, typically a blood sample. Cytogenetic testing may also be performed on products of conceptions in the event of a loss or termination.

1.2.5 Prenatal genetic screening in Ontario, Canada

Prenatal screening for genetic conditions such as Down syndrome has been offered in Ontario since 1993.[31] The goal of the screening program is to provide quality screening to pregnant women and pregnant individuals who desire to know the probability that the pregnancy has the genetic condition screened.

In 2018, the Ontario Ministry of Health and Long-Term Care introduced Prenatal Screening Ontario, a provincial program mandated to monitor the quality of the prenatal genetic screening system and enhance access for pregnant individuals across Ontario.[32,33] This program is housed within the Better Outcomes Registry & Network (BORN) Ontario, the provincial prescribed registry for perinatal care.[32] This registry collects data on all pregnancies and births in Ontario, providing a population-level data set that supports analyses by Prenatal Screening Ontario to inform care, policy, and research.[34]

Ontario's prenatal genetic screening program currently formally screens for trisomy 21 (Down syndrome) and trisomy 18 (Edwards syndrome) in the form of a publicly funded mixed contingent model (Figure 1.1). Traditional multiple marker screening is offered to all pregnant individuals as a first-tier test. The specific modalities of multiple marker screening in Ontario have changed numerous times over the years and are presented in Table 1.1. As of 2024, take the form of enhanced first trimester screening (eFTS) or second trimester maternal serum screening Quad (MSS-Quad). eFTS consists of a nuchal translucency ultrasound and a blood draw to measure serum biomarkers: beta-human chorionic gonadotrophin (bHCG), pregnancy-associated plasma protein A (PAPP-A), Alpha fetoprotein (AFP)+/- Placental growth factor (PlGF) in the first trimester. MSS-Quad consists only of a second trimester blood draw including four biomarkers: Alpha fetoprotein (AFP), beta-human chorionic gonadotrophin (bhCG), Unconjugated estriol (UE3), and inhibin A; it is typically offered to pregnant individuals who cannot access a nuchal translucency ultrasound or who present later to care. The sensitivity of multiple marker screening in Ontario to detect trisomy 21 is 86.3%, with a specificity of 95.0% and a positive predictive value of 3.9%.[16]

Table 1.1. Multiple Marker Screening Modalities in Ontario

Modality	Biomarkers	Screening cutoff for trisomy 21	Screening cutoff for trisomy 18	Time during which the modality was offered
4-marker enhanced First Trimester Screening (4-marker eFTS)	Nuchal translucency, total beta human chorionic gonadotropin, alpha fetoprotein, pregnancy-associated plasma protein A. **alpha fetoprotein is not used in the risk calculation for trisomy 18*	1 in 350	1 in 200	May of 2017 - current
5-marker enhanced First Trimester Screening (5-marker eFTS)	Nuchal translucency, free beta human chorionic gonadotropin, alpha fetoprotein, pregnancy-associated plasma protein A, placental growth factor. **alpha fetoprotein and placental growth factor are not used in the risk calculation for trisomy 18*	1 in 350	1 in 200	April of 2016 - current
First Trimester Screening (FTS)	Nuchal translucency, total or beta human chorionic gonadotropin, pregnancy-associated plasma protein A.	1 in 350	1 in 200	2001 to January of 2018
Maternal Serum Screening (MSS-Quad)	alpha fetoprotein, total chorionic gonadotrophin, unconjugated estriol, inhibin A.	1 in 350, 1 in 200 prior to April 2020	1 in 200	2003 - current
Integrated Prenatal Screening (IPS)	First trimester: nuchal translucency, pregnancy-associated plasma protein A Second trimester: alpha fetoprotein, total chorionic gonadotrophin, unconjugated estriol.	1 in 200	1 in 200	2000 to January of 2018
Serum Integrated Prenatal Screening (SIPS)	First trimester: pregnancy-associated plasma protein A Second trimester: alpha fetoprotein, total chorionic gonadotrophin, unconjugated estriol.	1 in 200	1 in 200	2003 to January of 2018

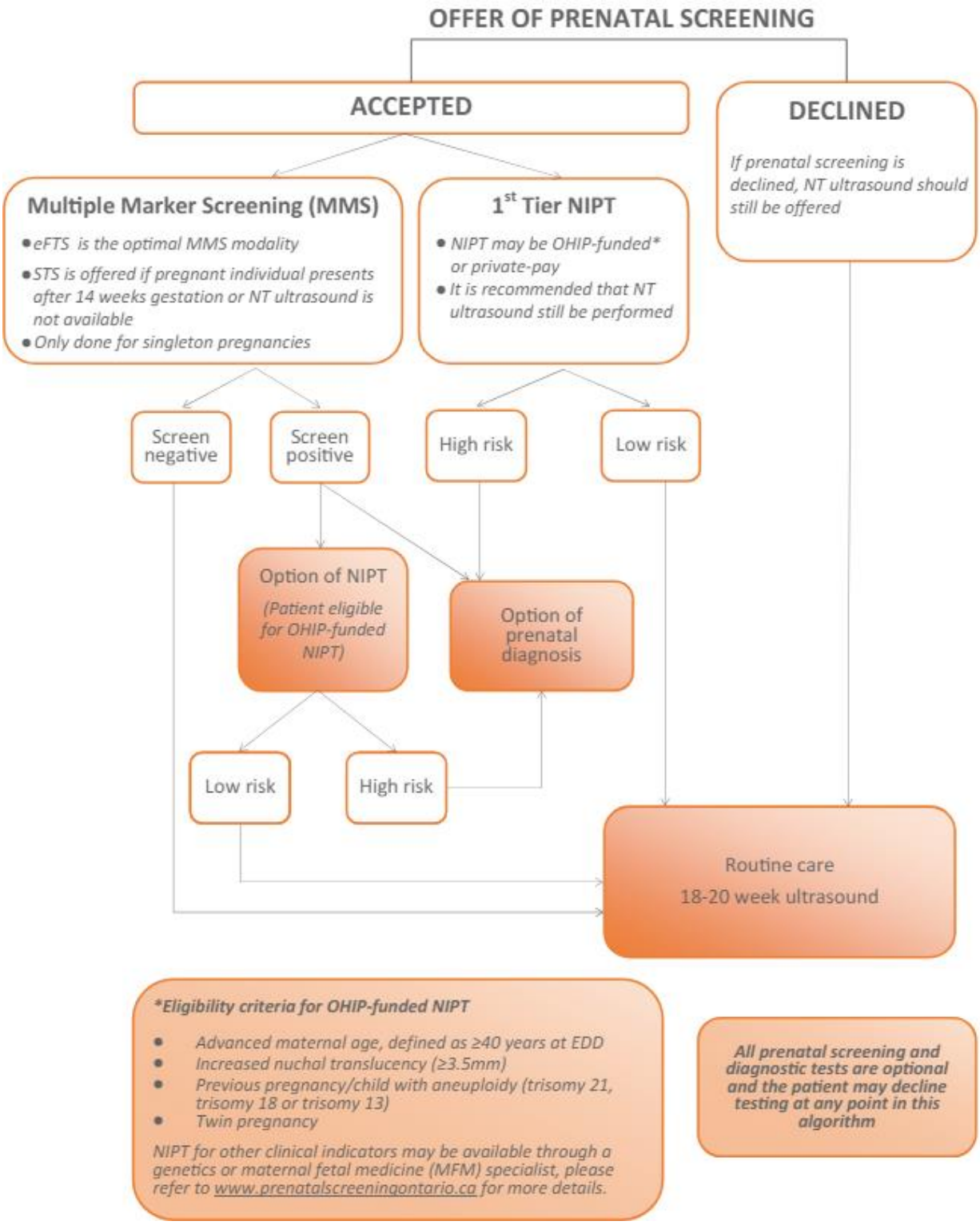
cfDNA screening is offered as a second-tier test following a screen positive multiple marker screen. For pregnancies deemed at higher risk for one of these aneuploidies (e.g., age of the pregnant individuals greater or equal to 40 years at estimated date of delivery, previous pregnancy with aneuploidy, ultrasound findings suggestive of chromosomal abnormalities), cfDNA screening is offered as a first-tier test. cfDNA screening became publicly funded for these indications in Ontario in 2014, and the analysis was patriated in 2016 to two dedicated

accredited laboratories in Ontario. Pregnant individuals not meeting the eligibility criteria for publicly funded cfDNA screening may self-pay at a cost of approximately $500 (as of 2024). Thus, the two laboratories established in Ontario perform all publicly funded screens, as well as self-paid screens. The laboratories use two different technologies: one laboratory uses the Single Nucleotide Polymorphism (SNP) Based Analysis while the other performs Chromosome Specific Sequencing through their Digital Analysis of Selected Regions (DANSR) technology.[24,35] The difference in technology allows one laboratory (the SNP based approach) to screen for triploidy, while the other cannot. Both laboratories also offer different testing for microdeletion syndromes: one is restricted to the 22q11.2 deletion, whereas the other offers screening for 22q11.2 deletion alone or as part of an expanded panel also including Cri-du-chat, 1p36 deletion, Angelman, and Prader-Willi syndromes. Given that current national and some international guidelines recommend against screening for microdeletions and microduplications,[36,37] screening for these conditions is not part of the publicly funded screening algorithm, and patients wishing to receive screening for these conditions must pay an additional cost.

Publicly-funded cfDNA screening in Ontario has a sensitivity of 99.8%, a specificity of 99.9% and a positive predictive value of 93.3% for trisomy 21.[16] The positive predictive value is considerably superior compared to multiple marker screening, which is why Ontario has implemented cfDNA screening as a mixed contingent model.

Confirmatory testing through cytogenetic analyses is required to establish the diagnosis following a screen positive result. There are a total of 9 cytogenetic laboratories in Ontario, housed in tertiary care centres: Children's Hospital of Eastern Ontario (CHEO), Mount Sinai Hospital, North York General Hospital, Credit Valley Hospital, London Health Sciences Centre, Hamilton Health Sciences, The Hospital for Sick Children, Health Sciences North, and Kingston Health Sciences Centre.

Figure 1.1. Prenatal Screning Algorithm Offered in Ontario, Canada



eFTS, enhanced first trimester screening; MSS, Maternal Serum Screening; NIPT, Noninvasive Prenatal Testing; OHIP, Ontario Health Insurance Plan; EDD, Estimated Date of Delivery

Illustration adapted from Prenatal Screening Ontario, permission for use granted

1.3 Rationale

1.3.1 Concepts of unanticipated or unclear prenatal genetic screening results

Although the formal goal of most prenatal genetic screening programs is to detect trisomy 21, the screens performed may yield unanticipated or unclear results, including results that are not targets of the screening but that could be clinically meaningful. Counselling pregnant individuals in these scenarios regarding the clinical implications of such findings can be difficult and limited, due to gaps in the literature.

Providing appropriate pre-test counselling to ensure informed decision making among pregnant individuals in general is challenging given the ever expanding testing options for prenatal genetic screening and given the wealth of information on other topics related to prenatal care that need to be shared with the patient.[38]

A previous study performed in Ontario reported that 28.1% of pregnant individuals surveyed did not receive any counselling before having prenatal genetic screening and 29.5% did not know it was optional.[39] In another study, only 54.0% of pregnant individuals reported the goal of their ultrasound was to screen for congenital anomalies.[40] The findings of these studies demonstrate a major gap in informed decision making, leading to patients potentially being unprepared to receive abnormal or unclear results.

Pregnant individuals may seek prenatal screening to offer reassurance that the fetus is healthy, thus not considering the eventuality that an abnormal or unclear result could arise.[41–43] These unanticipated or unclear results and the lack of information provided before screening can cause anxiety and confusion.[41] It is important to understand the uncertainty around these findings to identify the best way to address these results and offer the best care.

The medical community has struggled with concepts of uncertainty at different levels of care and even to define uncertainty in medical practice.[44] Uncertainty needs to be clearly defined to determine its source and impact in order to bridge gaps in knowledge. To address this, Han et al. developed a conceptual taxonomy of uncertainty encountered in healthcare.[44]

The taxonomy of uncertainty developed by Han et al. classifies uncertainty in healthcare into three dimensions: 1) sources of uncertainty (probability, ambiguity, complexity), 2) the issues caused by uncertainty (scientific, practical, personal), and 3) the locus of uncertainty (patient or clinician).[44] Among the sources of uncertainty, the concept of ***probability*** refers to the uncertainty of a future outcome, where it is expressed as a probability rather than a definitive result, ***ambiguity*** refers to a paucity of reliable, valid, or appropriate evidence for the risk estimates, and ***complexity*** represents uncertainty arising from aspects that can be difficult to comprehend. The ***scientific*** issues caused by uncertainty are data-centered and include concepts of uncertainty surrounding diagnosis, prognosis, causal explanations, and treatment recommendations. The ***practical*** issues of uncertainty are related to the healthcare system in terms of its structure and the processes of care. The ***personal*** issues of uncertainty pertain to concepts of uncertainty that are patient-centered and include psychosocial as well as existential concepts of uncertainty. The third dimension of the taxonomy defines where the uncertainty lies, or the ***locus*** of uncertainty, which can be with the patient or the clinician.

This taxonomy can easily be adapted to concepts of uncertainty in prenatal genetic screening, presented in Figure 1.2.

Figure 1.2. Adaptation of Taxonomy of Uncertainty by Han et al. to Unanticipated of Unclear Results in Prenatal Genetic Screening

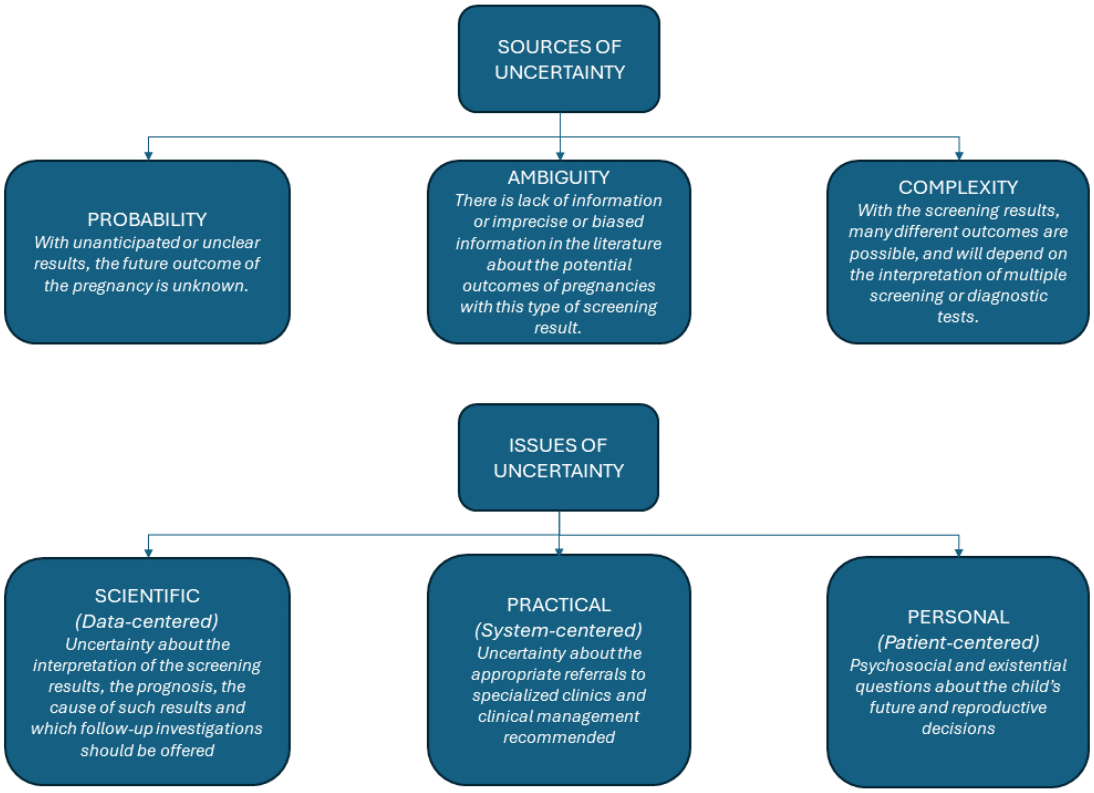


Image adapted from Han et al. 2011[44]

The proposed research project will aim to further our understanding of the implications of unanticipated or unclear results occurring during prenatal genetic screening, such as an increased nuchal translucency measurement or a multiple marker screening result positive for both trisomies 21 and 18 at once. The sources of uncertainty for these questions can mostly be attributed to the dimension of probability, where there is indeterminacy of the future outcome of pregnancies with these types of prenatal genetic screening results. The issues caused by these results are in the scientific and practical domains, with unclear prognosis and recommendations for clinical management in the context of such results; and contribute to personal uncertainty related to questions about reproductive decisions and the potential future of a child. While uncertainty about the potential future of the child to be born will always exist

in the prenatal context, the goal should be to bridge the gap in scientific and practical uncertainty to allow providers to provide evidence-based care to their patients.

The goal of the studies included in this thesis was therefore to provide evidence that can be used in counselling and facilitate informed decision making for patients and can provide a foundation for studies to inform policy decisions about screening programs.

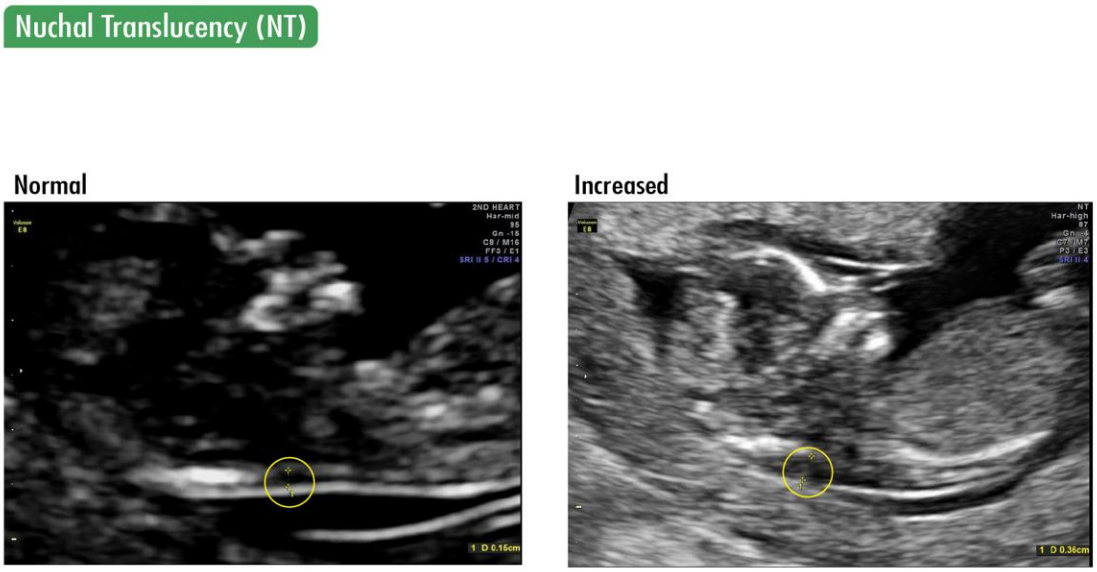
1.3.2 Increased nuchal translucency measurement

Nuchal translucency is a measure of subcutaneous fluid collection at the back of the fetal neck. It can be measured when the fetus has a crown-rump length of 45 mm to 84 mm, which corresponds to approximately 11 weeks to 13 weeks and 6 days gestation (Figure 1.3).[4] The ultrasound examination during which the nuchal translucency is measured is not typically offered as a stand-alone examination, but rather as one of the components of multiple marker screening for Down syndrome, and this is how it is usually presented to pregnant individuals. It may, thus, suggest to pregnant individuals that the examination screens for Down syndrome alone, and be a source of shock and confusion if they are told that follow-up investigations for conditions beyond Down syndrome are recommended when the nuchal translucency measurement is increased. Indeed, an increased nuchal translucency, frequently defined as a measurement greater or equal to 3.5 mm, is associated with chromosomal abnormalities other than Down syndrome, monogenic disorders, as well as congenital structural anomalies, most notably heart defects.[37]

The nuchal translucency level at which there is an increased risk of chromosomal abnormalities is important to define, as it determines which pregnant individuals are offered further investigations including prenatal diagnosis. The traditional cutoff of 3.5 mm represents a measurement greater than the 99th percentile across all gestational ages at which the nuchal translucency can be measured.[4] Yet, recent studies have reported an increased risk of chromosomal abnormalities in pregnancies with nuchal translucencies below 3.5 mm and some authors recommend adopting a lower cutoff to offer chromosomal investigations.[45–50] In light of these recent findings reported in the literature, the clinical community in Ontario has shown

interest in revisiting the nuchal translucency measurement level at which prenatal diagnosis should be offered.

Figure 1.3. Illustration of Nuchal Translucency Measurement



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Defining the probability that a pregnancy with an increased nuchal translucency will result in an adverse outcome is also challenging. Most studies that have investigated this question have been descriptive and have only included pregnancies with an increased nuchal translucency and normal cytogenetic results on prenatal diagnosis.[51–59] There is no information on the outcome of the pregnancies that have not had prenatal diagnosis, creating selection bias and risk measures that are difficult to apply when counselling a pregnant individual at the time the increased nuchal translucency is identified.

Therefore, more robust evidence is critically needed to guide pregnant individuals when an increased nuchal translucency measurement is identified, and to define whether the current cutoff at which investigations for chromosomal abnormalities are offered is appropriate.

1.3.3 Double-positive multiple marker screening results

In a small number of pregnancies, multiple marker screening results are positive for both trisomy 21 and trisomy 18; this is referred to as a double-positive result. A study of pregnancies screened by the California Prenatal Screening Program reported this type of result in 0.4% of pregnancies screened.[60]

The implications of this type of result remain unclear, as only one study analyzed the outcomes of a small cohort of 33 pregnancies with a double-positive result with normal cytogenetic results on prenatal diagnosis.[61] This study reported that these pregnancies were at increased risk of spontaneous abortion, anomalies on ultrasound, and were associated with a lower gestational age at birth, compared to pregnancies with screen negative results matched on age of the pregnant individual.[61]

Because of the limited information in the literature on pregnancies with double-positive multiple marker screening results, counselling of pregnant individuals experiencing this clinical scenario is particularly challenging. A large study of the association between a double-positive result on multiple marker screening and adverse pregnancy outcomes is needed to provide guidance about the best follow-up care for these pregnant individuals, including whether a low-risk cfDNA screening result is reassuring, or if there are risks of chromosomal abnormalities not detectable by cfDNA screening.

1.3.4 Perinatal and Obstetrical Outcomes

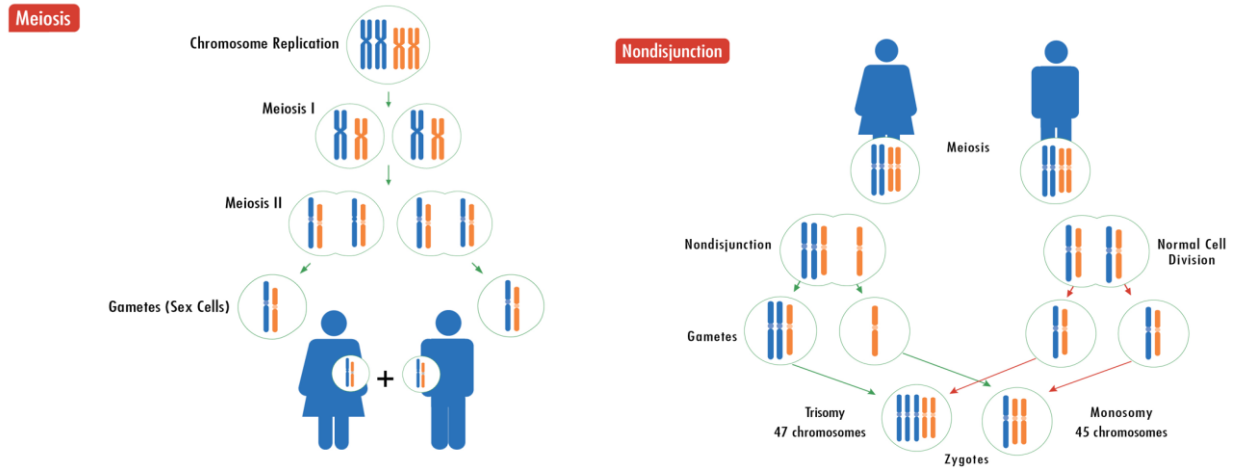
1.3.4.1 Chromosomal abnormalities

1.3.4.1.1 Aneuploidies

An aneuploidy occurs when there is an imbalance in the number of chromosomes, either with a full extra chromosome (**trisomy**) or a missing chromosome (**monosomy**). It is estimated that an aneuploidy occurs in at least 5% of recognized conceptions.[11] Conversely, the term euploid refers to the expected number of chromosomes in the cell, 23 pairs of chromosomes.[11]

The most common mechanism leading to aneuploidy is non-disjunction that occurs before conception, during the creation of the gamete cells. The genetic material is stored in cells in a diploid form (two pairs of chromosomes) but is then divided into haploid cells (containing a single copy of each chromosome), the gametes, so that the genetic material is diploid, with half of the genetic material from the egg and the other half from the sperm. Nondisjunction occurs when the chromosomes fail to separate into haploid cells, resulting in a gamete with an unbalanced number of chromosomes, either 47 chromosomes, resulting in a trisomy, or 45 chromosomes, resulting in a monosomy (Figure 1.4).[11]

Figure 1.4. Illustration of Meiosis and Nondisjunction Leading to Aneuploidy.



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A triploidy occurs when there are 3 copies of each chromosome. This can originate from the fertilization of an egg by two sperms or from a diploid (two copies of chromosomes) egg or sperm, when it should be haploid (single copy of chromosomes). Triploidy occurs in 1-3% of recognized conceptions, and is not compatible with life, typically resulting in an early pregnancy loss.[11]

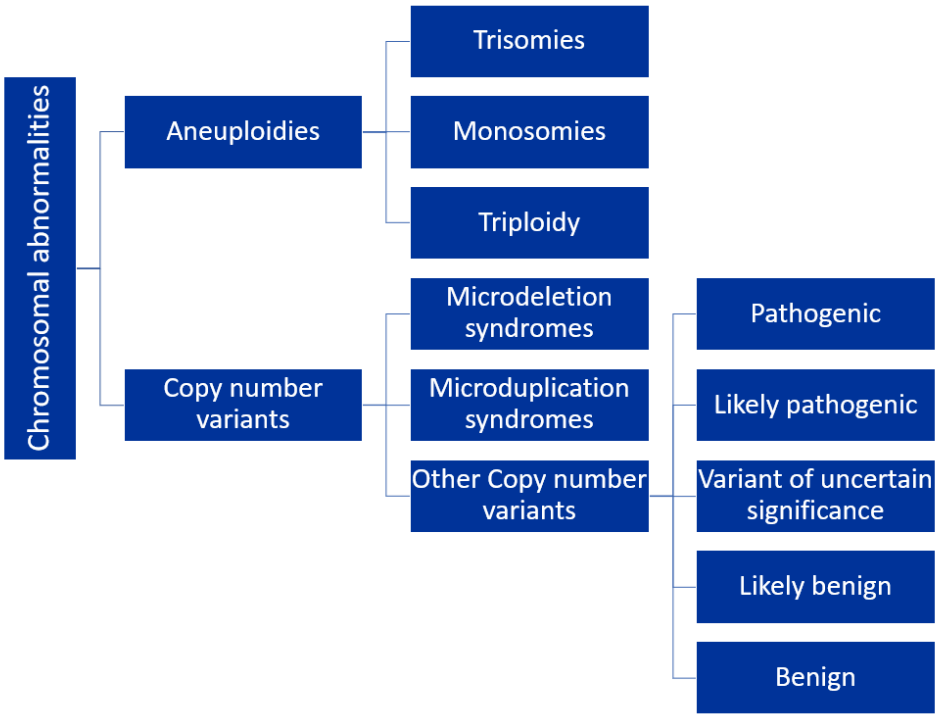
1.3.4.1.2 Copy number variants

Copy number variants (CNVs) are small variations in the genome, of sections either duplicated or deleted. They can be found in healthy individuals, mostly smaller variants, and can represent normal variation between individuals, or can be associated with clinically recognizable syndromes. Small CNVs from 1000 nucleotide base pairs up to hundreds of kilobase pairs can be

found in 5-10% of the general population and can be inherited.[11] The clinical manifestations of these CNVs will depend on the genes included in the region, if they are in excess or deficit, and on the size of the deletion or duplication. CNVs are classified by degree of pathogenicity (Pathogenic, Likely pathogenic, Variant of uncertain significance (VUS), Likely benign, Benign) according to professional guidelines.[62] The Society of Obstetricians and Gynaecologists of Canada (SOGC)-Canadian College of Medical Geneticists (CCMG) recommend against the reporting of copy number variants of unknown significance or very small deletions or duplications in the prenatal screening context in order to minimize uncertainty and parental anxiety.[30]

Microdeletion or microduplication syndromes are recurring CNVs, with consistent breakpoints in the genome that occur because of the architecture of the genome surrounding these regions (summary, Figure 1.5).[63]

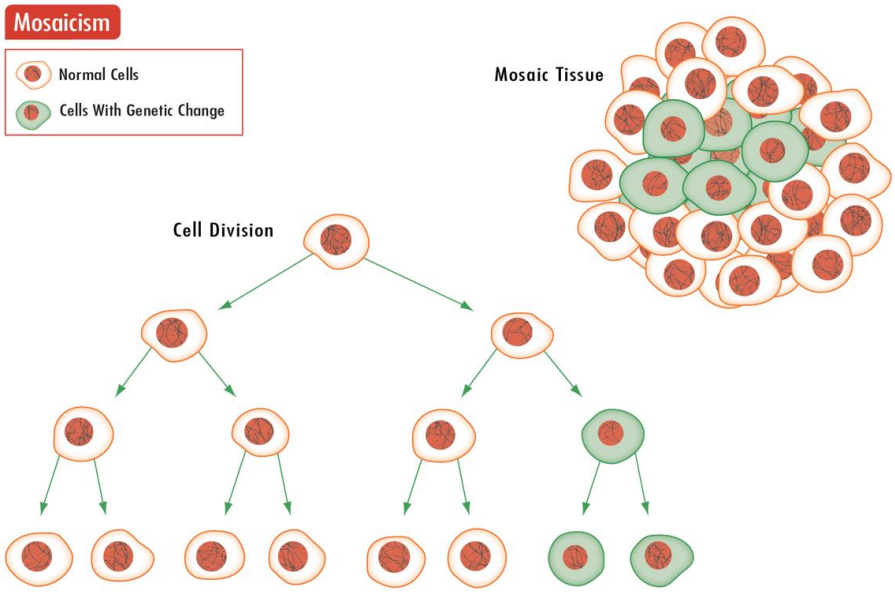
Figure 1.5. Illustration of Types of Chromosomal Abnormalities and Terminology.



1.3.4.1.3 Mosaicism

Mosaicism occurs when there is more than one cell lineage, and results in chromosomal abnormalities in only a portion of the genetic material (Figure 1.6).[11] It can be confined to the placenta (confined placental mosaicism), to the fetus, or be present in both.[11] When mosaicism is identified, careful consideration of the origin of the genetic material tested must therefore be taken into account to correctly interpret the results. At times, follow-up investigations with a sample originating from a different type of tissue is necessary (e.g., amniocentesis to follow-up mosaicism identified on chorionic villus sampling).[11]

Figure 1.6. Illustration of Mosaicism.



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1.3.4.4 Cytogenetic testing

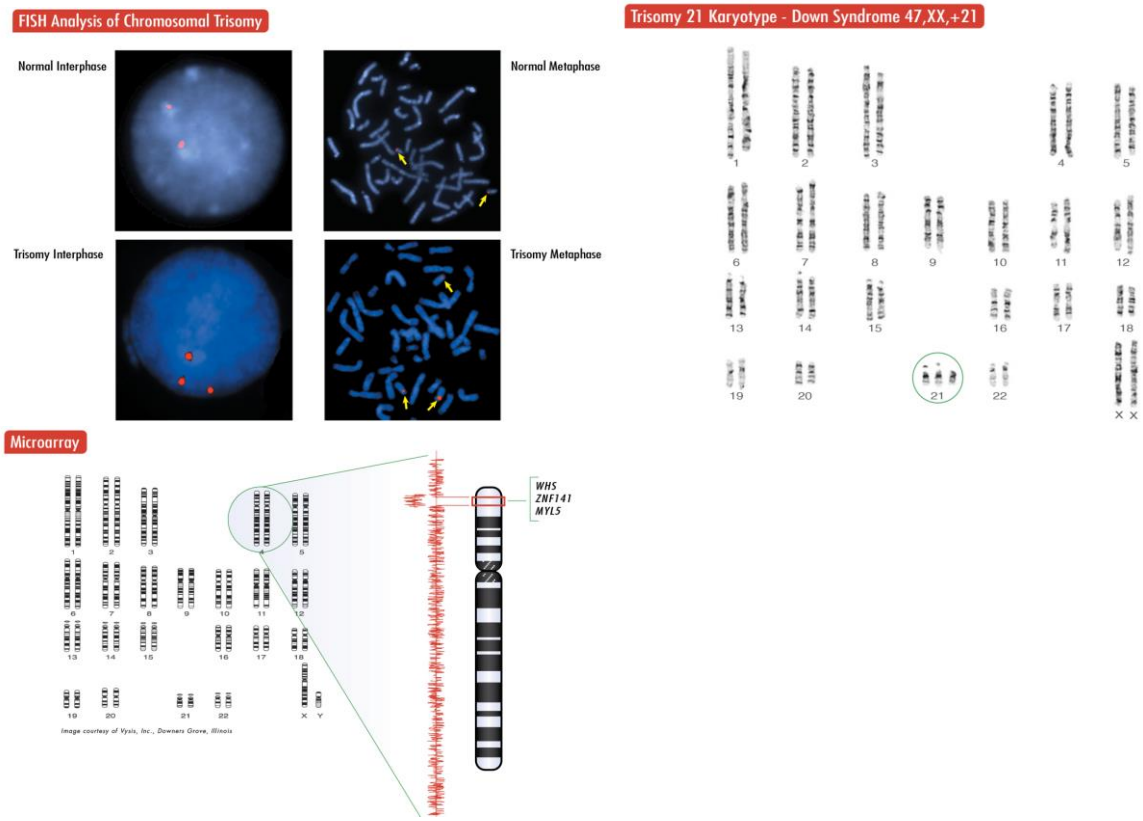
Fluorescent In Situ Hybridization (FISH): FISH analysis uses a probe to detect specific regions of the genome (Figure 1.7). It allows for the quick identification of the presence or absence of specific microdeletions for instance and can be used for Rapid Aneuploidy Detection (RAD) for aneuploidies of interest such as trisomies 21, 18, 13 and sex chromosome aneuploidies.

Qualitative Fluorescent Polymerase Chain Reaction (QF-PCR): QF-PCR is also commonly used in Rapid Aneuploidy Detection for aneuploidies of interest such as trisomies 21, 18, 13 and sex chromosome aneuploidies.[64]

Karyotype: A karyotype is performed to examine all chromosomes in a cell in metaphase. Specific staining procedures are done, and the chromosomes can be identified through recognizable banding patterns.[11] Aneuploidies, chromosomal rearrangements and larger deletions or duplications can be identified through a karyotype.[11]

Microarray: Analyses using microarray technologies (comparative genome hybridization, single nucleotide polymorphism (SNP) arrays) also examine all chromosomes, but at a greater resolution than a karyotype.[11] This allows the detection of copy number variants, small microdeletions or microduplications.[11]

Figure 1.7. Illustration of Cytogenetic Tests.



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1.3.4.5 Prevalence of chromosomal abnormalities

Determining the prevalence of chromosomal abnormalities is quite challenging, primarily because diagnostic investigations are not performed among the general population, but rather only when a chromosomal abnormality is suspected, thereby making the group tested at high-risk and not representative of the general population. This is especially true for the prevalence of chromosomal abnormalities in the antenatal period, as cytogenetic diagnosis would require a prenatal diagnostic procedure associated with a risk of pregnancy loss and would therefore not be routinely offered unless clinically indicated.[29] Studies have nonetheless attempted to define the prevalence of chromosomal abnormalities in the general population by reporting on the findings in for instance, prenatal diagnostic procedures performed following a request from the pregnant individual. A 2021 study by Stern et al. reported a chromosomal finding in 15 (2.2%) of the 673 prenatal diagnostic procedures performed following parental request overall, and 7 (1.0%) when excluding variants of unknown significance.[65]

1.3.4.2 Adverse perinatal outcomes

1.3.4.3.1 Pregnancy loss

Pregnancy loss is also referred to as miscarriage or spontaneous abortion and is defined as the loss of an intrauterine pregnancy that occurred before time of viability.[66] In Canada, this is defined as a loss before the 20th week of gestation.[67] The incidence of pregnancy losses is challenging to measure, as some losses may occur before the individual is even aware of the pregnancy in the case of a spontaneous pregnancy.[68] The Public Health Agency of Canada reports that 15-25% of pregnancies will result in a miscarriage.[67] A high proportion of these early losses can be attributed to chromosomal abnormalities,[69] but cytogenetic testing is not routinely performed on all products of conception, and therefore these early losses may not be captured. Indeed, early pregnancy losses are generally challenging to capture in the context of a registry. For example, in the BORN registry in Ontario, the context for this dissertation, while

losses >20 weeks gestation or >500g are routinely collected, only some of the pregnancy losses <20 weeks are captured.

1.3.4.3.2 Pregnancy termination

Pregnancy terminations, or induced abortions, can occur for a variety of reasons, including personal or social reasons, but also medical indications, in instances where a diagnosis of a condition not compatible with life has been established, where there are structural anomalies, or due to concern for the health of the pregnant individual.[67] The reasons for the termination cannot always be identified in the context of research through secondary use of data documenting terminations, and therefore the clinical implications of this event can be difficult to establish. In the context of prenatal screening, individuals should be offered prenatal diagnosis confirming the diagnosis before making reproductive decisions to avoid the scenario where the pregnancy would be interrupted on the basis of a false positive screening result. Pregnant individuals may however choose to end the pregnancy based on ultrasound findings alone, without an established diagnosis. Cytogenetic testing can be performed on products of conception if it is indicated. The Public Health Agency of Canada reported a pregnancy termination rate of 0.6 per 1,000 births in 2014 for pregnancies of greater than 500 g and 20 weeks gestation, but the incidence of terminations in early pregnancy is more difficult to establish. The Canadian Institute for Health Information reported a total of 87,595 induced abortions in Canada in 2021, 34,903 of which occurred in Ontario.[70]

1.3.4.3.3 Stillbirth

While different definitions of stillbirth exist, in Canada a stillbirth is defined as the death of the fetus after 20 completed weeks of gestation or with a birth weight greater than or equal to 500g.[67,71] Stillbirths occur during the antepartum period and are often referred to as intrauterine fetal death or intrauterine fetal demise, or can occur during the intrapartum period. The incidence of stillbirths varies widely by country, with the vast majority occurring in low and middle-income countries.[72] The Public Health Agency of Canada reported a rate of

stillbirth of 8.3 per 1,000 births in 2017.[67] Unfortunately, the cause of most stillbirths is unknown, but approximately 10-20% are estimated to be attributable to chromosomal abnormalities.[73]

1.3.4.3.4 Preeclampsia

Preeclampsia is a hypertensive disorder of pregnancy that is estimated to occur in 4.6% (95% confidence interval 2.7–8.2) of pregnancies globally.[74] it is diagnosed following the onset of hypertension after 20 weeks of completed gestation, accompanied by the onset of one or more of the following conditions: proteinuria, organ dysfunction in the pregnant individual, or uteroplacental dysfunction.[75]

The exact pathophysiology of preeclampsia has yet to be determined, but many theories refer to issues during the process of placental implantation.[76,77] Important risk factors associated with preeclampsia include nulliparity, older age, multiple gestations, higher body mass index, conception through assisted reproductive technologies, and personal or family history of preeclampsia.[77,78]

Preeclampsia can lead to acute and long-term complications for the pregnant individual and the fetus. Namely, for the pregnant individual, there is an increased risk of placental abruption, renal failure, complications involving the central nervous system, and ultimately death.[76] Studies have also reported an association with a long-term post-pregnancy increased risk of cardiovascular disease.[68]

Further, preeclampsia can have important consequences for the fetus, related to the risk of preterm birth and intrauterine growth restriction.[68]

1.3.4.3.5 Mode of delivery

Delivery by cesarean section is a common surgical procedure, representing the most frequent inpatient procedure to take place in North America, and has significantly increased in frequency over the years.[68,79] While a vaginal delivery is preferable due to its associated lower risk of

complications and shorter hospital stays, sometimes a cesarean delivery is medically indicated due to indications such as fetal distress.[68,79] The World Health Organization has stated that rates of cesarean section should not be higher than 10-15%.[80] The Canadian Institute for Health Information reported a rate of cesarean section of 31.0 per 100 births (95%CI 30.8-31.2) in Canada in 2020, and 31.9 (95%CI 31.7-32.2) in Ontario.[81]

1.3.4.3.6 Preterm birth

Preterm birth is defined as a live birth occurring at less than 37 weeks of gestation, and is the leading cause of infant morbidity and mortality globally.[68,82] The incidence of preterm birth varies globally, representing overall 9.9% of live births worldwide.[83] The Public Health Agency of Canada reported in 2017 that 8.2% of live births in Canada were preterm.[84]

Preterm births can occur spontaneously, and can be caused by premature preterm rupture of the membranes, or preterm labour.[85] In other instances, preterm birth can be induced due to pregnancy-related conditions such as preeclampsia, or fetal conditions such as intrauterine growth restriction.[85]

Neonates born prematurely can experience higher rates of respiratory and feeding difficulties, seizures, hypoglycemia, jaundice, and are more likely to experience rehospitalisation.[86]

Beyond the increased risk of infant mortality, preterm birth, particularly very preterm birth (defined as a birth at less than 28 weeks gestation), can also result in important sequelae for the infant and have an impact well into adulthood.[86] Indeed, there is an increased risk of neurodevelopmental conditions including intellectual disability, cerebral palsy, developmental delay, and visual and hearing deficits.

1.3.4.3.7 Fetal Growth Restriction, Small for gestational age

Fetal Growth Restriction (FGR), or Intrauterine Growth Restriction (IUGR) is defined by the Society of Obstetrician and Gynaecologists of Canada as "*a condition in which a fetus does not attain its genetically conferred growth potential because of an underlying pathology*".[87] The

growth restriction can be caused by pregnancy-related, fetal (including congenital anomalies), or placental factors and is associated with increased neonatal mortality and morbidity, and can even contribute to adult chronic disease.[68,88,89] The diagnosis of fetal growth restriction can be challenging, and is easily missed unless multiple accurate measures of fetal weight are taken over time.[68,90,91]

Historically, fetal growth restriction was defined as births under the 10th percentile for each gestational age.[90] Today, small for gestational age is commonly used as a proxy for growth restriction, however it must be noted that this metric will include healthy neonates who are simply constitutionally smaller than what is expected, and should not be interpreted definitively as fetal growth restriction.[90] Being small for gestational age is still, however, associated with neonatal mortality and morbidity.[92] A neonate is said to be small for gestational age when the birth weight is below the 10th percentile corresponding to their gestational age and sex.[82]

Another measure commonly used is low birth weight, defined as a weight of less than 2,500g.[68,92] However, a significant proportion of neonates with low birth weight will have experienced low birth weight due to a premature delivery, rather than representing a symptom of growth restriction. Low birth weight is associated with infant mortality and morbidity.[92]

The indicator of fetal growth restriction considered in the studies presented in this dissertation was small for gestational age. Although we recognize the limitations of this measurement, the data available only allowed us to systematically measure this indicator for all births.

1.3.4.3.8 APGAR score

The APGAR score is a metric used at birth to determine the overall well-being of the neonate.[68] A score of either 0, 1, or 2 is allocated for the following 5 components: heart rate, respiration, muscle tone, reflexes, skin color. It is calculated 1 minute after the birth, and repeated at 5 minutes.[68] The score is interpreted as normal if it is greater or equal to 7 at 1 minute, and greater or equal to 9 at 5 minutes.[68]

1.3.4.3.9 Admission to neonatal intensive care unit

Newborns can be transferred to the neonatal intensive care unit, either within the same hospital in which the delivery took place, or transferred to a different hospital if the birthing hospital does not provide the level of care required. Some hospitals have the practice of routinely transferring newborns to the neonatal intensive care unit for a period of observation. In Canada, a 2011 study reported that overall 11.1% of live births were admitted to the neonatal intensive care unit, and 13.3% in Ontario.[93] In Ontario, 35.6% of the neonates transferred to a neonatal intensive care unit were admitted for less than 24 hours.[93]

Admission to the neonatal intensive care unit was used as a general proxy for neonatal health conditions or complications in this dissertation. Because, as mentioned, some hospitals in Ontario have a policy of routinely transferring neonates to the intensive care unit for a short period of observation, this outcome was further refined by analyzing the length of stay in the neonatal intensive care unit.

1.3.4.3.10 Neonatal death

A neonatal death is defined as such if it occurs under 28 days postnatally.[67] To distinguish from stillbirths, the neonatal death must occur following a live birth.[94] In Canada, neonatal deaths occurred at a rate of 3.5 per 1,000 live births in 2022, and are largely driven by prematurity (37.6%) and congenital anomalies (21.4%).[67,95]

For the studies included in this dissertation, information on neonatal death was available if it occurred during the admission for the delivery. A neonatal death in our case was therefore defined as a death that occurred following delivery or during the birth admission after a live birth was recorded, under 28 days postnatally.

1.4 Research Aims, Objectives, and Hypothesis

The aim of this doctoral thesis project was to generate high-quality evidence to improve our understanding of the implications of unanticipated or unclear prenatal genetic screening results by studying specific clinical scenarios for which there was currently only limited and/or biased evidence available in the literature, hindering patient counselling.

The objectives of the thesis were:

1) To determine cytogenetic outcomes (Manuscript 1) and pregnancy outcomes (Manuscript 2) following an increased nuchal translucency measurement on a population-level; and
2) To examine the outcomes of pregnancies with a double-positive multiple marker screening result in which trisomies 21 and 18 have been excluded using a population-based retrospective cohort study design (Manuscript 3).

The main hypothesis for this dissertation was that unanticipated or unclear prenatal genetic screening results can be associated with an increased risk of chromosomal abnormalities and adverse perinatal outcomes and may guide clinical management.

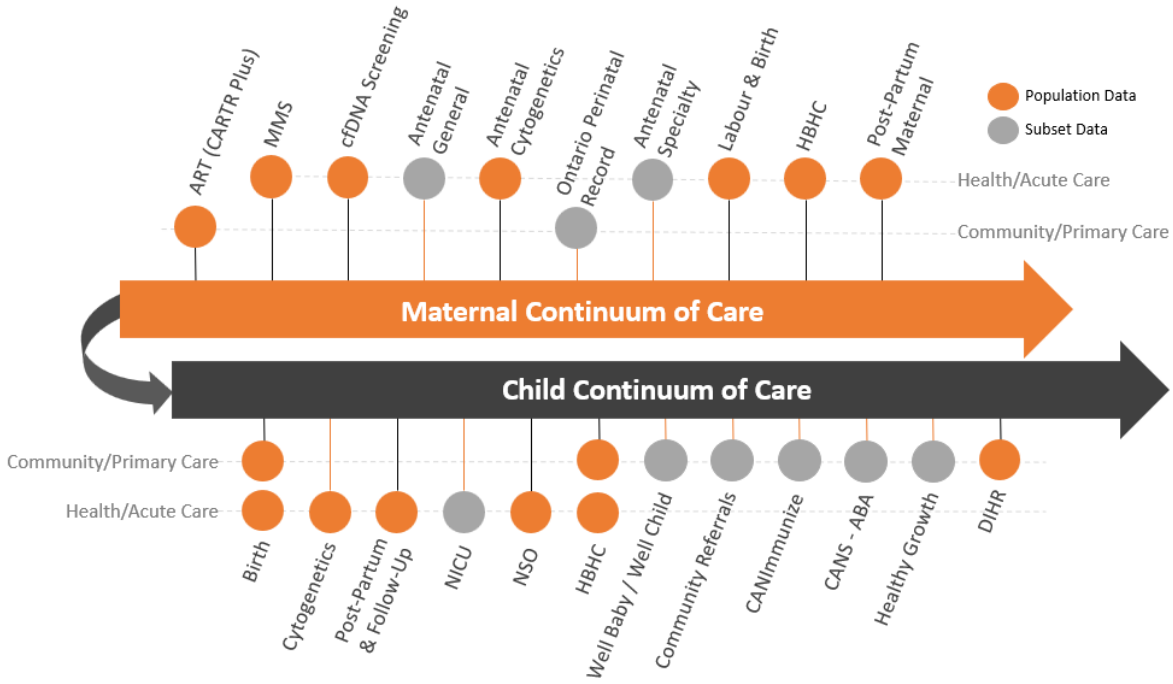
1.5 Overview of General Methodology

1.5.1 Data Sources

The data source used to conduct the studies presented in this dissertation is Ontario's prescribed maternal and child registry, the Better Outcomes Registry & Network (BORN). The registry collects data directly from all prenatal genetic screening laboratories in Ontario, both multiple marker screening (Mount Sinai Hospital, North York General Hospital, Credit Valley Hospital) and cfDNA screening (Dynacare, LifeLabs), as well as the 9 provincial cytogenetic laboratories (CHEO, Mount Sinai Hospital, The Hospital for Sick Children, Credit Valley Hospital, London Health Sciences Centre, Health Sciences North, North York General Hospital, Hamilton

Health Sciences Centre, and Kingston Health Sciences Centre).[34] Pregnancy and birth outcomes are captured in the registry for nearly all births in Ontario, approximately 140 000 each year.[96] The data sources for the registry are illustrated in Figure 1.8.

Figure 1.8. Data sources From the Better Outcomes Registry & Network (BORN) Ontario.



ART; Assisted Reproductive Therapy, MMS; Multiple Marker Screening, cfDNA screening; cell-free DNA screening, HBHC; Healthy Babies Healthy Children, NICU; Neonatal Intensive Care Unit, NSO; Newborn Screening Ontario, CANS-ABA; Child and Adolescent Needs and Strengths – Applied Behaviour Analysis, DIHR; Digital Health Immunization Repository
**The BORN Registry receives the Discharge Abstract Database metadata (DAD) from the Canadian Institute for Health Information (CIHI) annually, and these data are linked and incorporated within the registry*

Illustration adapted from BORN Ontario, permission for use granted

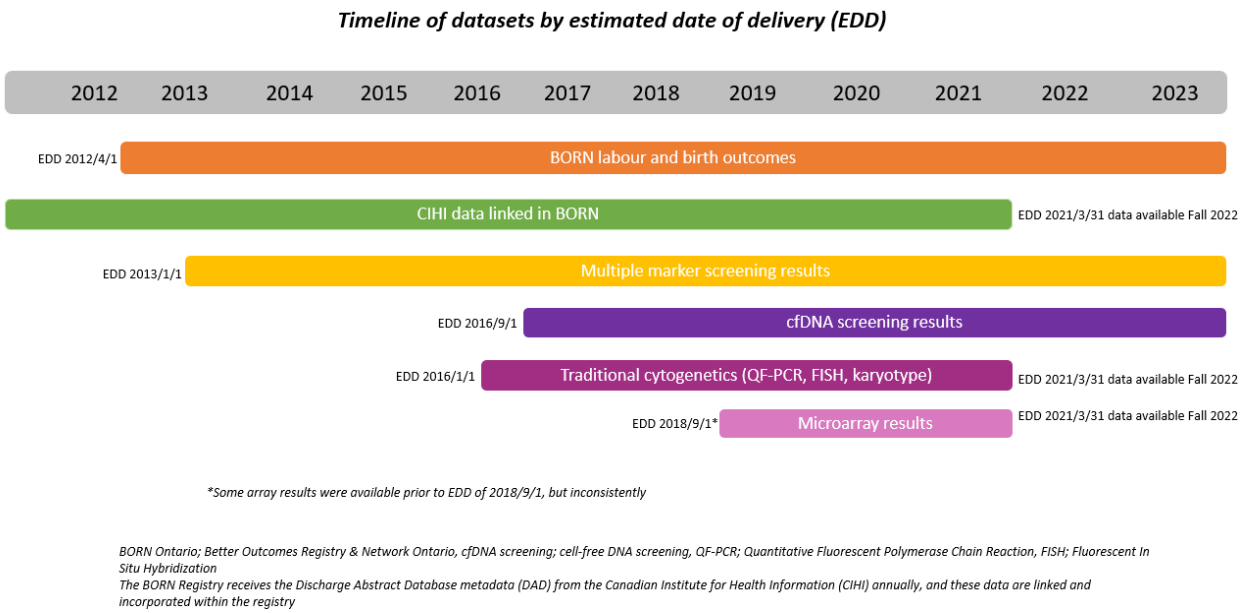
1.5.2 Study Design and Study Population

Population-based cohort studies were conducted with BORN data to obtain the statistical power needed to study the rare exposures and outcomes of interest for the studies. A population-based approach also allowed us to understand the different care pathways involved in Ontario's prenatal genetic screening system and how pregnant individuals use this system, which is not always as planned or expected by policy makers.[16] The study cohorts included singleton pregnancies with an estimated date of delivery from September 1, 2016 to March 31,

2021. The availability of each dataset based on estimated date of delivery is presented in Figure 1.9.

Twin pregnancies and higher-order multiples were excluded from the studies as the prenatal genetic screening options are different, and the limited number of twins and higher-order multiples precluded a stratification based on the number of fetuses.

Figure 1.9. Timeline of Datasets by Estimated Date of Delivery (EDD).



1.5.3 Analytic Approach

The modified Poisson regression model with robust variance estimation was selected to conduct the analyses. This model can use the Poisson distribution for a binary outcome when applying a robust variance estimator to the regression parameters to obtain the appropriate standard errors. The intent in selecting this model was to generate risk ratios rather than odds ratios for ease of interpretation, and to generate both relative and absolute risk estimates.[97,98] Both measures of association were reported for all three studies as they provided different information important for the clinical interpretation of the study findings: absolute measures of

risk will be more appropriate to discuss the public health implications of the findings, while the relative measures contribute information about the strength of the associations.[99] The modified Poisson regression model was selected rather than a log-binomial model given it is less prone to issues of nonconvergence.[100] Further, the model can accommodate clustered data, which allowed us to account for individuals with more than one pregnancy included in the dataset.[97]

Potential confounders to include in the models were identified *a priori* through directed acyclic graphs (DAGs), which were used in accordance with recent guidelines.[101]

More details about methods specific to each study can be found in the corresponding chapters in the subsequent sections of this dissertation.

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Chapter 2. Manuscript 1: Cytogenetic outcomes following an increased nuchal translucency measurement

Title of manuscript: Ultrasonographic Fetal Nuchal Translucency Measurements and Cytogenetic Outcomes

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Publication status: The manuscript presented in this chapter was published by JAMA Network Open on March 26, 2024.

Citation details:

Bellai-Dussault K, Dougan SD, Fell DB, Little J, Meng L, Okun N, Walker MC, Armour CM, Potter BK. Ultrasonographic Fetal Nuchal Translucency Measurements and Cytogenetic Outcomes. *JAMA Netw Open.* 2024 Mar 4;7(3):e243689. doi: 10.1001/jamanetworkopen.2024.3689. PMID: 38530313; PMCID: PMC10966411.

2.1 Preface

This chapter presents the first manuscript in which we examined the association between nuchal translucency measurements and chromosomal abnormalities using a population-based retrospective cohort study with data from Ontario's prescribed perinatal registry, BORN Ontario. The manuscript in this chapter is presented as the final version submitted to the journal. The published manuscript is presented in Appendix C. The protocol for this study was approved by the research ethics board of the Children's Hospital of Eastern Ontario (protocol #22/03PE) and University of Ottawa (protocol #H-06-22-8234).

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Contribution Statement

Kara Bellai-Dussault led the study design, performed all analyses including extensive data quality reviews in the secure environment of the registry, interpreted the findings, and drafted the manuscript. The specific contributions for the other authors for this article are listed in the manuscript on pages 59-60.

Question

Is there an association between nuchal translucency measurements less than 3.5 mm and chromosomal anomalies?

Findings

In this population-based cohort study including 414 268 singleton pregnancies in Ontario, Canada, a significantly increased risk of chromosomal anomalies was associated with each increasing level of nuchal translucency measurement, compared with a reference group of pregnancies with nuchal translucencies less than 2.0 mm.

Meaning

The findings of this cohort study suggest that pregnancies with nuchal translucency measurements greater than 2.0 mm are at increased risk of chromosomal anomalies, indicated that the widely used threshold of 3.5 mm may need to be reexamined.

2.2 Abstract

IMPORTANCE

Ultrasonographic measurement of fetal nuchal translucency is used in prenatal screening for trisomies 21 and 18 and other conditions. A cutoff of 3.5 mm or greater is commonly used to offer follow-up investigations, such as prenatal cell-free DNA (cfDNA) screening or cytogenetic testing. Recent studies showed a possible association with chromosomal anomalies for levels less than 3.5 mm, but extant evidence has limitations.

OBJECTIVE

To evaluate the association between different nuchal translucency measurements and cytogenetic outcomes on a population level.

DESIGN, SETTING, AND PARTICIPANTS

This population-based retrospective cohort study used data from the Better Outcomes Registry & Network, the perinatal registry for Ontario, Canada. All singleton pregnancies with an estimated date of delivery from September 1, 2016, to March 31, 2021, were included. Data were analyzed from March 17 to August 14, 2023.

EXPOSURES

Nuchal translucency measurements were identified through multiple-marker screening results.

MAIN OUTCOMES AND MEASURES

Chromosomal anomalies were identified through all Ontario laboratory-generated prenatal and postnatal cytogenetic tests. Cytogenetic testing results, supplemented with information from cfDNA screening and clinical examination at birth, were used to identify pregnancies without chromosomal anomalies. Multivariable modified Poisson regression with robust variance estimation and adjustment for gestational age was used to compare cytogenetic outcomes for

pregnancies with varying nuchal translucency measurement categories and a reference group with nuchal translucency less than 2.0 mm.

RESULTS

Of 414 268 pregnancies included in the study (mean [SD] age of the pregnant individual at estimated delivery date, 31.5 [4.7] years), 359 807 (86.9%) had a nuchal translucency less than 2.0 mm; the prevalence of chromosomal anomalies in this group was 0.5%. An increased risk of chromosomal anomalies was associated with increasing nuchal translucency measurements, with an adjusted risk ratio (aRR) of 20.33 (95% CI, 17.58-23.52) and adjusted risk difference (aRD) of 9.94% (95% CI, 8.49%-11.39%) for pregnancies with measurements of 3.0 to less than 3.5 mm. The aRR was 4.97 (95% CI, 3.45-7.17) and the aRD was 1.40% (95% CI, 0.77%-2.04%) when restricted to chromosomal anomalies beyond the commonly screened aneuploidies (excluding trisomies 21, 18, and 13 and sex chromosome aneuploidies).

CONCLUSIONS AND RELEVANCE

In this cohort study of 414 268 singleton pregnancies, those with nuchal translucency measurements less than 2.0 mm were at the lowest risk of chromosomal anomalies. Risk increased with increasing measurements, including measurements less than 3.5 mm and anomalies not routinely screened by many prenatal genetic screening programs.

2.3 Introduction

Since the 1990s, ultrasonographic measurement of nuchal translucency—a collection of fluid behind the fetal neck[1]—has been included in prenatal genetic screening offered to pregnant individuals to identify trisomies 21 and 18, typically as a part of multiple-marker screening.[2,3] In addition to these aneuploidies, increased fetal nuchal translucency is associated with other chromosomal anomalies, single gene conditions, and structural defects.[4] The current practice in many jurisdictions, including Ontario, Canada, is to offer follow-up investigations when nuchal translucency is greater than or equal to 3.5 mm, which theoretically corresponds to the 99th percentile for all gestational ages.[5-9] These follow-up investigations may include prenatal cell-free DNA (cfDNA) screening and/or confirmatory diagnostic testing through cytogenetic analysis.

Many studies of the association between nuchal translucency measurement and chromosomal anomalies beyond trisomies 21 and 18 have been beset by methodological limitations such as selection bias, for example, by focusing on narrow or high-risk populations (eg, based at tertiary institutions)[10-14] or including only pregnant individuals who elected to have a prenatal diagnosis.[15-18] Studies have also been limited by failing to include a low nuchal translucency reference group, including a historical reference group only, having a small sample size, or including only prenatal cytogenetic testing results.[19-23] A small number of these studies[19,20,22] have provided preliminary evidence that pregnancies with nuchal translucency measurements that are elevated but still lower than 3.5 mm could also be at increased risk of clinically significant chromosomal anomalies. These findings require confirmation using robust methodological approaches in large, unselected samples with comprehensive follow-up to adequately assess the risk of chromosomal anomalies across the entire range of nuchal translucency measurements. In this study, we aimed to evaluate the association between all levels of nuchal translucency measurements and cytogenetic outcomes among pregnancies in Ontario, Canada, identified through a population-based provincial registry.

2.4 Methods

This study received approval from the research ethics boards of the Children's Hospital of Eastern Ontario and the University of Ottawa. The requirement of informed consent was waived owing to the use of deidentified patient data. All cell counts of less than 6 were suppressed to comply with the privacy requirements of the registry. The study followed the Reporting of Studies Conducted Using Observational Routinely–Collected Health Data (RECORD) reporting guideline.

Data Source

Better Outcomes Registry & Network (BORN) Ontario is a prescribed perinatal registry that collects data directly from all multiple-marker screening, cfDNA screening, and cytogenetics laboratories in Ontario.[24] Pregnancy and birth outcomes are also captured, including information on the clinical birth examination and linkage to discharge data from all hospitals through the discharge abstract database of the Canadian Institute for Health Information (CIHI). This enabled comprehensive ascertainment of pregnancy outcomes. Additional details on data sources are provided in Supplementary Table 2.1.

Setting and Study Population

Ontario offers a publicly funded screening program in which all pregnant individuals have access to multiple-marker screening in the first trimester, most often including a nuchal translucency measurement.[25] Cell-free DNA screening or cytogenetic testing is offered if the screen result is positive or as a first-tier screen if specific eligibility criteria are met.[26] Individuals may also self-pay for cfDNA screening.[26]

Nuchal translucencies are measured at crown-rump lengths of 45 to 84 mm by sonographers registered in Ontario's Nuchal Translucency Quality Assurance program.[27] This study included all singleton pregnancies in Ontario with a valid multiple-marker screening test including a nuchal translucency and with an estimated date of delivery (EDD) from September 1, 2016, to March 31, 2021 (Supplementary Figure 2.1). While racial origin is recorded in the BORN

database, we did not incorporate this information into our analysis as it was not expected to influence the associations studied.

Study Exposure

Nuchal translucency measurements for all pregnancies were identified from multiple-marker screening results. The reference group was defined as pregnancies with a measurement less than 2.0 mm, compared with pregnancies with the following categories of nuchal translucency measurements: 2.0 to less than 2.5 mm, 2.5 to less than 3.0 mm, 3.0 to less than 3.5 mm, 3.5 to less than 5.0 mm, 5.0 to less than 6.5 mm, and 6.5 mm or greater.

Study Outcome

Pregnancies with chromosomal anomalies were identified through cytogenetic testing results submitted by all Ontario cytogenetic laboratories to the BORN registry. The primary outcome was defined as any chromosomal anomaly identified on cytogenetic testing, during pregnancy or postnatally, including microarray analysis. As secondary outcomes, we stratified chromosomal anomalies by whether or not the condition is routinely tested through cfDNA screening in Ontario (trisomies 21, 18, and 13 and sex chromosome aneuploidies).

Because only a small number of pregnancies have cytogenetic investigations, we supplemented our outcome data with information from other sources (pregnancies with and without cytogenetic testing are described in Supplementary Table 2.2). Specifically, to identify pregnancies without chromosomal anomalies, we first used cytogenetic testing results if performed. If no cytogenetic testing results were available, we used cfDNA screening results, if performed; although a low-risk cfDNA screening result cannot be used clinically to exclude these conditions (trisomies 21, 18, and 13 and sex chromosome aneuploidies), for the purposes of this research, it was considered a reasonable proxy given its negative predictive value of greater than 99.9%.[28] Finally, for pregnancies with no cytogenetic testing and no cfDNA screening, we used results from the clinical examination at birth to exclude conditions typically clinically diagnosable at birth, relying on both BORN and CIHI data (Supplementary Table 2.3).

Statistical Analysis

Data were analyzed from March 17 to August 14, 2023. The study population was described using means (SDs) for continuous variables and frequencies and proportions for categorical variables. We used multivariable modified Poisson regression models with robust variance estimation and adjustment for gestational age at screening to compare the risk of chromosomal anomalies across pregnancies with varying categories of nuchal translucency measurements with the reference category (<2.0 mm).[29] This model also allowed us to account for clustering for individuals with more than one pregnancy within the study period.[30] Gestational age at screening was identified a priori as a potential confounder through directed acyclic graphs, as nuchal translucency measurements are on a continuum and will change with gestational age (Supplementary Figure 2.2). A post hoc analysis with additional adjustment for age of the pregnant individual was also performed. Adjusted risk ratios (aRRs) and risk differences (aRDs) were reported with 95% CIs.

We conducted the following sensitivity analyses to evaluate the potential impact of incomplete or inaccurate ascertainment of the exposure and outcome and to address losses to follow-up. All analyses were performed using SAS, version 9.4 (SAS Institute Inc), and 2-tailed $P < .05$ was considered statistically significant.

Exposure Measurement Source

When a very high nuchal translucency measurement or cystic hygroma is identified, some pregnant individuals may not complete the multiple-marker screening process and, thus, may not be ascertained by the laboratories. We therefore performed a sensitivity analysis identifying pregnancies with increased nuchal translucency measurements through other sources available within the registry, including data obtained from consultations with genetics or maternal fetal medicine clinics and from documented clinical indications for testing obtained from cytogenetic laboratories (Supplementary Table 2.4).

Exposure Definition

Some studies rely on percentiles of the nuchal translucency measurement rather than absolute values. Therefore, we categorized nuchal translucency measurements as less than 90th, 90th to less than 95th, 95th to less than 99th, and 99th percentile or greater[10,21] in an additional sensitivity analysis (Supplementary Table 2.5).

Losses to Follow-Up

For some pregnancies, no outcome was recorded. These may reflect pregnancy losses or terminations in the absence of follow-up cfDNA screening or cytogenetic testing or pregnant individuals who had multiple-marker screening in Ontario but subsequently received care outside the province. Because of the unclear nature of the outcome for these pregnancies, we performed a sensitivity analysis in which we randomly classified the losses to follow-up to having twice the prevalence of chromosomal anomalies compared with pregnancies in the same category of nuchal translucency measurement for which an outcome was recorded or half the prevalence (Supplementary Table 2.6). A further sensitivity analysis included all pregnancies with varying assumptions of risk of chromosomal anomalies for those lost to follow-up based on the pregnancy outcome (Supplementary Table 2.7).

Outcome

Complete cytogenetic data for microarray testing was only available since January 2018. Therefore, we conducted an analysis restricted to pregnancies with EDD from September 1, 2018, to March 31, 2021 (Supplementary Table 2.8).

Time Period

An additional sensitivity analysis excluded pregnancies with an EDD from April 1, 2020, to March 31, 2021. This exclusion accounted for potential effects of the COVID-19 pandemic on prenatal care practices (Supplementary Table 2.9).

2.5 Results

From 643 146 singleton pregnancies in Ontario during the study period, 414 268 were eligible for the analysis (mean [SD] age of the pregnant individual at EDD, 31.5 [4.7] years). Of these, 359 807 pregnancies (86.9%) had a nuchal translucency measurement less than 2.0 mm; 43 219 (10.4%), from 2.0 to less than 2.5 mm; 7474 (1.8%), from 2.5 to less than 3.0 mm; 1789 (0.4%), from 3.0 to less than 3.5 mm; 1088 (0.3%), from 3.5 to less than 5.0 mm; 404 (0.1%), from 5.0 to less than 6.5 mm; and 487 (0.1%), 6.5 mm or greater (Table 2.1). The mean (SD) age of the pregnant individual at EDD increased across nuchal translucency categories, from 31.5 (4.7) years for pregnant individuals with nuchal translucency measurements less than 2.0 mm to 33.3 (5.4) years for those with measurements 6.5 mm or greater. We excluded 225 264 pregnancies without a valid multiple-marker screening test including a nuchal translucency measurement, 158 with no screening result report issued, and 3456 where the measurement was performed outside the gestational age range corresponding to the crown-rump length of 45 to 84 mm.

Figure 1 and Figure 2 describe the uptake of follow-up investigations (cfDNA screening, cytogenetic testing) and pregnancy outcomes among pregnant individuals with nuchal translucency measurements less than 3.5 mm and 3.5 mm or greater, respectively. Of pregnancies with a nuchal translucency measurement 3.5 mm or greater, 1654 (83.6%) underwent follow-up investigations prenatally, compared with 44 849 (10.9%) of pregnancies with a measurement less than 3.5 mm.

Among those with nuchal translucencies 3.5 mm or greater, 414 (20.9%) had both cfDNA screening and prenatal diagnosis with cytogenetic testing, compared with 1747 (0.4%) among pregnancies with a measurement less than 3.5 mm. Of chromosomal anomalies identified in pregnancies with measurements of 3.5 mm or greater, 496 (72.5%) were identified prenatally, compared with 947 (35.3%) for measurements less than 3.5 mm (Supplementary Table 2.10).

Among pregnancies with cytogenetic testing results (n = 15 755), the proportion with chromosomal anomalies increased across nuchal translucency measurement categories, from

1913 pregnancies (16.6%) with nuchal translucency less than 2.0 mm to 256 pregnancies (70.1%) with a measurement 6.5 mm or greater (Table 2.2). To ascertain potential chromosomal anomalies in pregnancies that did not receive cytogenetic testing, 38 041 (98.2%) received a low-risk cfDNA screening result (Table 2.2). Next, among pregnancies with no cfDNA screening performed, we identified 331 638 documented live births with no notable clinical findings reported (Table 2.2). These results were used to estimate the proportion of pregnancies in the study with chromosomal anomalies, increasing from 1913 (0.5%) in pregnancies with nuchal translucency less than 2.0 mm to 256 (52.6%) in pregnancies with nuchal translucency 6.5 mm or greater (Table 2.2).

The risk of chromosomal anomalies increased with increasing nuchal translucency measurements (Table 2.3). The risk was markedly increased in pregnancies with nuchal translucency measurements from 3.0 to less than 3.5 mm relative to less than 2.0 mm (aRD, 9.94% [95% CI, 8.49%-11.39%]; aRR, 20.33 [95% CI, 17.58-23.52]).

The proportion of pregnancies excluded from the primary analysis due to an unknown outcome was associated with nuchal translucency category, from 9281 (2.6%) in the group with measurements less than 2.0 mm to 48 (9.9%) in the group with measurements 6.5 mm or greater (Table 2.2). We therefore conducted a sensitivity analysis randomly classifying the pregnancies lost to follow-up to have twice the prevalence of chromosomal anomalies compared with those for which an outcome was recorded within the given nuchal translucency measurement category, and results were mildly accentuated. The analysis assuming half the prevalence showed mildly attenuated results (Supplementary Table 2.6).

Table 2.3 further categorizes chromosomal anomalies into a group of conditions routinely screened by cfDNA screening (trisomies 21, 18, and 13 and sex chromosome aneuploidies) and a group of conditions beyond the cfDNA screening options consistently available in Ontario (other autosomal aneuploidies, triploidy, mosaic autosomal and sex chromosome aneuploidies, and copy number variants). The risk of chromosomal anomalies routinely screened by cfDNA screening increased with increasing nuchal translucency measurements: for the nuchal

translucency category of 3.0 to less than 3.5 mm relative to less than 2.0 mm, the aRD was 8.62% (95% CI, 7.27%-9.96%) and the aRR was 52.15 (95% CI, 43.98-61.84). The risk also increased but with weaker magnitude for the subgroup with other chromosomal anomalies (detailed in Supplementary Table 2.2): for nuchal translucency category of 3.0 to less than 3.5 mm relative to less than 2.0 mm, the aRD was 1.40% (95% CI, 0.77%-2.04%) and the aRR was 4.97 (95% CI, 3.45-7.17).

All additional sensitivity analyses showed comparable findings to the primary analysis, including analyses incorporating pregnancies with nuchal translucency measurements identified by sources other than multiple-marker screening (Supplementary Table 2.4); analyses restricted to a timeline with complete capture of microarray data, with an EDD from September 1, 2018, to March 31, 2021 (Supplementary Table 2.8); analyses excluding pregnancies with an EDD from April 1, 2020, to March 31, 2021, to assess potential effects of the COVID-19 pandemic (Supplementary Table 2.9); and post hoc analyses additionally adjusting for age of the pregnant individual (Supplementary Table 2.12).

Finally, for the pregnancies included in our study, the 99th percentile for nuchal translucency measurement was 2.8 mm, the 95th percentile was 2.2 mm, and the 90th percentile was 2.0 mm. When defining the exposure by nuchal translucency percentile, pregnancies with a measurement greater than the 99th percentile had a risk of any chromosomal anomaly 34.9 times greater than pregnancies with a measurement less than the 90th percentile (Supplementary Table 2.5).

2.6 Discussion

This population-based cohort study leveraged linked multiple-marker screening, cytogenetic testing, cfDNA screening, and birth registry data capturing pregnancy outcomes and findings from the clinical examination at birth to quantify the association of increased risk of chromosomal anomalies with increasing nuchal translucency measurement. We found a

strongly increased risk of chromosomal anomalies with increased nuchal translucency relative to values less than 2.0 mm, particularly for measurements of 3.0 mm or higher. The findings were consistent through several sensitivity analyses.

To our knowledge, this is the first population-based study assessing the risk of chromosomal anomalies across all levels of nuchal translucency measurements and incorporating information from antenatal as well as postnatal cytogenetic testing, cfDNA screening, pregnancy outcomes, and newborn clinical examinations. Indeed, most studies on this topic have focused on high-risk settings or have included only prenatal cytogenetic testing and, therefore, only represent a small proportion of pregnant individuals undergoing nuchal translucency ultrasonography.[11,13,14,16-18,21,31] The concern is that pregnant individuals who opted for cytogenetic testing in these studies may have had a higher risk of chromosomal anomalies, as additional findings beyond nuchal translucency measurement may have led them to have prenatal diagnostic testing; excluding pregnancies at lower risk for which outcomes are not available would, therefore, tend to overestimate the risk. This is particularly important when investigating nuchal translucency measurement values that would not independently trigger an offer of a follow-up investigation. As expected, we observed a substantial difference in the proportion of pregnant individuals who elected to have follow-up investigations prenatally in the group with nuchal translucency measurements less than 3.5 mm (10.9%) compared with the group with measurements of at least 3.5 mm (83.6%), illustrating the importance of including outcomes beyond cytogenetic results from prenatal diagnosis.

Our finding that nuchal translucency values below 3.5 mm, particularly those from 3.0 to less than 3.5 mm, are associated with chromosomal anomalies relative to values less than 2.0 mm, has important implications for prenatal genetic screening and counselling. Beyond the common aneuploidies (trisomies 21, 13, and 18 and sex chromosome aneuploidies) routinely identified by cfDNA screening in Ontario and included in many screening programs internationally,[32] we report that increased nuchal translucency measurements also yield an increased risk for other chromosome anomalies, although weaker. This has important policy implications given that in

some jurisdictions, prenatal cfDNA screening is offered following the identification of an increased nuchal translucency measurement, whereas in others, cfDNA screening is part of first-tier prenatal screening, with or without accompanying nuchal translucency measurement. Screening programs should consider the value of nuchal translucency measurements when making decisions about whether to replace such measurements with cfDNA screening alone and may wish to reexamine the threshold of nuchal translucency at which diagnostic investigations are offered.[10,32] Further research, including economic modeling to estimate benefits and costs, is needed to inform the best options for policy and practice.[10,32]

The results of this study also have implications for the quality assurance of nuchal translucency: the 99th percentile for nuchal translucency measurement was well below the expected 3.5 mm, implying that using a cutoff of 3.5 mm to offer follow-up investigations may not be sufficient. While the increased risk of chromosomal anomalies in pregnancies with nuchal translucencies less than 3.5 mm could be partly due to chronic undermeasurement, it is unlikely that this would be the only factor. Indeed, some jurisdictions have adopted thresholds to offer follow-up investigations much lower than the 99th percentile (eg, 95th percentile in Finland, the Netherlands, Germany, and Switzerland).[33] These findings also highlight the importance of a robust quality assurance program to support nuchal translucency measurement, as chronic undermeasurement can reduce the screening sensitivity.[34-39]

Limitations

An inherent limitation of this study is that cytogenetic outcomes were not available for all pregnancies, as cytogenetic testing is only offered under specific clinical indications. We therefore included additional information from cfDNA screening and pregnancy outcomes recorded in the birth registry to maximize ascertainment of chromosomal anomalies. Moreover, sensitivity analyses with varying assumptions about outcome ascertainment yielded the same conclusions.

Additionally, some chromosomal anomalies may not have clinically significant features at birth, prompting postnatal cytogenetic investigations, such that an infant could possibly be misclassified as not having a chromosomal anomaly. Because of the difference in ascertainment of pregnancies with nuchal translucencies less than 3.5 mm and 3.5 mm or greater, it is possible that we overestimated the risk of chromosomal anomalies in pregnancies with measurements of 3.5 mm or greater. For this reason, our findings allow us to draw conclusions on the association between nuchal translucency measurements and chromosomal anomalies that have clear features at birth, whereas more careful consideration is needed for chromosomal anomalies for which a clear phenotype is not expected at birth. Additionally, single-gene conditions associated with increased nuchal translucency measurements such as RASopathies are not captured in the registry and were therefore not included.[40] Although there is evidence that some factors may influence the choice to have prenatal genetic screening (eg, age of the pregnant individual, rural residence),[25,41] there is no reason to expect the association between the nuchal translucency measurement and chromosomal anomalies to differ in this population excluded from our study.

2.7 Conclusions

The findings of this population-based cohort study suggest that increased nuchal translucency measurements were associated with increased risk of chromosomal anomalies, even at values below the currently used standard threshold of 3.5 mm. These findings have important policy implications for setting the threshold nuchal translucency value such that further investigations may be offered to pregnant individuals and, in turn, contribute to determining the best approach to offering high-quality prenatal screening to pregnant individuals in Ontario and around the world.

Article Information

Accepted for Publication: January 11, 2024.

Published: March 26, 2024. doi:10.1001/jamanetworkopen.2024.3689

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Administrative, technical, or material support: Bellai-Dussault, Dougan, Walker, Armour, Potter.

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Conflict of Interest Disclosures: Ms Bellai-Dussault reported receiving grant funding from the Canadian Institute of Health Research (CIHR) during the conduct of the study. Dr Fell reported being employed by the University of Ottawa and having an academic appointment at the Children's Hospital of Eastern Ontario Research Institute during the conduct of the study; although she maintains those academic affiliations, she is now employed by Pfizer and works on an unrelated topic. Dr Little reported receiving grant funding from the Ontario Research Fund Genome Canada–CIHR outside the submitted work. Dr Okun reported serving as Comedical Director of Prenatal Screening Ontario for Better Outcomes Registry & Network Ontario during the conduct of the study. No other disclosures were reported.

Funding/Support: This study was supported by Frederick Banting and Charles Best Canada Graduate Scholarships Doctoral Award FBD-170866 from the CIHR (Ms Bellai-Dussault). Role of the Funder/Sponsor: The CIHR had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

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Figure 2.1. Investigations Following a Nuchal Translucency Measurement Ultrasound Under Current Practice: Pregnancies with Measurements <3.5 mm.

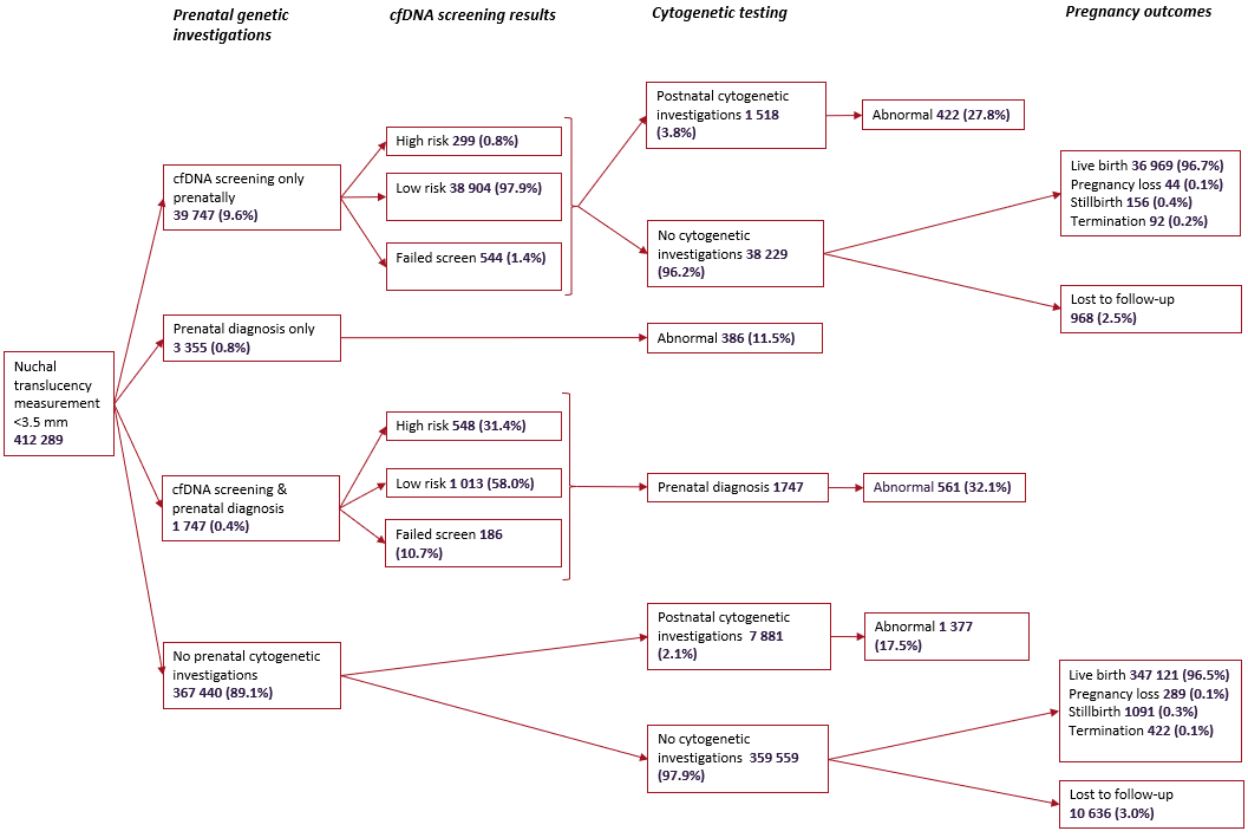


Figure 2.2. Investigations Following a Nuchal Translucency Measurement Ultrasound Under Current Practice: Pregnancies with Measurements ≥3.5 mm.

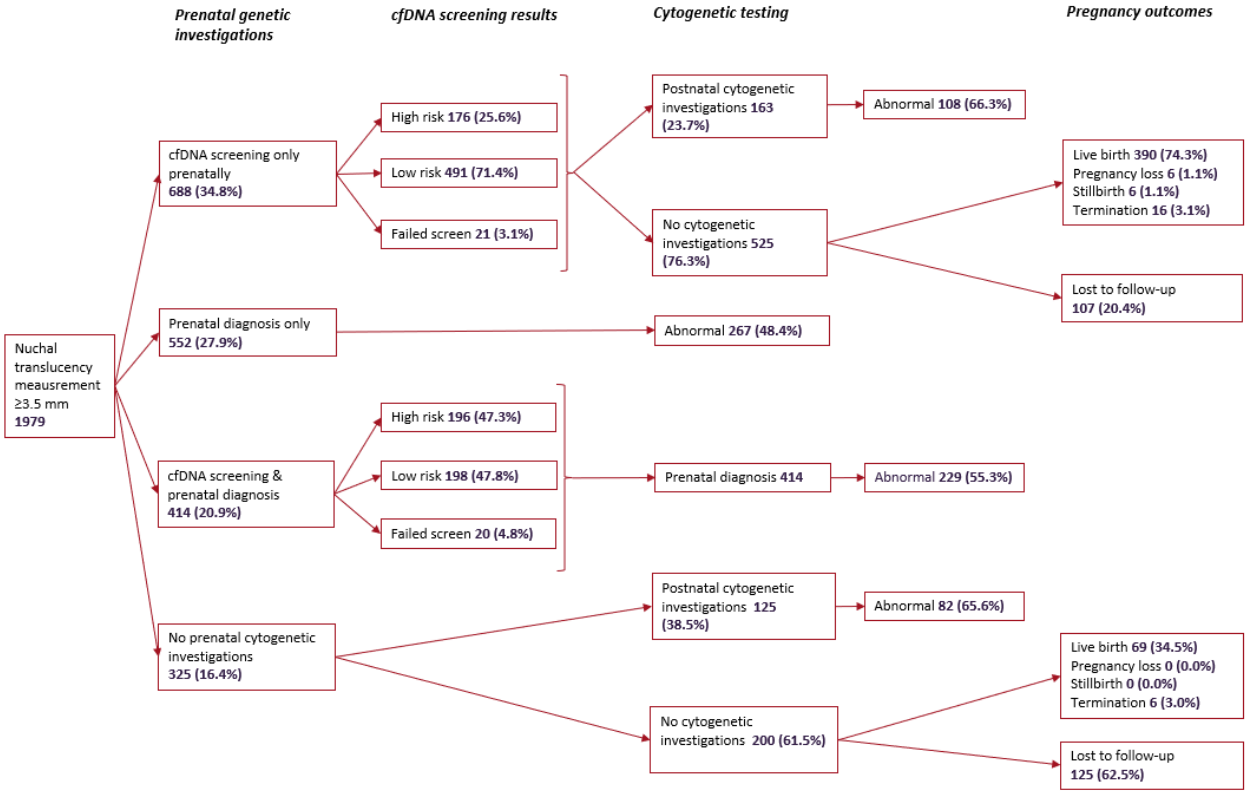


Table 2.1. Characteristics of Study Population by Nuchal Translucency Measurement.

Characteristic	All pregnancies with nuchal translucency measurement	Nuchal translucency measurement (mm)						
		<2.0	2.0-<2.5	2.5-<3.0	3.0-<3.5	3.5-<5.0	5.0-<6.5	≥6.5
Total pregnancies	**414 268**	**359 807**	**43 219**	**7 474**	**1 789**	**1 088**	**404**	**487**
Age at EDD, years Mean (SD)	**31.5 (4.7)**	31.5 (4.7)	31.6 (4.8)	31.9 (4.8)	32.2 (4.8)	32.8 (5.0)	33.0 (5.5)	33.3 (5.4)
Gestational age at screening, days Mean (SD)	**87.8 (3.3)**	87.5 (3.3)	90.0 (2.6)	90.0 (3.0)	88.9 (3.4)	87.3 (3.6)	86.4 (3.6)	86.9 (3.1)
Crown Rump Length, mm Mean (SD)	**62.5 (8.3)**	61.7 (8.1)	68.5 (7.5)	68.8 (8.2)	65.6 (8.9)	61.6 (9.0)	59.2 (8.7)	60.3 (7.5)
Missing	***199***	*134*	*53*	*10*	*<6*	*0*	*0*	*<6*
Weight of pregnant individual, kg Mean (SD)	**68 (17.0)**	67.9 (16.9)	68.5 (17.4)	68.3 (17.3)	68.1 (17.0)	67.2 (16.6)	68.8 (17.8)	67.0 (14.0)
Missing	***18 316***	*14 783*	*2 009*	*448*	*209**	*297**	*227**	*343**
Parity, No. (%)								
Nulliparous	**183 587 (46.2)**	161 994 (46.8)	17 689 (42.7)	2 847 (40.3)	595 (37.5)	314 (39.4)	81 (46.0)	67 (45.6)
Primiparous	**143 190 (36.0)**	123 661 (35.7)	15 760 (38.0)	2 739 (38.7)	614 (38.7)	320 (40.2)	56 (31.8)	40 (27.2)
Multiparous	**70 967 (17.8)**	60 848 (17.6)	8 012 (19.3)	1 487 (21.0)	378 (23.8)	163 (20.5)	39 (22.2)	40 (27.2)
Missing	***16 524***	*13 304*	*1 758*	*401*	*202**	*291**	*228**	*340**
Type of conception, No. (%)								
Spontaneous conception	**374 873 (96.0)**	326 450 (96.0)	3 8712 (95.9)	6 685 (95.9)	1 568 (96.1)	876 (96.4)	275 (96.8)	307 (97.5)
IVF	**12 025 (3.1)**	10 429 (3.1)	1 289 (3.2)	220 (3.2)	49 (3.0)	22 (2.4)	8 (2.8)	8 (2.5)
Other ART	**3 659 (0.9)**	3 188 (0.9)	378 (0.9)	66 (0.9)	15 (0.9)	11 (1.2)	<6 (S)	0 (0.0)
Missing	***23 711***	*19 740*	*2,840*	*503*	*157*	*179**	*120**	*172**

**Missing data for more than 10.0% of the pregnancies*

EDD, estimated date of delivery; SD, standard deviation; IVF, in vitro fertilization; ART, assisted reproductive technology.
There were no missing values for age of the pregnant individual and gestational age at screening.

Table 2.2. Chromosomal and Pregnancy Outcomes by Nuchal Translucency Measurement.

	Total	Nuchal translucency measurement (mm)						
		<2.0	2.0-<2.5	2.5-<3.0	3.0-<3.5	3.5-<5.0	5.0-<6.5	≥6.5
Cytogenetic testing results								
Pregnancies with cytogenetic results, No.	**15 755**	**11 552**	**1 875**	**653**	**421**	**602**	**287**	**365**
Unknown result, No. (%)	217 (1.4)	180 (1.6)	22 (1.2)	<6 (S)	<6 (S)	<6 (S)	<6 (S)	<6 (S)
No chromosomal anomaly identified, No. (%)	12 106 (76.8)	9 459 (81.9)	1 397 (74.5)	450 (68.9)	241 (57.2)	341 (56.6)	112 (39.0)	106 (29.0)
Chromosomal anomaly, No. (%)	3 432 (21.8)	1 913 (16.6)	456 (24.3)	198 (30.3)	179 (42.5)	256 (42.5)	174 (60.6)	256 (70.1)
Identifiable on cfDNA screening[a], No. (% of 3 432)	*1 690 (49.2)*	*543 (28.4)*	*241 (52.9)*	*154 (77.8)*	*149 (83.2)*	*215 (84.0)*	*153 (87.9)*	*235 (91.8)*
Not identifiable on cfDNA screening[b], No. (% of 3 432)	*1 742 (50.8)*	*1 370 (71.6)*	*215 (47.1)*	*44 (22.2)*	*30 (16.8)*	*41 (16.0)*	*21 (12.1)*	*21 (8.2)*
cfDNA screening results for pregnancies without cytogenetic testing results								
Pregnancies with cfDNA screening, No.	**38 754**	**30 551**	**5 310**	**1 701**	**667**	**398**	**69**	**58**
Unknown/uninformative result, No. (%)	495 (1.3)	404 (1.3)	58 (1.1)	14 (0.8)	<6 (S)	8 (2.0)	<6 (S)	<6 (S)
Low-risk result, No. (%)	38 041 (98.2)	30 071 (98.4)	5 234 (98.6)	1 674 (98.4)	645 (96.7)	354 (88.9)	44 (63.8)	19 (32.8)
High-risk result, No. (%)	218 (0.6)	76 (0.2)	18 (0.3)	13 (0.8)	18 (2.7)	36 (9.0)	21 (30.4)	36 (62.1)
Pregnancy outcomes for pregnancies with neither cytogenetic testing nor cfDNA screening results								
Pregnancies with no follow-up testing, No.	**359 759**	**317 704**	**36 034**	**5 120**	**701**	**88**	**48**	**64**
Lost to follow-up, No. (%)	10 761 (3.0)	9 281 (2.9)	1 132 (3.1)	181 (3.5)	42 (6.0)	38 (43.2)	39 (81.3)	48 (75.0)
Live birth, No. (%)	347 190 (96.5)	306 838 (96.6)	34 727 (96.4)	4 901 (95.7)	655 (93.4)	49 (55.7)	7 (14.6)	13 (20.3)
Clinical findings at birth[c], No. (% of 347 190)	15 552 (4.5)	13 919 (4.5)	1 391 (4.0)	203 (4.1)	33 (5.0)	<6 (S)	<6 (S)	<6 (S)
Pregnancy loss, termination, or stillbirth, No. (%)	1 808 (0.5)	1 585 (0.5)	175 (0.5)	38 (0.7)	<6 (S)	<6 (S)	<6 (S)	<6 (S)
Chromosomal anomalies in the full sample								
Pregnancies in the full sample, No.	**414 268**	**359 807**	**43 219**	**7 474**	**1 789**	**1 088**	**404**	**487**
No chromosomal anomaly identified[d], No. (%)	382 478 (92.3)	332 945 (92.5)	40 046 (92.7)	6 846 (91.6)	1 522 (85.1)	771 (70.9)	179 (44.3)	169 (34.7)
Chromosomal anomaly[e] No. (%)	3 432 (0.8)	1 913 (0.5)	456 (1.1)	198 (2.6)	179 (10.0)	256 (23.5)	174 (43.1)	256 (52.6)
Excluded, No. (%)	28 358 (6.8)	24 949 (6.9)	2 717 (6.3)	430 (5.8)	88 (4.9)	61 (5.6)	51 (12.6)	62 (12.7)
Lost to follow-up	*10 761 (2.6)*	*9 281 (2.6)*	*1 132 (2.6)*	*181 (2.4)*	*42 (2.3)*	*38 (3.5)*	*39 (9.7)*	*48 (9.9)*
Excluded for other reason[f]	*17 597 (4.2)*	*15 668 (4.4)*	*1 585 (3.7)*	*249 (3.3)*	*46 (2.6)*	*23 (2.1)*	*12 (3.0)*	*14 (2.9)*

[a]chromosomal anomaly identifiable on cfDNA screening: trisomies 21, 18, 13, sex chromosome aneuploidies

[b]chromosomal anomaly not identifiable on cfDNA screening: all other chromosomal anomalies, including other autosomal aneuploidies, mosaic aneuploidies, copy number variants. A detailed list of all chromosomal anomalies is available in the supplementary materials.

[c]clinical findings at birth: any congenital structural anomaly identified at birth.

[d]no chromosomal anomaly: normal cytogenetic results OR no cytogenetic results but low-risk cfDNA results OR no follow-up testing results but documented live birth with no clinical findings.

[e]chromosomal anomaly: based on cytogenetic testing results.

[f]pregnancies excluded because resulted in pregnancy loss, stillbirth, termination, live birth with clinical findings or a high-risk cfDNA screening result.

Table 2.3. Chromosomal Anomalies by Nuchal Translucency Measurement.

Nuchal translucency measurement	*Main analysis*				*Subgroup analysis*			
	Crude model		*Adjusted model**		*Adjusted model** *Conditions identifiable on cfDNA screening (Trisomies 21, 18, 13, sex chromosome aneuploidies)*		*Adjusted model** *Conditions not identifiable on cfDNA screening (All other chromosomal anomalies)*	
	Risk Difference % (95% CI)	Risk Ratio (95% CI)	Risk Difference % (95% CI)	Risk Ratio (95% CI)	Risk Difference % (95% CI)	Risk Ratio (95% CI)	Risk Difference % (95% CI)	Risk Ratio (95% CI)
< 2.0 mm	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
2.0-<2.5 mm	0.55 (0.45-0.66)	1.97 (1.78-2.18)	0.61 (0.51-0.71)	2.39 (2.14-2.66)	0.45 (0.37-0.52)	4.21 (3.62-4.91)	0.16 (0.08-0.23)	1.48 (1.27-1.73)
2.5-<3.0 mm	2.24 (1.85-2.63)	4.92 (4.26-5.69)	2.26 (1.88-2.63)	5.93 (5.11-6.88)	2.01 (1.67-2.34)	14.76 (12.33-17.65)	0.27 (0.08-0.46)	1.82 (1.34-2.47)
3.0-<3.5 mm	9.95 (8.49-11.41)	18.42 (15.92-21.31)	9.94 (8.49-11.39)	20.33 (17.58-23.52)	8.62 (7.27-9.96)	52.15 (43.98-61.84)	1.40 (0.77-2.04)	4.97 (3.45-7.17)
3.5-<5.0 mm	24.36 (21.71-27.00)	43.63 (38.88-48.96)	24.31 (21.67-26.96)	42.94 (38.28-48.16)	21.10 (18.60-23.61)	107.31 (93.20-123.56)	3.50 (2.27-4.72)	10.15 (7.37-13.98)
5.0-<6.5 mm	48.72 (43.51-53.94)	86.28 (76.91-96.80)	48.68 (43.46-53.89)	80.85 (71.91-90.90)	43.97 (38.79-49.15)	208.01 (179.77-240.69)	5.15 (2.66-7.64)	14.17 (9.02-22.25)
≥ 6.5 mm	59.66 (54.99-64.33)	105.44 (96.40-115.32)	59.64 (54.97-64.31)	101.88 (92.79-111.88)	55.79 (51.06-60.53)	276.14 (244.84-311.44)	4.30 (2.18-6.41)	12.11 (7.69-19.07)

**Model adjusted for gestational age at screening*

Outcome determined by cytogenetic testing, not having the outcome is determined by normal cytogenetic testing if performed, normal cfDNA screen if condition was tested and by live birth without clinical findings if condition can be clinically diagnosed.

2.9 Supplementary materials

Supplementary Table 2.1. Description of Data Sources

Better Outcomes Registry & Network (BORN) Ontario	BORN Ontario is a prescribed registry for the province of Ontario, Canada, designed to capture various encounters the pregnant individual and child have with the healthcare system. The three laboratories that provide all multiple marker screening in Ontario transfer records directly to BORN on a weekly basis, and include information on nuchal translucency measurements, biomarkers, and clinical information from the pregnant individual. There are two cell-free DNA screening laboratories established in Ontario that provide publicly funded screening, as well as privately paid screening and both contribute to the registry. All 9 cytogenetic laboratories in Ontario provide data to the registry through quarterly transfers and include all tests performed both prenatally as well as postnatally and on products of conception. The registry also captures all hospital births and home births with a midwife in the province. Additional information on BORN can be found in the 2021 publication by Murphy et al. as well as on the www.bornontario.ca website.

Canadian Institute for Health Information (CIHI)	The CIHI Discharge Abstract Database metadata (DAD) contains information from hospital discharges in Ontario, as well as other provinces and territories of Canada.
	Data from CIHI was used in this study to supplement information on pregnancy outcomes including congenital anomalies. More information on CIHI can be found at https://www.cihi.ca/en/discharge-abstract-database-metadata-dad.
	The following variables were supplemented using CIHI data:
	- Pregnancy outcome
	  ○ Fetal demise - ICD10 code : P95
	  ○ Spontaneous abortion – ICD10 codes: O03;P01.8
	  ○ Termination of Pregnancy – ICD10 codes: O04, O05, O06, P96.4
	  ○ Congenital anomalies – ICD10 codes from Q00 to Q99

Supplementary Table 2.2. Characteristics of Pregnancies with and Without Cytogenetic Testing.

Characteristic	Pregnancies with nuchal translucency measurement	Nuchal translucency measurement					
		<2.0 mm		2.0-<3.5 mm		≥3.5 mm	
		Did not have cytogenetic testing	Had cytogenetic testing	Did not have cytogenetic testing	Had cytogenetic testing	Did not have cytogenetic testing	Had cytogenetic testing
Total	**414,268**	**348,255**	**11,552**	**49,533**	**2,949**	**725**	**1,254**
Age at EDD, years Mean (SD)	**31.5 (4.7)**	31.4 (4.7)	32.5 (5.1)	31.6 (4.8)	33.4 (5.2)	32.2 (5.4)	33.4 (5.1)
Gestational age at screening, days Mean (SD)	**87.8 (3.3)**	87.5 (3.3)	87.3 (3.3)	90 (2.7)	88.8 (3.1)	87.3 (3.7)	86.9 (3.5)
Crown Rump Length, mm Mean (SD)	**62.5 (8.3)**	61.7 (8.1)	61.2 (8.1)	68.6 (7.6)	65.4 (8.3)	61.4 (9.0)	60.4 (8.4)
Weight of pregnant individual, kg Mean (SD)	**68 (17.0)**	67.8 (16.9)	69.3 (18.5)	68.5 (17.3)	68.5 (18.2)	68.5 (17.4)	66.6 (15.6)
Parity, No. (%)							
Nulliparous	**183587 (46.2)**	157256 (46.8)	4738 (44.7)	20201 (42.4)	930 (37.7)	222 (45.5)	240 (38.0)
Primiparous	**143190 (36.0)**	119978 (35.7)	3683 (34.8)	18194 (38.2)	919 (37.2)	171 (35.0)	245 (38.8)
Multiparous	**70967 (17.8)**	58681 (17.5)	2167 (20.5)	9258 (19.4)	619 (25.1)	95 (19.5)	147 (23.3)
Missing	***16,524***	*12,340*	*964*	*1,880*	*481**	*237**	*622**
Conception, No. (%)							
Spontaneous conception	**374873 (96.0)**	316437 (96.0)	10013 (94.6)	44468 (95.9)	2497 (95.3)	546 (95.6)	912 (97.3)
IVF	**12025 (3.1)**	9970 (3.0)	459 (4.3)	1465 (3.2)	93 (3.5)	17 (3.0)	21 (2.2)
Other ART	**3659 (0.9)**	3075 (0.9)	113 (1.1)	429 (0.9)	30 (1.1)	8 (1.4)	<6 (S)
Missing	***23,711***	*18,773*	*967*	*3,171*	*329**	*154**	*317**
Smoking status, No. (%)							
Nonsmoker	**358781 (91.8)**	302150 (92.0)	9888 (91.4)	42473 (90.5)	2533 (91.0)	629 (92.2)	1108 (93.9)
Smoker	**32117 (8.2)**	26334 (8.0)	930 (8.6)	4477 (9.5)	251 (9.0)	53 (7.8)	72 (6.1)
Missing	**23,370**	19,771	734	2,583	165	43	74

**Missingness >10.0%*

SD, standard deviation; IVF, in vitro fertilization; ART, Assisted reproductive technology

Supplementary Table 2.3. Method of Ascertainment of Outcome.

Chromosomal anomaly	Traditional cytogenetic testing[a]	Microarray testing	cfDNA screening[b]	Newborn clinical exam
Conditions formally screened by program				
trisomy 21 and 18	Tested	Tested	NPV >99.9[c]	Diagnosis may be excluded [d]
Secondary findings of the screening program				
trisomy 13	Tested	Tested	NPV >99.9 [c]	Diagnosis may be excluded [d]
other autosomal aneuploidies	Tested	Tested	Not tested	Diagnosis may be excluded [d]
mosaic autosomal aneuploidies	Tested	Can be ascertained, unless low-level[e]	Not tested	Cannot exclude diagnosis [f]
triploidy	Tested	Tested	Tested in only one cfDNA screening platform[b]	Diagnosis may be excluded [d]
monosomy x	Tested	Tested	May be tested (opt-in or automatically tested depending on platform) [b]	Cannot exclude diagnosis [f]
mosaic monosomy x	Tested	Can be ascertained, unless low-level[e]	Not tested	Cannot exclude diagnosis [f]
other sex chromosome aneuploidies	Tested	Tested	May be tested (opt-in or automatically tested depending on platform) [b]	Cannot exclude diagnosis [f]
mosaic other sex chromosome aneuploidies	Tested	Can ascertain mosaicism, unless low- level[e]	Not tested	Cannot exclude diagnosis [f]
22q11.2 deletion, Cri-du-Chat, Angelman/Prader-Willi, 1p36 deletion syndromes	Not routinely ascertained	Tested	May be tested (opt in, private pay), NPV unclear[b]	Cannot exclude diagnosis [f]
Other copy number variants	Large deletions or duplications only [g]	Tested	Not tested	Cannot exclude diagnosis [f]

NPV, Negative predictive value

[a] *Traditional cytogenetic testing refers to rapid aneuploidy detection techniques (fluorescence in situ hybridization (FISH) or quantitative fluorescence-PCR (QFPCR)), or karyotype.*

[b] *Two cfDNA screening laboratories are established in Ontario, one using Single Nucleotide Polymorphism (SNP) Based Analysis while the other performs Chromosome Specific Sequencing through their Digital Analysis of Selected Regions (DANSR) technology. At the time of the study, one laboratory automatically screened for sex chromosome aneuploidies, while the other allowed to opt in. Only the SNP-based technology can screen for triploidy. The option to self-paid for microdeletion syndromes was also available.*

[c] *Program Report, Prenatal Screening Ontario. Published online December 2021. Accessed July 19, 2023. https://www.bornontario.ca/en/pso/resources/Remediated-PDFs-2020/PSO-Program-Report---FINAL-Dec-8-2021.pdf*

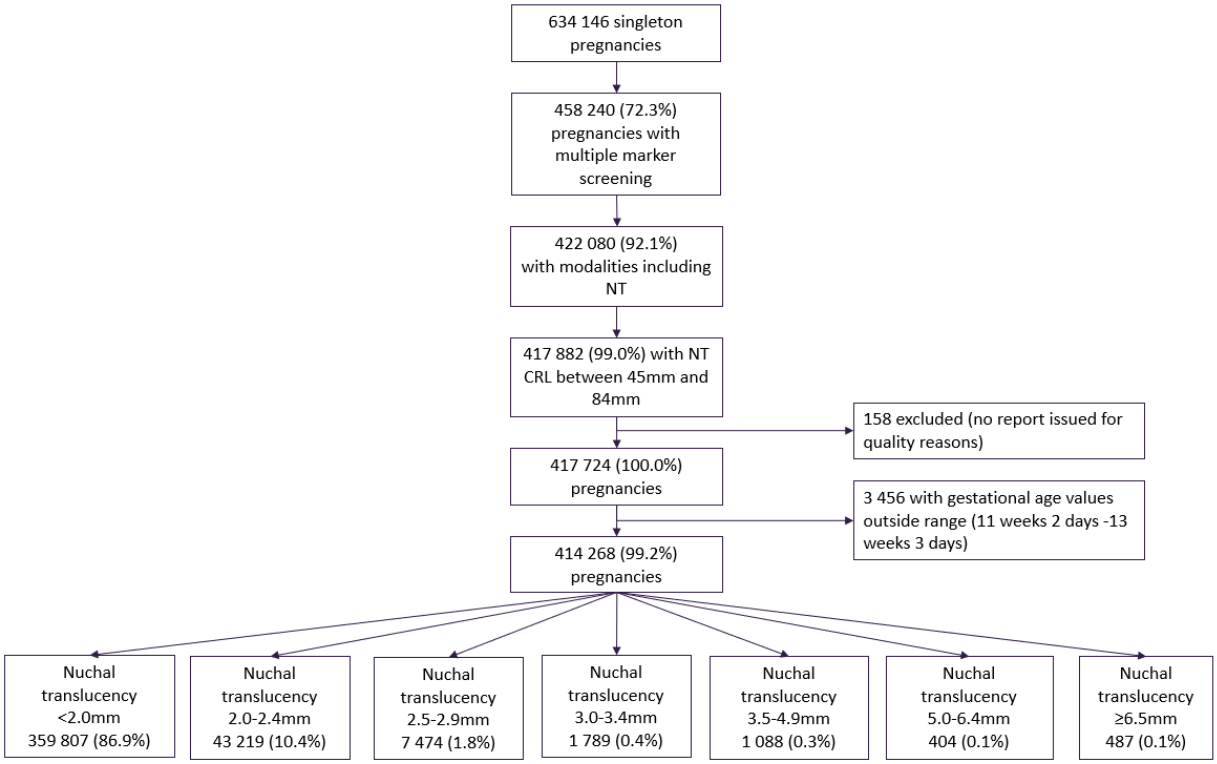
[d] *Recognizable clinical findings are expected at birth. Nussbaum R, McInnes R, Willard H. Thompson & Thompson Genetics in Medicine. Eight Edition. ELSEVIER; 2016.*

[e] *Armour CM, Dougan SD, Brock JA, et al. Practice guideline: joint CCMG-SOGC recommendations for the use of chromosomal microarray analysis for prenatal diagnosis and assessment of fetal loss in Canada. J Med Genet. 2018;55(4):215-221. Doi:10.1136/jmedgenet-2017-105013*

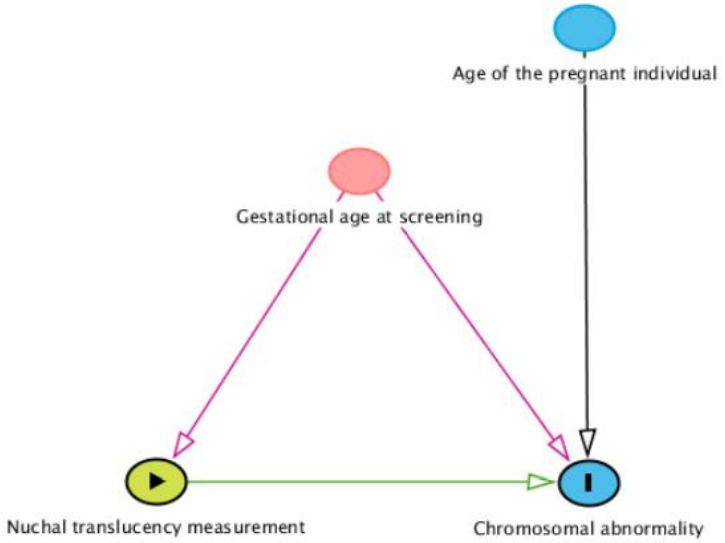
[f] *Clinical findings of this condition may not be easily and clearly identifiable at birth due to phenotype variability and syndromes that are less recognizable. Nussbaum R, McInnes R, Willard H. Thompson & Thompson Genetics in Medicine. Eight Edition. ELSEVIER; 2016.*

[g] *Microarray can detect smaller deletions and duplications not identifiable on karyotype: Wapner RJ, Martin CL, Levy B, et al. Chromosomal Microarray versus Karyotyping for Prenatal Diagnosis. N Engl J Med. 2012;367(23):2175-2184. doi:10.1056/NEJMoa1203382*

Supplementary Figure 2.1. Inclusion Flow.



Supplementary Figure 2.2. Directed Acyclic Graph for Relationship Between Nuchal Translucency Measurement and Chromosomal Anomalies.



Note: Adjustment for factors that incidentally would affect the nuchal translucency measurement and would also be related to chromosomal anomalies were made to the regression models; only gestational age met this criterion. A post-hoc analysis also included adjustment for age of the pregnant individual and is presented in Supplementary Table 10.

Supplementary Table 2.4. Regression Models Including Pregnancies Identified Through Other Data Sources Beyond Multiple Marker Screening.

Nuchal translucency measurement	***Any chromosomal anomaly***			
	Risk Difference % (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	Ref.		Ref.	
2.0-<2.5 mm	0.55	(0.45-0.66)	1.97	(1.78-2.18)
2.5-<3.0 mm	2.24	(1.85-2.63)	4.92	(4.26-5.68)
3.0-<3.5 mm	9.95	(8.49-11.41)	18.42	(15.92-21.31)
≥ 3.5 mm	35.83	(33.85-37.82)	63.72	(59.39-68.37)

Outcome determined by cytogenetic testing, not having the outcome is determined by normal cytogenetic testing if performed, normal cfDNA screen if condition was tested and by live birth without clinical findings if condition can be clinically diagnosed.

Crude model is presented, as pregnancies with nuchal translucency measurements identified outside the multiple marker screening data set did not have information on gestational age at screening.

Nuchal translucency measurements categories were collapsed, as the additional data sources only provided whether the nuchal translucency measurement was 3.0-<3.5 mm or ≥ 3.5 mm

Supplementary Table 2.5. Regression Model with Varying Definition of Exposure – by Nuchal Translucency Percentile.

Nuchal translucency percentile	***Any chromosomal anomaly***							
	Crude model				*Adjusted model**			
	Risk Difference % (95% CI)		Risk Ratio (95% CI)		Risk Difference % (95% CI)		Risk Ratio (95% CI)	
<90th percentile	Ref.		Ref.		Ref.		Ref.	
90-95th percentile	0.38	(0.26-0.50)	1.67	(1.46-1.91)	0.44	(0.32-0.57)	2.12	(1.84-2.43)
95-99th percentile	1.12	(0.94-1.29)	2.96	(2.64-3.30)	1.15	(0.98-1.32)	3.82	(3.39-4.29)
≥ 99th percentile	17.75	(16.69-18.82)	32.08	(29.80-34.53)	17.73	(16.66-18.79)	34.90	(32.43-37.57)

Outcome determined by cytogenetic testing, not having the outcome is determined by normal cytogenetic testing if performed, normal cfDNA screen if condition was tested and by live birth without clinical findings if condition can be clinically diagnosed.

**Model adjusted for gestational age at screening*

Supplementary Table 2.6A. Sensitivity Analysis for Losses to Follow-Up.

Nuchal translucency measurement	Number of pregnancies for which an outcome was recorded	Prevalence of chromosomal anomaly among those for which an outcome was recorded	Number of pregnancies for which NO outcome was recorded	Number of pregnancies randomized to having a chromosomal anomaly. Assumption of half the prevalence of chromosomal anomalies compared to those for which an outcome is available.	Number of pregnancies randomized to having a chromosomal anomaly. Assumption of twice the prevalence of chromosomal anomalies compared to those for which an outcome is available.
	n (%)	n (%)	n (%)	n (%)	n (%)
< 2.0 mm	334858 (97.3)	1913 (0.6)	9281 (2.7)	26 (0.3)	103 (1.1)
2.0-<2.5 mm	40502 (97.3)	456 (1.1)	1132 (2.7)	6 (0.5)	25 (2.2)
2.5-<3.0 mm	7044 (97.5)	198 (2.7)	181 (2.5)	3 (1.4)	10 (5.5)
3.0-<3.5 mm	1701 (97.6)	179 (10.3)	42 (2.4)	3 (5.1)	9 (20.5)
3.5-<5.0 mm	1027 (96.4)	256 (24.0)	38 (3.6)	5 (12.0)	18 (48.1)
5.0-<6.5 mm	353 (90.1)	174 (44.4)	39 (9.9)	9 (22.2)	35 (88.8)
≥ 6.5 mm	425 (89.9)	256 (54.1)	48 (10.1)	13 (27.1)	48 (100.0)

Example

1913 (0.6%) of the 334 858 pregnancies with nuchal translucency measurement < 2.0 mm for which an outcome was recorded had a chromosomal anomaly. In this nuchal translucency category, 9281 (2.7%) pregnancies had no outcome recorded. In the sensitivity analyses, pregnancies lost to follow-up were randomized to having 1) half the prevalence of chromosomal anomalies (0.3% = 26 pregnancies randomly assigned to having a chromosomal anomaly) or 2) twice the prevalence of chromosomal anomalies (1.1% = 103 pregnancies randomly assigned to having a chromosomal anomaly).

Supplementary Table 2.6B. Sensitivity Analysis for Losses to Follow-Up.

Nuchal translucency measurement	*Main analysis*				*Sensitivity analysis***			
	Crude model		*Adjusted model**		*Adjusted model* Pregnancies lost to follow-up assumed to have half the prevalence of chromosomal anomalies*		*Adjusted model* Pregnancies lost to follow-up assumed to have twice the prevalence of chromosomal anomalies*	
	Risk Difference % (95% CI)	Risk Ratio (95% CI)	Risk Difference % (95% CI)	Risk Ratio (95% CI)	Risk Difference % (95% CI)	Risk Ratio (95% CI)	Risk Difference % (95% CI)	Risk Ratio (95% CI)
< 2.0 mm	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
2.0-<2.5 mm	0.55 (0.45-0.66)	1.97 (1.78-2.18)	0.61 (0.51-0.71)	2.39 (2.14-2.66)	0.60 (0.50-0.70)	2.38 (2.14-2.65)	0.63 (0.52-0.73)	2.37 (2.13-2.63)
2.5-<3.0 mm	2.24 (1.85-2.63)	4.92 (4.26-5.69)	2.26 (1.88-2.63)	5.93 (5.11-6.88)	2.22 (1.85-2.59)	5.91 (5.09-6.85)	2.31 (1.94-2.69)	5.87 (5.08-6.79)
3.0-<3.5 mm	9.95 (8.49-11.41)	18.42 (15.92-21.31)	9.94 (8.49-11.39)	20.33 (17.58-23.52)	9.81 (8.38-11.23)	20.30 (17.56-23.46)	10.19 (8.74-11.64)	20.21 (17.54-23.29)
3.5-<5.0 mm	24.36 (21.71-27.00)	43.63 (38.88-48.96)	24.31 (21.67-26.96)	42.94 (38.28-48.16)	23.90 (21.32-26.48)	42.82 (38.21-47.99)	25.10 (22.48-27.73)	43.27 (38.74-48.33)
5.0-<6.5 mm	48.72 (43.51-53.94)	86.28 (76.91-96.80)	48.68 (43.46-53.89)	80.85 (71.91-90.90)	46.07 (41.13-51.01)	76.76 (68.24-86.35)	52.68 (47.74-57.62)	84.55 (76.16-93.85)
≥ 6.5 mm	59.66 (54.99-64.33)	105.44 (96.40-115.32)	59.64 (54.97-64.31)	101.88 (92.79-111.88)	56.29 (51.81-60.76)	97.49 (88.76-107.10)	63.66 (59.33-68.00)	106.09 (97.57-115.35)

**Model adjusted for gestational age at screening*

***The sensitivity analyses randomly classified the losses to follow-up to having 1) half and 2) twice the prevalence of chromosomal anomalies compared to pregnancies in the same category of nuchal translucency measurement for which an outcome was recorded. (See supplementary Table 5A for details.)*

Supplementary Table 2.7. Regression Models Including All Pregnancies with Nuchal Translucency Measurements. (See Full List of Assumptions in Table Legend.)

Nuchal translucency measurement	***Any chromosomal anomaly***							
	Crude model				*Adjusted model**			
	Risk Difference % (95% CI)		Risk Ratio (95% CI)		Risk Difference % (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	Ref.		Ref.		Ref.		Ref.	
2.0-<2.5 mm	0.47	(0.32-0.63)	1.22	(1.15-1.30)	0.63	(0.47-0.79)	1.35	(1.26-1.44)
2.5-<3.0 mm	1.81	(1.37-2.25)	1.86	(1.66-2.08)	1.90	(1.47-2.34)	2.05	(1.82-2.30)
3.0-<3.5 mm	9.52	(8.04-11.01)	5.51	(4.84-6.28)	9.52	(8.04-10.99)	5.81	(5.10-6.61)
3.5-<5.0 mm	23.60	(21.00-26.21)	12.18	(10.98-13.51)	23.54	(20.94-26.13)	12.10	(10.92-13.42)
5.0-<6.5 mm	46.16	(41.28-51.03)	22.87	(20.62-25.36)	46.07	(41.20-50.94)	21.97	(19.80-24.37)
≥ 6.5 mm	55.95	(51.54-60.36)	27.51	(25.41-29.78)	55.91	(51.50-60.32)	27.01	(24.93-29.27)

Outcome determined by cytogenetic testing, not having the outcome is determined by normal cytogenetic testing if performed, normal cfDNA screen if condition was tested and by live birth without clinical findings if condition can be clinically diagnosed

If the outcome was not recorded, pregnancies were assumed to have the same risk of chromosomal anomalies as pregnancies with the same outcome, for which cytogenetic testing was performed. E.g., for the pregnancies that resulted in a pregnancy loss and had cytogenetic testing, 21.6% had a chromosomal anomaly, therefore 21.6% of pregnancies for which a pregnancy loss was recorded, but no cytogenetic testing was performed were randomized to have a chromosomal anomaly. The same process was followed for pregnancies for which cytogenetic outcomes were unavailable, but where a stillbirth, termination, live birth with clinical findings or loss to follow-up were recorded.

Pregnancies for which no outcome was recorded, but where a cfDNA screening result was high-risk, a positive predictive value of 90.0% was assumed and 90.0% of the pregnancies were randomized to have a chromosomal anomaly.

**Model adjusted for gestational age at screening.*

Supplementary Table 2.8. Regression Models Including Only Pregnancies with an Estimated Date of Delivery from September 1, 2018, to March 31, 2021 (Timeline with Complete Capture of Microarray Testing).

Nuchal translucency measurement	***Any chromosomal anomaly***							
	Crude model				*Adjusted model**			
	Risk Difference % (95% CI)		Risk Ratio (95% CI)		Risk Difference % (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	Ref.		Ref.		Ref.		Ref.	
2.0-<2.5 mm	0.52	(0.39-0.66)	1.98	(1.72-2.27)	0.58	(0.45-0.71)	2.41	(2.08-2.78)
2.5-<3.0 mm	2.20	(1.70-2.71)	5.12	(4.21-6.21)	2.22	(1.73-2.71)	6.19	(5.07-7.56)
3.0-<3.5 mm	10.56	(8.57-12.56)	20.74	(17.15-25.07)	10.56	(8.58-12.55)	22.99	(19.02-27.79)
3.5-<5.0 mm	24.85	(21.33-28.36)	47.42	(40.76-55.16)	24.80	(21.30-28.31)	46.94	(40.39-54.54)
5.0-<6.5 mm	50.91	(44.11-57.70)	96.10	(83.09-111.16)	50.87	(44.08-57.66)	89.97	(77.41-104.58)
≥ 6.5 mm	61.48	(55.56-67.40)	115.86	(103.43-129.77)	61.46	(55.53-67.38)	110.47	(97.98-124.55)

Outcome determined by cytogenetic testing, not having the outcome is determined by normal cytogenetic testing if performed, normal cfDNA screen if condition was tested and by live birth without clinical findings if condition can be clinically diagnosed

**Model adjusted for gestational age at screening*

Supplementary Table 2.9. Regression Models Excluding Pregnancies with an Estimated Date of Delivery from April 1, 2020, to March 31, 2021 (Accounting for Potential Changes in Practice During the COVID-19 Pandemic).

Nuchal translucency measurement	***Any chromosomal anomaly***							
	Crude model				*Adjusted model**			
	Risk Difference % (95% CI)		Risk Ratio (95% CI)		Risk Difference % (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	Ref.		Ref.		Ref.		Ref.	
2.0-<2.5 mm	0.50	(0.38-0.62)	1.85	(1.64-2.08)	0.56	(0.44-0.68)	2.22	(1.96-2.51)
2.5-<3.0 mm	2.04	(1.61-2.46)	4.43	(3.74-5.25)	2.05	(1.64-2.47)	5.29	(4.44-6.30)
3.0-<3.5 mm	9.38	(7.78-10.99)	16.80	(14.20-19.88)	9.38	(7.78-10.97)	18.47	(15.61-21.85)
3.5-<5.0 mm	23.99	(20.99-27.00)	41.40	(36.28-47.25)	23.96	(20.95-26.96)	40.73	(35.70-46.47)
5.0-<6.5 mm	45.08	(38.95-51.20)	76.91	(66.65-88.74)	45.02	(38.90-51.15)	71.89	(62.22-83.05)
≥ 6.5 mm	59.28	(53.86-64.70)	100.83	(90.92-111.83)	59.26	(53.84-64.68)	97.93	(88.00-109.01)

Outcome determined by cytogenetic testing, not having the outcome is determined by normal cytogenetic testing if performed, normal cfDNA screen if condition was tested and by live birth without clinical findings if condition can be clinically diagnosed

**Model adjusted for gestational age at screening*

Supplementary Table 2.10. Time of Cytogenetic Testing of Chromosomal Anomalies Identified by Nuchal Translucency Measurement.

Time of cytogenetic testing for chromosomal anomalies identified	Nuchal translucency measurement (mm)							
	<2.0	2.0-<2.5	2.5-<3.0	3.0-<3.5	3.5-<5.0	5.0-<6.5	≥6.5	Total
Prenatal diagnosis, n (%)	490 (26.4)	204 (45.3)	119 (60.7)	134 (75.7)	196 (76.9)	135 (78.0)	165 (64.5)	1443 (42.9)
Postnatal cytogenetic testing, n (%)	1369 (73.6)	246 (54.7)	77 (39.3)	43 (24.3)	59 (23.1)	38 (22.0)	91 (35.5)	1923 (57.1)
Total	**1859**	**450**	**196**	**177**	**255**	**173**	**256**	**3366**

**For 66 cytogenetic tests performed, the timing of the test was unknown*

Supplementary Table 2.11. Chromosomal and Pregnancy Outcomes by Nuchal Translucency Measurement – Detailed Results.

Cytogenetic testing results	Pregnancies with cytogenetic testing	Nuchal translucency measurement (mm)						
		<2.0	2.0-<2.5	2.5-<3.0	3.0-<3.5	3.5-<5.0	5.0-<6.5	≥6.5
Total pregnancies with cytogenetic testing, No.	**15,755**	**11,552**	**1,875**	**653**	**421**	**602**	**287**	**365**
Unknown results, No. (%)	**217 (1.4)**	**180 (1.6)**	**22 (1.2)**	**<6 (S)**	**<6 (S)**	**<6 (S)**	**<6 (S)**	**<6 (S)**
No chromosomal anomaly identified, No. (%)	**12106 (76.8)**	**9459 (81.9)**	**1397 (74.5)**	**450 (68.9)**	**241 (57.2)**	**341 (56.6)**	**112 (39.0)**	**106 (29.0)**
Chromosomal anomalies identified results, No. (%)	**3432 (21.8)**	**1913 (16.6)**	**456 (24.3)**	**198 (30.3)**	**179 (42.5)**	**256 (42.5)**	**174 (60.6)**	**256 (70.1)**
Trisomy 21	**1024 (29.8)**	299 (15.6)	180 (39.5)	127 (64.1)	117 (65.4)	151 (59.0)	74 (42.5)	76 (29.7)
Trisomy 18	**293 (8.5)**	110 (5.8)	20 (4.4)	11 (5.6)	13 (7.3)	32 (12.5)	50 (28.7)	57 (22.3)
Trisomy 13	**136 (4.0)**	39 (2.0)	23 (5.0)	10 (5.1)	10 (5.6)	25 (9.8)	16 (9.2)	13 (5.1)
Monosomy X	**114 (3.3)**	10 (0.5)	<6 (S)	0 (0.0)	<6 (S)	<6 (S)	11 (6.3)	83 (32.4)
Other sex chromosome aneuploidies	**123 (3.6)**	85 (4.4)	15 (3.3)	6 (3.0)	6 (3.4)	<6 (S)	<6 (S)	6 (2.3)
Triploidy	**61 (1.8)**	52 (2.7)	0 (0.0)	0 (0.0)	<6 (S)	<6 (S)	<6 (S)	<6 (S)
Mosaic or partial trisomies 21, 18 or 13	**59 (1.7)**	45 (2.4)	7 (1.5)	<6 (S)	<6 (S)	0 (0.0)	<6 (S)	0 (0.0)
Mosaic or partial sex chromosome aneuploidies	**154 (4.5)**	119 (6.2)	20 (4.4)	<6 (S)	<6 (S)	8 (3.1)	0 (0.0)	<6 (S)
22q11.2 microdeletion syndrome	**53 (1.5)**	40 (2.1)	<6 (S)	0 (0.0)	0 (0.0)	<6 (S)	0 (0.0)	0 (0.0)
Cri-du-Chat syndrome	**<6 (S)**	0 (0.0)	0 (0.0)	<6 (S)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Angelman or Prader-Willi syndrome	**12 (0.3)**	12 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
1p36 deletion	**<6 (S)**	<6 (S)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Other autosomal aneuploidies	**<6 (S)**	<6 (S)	0 (0.0)	<6 (S)	<6 (S)	<6 (S)	0 (0.0)	0 (0.0)
Other mosaic autosomal aneuploidies	**12 (0.3)**	<6 (S)	<6 (S)	<6 (S)	<6 (S)	<6 (S)	0 (0.0)	<6 (S)
Other chromosomal anomaly	**1384 (40.3)**	1095 (57.2)	175 (38.4)	37 (18.7)	22 (12.3)	27 (10.5)	14 (8.0)	14 (5.5)
Pathogenic finding	***421***	*311*	*59*	*12*	*10*	*13*	*10*	*6*
Likely Pathogenic finding	***91***	*76*	*8*	*<6 (S)*	*<6 (S)*	*<6 (S)*	*<6 (S)*	*0*
Variant of Uncertain Significance	***717***	*598*	*85*	*21*	*<6 (S)*	*<6 (S)*	*<6 (S)*	*<6 (S)*
No interpretation available	***155***	*108*	*23*	*<6 (S)*	*6*	*7*	*<6 (S)*	*6*
Results from cfDNA screening for those without cytogenetic testing	**Pregnancies with cfDNA (but no cytogenetic testing)**	**Nuchal translucency measurement (mm)**						
		<2.0	**2.0-<2.5**	**2.5-<3.0**	**3.0-<3.5**	**3.5-<5.0**	**5.0-<6.5**	**≥6.5**
Total pregnancies with cfDNA screening, No.	**38,754**	**30,551**	**5,310**	**1,701**	**667**	**398**	**69**	**58**
No results, No. (%)	**495 (1.3)**	404 (1.3)	58 (1.1)	14 (0.8)	<6 (S)	8 (2.0)	<6 (S)	<6 (S)
Low risk, No. (%)	**38041 (98.2)**	30071 (98.4)	5234 (98.6)	1674 (98.4)	645 (96.7)	354 (88.9)	44 (63.8)	19 (32.8)
High risk T21, No. (%)	**103 (0.3)**	18 (0.1)	15 (0.3)	12 (0.7)	16 (2.4)	21 (5.3)	9 (13.0)	12 (20.7)
High risk T18	**38 (0.1)**	13 (0.0)	0 (0.0)	<6 (S)	<6 (S)	7 (1.8)	7 (10.1)	9 (15.5)
High risk T13, No. (%)	**15 (0.0)**	6 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	<6 (S)	<6 (S)	<6 (S)
High risk monosomy X, No. (%)	**20 (0.1)**	6 (0.0)	<6 (S)	0 (0.0)	0 (0.0)	<6 (S)	0 (0.0)	11 (19.0)
High risk other SCA, No. (%)	**18 (0.0)**	16 (0.1)	0 (0.0)	0 (0.0)	0 (0.0)	<6 (S)	<6 (S)	0 (0.0)
High risk triploidy, No. (%)	**22 (0.1)**	16 (0.1)	<6 (S)	0 (0.0)	0 (0.0)	<6 (S)	<6 (S)	0 (0.0)
High risk microdeletion, No. (%)	**<6 (S)**	<6 (S)	0 (0.0)	0 (0.0)	<6 (S)	0 (0.0)	0 (0.0)	0 (0.0)

Supplementary Table 2.12. Post-hoc analysis including additional adjustment for age of the pregnant individual at estimated date of delivery.

Nuchal translucency measurement	***Any chromosomal anomaly***									
	Crude model				*Model adjusted for gestational age at screening and age of the pregnant individual (continuous)**		*Model adjusted for gestational age at screening and age of the pregnant individual (categorical)***			
	Risk Difference % (95% CI)		Risk Ratio (95% CI)		Risk Ratio (95% CI)		Risk Difference % (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	Ref.		Ref.	Ref.	Ref.		Ref.		Ref.	Ref.
2.0-<2.5 mm	0.55	(0.45-0.66)	1.97	(1.78-2.18)	2.34	(2.10-2.61)	0.53	(0.43-0.63)	2.35	(2.11-2.61)
2.5-<3.0 mm	2.24	(1.85-2.63)	4.92	(4.26-5.69)	5.7	(4.92-6.61)	2.06	(1.70-2.42)	5.74	(4.96-6.66)
3.0-<3.5 mm	9.95	(8.49-11.41)	18.42	(15.92-21.31)	19.12	(16.57-22.06)	9.81	(8.37-11.25)	19.46	(16.85-22.47)
3.5-<5.0 mm	24.36	(21.71-27.00)	43.63	(38.88-48.96)	38.92	(34.80-43.53)	24.13	(21.49-26.76)	39.06	(34.86-43.76)
5.0-<6.5 mm	48.72	(43.51-53.94)	86.28	(76.91-96.80)	72.15	(64.30-80.96)	48.49	(43.28-53.69)	70.99	(63.09-79.87)
≥ 6.5 mm	59.66	(54.99-64.33)	105.44	(96.40-115.32)	87.96	(79.70-97.09)	59.47	(54.81-64.14)	87.23	(78.83-96.54)

**The model adjusted for age of the pregnant individuals using the continuous variable did not converge for the adjusted risk differences.*

***The age categories were defined as follows: (<25 years at estimated date of delivery, 25-<30 years , 30-<35 years, 35-<40 years, ≥40 years)*

Chapter 3. Manuscript 2: Pregnancy outcomes by nuchal translucency measurement

Title of manuscript: Outcomes of pregnancies with varying levels of nuchal translucency measurements: a population-based retrospective study in Ontario, Canada.

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Publication status: The manuscript presented in this chapter has been submitted for peer-review.

3.1 Preface

The manuscript presented in this chapter builds on the findings of the first manuscript to assess pregnancy and perinatal outcomes of pregnancies in association with nuchal translucency measurements. The aim of the study was to examine the association between nuchal translucency measurements and a composite of pregnancy loss, termination, stillbirth, or neonatal death. The secondary objective was to investigate the association between nuchal translucency measurements and adverse perinatal outcomes in pregnancies that resulted in a live birth. This study received approval by the Children's Hospital of Eastern Ontario Research Ethics Board (protocol #22/03PE) and University of Ottawa Research Ethics Board (protocol #H-06-22-8234).

Contribution Statement

Kara Bellai-Dussault designed the study, performed all analyses, interpreted the findings, and drafted the manuscript. The co-authors Beth Potter, Shelley Dougan, Deshayne Fell, Carolina Lavin Venegas, Julian Little, Lynn Meng, Mark Walker, Nan Okun, and Christine Armour were involved in the conceptualization of study, interpretation of the results, and critical review of the article. Additionally, Lynn Meng provided support in obtaining the data set and advised on data-related practices and Carolina Lavin Venegas provided administrative support in the research ethics board application. All authors made significant contributions to the manuscript, and approved the final version submitted to the journal.

What are the key findings of this work?

In this population-based cohort study, we assessed pregnancy outcomes for all levels of nuchal translucency measurements, compared to pregnancies with measurements <2.0 mm. We found that pregnancies with increased nuchal translucency measurements are less likely to result in a live birth, even with the exclusion of chromosomal abnormalities.

What are the clinical implications of this work?

This study provides robust evidence of the increased risk of pregnancy loss, termination, stillbirth, or neonatal death with increasing levels of nuchal translucency. This study provides valuable information for patient counseling and illustrates the importance of nuchal translucency ultrasound in a changing landscape of prenatal screening options.

3.2 Abstract

Objectives

To investigate the association between ultrasound fetal nuchal translucency measurements and pregnancy outcome, specifically, a composite of pregnancy loss, termination, stillbirth, or neonatal death. We also investigated the association between nuchal translucency measurements and the risk of adverse perinatal outcomes among pregnancies resulting in a live birth.

Methods

This was a population-based retrospective cohort study conducted with data from the prescribed provincial perinatal registry in Ontario, Canada, Better Outcomes Registry & Network. All singleton pregnancies with an estimated date of delivery from September 1, 2016, to March 31, 2021, and a valid multiple marker screening result including a nuchal translucency were included. Nuchal translucency measurements were grouped into categories (2.0-<2.5 mm, 2.5-<3.0 mm, 3.0-<3.5 mm, 3.5-<5.0 mm, 5.0-<6.5 mm, ≥6.5 mm) and compared to a reference group of pregnancies with a measurement <2.0 mm. We used multivariable modified Poisson regression models with robust variance estimation to estimate associations between nuchal translucency measurement and pregnancy outcome, with adjustment for age of the pregnant individual at estimated date of delivery and gestational age at screening.

Results

There were 414 268 singleton pregnancies included in the study. The risk of pregnancy loss, termination, stillbirth, or neonatal death increased with increasing levels of nuchal translucency measurements, with an adjusted risk ratio (aRR) of 11.9 (95% confidence interval (CI) 9.9,14.3) in the group with measurements 3.5-<5.0 mm. When pregnancies with diagnosed chromosomal abnormalities were excluded, this association remained strong, with an aRR of 6.4 (95%CI 4.8,8.5). Among pregnancies with a live birth, those with a higher nuchal translucency measurement (>5.0 mm versus <2.0 mm) were also at increased risk of adverse perinatal outcomes such as admission to the neonatal intensive care unit and APGAR score <7.

Conclusion

In this population-based study using robust methods to reduce the risk of selection bias, we found that pregnancies with increased nuchal translucency measurements are less likely to result in a live birth, even with the exclusion of chromosomal abnormalities. Pregnancies with increased nuchal translucency measurements that resulted in a live birth may also be at increased risk of adverse perinatal outcomes.

3.3 Introduction

Fetal nuchal translucency results from an accumulation of fluid located posteriorly to the fetal neck and is commonly used as a marker for chromosomal abnormalities, single-gene conditions, and structural defects.[1] In many prenatal screening programs in Canada and internationally, pregnant individuals are offered multiple marker screening to detect trisomies 21 and 18, often incorporating information from this nuchal translucency ultrasound.[2] When an increased nuchal translucency is identified (traditionally defined as a measurement greater or equal to 3.5 mm^3), follow-up investigations such as prenatal diagnosis with cytogenetic testing or cell-free DNA (cfDNA) screening are offered to pregnant individuals to confirm chromosomal abnormalities.

Beyond the detection of chromosomal abnormalities, studies have also reported an increased risk of pregnancy loss with elevated nuchal translucency measurements.[4–6] However, studies investigating the outcomes of pregnancies with increased nuchal translucency have largely focused only on the minority of pregnancies where individuals received cytogenetic testing and/or additional ultrasound investigations conducted at tertiary care centres, and had normal results.[7–9] To fully understand the association of nuchal translucency levels with pregnancy outcomes, there is a need to investigate this relationship at the population level, i.e., among all pregnancies receiving a nuchal translucency measurement as part of prenatal screening; and to understand whether increased nuchal translucency levels are associated with additional risks in the perinatal period. The primary objective of the present study was to estimate the association between nuchal translucency measurements and pregnancy outcomes in the full screened population in Ontario by defining whether the pregnancy resulted in a loss, termination, stillbirth, or neonatal death. Understanding this association in an unselected sample of pregnant individuals, regardless of follow-up confirmatory testing received, can support the interpretation of nuchal translucency measurement results at the time they are received. Our secondary objective was to estimate the relationship (overall and for pregnancies without diagnosed chromosomal abnormalities) between nuchal translucency measurements and outcomes indicative of perinatal complications (preterm birth, admission to the neonatal intensive care unit, APGAR score <7 and small for gestational age), for those pregnancies resulting in a live birth.

3.4 Methods

This population-based cohort study was approved by the research ethics board of the Children's Hospital of Eastern Ontario (protocol # 22/03PE) and the University of Ottawa (protocol # H-06-22-8234). Given that this study was conducted with data from a prescribed registry, obtaining individual patient consent was not required. To comply with privacy requirements, numbers <6 are not presented. Reporting was guided by the RECORD statement.[10]

Data sources

The Better Outcomes Registry & Network (BORN) is Ontario's prescribed perinatal registry and collects data directly from all prenatal screening and diagnostic laboratories in the province.[96] BORN Ontario also collects information on pregnancy and postnatal outcomes from labour and delivery units and neonatal intensive care units.[11] BORN data are linked to the hospital Discharge Abstract Database from the Canadian Institute for Health Information (CIHI), providing supplemental information from all Ontario hospitals births on pregnancy outcomes and perinatal outcomes, including congenital anomalies (Supplementary Table 3.1).[12]

Setting and study population

In Ontario, prenatal genetic screening is publicly funded in the form of a mixed contingent model where multiple marker screening, including a nuchal translucency measurement in more than 90% of screens, is offered to all pregnant individuals and cfDNA screening is offered to those who meet specific eligibility criteria.[13] The province has considerable variability in terms of socio-economic status, race/ethnicity, and geography, which renders varying access to some aspects of prenatal care (e.g., nuchal translucency ultrasound is more difficult to access in some remote regions).

All sonographers providing nuchal translucency measurements in the context of multiple marker screening are enrolled in Ontario's Nuchal Translucency Quality Assurance Program.[14]

We included all singleton pregnancies with an estimated date of delivery from September 1, 2016, to March 31, 2021, and with a multiple marker screening test result including a nuchal

translucency measurement. Pregnancies for which the nuchal translucency was measured outside the internationally recognized range of 45-84 mm crown-rump length were excluded (Figure 3.1).[15]

Study exposure

Nuchal translucency measurements were identified through multiple marker screening results in the registry and categorized as follows: <2.0 mm (reference group), 2.0-<2.5 mm, 2.5-<3.0 mm, 3.0-<3.5 mm, 3.5-<5.0 mm, 5.0-<6.5 mm, and ≥6.5 mm.

Study outcome

Our primary outcome of interest was pregnancy outcome, defined as a composite of pregnancy loss, termination, stillbirth, or a neonatal death that occurred during the index pregnancy delivery admission. The outcome was identified through the registry's birth encounter data with supplementation from hospital discharge data. Pregnancies with diagnosed chromosomal abnormalities were identified based on prenatal or postnatal cytogenetic testing.

Individual secondary outcomes were identified among the subgroup of pregnancies that resulted in a live birth and included: preterm birth, defined as a birth before 37 weeks' gestation; admission to the neonatal intensive care unit (NICU) for more than 12 hours; 5-minute APGAR score below 7; and small for gestational age, defined as smaller than the 10th percentile for the corresponding gestational age[16].

Statistical analysis

We used means and standard deviations to describe continuous variables, and frequencies and proportions to describe categorical variables. We used multivariable modified Poisson regression models with robust variance estimation to generate risk ratios as well as risk differences to compare pregnancy outcomes among pregnancies with varying levels of nuchal translucency measurements relative to the reference group of pregnancies with measurements <2.0 mm. This analysis accounted for non-independence of individuals with more than one pregnancy during the study period. We identified gestational age at screening and age of the pregnant individual at estimated date of delivery as potential confounders a priori and therefore included these variables as covariates in the adjusted models. The primary analysis

was repeated excluding pregnancies with identified chromosomal abnormalities, to assess if any association was solely due to the increased risk of chromosomal abnormalities with increasing nuchal translucency.

While we mitigated against selection bias by including all pregnancies in the screened population, some potential for residual bias remained given that there were pregnancies for which no outcome was available in our registry data. This was particularly important to examine in sensitivity analyses given that the proportion of such pregnancies increased with increasing nuchal translucency measurements. These pregnancies that were lost to follow-up could represent early pregnancy losses or terminations, or pregnant individuals who had multiple marker screening in Ontario but subsequently received care outside the province. Thus, in two sensitivity analyses we randomly assigned pregnancies for which no outcome was recorded (lost to follow-up) to either experiencing or not experiencing the outcome. This was done under assumptions that pregnancies lost to follow-up had half the risk of the composite pregnancy outcome compared to those for which an outcome was recorded within the same category of nuchal translucency measurement, or twice the risk, respectively (Supplementary Figure 3.1). This analysis was also conducted with the exclusion of pregnancies with identified chromosomal abnormalities (Supplementary Figure 3.2).

We conducted a further sensitivity analysis to qualitatively evaluate the potential for bias in analyses that are restricted to only those pregnancies receiving cytogenetic testing, with normal results, as has been common practice in studies on this topic (Supplementary Table 3.2).

While the registry routinely captures pregnancy outcomes after 20 weeks' gestation, early losses or terminations before 20 weeks' gestation are not systematically captured. Pregnancies with an increased nuchal translucency measurement (defined as a measurement greater or equal to 3.5 mm) may be subject to closer ascertainment of these outcomes. A sensitivity analysis including only those pregnancies with outcomes recorded after 20 weeks' gestation was, therefore, performed to account for this potential ascertainment bias (Supplementary Table 3.3).

Additional sensitivity analyses explored different lengths of stay for NICU admissions and are presented in the supplementary materials (Supplementary Table 3.4).

We performed all study analyses using SAS, version 9.4 (SAS Institute Inc, Cary, NC).

3.5 Results

From 643 146 singleton pregnancies recorded in the registry during the study period, 414 268 pregnant individuals received a valid multiple marker screening result that included a nuchal translucency measurement and were included in this study. Most pregnancies (359 807, 86.9%) had a nuchal translucency measurement <2.0 mm and comprised the reference group, while 10.4% were in the category 2.0-<2.5 mm, 1.8% in 2.5-<3.0 mm, 0.4% in 3.0-<3.5 mm, 0.3% in 3.5-<5.0 mm, 0.1% in 5.0-<6.5 mm and 0.1% in ≥6.5 mm (Table 3.1). Mean age of the pregnant individual increased with increasing nuchal translucency categories; no other trends in characteristics of the population by nuchal translucency were apparent. The proportion of pregnancies with missing data regarding parity and method of conception increased with increasing nuchal translucency measurement; the distribution of these characteristics should therefore be interpreted with caution.

The proportion of pregnancies experiencing the composite outcome of pregnancy loss, termination, stillbirth, or neonatal death among those for which an outcome was recorded increased with increasing nuchal translucency measurements, from 1.0% in the group with measurements <2.0 mm to 57.7% in the group with the highest nuchal translucency measurements (≥6.5 mm) (Table 3.2). After excluding pregnancies for which a chromosomal abnormality was identified, the same trend was observed with some attenuation in magnitude of the increase: 1.0% in the group with nuchal translucencies <2.0 mm experienced the composite outcome of either pregnancy loss, termination, stillbirth, or neonatal death, increasing to 41.8% in the group with measurements ≥6.5 mm. The proportion of pregnancies for which outcomes were unavailable (lost to follow-up) increased with nuchal translucency measurements, from 3.0% in pregnancies with a measurement <2.0 mm to 65.5% in the group with a measurement ≥6.5 mm. With increasing nuchal translucency, the proportion of

pregnancies experiencing each individual outcome included in the composite also increased. The most common of these component outcomes were pregnancy loss and termination; pregnancy termination comprised an increasing proportion of outcomes in the composite with increasing nuchal translucency.

Results for primary outcomes

The analysis of the full cohort of pregnancies showed an increased risk of the composite of pregnancy loss, termination, stillbirth, or neonatal death with increasing nuchal translucency measurements (Figure 3.2), with an aRD of 11.2% (95%CI 9.0,13.5) and aRR of 11.9 (95%CI 9.9,14.3) for pregnancies with nuchal translucency measurements from 3.5-<5.0 mm. Sensitivity analyses conducted to account for losses to follow-up showed similar results with slight attenuation or accentuation of the association depending on whether the losses to follow-up were assumed to have either half or twice the risk of the composite outcome compared to those for which an outcome was recorded, respectively, within each category of nuchal translucency measurement (Supplementary Figure 3.1).

The regression models were also performed with the exclusion of pregnancies for which a chromosomal abnormality had been identified to determine if the nuchal translucency measurement was associated with pregnancy outcomes independently of chromosomal abnormalities. Although attenuated, the association remained significant in all groups relative to the reference category of <2.0 mm, apart from pregnancies with a nuchal translucency measurement between 2.0-<2.5 mm (Figure 3.2).

The sensitivity analysis restricted to pregnancies with cytogenetic testing results available, and with normal results, yielded different findings, with a very attenuated association between nuchal translucency and the composite outcome of pregnancy loss, termination, stillbirth, or neonatal death that was apparent only in pregnancies with nuchal translucency measurements ≥5.0 mm (Supplementary Table 3.2).

The results of the sensitivity analysis including only pregnancy outcomes captured after 20 weeks' gestation were mildly attenuated, but not qualitatively different from the main findings (Supplementary Table 3.3).

Results for secondary outcomes

We also performed a series of analyses to assess the relationship between nuchal translucency measurements and perinatal outcomes among pregnancies that resulted in a live birth. Pregnancies with very high nuchal translucency measurements (i.e., ≥5.0 mm) were at risk of some adverse perinatal outcomes such as neonatal intensive care unit admission or having an APGAR score below 7, even when pregnancies with identified chromosomal abnormalities were excluded (Table 3.3).

3.6 Discussion

Principal findings

In this population-based study of pregnancies receiving prenatal screening, the risk of a composite of pregnancy loss, termination, stillbirth, or neonatal death increased with increasing nuchal translucency measurements. This association remained, though attenuated, when excluding pregnancies with identified chromosomal abnormalities. This association also persisted in sensitivity analyses with different assumptions about the pregnancies for which no outcome was recorded, which was important given that the proportion of pregnancies with an unknown outcome was positively associated with the nuchal translucency measurement. Further, among pregnancies that resulted in a live birth, our study showed a potential association between higher nuchal translucency measurement (>5.0 mm) and some adverse perinatal outcomes such as admission to the neonatal intensive care unit and APGAR scores below 7.

Comparison with previous studies

Because previous studies have typically focused on selected groups of pregnant individuals seen in tertiary care centres, or who opted to have diagnostic investigations prenatally, the results of this study are difficult to situate in the context of existing literature. Among studies performed in unselected pregnancies, which would be more comparable to our results, Cheng et al. reported that 70.6% of pregnancies with nuchal translucency measurements ≥3.0 mm resulted in a live birth after excluding chromosomal abnormalities, whereas in our study 80.0% (2 323/2

903) of pregnancies with measurements ≥3.0 mm had a recorded live birth.[17] Westin et al. calculated a six-fold increased risk of adverse outcome in pregnancies with measurements ≥3.0 mm compared to pregnancies with measurements <3.0mm, mirroring the six-fold increase in our study (6.2% compared to 1.0%).[18] Additionally, in descriptive studies of unselected pregnancies with very high nuchal translucency measurements of ≥6.5mm, after excluding those with identified chromosomal abnormalities, Scott et al. and Pitkanen et al. reported 29.6% (8/27) and 20.0% (1/5) resulted in a live birth, respectively, compared to 24.7% (57/231) in our study.[19,20] Shakoor et al. and Tahmasebpour et al. included multiple detailed thresholds but were limited to a small sample size with increasing levels of nuchal translucency; a strength of the study by Shakoor et al. was including a reference group of pregnancies <2.5 mm where 94.6% (1 780/1 882) resulted in a live birth, similarly to the 96.1% (358 074/400 657) in our study.[21,22] None of the other studies cited here or otherwise identified by us included a reference group.

Clinical and research implications

This study represents, to our knowledge, the first report of all pregnancy outcomes by nuchal translucency measurement at a population level, generating more accurate and generalizable risk estimates applicable to the general population. This provides valuable information for patient counselling on the elevated risk of adverse pregnancy outcomes and some perinatal outcomes when an increased nuchal translucency is identified, including when chromosomal abnormalities are not diagnosed. These results also provide important information for the counselling of pregnant individuals who decline prenatal diagnostic investigations following the nuchal translucency measurement but are still interested in the meaning of the nuchal translucency measurement for the pregnancy outcome. Further, our findings illustrate the importance of the nuchal translucency ultrasound beyond its role in screening for chromosomal abnormalities at a time where prenatal screening programs may be considering moving away from multiple marker screening algorithms that include a nuchal translucency measurement. For example, some programs offering universal cell-free DNA screening are proceeding to discontinue the offer of nuchal translucency.[23]

While in theory the 99th percentile for nuchal translucency measurement is measured at 3.5 mm, in this study pregnancies with measurements ≥3.5 mm represented only 0.5% (1 979) of the study population. Although some pregnancies with very high nuchal translucency measurements may have been excluded from the study by discontinuing the multiple marker screening process this points to a chronic undermeasurement of nuchal translucency as has been reported by other jurisdictions and underlines the importance of continued quality assurance of nuchal translucency.[24,25]

Strengths and limitations

Key strengths of our study include the ascertainment of pregnancies in the first trimester, at the time of the nuchal translucency ultrasound; the population-based nature of the data; and the large sample size, including 1 979 pregnancies with nuchal translucency measurements of 3.5 mm or greater. These features allowed us to overcome key limitations of previous studies on this topic, including: (i) restriction to outcomes among pregnant individuals referred to specialty care and seen at academic centers,[4,5,7,8,26–36] where survivor bias could mask an association between increased nuchal translucency measurements and early pregnancy loss; (ii) restriction to pregnant individuals who opted to have prenatal diagnosis and had normal cytogenetic investigations,[7–9,26,28,34,35,37,37–40] which could lead to selection bias to the extent that those receiving prenatal diagnosis are more likely to have additional clinical indications beyond an elevated nuchal translucency prompting the procedure; and (iii) small sample sizes, including fewer than 200 pregnancies with increased nuchal translucency measurements,[4,8,9,17,19–22,26,27,29,34,37–39,41–44] rendering it challenging to understand the association of narrow bands of nuchal translucency measurement with rare outcomes. This study also had several limitations. Information on pregnancy outcomes was missing for some individuals included in the study. To address this limitation, we conducted sensitivity analyses with the assumptions that these losses to follow-up either experienced the composite outcome of pregnancy loss, termination, stillbirth, or neonatal death at either half or twice the risk of those for which an outcome was recorded within each corresponding nuchal translucency category. The results did not change the interpretation of the associations. Further, not all pregnancies in our analysis received cytogenetic testing, as expected on a population level, therefore, we may have failed to identify

some individuals who had a chromosomal abnormality. This misclassification, particularly for pregnancies not resulting in a live birth, may have overestimated the risk estimates in the subgroup analyses where pregnancies with chromosomal abnormalities were excluded.

Additionally, the registry does not systematically capture findings identified on the 18-to-22-week detailed ultrasound and fetal echocardiogram that are routinely offered in Ontario when an increased nuchal translucency measurement is identified (≥3.5 mm), nor the diagnosis of single-gene conditions, which precluded us from incorporating this information in our analysis. Further studies on a population level are required to understand the role of structural defects and single-gene conditions in the increased risk of perinatal complications with increasing nuchal translucency levels, as we hypothesize these conditions would play an important role in the underlying pathophysiological explanation of the association between nuchal translucency values and perinatal complications when chromosomal abnormalities have been excluded.

Finally, not all pregnant individuals in Ontario receive multiple marker screening. Although there is evidence that there are differences among pregnant individuals who do and do not receive prenatal genetic screening (e.g. maternal age, living in rural areas of Ontario)[13,45] there is no reason to expect the association between the nuchal translucency measurement and pregnancy outcome to differ in this population excluded from our study.

3.7 Conclusions

This study reports on the increasing risk of a composite of pregnancy loss, termination, stillbirth, or neonatal death with increasing nuchal translucency measurements on a population level. This association was found to persist when pregnancies with chromosomal abnormalities were excluded, and under different scenarios accounting for the pregnancies for which no outcome was recorded. Pregnancies with high nuchal translucency measurements were also at risk for some adverse perinatal health outcomes even if cytogenetic investigations were normal. These findings will provide valuable information for patient counselling on overall pregnancy outcomes, rather than specifically in the situation where prenatal diagnosis has already taken place and resulted in normal findings.

Acknowledgements

Source of funding:

Canadian Institutes of Health Research: Doctoral Award Frederick Banting and Charles Best Canada Graduate Scholarships (Funding Reference Number FBD-170866)

There was no involvement from the funding source in the design, collection, analysis, interpretation of the study findings or manuscript preparation.

Conflict of interest:

During the conduct of this work, DBF was employed by the University of Ottawa and had an academic appointment at the Children's Hospital of Eastern Ontario Research Institute. Although she maintains those academic affiliations, she is now employed by Pfizer and works on an unrelated topic.

The other authors report no conflict of interest.

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Figure 3.1. Inclusion Flow for Pregnancies Included In the Study.

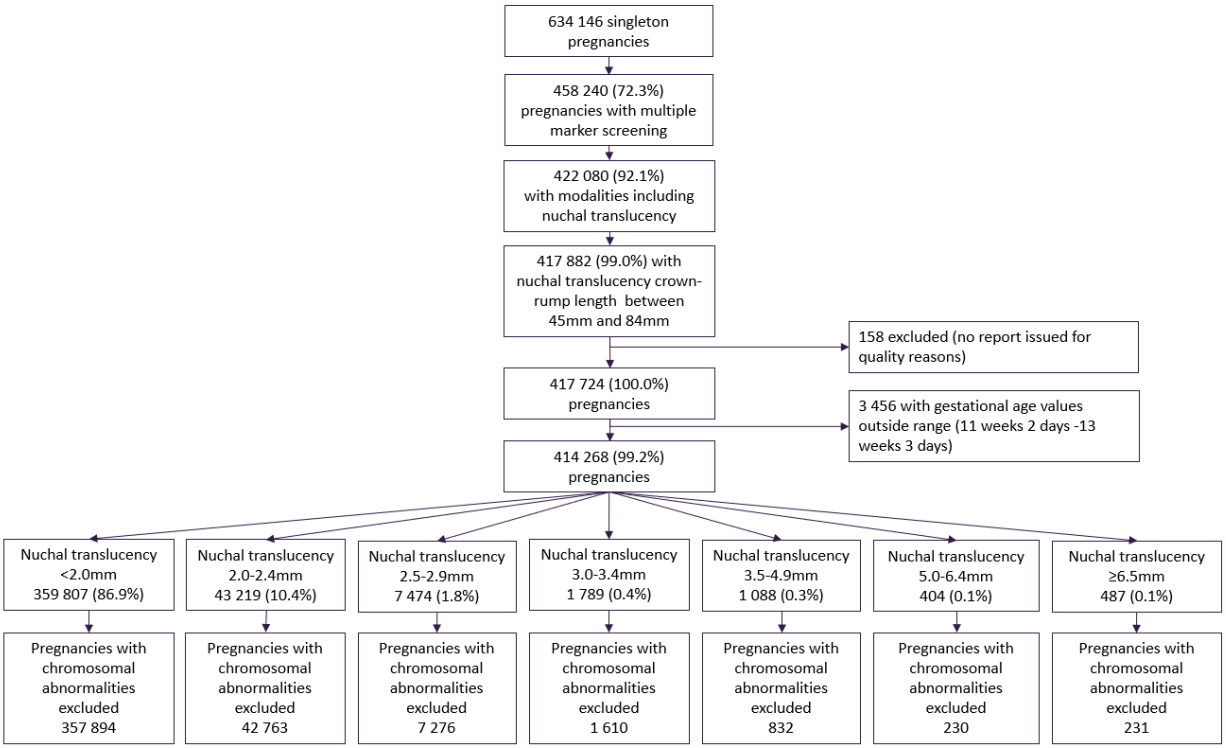


Table 3.1. Characteristics of Study Population by Nuchal Translucency Measurement.

Characteristic	Pregnancies with nuchal translucency measurement	Nuchal translucency measurement (mm)						
		<2.0	2.0-<2.5	2.5-<3.0	3.0-<3.5	3.5-<5.0	5.0-<6.5	≥6.5
Total	**414 268**	**359 807**	**43 219**	**7 474**	**1 789**	**1 088**	**404**	**487**
Age, EDD Mean years (SD)	**31.5 (4.7)**	31.5 (4.7)	31.6 (4.8)	31.9 (4.8)	32.2 (4.8)	32.8 (5.0)	33.0 (5.5)	33.3 (5.4)
Gestational age at screening Mean days (SD)	**87.8 (3.3)**	87.5 (3.3)	90.0 (2.6)	90.0 (3.0)	88.9 (3.4)	87.3 (3.6)	86.4 (3.6)	86.9 (3.1)
Weight of pregnant individual Mean kg (SD)	**68 (17.0)**	67.9 (16.9)	68.5 (17.4)	68.3 (17.3)	68.1 (17.0)	67.2 (16.6)	68.8 (17.8)	67.0 (14.0)
Parity, n(%)								
Nulliparous	**183 587 (46.2)**	161 994 (46.8)	17 689 (42.7)	2 847 (40.3)	595 (37.5)	314 (39.4)	81 (46.0)	67 (45.6)
Primiparous	**143 190 (36.0)**	123 661 (35.7)	15 760 (38.0)	2 739 (38.7)	614 (38.7)	320 (40.2)	56 (31.8)	40 (27.2)
Multiparous	**70 967 (17.8)**	60 848 (17.6)	8 012 (19.3)	1 487 (21.0)	378 (23.8)	163 (20.5)	39 (22.2)	40 (27.2)
Missing	***16 524***	*13 304*	*1 758*	*401*	*202**	*291**	*228**	*340**
Conception, n(%)								
Spontaneous	**374 873 (96.0)**	326 450 (96.0)	38 712 (95.9)	6 685 (95.9)	1 568 (96.1)	876 (96.4)	275 (96.8)	307 (97.5)
IVF	**1 2025 (3.1)**	10 429 (3.1)	1 289 (3.2)	220 (3.2)	49 (3.0)	22 (2.4)	8 (2.8)	8 (2.5)
Other ART	**3 659 (0.9)**	3 188 (0.9)	378 (0.9)	66 (0.9)	15 (0.9)	11 (1.2)	<6 (S)	0 (0.0)
Missing	***23 711***	*19 740*	*2 840*	*503*	*157*	*179**	*120**	*172**
Smoking, n(%)								
Nonsmoker	**358 781 (91.8)**	312 038 (92.0)	37 006 (90.5)	6 458 (90.3)	1 542 (90.5)	953 (92.9)	364 (94.5)	420 (93.1)
Smoker	**32 117 (8.2)**	27 264 (8.0)	3 874 (9.5)	693 (9.7)	161 (9.5)	73 (7.1)	21 (5.5)	31 (6.9)
Missing	***23 370***	*20 505*	*2 339*	*323*	*86*	*62*	*19*	*36*
Neighborhood Income Quintile, n(%)								
First	**84 356 (20.7)**	73 001 (20.6)	9 057 (21.3)	1 564 (21.3)	343 (19.6)	217 (20.4)	91 (23.0)	83 (17.4)
Second	**84 977 (20.9)**	73 596 (20.8)	8 929 (21.0)	1 647 (22.4)	382 (21.8)	224 (21.1)	95 (24.0)	104 (21.8)
Third	**87 373 (21.4)**	76 165 (21.5)	8 984 (21.2)	1 476 (20.1)	365 (20.9)	209 (19.7)	63 (15.9)	111 (23.3)
Fourth	**83 373 (20.5)**	72 711 (20.5)	8 496 (20.0)	1 407 (19.1)	364 (20.8)	216 (20.3)	79 (19.9)	100 (21.0)
Fifth	**67 279 (16.5)**	58 379 (16.5)	7 001 (16.5)	1 259 (17.1)	296 (16.9)	197 (18.5)	68 (17.2)	79 (16.6)
Missing	***6 910***	*5 955*	*752*	*121*	*39*	*25*	*8*	*10*
Neighborhood Education Quintile, n(%)								
First	**51 317 (13.6)**	44 203 (13.5)	5 677 (14.5)	968 (14.3)	236 (14.6)	143 (14.6)	43 (12.1)	47 (10.5)
Second	**69 246 (18.4)**	59 896 (18.3)	7 431 (19.0)	1 259 (18.5)	333 (20.7)	165 (16.8)	77 (21.8)	85 (18.9)
Third	**83 534 (22.2)**	72 957 (22.3)	8 396 (21.4)	1 474 (21.7)	313 (19.4)	218 (22.2)	72 (20.3)	104 (23.2)
Fourth	**94 749 (25.2)**	82 688 (25.3)	9 496 (24.2)	1 665 (24.5)	422 (26.2)	264 (26.9)	97 (27.4)	117 (26.1)
Fifth	**77 202 (20.5)**	66 954 (20.5)	8 165 (20.8)	1 423 (21.0)	308 (19.1)	191 (19.5)	65 (18.4)	96 (21.4)
Missing	***38 220***	*33 109*	*4 054*	*685*	*177*	*107*	*50**	*38*

**Missing data for more than 10.0% of the pregnancies*

ART, Assisted reproductive technology; EDD, Estimated date of delivery; IVF, in vitro fertilization; SD, standard deviation.

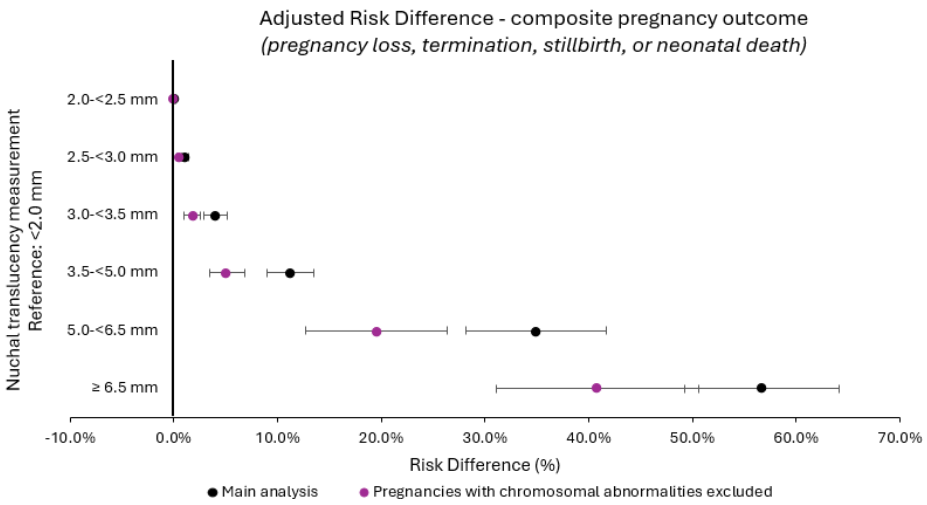
Table 3.2. Overall Pregnancy Outcomes by Nuchal Translucency Measurement Category.

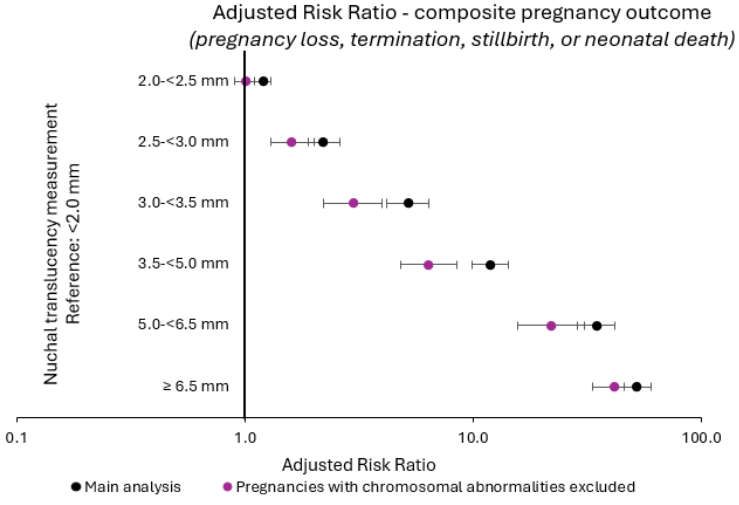
	Overall	Nuchal translucency measurement (mm)						
		<2.0	2.0-<2.5	2.5-<3.0	3.0-<3.5	3.5-<5.0	5.0-<6.5	≥6.5
A. All pregnancies	**414 268**	**359 807**	**43 219**	**7 474**	**1 789**	**1 088**	**404**	**487**
Pregnancies with a recorded outcome	**400 625 (96.7)**	**348 947 (97.0)**	**41 753 (96.6)**	**7 133 (95.4)**	**1 608 (89.9)**	**824 (75.7)**	**192 (47.5)**	**168 (34.5)**
Live birth without neonatal death, n (%)	**396148 (98.9)**	345404 (99.0)	41313 (98.9)	6988 (98.0)	1527 (95.0)	722 (87.6)	123 (64.1)	71 (42.3)
Total composite outcome	**4 477 (1.1)**	3 543 (1.0)	440 (1.1)	145 (2.0)	81 (5.0)	102 (12.4)	69 (35.9)	97 (57.7)
Pregnancy loss, n (%)	***468 (0.1)***	*381 (0.1)*	*37 (0.1)*	*14 (0.2)*	*6 (0.4)*	*8 (1.0)*	*6 (3.1)*	*16 (9.5)*
Stillbirth, n (%)	***1 602 (0.4)***	*1 356 (0.4)*	*168 (0.4)*	*40 (0.6)*	*9 (0.6)*	*8 (1.0)*	*11 (5.7)*	*10 (6.0)*
Termination, n (%)	***1 396 (0.3)***	*961 (0.3)*	*134 (0.3)*	*66 (0.9)*	*54 (3.4)*	*73 (8.9)*	*45 (23.4)*	*63 (37.5)*
Neonatal death, n (%)	***1 011 (0.3)***	*845 (0.2)*	*101 (0.2)*	*25 (0.4)*	*12 (0.7)*	*13 (1.6)*	*7 (3.6)*	*8 (4.8)*
Pregnancies with no recorded outcome (lost to follow-up)	**13 643 (3.3)**	**10 860 (3.0)**	**1 466 (3.4)**	**341 (4.6)**	**181 (10.1)**	**264 (24.3)**	**212 (52.5)**	**319 (65.5)**
B. Pregnancies with chromosomal abnormalities excluded								
Total pregnancies, chromosomal abnormalities excluded	**410 836**	**357 894**	**42 763**	**7 276**	**1 610**	**832**	**230**	**231**
Pregnancies with a recorded outcome	**398 235 (96.9)**	**347 338 (97.1)**	**41 414 (96.8)**	**7 006 (96.3)**	**1 529 (95.0)**	**718 (86.3)**	**132 (57.4)**	**98 (42.4)**
Live birth without neonatal death, n (%)	**394 302 (99.0)**	344 022 (99.0)	41 052 (99.1)	6 905 (98.6)	1 487 (97.3)	674 (93.9)	105 (79.5)	57 (58.2)
Total composite outcome	**3 933 (1.0)**	3 316 (1.0)	362 (0.9)	101 (1.4)	42 (2.7)	44 (6.1)	27 (20.5)	41 (41.8)
Pregnancy loss, n (%)	***432 (0.1)***	*372 (0.1)*	*33 (0.1)*	*14 (0.2)*	*<6 (S)*	*<6 (S)*	*<6 (S)*	*<6 (S)*
Stillbirth, n (%)	***1 549 (0.4)***	*1 327 (0.4)*	*164 (0.4)*	*35 (0.5)*	*7 (0.5)*	*6 (0.8)*	*<6 (S)*	*<6 (S)*
Termination, n (%)	***1 001 (0.3)***	*804 (0.2)*	*76 (0.2)*	*31 (0.4)*	*22 (1.4)*	*26 (3.6)*	*15 (11.4)*	*27 (27.6)*
Neonatal death, n (%)	***951 (0.2)***	*813 (0.2)*	*89 (0.2)*	*21 (0.3)*	*8 (0.5)*	*8 (1.1)*	*<6 (S)*	*7 (7.1)*
Pregnancies with no recorded outcome (lost to follow-up)	**12 601 (3.1)**	**10 556 (3.0)**	**1 349 (3.2)**	**270 (3.7)**	**81 (5.0)**	**114 (13.7)**	**98 (42.6)**	**133 (57.6)**

Figure 3.2. Pregnancy Outcomes by Nuchal Translucency Measurement, Main Analysis and Analysis Excluding Pregnancies with Identified Chromosomal Abnormalities.

Nuchal translucency measurement	***Composite of pregnancy loss, termination, stillbirth, or neonatal death***									
	*Adjusted model**					*Adjusted model, pregnancies with chromosomal abnormalities excluded**				
	n (%)	Risk Difference (95% CI)		Risk Ratio (95% CI)		n (%)	Risk Difference (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	3 543 (1.0)	Ref.		Ref.		3 316 (1.0)	Ref.		Ref.	
2.0-<2.5 mm	440 (1.1)	0.1	(0.0-0.2)	1.2	(1.0-1.3)	362 (0.9)	0.0	(-0.1-0.1)	1.0	(0.9-1.1)
2.5-<3.0 mm	145 (2.0)	1.0	(0.7-1.4)	2.2	(1.9-2.6)	101 (1.4)	0.5	(0.3-0.8)	1.6	(1.3-2.0)
3.0-<3.5 mm	81 (5.0)	4.0	(2.9-5.1)	5.2	(4.2-6.4)	42 (2.7)	1.8	(1.0-2.6)	3.0	(2.2-4.0)
3.5-<5.0 mm	102 (12.4)	11.2	(9.0-13.5)	11.9	(9.9-14.3)	44 (6.1)	5.1	(3.4-6.8)	6.4	(4.8-8.5)
5.0-<6.5 mm	69 (35.9)	34.9	(28.1-41.7)	34.8	(28.8-42.0)	27 (20.5)	19.5	(12.7-26.4)	21.9	(15.6-30.8)
≥ 6.5 mm	97 (57.7)	56.6	(49.2-64.1)	52.7	(46.0-60.4)	41 (41.8)	40.8	(31.1-50.6)	42.1	(33.2-53.4)

**Model adjusted for gestational age at nuchal translucency measurement and maternal age at EDD*





**More details on number of cases, crude and adjusted models presented in Supplementary Figures 3.1 and 3.2.*

Table 3.3. Perinatal Outcomes by Nuchal Translucency Measurement Among Pregnancies with a Recorded Live Birth.

	Main model, full analytical sample			*Sample excluding chromosomal abnormalities*		
	No. (%)	**RD, %, (95% CI)**	**RR, (95% CI)**	**No. (%)**	**RD, %, (95% CI)**	**RR, (95% CI)**
Preterm birth						
NT <2.0 mm	21 022 (6.1)	Ref.	Ref.	20 796 (6.1)	Ref.	Ref.
NT 2.0-<2.5 mm	2 363 (5.7)	-0.4 (-0.6,-0.1)	0.9 (0.9,1.0)	2 318 (5.7)	-0.4 (-0.6,-0.2)	0.9 (0.9,1.0)
NT 2.5-<3.0 mm	391 (5.6)	-0.5 (-1.1,0.1)	0.9 (0.8,1.0)	379 (5.5)	-0.6 (-1.1,0.0)	0.9 (0.8,1.0)
NT 3.0-<3.5 mm	97 (6.4)	0.3 (-1.0,1.5)	1.0 (0.9,1.3)	86 (5.8)	-0.3 (-1.5,0.9)	1.0 (0.8,1.2)
NT 3.5-<5.0 mm	57 (7.8)	1.8 (-0.2,3.7)	1.3 (1.0,1.7)	40 (6.0)	-0.1 (-1.9,1.7)	1.0 (0.7,1.3)
NT 5.0-<6.5 mm	18 (14.1)	8.0 (1.9,14.0)	2.3 (1.5,3.5)	14 (13.0)	6.9 (0.6,13.2)	2.1 (1.3,3.5)
NT ≥6.5 mm	9 (12.3)	6.2 (-1.3,13.8)	2.0 (1.1,3.7)	6 (10.0)	3.9 (-3.7,11.5)	1.6 (0.8,3.5)
Missing	*2 939*			*2 888*		
NICU admission						
NT <2.0 mm	34 322 (10.0)	Ref.	Ref.	33 875 (9.9)	Ref.	Ref.
NT 2.0-<2.5 mm	3 852 (9.4)	-0.6 (-0.9,-0.3)	0.9 (0.9,1.0)	3 771 (9.2)	-0.7 (-1.0,-0.4)	0.9 (0.9,1.0)
NT 2.5-<3.0 mm	720 (10.4)	0.4 (-0.4,1.1)	1.0 (0.9,1.1)	684 (10.0)	0.1 (-0.7, 0.8)	1.0 (0.9,1.1)
NT 3.0-<3.5 mm	173 (11.4)	1.4 (-0.2,3.0)	1.1 (0.9,1.3)	154 (10.4)	0.5 (-1.1,2.1)	1.1 (0.9,1.2)
NT 3.5-<5.0 mm	114 (15.9)	5.9 (3.2,8.5)	1.6 (1.3,1.9)	84 (12.5)	2.6 (0.1,5.1)	1.3 (1.0,1.5)
NT 5.0-<6.5 mm	31 (24.4)	14.4 (6.9,21.9)	2.4 (1.8,3.3)	19 (17.8)	7.9 (0.6,15.1)	1.8 (1.2,2.7)
NT ≥6.5 mm	20 (27.4)	17.4 (7.2,27.6)	2.7 (1.9,4.0)	12 (20.0)	10.1 (0.0,20.2)	2.0 (1.2,3.3)
Missing	*3495*			*3425*		
APGAR <7						
NT <2.0 mm	6 124 (1.8)	Ref.	Ref.	6 042 (1.8)	Ref.	Ref.
NT 2.0-<2.5 mm	784 (1.9)	0.1 (0.0,0.3)	1.1 (1.0,1.2)	765 (1.9)	0.1 (0.0,0.3)	1.1 (1.0,1.1)
NT 2.5-<3.0 mm	139 (2.0)	0.2 (-0.1,0.5)	1.1 (0.9,1.3)	135 (2.0)	0.2 (-0.1,0.5)	1.1 (0.9,1.3)
NT 3.0-<3.5 mm	27 (1.8)	0.0 (-0.7,0.7)	1.0 (0.7,1.4)	22 (1.5)	-0.3 (-0.9,0.3)	0.8 (0.6,1.3)
NT 3.5-<5.0 mm	23 (3.2)	1.5 (0.1,2.8)	1.8 (1.2,2.7)	17 (2.6)	0.8 (-0.4,2.0)	1.4 (0.9,2.3)
NT 5.0-<6.5 mm	10 (8.1)	6.3 (1.5,11.2)	4.5 (2.5,8.2)	8 (7.8)	6.0 (0.8,11.2)	4.4 (2.2,8.5)
NT ≥6.5 mm	8 (11.1)	9.3 (2.1,16.6)	6.2 (3.2,11.9)	8 (13.6)	11.8 (3.0,20.5)	7.6 (4.0,14.5)
Missing	*7311*			*7230*		
Small for gestational age						
NT <2.0 mm	34 101 (10.0)	Ref.	Ref.	33 777 (9.9)	Ref.	Ref.
NT 2.0-<2.5 mm	2 971 (7.3)	-2.7 (-3.0,-2.4)	0.7 (0.7,0.8)	2 921 (7.2)	-2.7 (-3.0,-2.5)	0.7 (0.7,0.8)
NT 2.5-<3.0 mm	455 (6.6)	-3.4 (-4.0,-2.8)	0.7 (0.6,0.7)	436 (6.4)	-3.5 (-4.1,-2.9)	0.6 (0.6,0.7)
NT 3.0-<3.5 mm	101 (6.7)	-3.3 (-4.6,-2.1)	0.7 (0.6,0.8)	89 (6.1)	-3.9 (-5.1,-2.7)	0.6 (0.5,0.7)
NT 3.5-<5.0 mm	58 (8.2)	-1.8 (-3.9,0.2)	0.8 (0.6,1.0)	48 (7.2)	-2.7 (-4.7,-0.7)	0.7 (0.6,1.0)
NT 5.0-<6.5 mm	16 (13.0)	3.0 (-2.9,9.0)	1.3 (0.8,2.1)	10 (9.7)	-0.2 (-5.9,5.5)	1.0 (0.5,1.8)
NT ≥6.5 mm	8 (11.4)	1.4 (-6.0,8.9)	1.1 (0.6,2.2)	6 (10.2)	0.2 (-7.5,8.0)	1.0 (0.5,2.2)
Missing	*5595*			*5510*		

CI, confidence interval; NICU, neonatal intensive care unit; NT, nuchal translucency measurement; RD, risk difference; Ref., reference group; RR, Risk ratio.

3.9 Supplementary materials

Supplementary Table 3.1. Description of data sources.

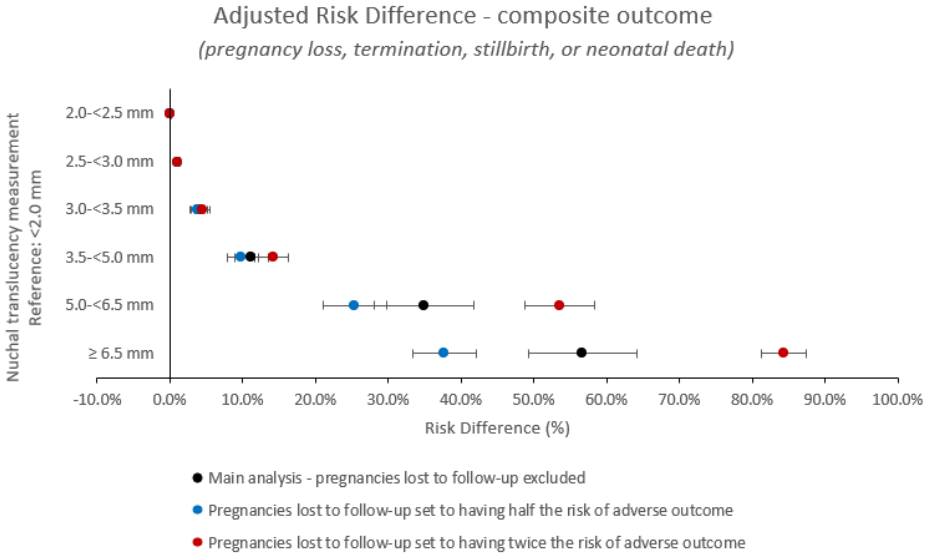
Better Outcomes Registry & Network (BORN) Ontario	Better Outcomes Registry & Network (BORN) Ontario is the provincial perinatal registry for Ontario Canada. Data from all multiple marker screening laboratories, cfDNA screening laboratories, as well as cytogenetic laboratories in Ontario are collected. Information is also collected on all hospital births as well as home births cared for by a midwife. The registry is described in more details in the 2021 publication by Murphy et al. and up-to-date information on data collection can be found on the www.bornontario.ca website.

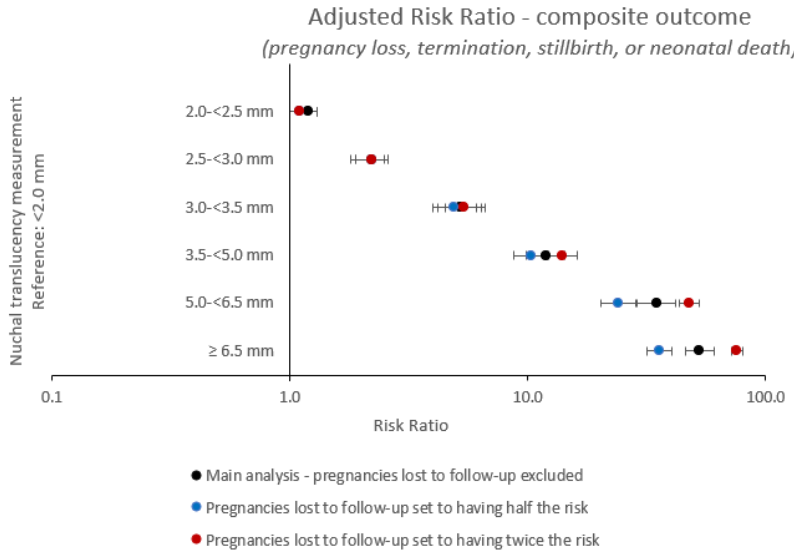
Canadian Institute for Health Information (CIHI) Discharge Abstract Database	The CIHI Discharge Abstract Database metadata (DAD) is a Canadian database that captures information from all hospital discharges. In this study data from CIHI birth admissions were used to supplement information from BORN Ontario for the following variables:
	- Pregnancy outcome
	  ○ Fetal demise - ICD10 code : P95
	  ○ Spontaneous abortion – ICD10 codes: O03;P01.8
	  ○ Termination of Pregnancy – ICD10 codes: O04, O05, O06, P96.4
	  ○ Congenital anomalies – ICD10 codes from Q00 to Q99

Supplementary Figure 3.1. Pregnancy Outcomes by Nuchal Translucency Measurement - Sensitivity Analyses with Varying Assumptions About the Outcome of Pregnancies Lost to Follow-up.

Nuchal translucency measurement (mm)	***Composite outcome of pregnancy loss, termination, stillbirth, or neonatal death***															
	Crude model				*Adjusted model**				*Adjusted model, pregnancies lost to follow-up assumed to have half the risk of those for which an outcome is recorded**				*Adjusted model, pregnancies lost to follow-up assumed to have twice the risk of those for which an outcome is recorded**			
	Risk Difference (95% CI)		Risk ratio (95% CI)		Risk Difference (95% CI)		Risk Ratio (95% CI)		Risk Difference (95% CI)		Risk Ratio (95% CI)		Risk Difference (95% CI)		Risk Ratio (95% CI)	
< 2.0	Ref.		Ref.		Ref.		Ref.		Ref.		Ref.		Ref.		Ref.	
2.0-<2.5	0.0	(-0.1,0.1)	1.0	(0.9,1.1)	0.1	(0.0,0.2)	1.2	(1.0,1.3)	0.1	(0.0,0.2)	1.1	(1.0,1.3)	0.1	(0.0,0.2)	1.1	(1.0,1.3)
2.5-<3.0	1.0	(0.7,1.4)	2.0	(1.7,2.4)	1.0	(0.7,1.4)	2.2	(1.9,2.6)	1.0	(0.7,1.3)	2.2	(1.8,2.5)	1.1	(0.8,1.4)	2.2	(1.9,2.6)
3.0-<3.5	4.0	(3.0,5.1)	5.0	(4.0,6.1)	4.0	(2.9,5.1)	5.2	(4.2,6.4)	3.8	(2.8,4.8)	4.9	(4.0,6.1)	4.5	(3.4,5.5)	5.4	(4.5,6.6)
3.5-<5.0	11.4	(9.1,13.6)	12.2	(10.1,14.7)	11.2	(9.0,13.5)	11.9	(9.9,14.3)	9.7	(7.9,11.6)	10.3	(8.7,12.2)	14.2	(12.1,16.3)	14.0	(12.1,16.1)
5.0-<6.5	34.9	(28.1,41.7)	35.4	(29.2,42.9)	34.9	(28.1,41.7)	34.8	(28.8,42.0)	25.4	(21.1,29.7)	24.1	(20.3,28.5)	53.5	(48.7,58.4)	47.7	(43.4,52.4)
≥ 6.5	56.7	(49.3,64.2)	56.9	(49.7,65.0)	56.6	(49.2,64.1)	52.7	(46.0,60.4)	37.7	(33.4,42.1)	35.8	(31.8,40.3)	84.3	(81.2,87.4)	75.7	(71.8,79.8)

**Model adjusted for gestational age at nuchal translucency measurement and age of the pregnant individual at estimated date of delivery*

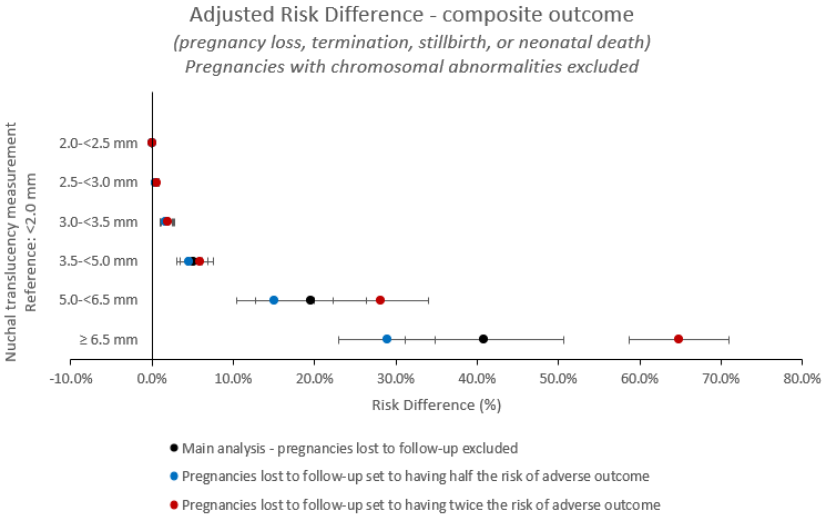


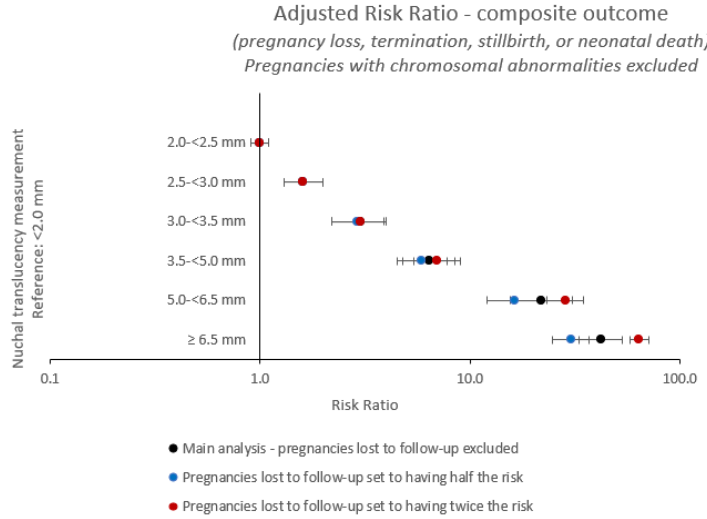


Supplementary Figure 3.2. Pregnancy Outcomes by Nuchal Translucency Measurement - Sensitivity Analyses with Varying Assumptions About the Outcome of Pregnancies Lost to Follow-up, with Exlusion of Pregnancies with Identified Chromosomal Abnormalities.

Nuchal translucency measurement (mm)	***Composite outcome of pregnancy loss, termination, stillbirth, or neonatal death - pregnancies with chromosomal abnormalities excluded***															
	Crude model				*Adjusted model**				*Adjusted model, pregnancies lost to follow-up assumed to have half the risk of those for which an outcome is recorded**				*Adjusted model, pregnancies lost to follow-up assumed to have twice the risk of those for which an outcome is recorded**			
	Risk Difference (95% CI)		Risk ratio (95% CI)		Risk Difference (95% CI)		Risk Ratio (95% CI)		Risk Difference (95% CI)		Risk Ratio (95% CI)		Risk Difference (95% CI)		Risk Ratio (95% CI)	
< 2.0	Ref.		Ref.		Ref.		Ref.		Ref.		Ref.		Ref.		Ref.	
2.0-<2.5	-0.1	(-0.2,0.0)	0.9	(0.8,1.0)	0.0	(-0.1,0.1)	1.0	(0.9,1.1)	0.0	(-0.1,0.1)	1.0	(0.9,1.1)	0.0	(-0.1,0.1)	1.0	(0.9,1.1)
2.5-<3.0	0.5	(0.2,0.8)	1.5	(1.2,1.8)	0.5	(0.3,0.8)	1.6	(1.3,2.0)	0.5	(0.3,0.8)	1.6	(1.3,2.0)	0.6	(0.3,0.9)	1.6	(1.3,2.0)
3.0-<3.5	1.8	(1.0,2.6)	2.9	(2.1,3.9)	1.8	(1.0,2.6)	3.0	(2.2,4.0)	1.7	(1.0,2.5)	2.9	(2.2,3.9)	1.9	(1.1,2.7)	3.0	(2.2,4.0)
3.5-<5.0	5.2	(3.4,6.9)	6.4	(4.8,8.6)	5.1	(3.4,6.8)	6.4	(4.8,8.5)	4.6	(3.1,6.2)	5.9	(4.5,7.8)	5.9	(4.2,7.6)	7.0	(5.4,9.0)
5.0-<6.5	19.5	(12.6,26.4)	21.4	(15.3,30.0)	19.5	(12.7,26.4)	21.9	(15.6,30.8)	15.1	(10.4,19.9)	16.3	(12.1,22.0)	28.1	(22.2,34.0)	28.4	(23.1,34.8)
≥ 6.5	40.9	(31.1,50.7)	43.8	(34.6,55.5)	40.8	(31.1,50.6)	42.1	(33.2,53.4)	28.9	(23.0,34.8)	30.3	(24.8,37.0)	64.8	(58.7,70.9)	63.9	(57.7,70.8)

**Model adjusted for gestational age at nuchal translucency measurement and age of the pregnant individual at estimated date of delivery*





Supplementary Table 3.2. Pregnancy Outcomes by Nuchal Translucency Measurement Including Only Pregnancies with Cytogenetic Testing and Normal Cytogenetic Results.

Nuchal translucency measurement	***Number of pregnancies with composite outcome***	***Composite outcome of pregnancy loss, termination, stillbirth, or neonatal death***							
		Crude model				*Adjusted model**			
		Risk Difference (95% CI)		Risk Ratio (95% CI)		Risk Difference (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	828 (9.1)	Ref.		Ref.		Ref.		Ref.	
2.0-<2.5 mm	87 (6.5)	-2.7	(-4.1,1.2)	0.7	(0.6,0.9)	-2.1	(-3.6,0.6)	0.8	(0.6,0.9)
2.5-<3.0 mm	38 (9.1)	-0.1	(-2.9,2.7)	1.0	(0.7,1.4)	0.3	(-2.5,3.2)	1.0	(0.8,1.4)
3.0-<3.5 mm	16 (7.2)	-1.9	(-5.4,1.5)	0.8	(0.5,1.3)	-1.4	(-5.0,2.2)	0.8	(0.5,1.3)
3.5-<5.0 mm	29 (9.3)	0.1	(-3.1,3.4)	1.0	(0.7,1.4)	0.0	(-3.2,3.2)	1.0	(0.7,1.4)
5.0-<6.5 mm	15 (19.0)	9.9	(1.2,18.5)	2.1	(1.3,3.3)	9.5	(0.9,18.0)	2.0	(1.3,3.2)
≥ 6.5 mm	29 (46.8)	37.7	(25.2,50.1)	5.1	(3.9,6.7)	37.6	(25.2,50.0)	5.1	(3.9,6.7)

**Model adjusted for gestational age at screening and age of the pregnant individual at estimated date of delivery.*

Supplementary Table 3.3. Pregnancy Outcomes After 20 weeks' Gestation by Nuchal Translucency Measurement.

Nuchal translucency measurement	***Number of pregnancies with composite outcome***	***Composite outcome of pregnancy loss, termination, stillbirth, or neonatal death recorded after 20 weeks gestation only***							
		Crude model				*Adjusted model**			
		Risk Difference (95% CI)		Risk Ratio (95% CI)		Risk Difference (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	3 266 (0.9)	Ref.		Ref.		Ref.		Ref.	
2.0-<2.5 mm	386 (0.9)	0.0	(-0.1,0.1)	1.0	(0.9,1.1)	0.1	(0.0,0.2)	1.1	(1.0,1.2)
2.5-<3.0 mm	104 (1.5)	0.5	(0.3,0.8)	1.6	(1.3,1.9)	0.6	(0.3,0.9)	1.7	(1.4,2.1)
3.0-<3.5 mm	51 (3.2)	2.3	(1.4,3.2)	3.5	(2.6,4.5)	2.3	(1.4,3.2)	3.6	(2.7,4.7)
3.5-<5.0 mm	45 (5.9)	4.9	(3.3,6.6)	6.3	(4.7,8.3)	4.8	(3.2,6.4)	6.2	(4.7,8.2)
5.0-<6.5 mm	36 (22.6)	21.7	(15.2,28.2)	24.2	(18.1,32.3)	21.7	(15.2,28.2)	24.2	(18.1,32.3)
≥ 6.5 mm	47 (39.8)	38.9	(30.1,47.7)	42.5	(34.0,53.2)	38.9	(30.0,47.7)	40.8	(32.5,51.1)

**Model adjusted for gestational age at screening and age of the pregnant individual at estimated date of delivery.*

Supplementary Table 3.4. Sensitivity Analyses with Overall Admission to the Neonatal Intensive Care Unit and Varying Lengths of Stay.

NICU admission	*Main model, full analytical sample*			*Sample excluding chromosomal abnormalities*		
	No. (%)	**RD, %, (95% CI)**	**RR, (95% CI)**	**No. (%)**	**RD, %, (95% CI)**	**RR, (95% CI)**
Overall						
NT <2.0 mm	41 242 (12.0)			40 697 (11.9)	Ref.	Ref.
NT 2.0-<2.5 mm	4 747 (11.5)	-0.5 (-0.8,-0.1)	1.0 (0.9,1.0)	4 639 (11.4)	-0.5 (-0.9, -0.2)	1.0 (0.9,1.0)
NT 2.5-<3.0 mm	896 (12.9)	0.9 (0.1,1.7)	1.1 (1.0,1.1)	857 (12.5)	0.6 (-0.2,1.4)	1.0 (1.0,1.1)
NT 3.0-<3.5 mm	215 (14.1)	2.1 (0.4, 3.9)	1.2 (1.0,1.3)	193 (13.0)	1.2 (-0.6,2.9)	1.1 (1.0,1.3)
NT 3.5-<5.0 mm	150 (20.8)	8.8 (5.8,11.7)	1.7 (1.5,2.0)	115 (17.1)	5.2 (2.4, 8.1)	1.4 (1.2,1.7)
NT 5.0-<6.5 mm	38 (29.7)	17.7 (9.8,25.6)	2.5 (1.9, 3.2)	25 (23.2)	11.3 (3.3,19.2)	1.9 (1.4,2.7)
NT ≥6.5 mm	31 (42.5)	30.5 (19.1,41.8)	3.5 (2.7, 4.6)	21 (35)	23.1 (11.0-35.2)	2.9 (2.1,4.2)
Missing	*2 937*			*2 886*		
Over 4 hours						
NT <2.0 mm	37 416 (10.9)	Ref.	Ref.	36 924 (10.8)	Ref.	Ref.
NT 2.0-<2.5 mm	4 269 (10.4)	-0.5 (-0.8,-0.2)	1.0 (0.9,1.0)	4 178 (10.2)	-0.6 (-0.9,-0.3)	0.9 (0.9,1.0)
NT 2.5-<3.0 mm	795 (11.5)	0.6 (-0.2,1.3)	1.1 (1.0,1.1)	757 (11.0)	0.2 (-0.5, 1.0)	1.0 (1.0,1.1)
NT 3.0-<3.5 mm	190 (12.5)	1.6 (-0.1,3.3)	1.1 (1.0,1.3)	170 (11.5)	0.7 (-0.9,2.3)	1.1 (0.9, 1.2)
NT 3.5-<5.0 mm	121 (16.8)	5.9 (3.2,8.7)	1.5 (1.3,1.8)	89 (13.3)	2.5 (-0.1,5.1)	1.2 (1.0,1.5)
NT 5.0-<6.5 mm	32 (25.2)	14.3 (6.7,21.9)	2.3 (1.7,3.1)	20 (18.7)	7.9 (0.5, 15.3)	1.7 (1.2,2.6)
NT ≥6.5 mm	23 (31.5)	20.6 (10.0,31.3)	2.9 (2.1,4.1)	15 (25.0)	14.2 (3.2,25.2)	2.3 (1.5,3.6)
Missing	*3495*			*3425*		
Over 6 hours						
NT <2.0 mm	36 141 (10.5)	Ref.	Ref.	35 670 (10.4)	Ref.	Ref.
NT 2.0-<2.5 mm	4 092 (10.0)	-0.6 (-0.9,-0.3)	0.9 (0.9,1.0)	4 007 (9.8)	-0.6 (-0.9,-0.3)	0.9 (0.9,1.0)
NT 2.5-<3.0 mm	760 (11.0)	0.4 (-0.3,1.2)	1.0 (0.9,1.1)	722 (10.5)	0.1 (-0.6, 0.8)	1.0 (0.9,1.1)
NT 3.0-<3.5 mm	183 (12.0)	1.5 (-0.1,3.1)	1.1 (0.9,1.3)	163 (11.0)	0.6 (-1.0,2.2)	1.1 (0.9,1.2)
NT 3.5-<5.0 mm	116 (16.1)	5.6 (2.9,8.3)	1.5 (1.3,1.8)	85 (12.7)	2.3 (-0.3,4.8)	1.2 (1.0,1.5)
NT 5.0-<6.5 mm	32 (25.2)	14.7 (7.1, 22.2)	2.4 (1.8,3.2)	20 (18.7)	8.3 (0.9,15.6)	1.8 (1.2,2.7)
NT ≥6.5 mm	21 (28.8)	18.2 (7.9,28.6)	2.7 (1.9,3.9)	13 (21.7)	11.2 (0.8,21.7)	2.1 (1.3,3.4)
Missing	*3495*			*3425*		
Over 12 hours						
NT <2.0 mm	34 322 (10.0)	Ref.	Ref.	33 875 (9.9)	Ref.	Ref.
NT 2.0-<2.5 mm	3 852 (9.4)	-0.6 (-0.9,-0.3)	0.9 (0.9,1.0)	3 771 (9.2)	-0.7 (-1.0,-0.4)	0.9 (0.9,1.0)
NT 2.5-<3.0 mm	720 (10.4)	0.4 (-0.4,1.1)	1.0 (0.9,1.1)	684 (10.0)	0.1 (-0.7, 0.8)	1.0 (0.9,1.1)
NT 3.0-<3.5 mm	173 (11.4)	1.4 (-0.2,3.0)	1.1 (0.9,1.3)	154 (10.4)	0.5 (-1.1,2.1)	1.1 (0.9,1.2)
NT 3.5-<5.0 mm	114 (15.9)	5.9 (3.2,8.5)	1.6 (1.3,1.9)	84 (12.5)	2.6 (0.1,5.1)	1.3 (1.0,1.5)
NT 5.0-<6.5 mm	31 (24.4)	14.4 (6.9,21.9)	2.4 (1.8,3.3)	19 (17.8)	7.9 (0.6,15.1)	1.8 (1.2,2.7)
NT ≥6.5 mm	20 (27.4)	17.4 (7.2,27.6)	2.7 (1.9,4.0)	12 (20.0)	10.1 (0.0,20.2)	2.0 (1.2,3.3)
Missing	*3495*			*3425*		

CI, confidence interval; NICU, neonatal intensive care unit; NT, nuchal translucency measurement; RD, risk difference; Ref., reference group; RR, Risk ratio.

Chapter 4. Manuscript 3: Double-positive multiple marker screening results

Title of manuscript: Pregnancies with 'double-positive' multiple marker screening results: a population-based study in Ontario, Canada.

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Publication status: The manuscript presented in this chapter was submitted for peer-review.

4.1 Preface

This chapter presents the results of our study investigating the outcomes of pregnancies with double-positive multiple marker screening results (results positive for both trisomy 21 and trisomy 18 at once) in which a diagnosis of trisomies 21 and 18 have been excluded. The main objective was to assess the risk of preterm birth among pregnancies with double-positive multiple marker screening results given the clinical importance of this outcome. Secondary objectives aimed to examine the association with double-positive results and chromosomal abnormalities other than trisomy 21 and 18, a composite outcome of pregnancy loss, termination, or stillbirth, as well as adverse perinatal outcomes among the pregnancies that resulted in a live birth. This study was approved by the research ethics board of the Children's Hospital of Eastern Ontario (protocol #22/05PE) and University of Ottawa (protocol #H-06-22-8269).

Contribution Statement

Kara Bellai-Dussault led the design of this study, performed the statistical analyses, interpreted the study results, and drafted the manuscript. The co-authors Beth Potter, Shelley Dougan, Deshayne Fell, Steven Hawken, Carolina Lavin Venegas, Julian Little, Lynn Meng, Mark Walker, Tianhua Huang, Nan Okun, and Christine Armour contributed to the conceptualization of the study, the interpretation of the study findings, and reviewed the manuscript. Additionally, Lynn Meng was involved in obtaining the data set and provided support for the more complex data analysis portions of the project. Carolina Lavin Venegas also provided administrative support for the research ethics board applications and reviewed the manuscript. Moreover, Steven Hawken provided advice on the statistical methods. All authors made significant contributions to this work, critically reviewed the manuscript, and have approved the final version that has been submitted for publication.

Why was this study conducted?

Information on the outcome of pregnancies with multiple marker screening results positive for both trisomies 21 and 18, or 'double-positive results' is extremely scarce, hindering patient counselling when this type of result is reported.

What are the key findings?

Pregnancies with double-positive multiple marker screening results were found to be at increased risk of preterm birth and other adverse perinatal outcomes, compared to pregnancies with screen-negative results, even when diagnosed chromosomal abnormalities were excluded.

What does this study add to what is already known?

We identified only one small previous comparative study on this topic. Our study provides population-based evidence on the outcomes of pregnancies with double-positive multiple marker screening results to inform clinical practice and patient counselling.

4.2 Abstract

Background

Multiple marker screening has been offered to pregnant individuals since the 1980s to screen for trisomies 21 and 18. On occasion, the result is 'double-positive'—a screening result that is unexpectedly positive for both trisomies 21 and 18. Although this occurs rarely, the paucity of available evidence about the outcomes of these pregnancies hinders patient counselling.

Objectives

This study aimed to investigate the association of double-positive prenatal screening results with preterm birth and other adverse perinatal outcomes.

Study Design

We conducted a population-based retrospective cohort study using province-wide perinatal registry data in Ontario, Canada. From pregnancies receiving multiple marker screening with an estimated date of delivery from September 1, 2016, to March 31, 2021, we identified those with double-positive screening results overall and where trisomies 21 and 18 were excluded through subsequent cytogenetic testing or a low-risk cell-free DNA screening result. These pregnancies were compared to pregnancies with screen negative results for both aneuploidies. The primary outcome was preterm birth. Secondary outcomes included chromosomal abnormalities other than trisomies 21 and 18, a composite outcome of pregnancy loss, stillbirth, or termination, and for pregnancies resulting in a live birth, admission to a neonatal intensive care unit, cesarean delivery, small for gestational age, and preeclampsia.

We used modified Poisson regression models with robust variance estimation to examine the association of double positive results with preterm birth and secondary outcomes while adjusting for characteristics of the pregnant individual (age, pre-pregnancy weight, racial origin, smoking, insulin dependent diabetes mellitus) and type of conception. We used multiple imputation to address missing covariate data.

Results

From 429 540 pregnancies with multiple marker screening results, 863 (0.2%) had a double-positive result; after restricting to those for which trisomies 21 and 18 were ruled out, there were 374 double-positive pregnancies included in the main analyses, 203 of which resulted in a live birth. Among the pregnancies in the double-positive group resulting in a live birth, the risk of preterm birth was increased compared to pregnancies with a screen negative result: adjusted risk ratio (aRR) 2.6 (95%CI 2.0-3.6), adjusted risk difference (aRD) 10.5% (95%CI 5.4-15.7). In a sensitivity analysis excluding all diagnosed chromosomal abnormalities, the risk of preterm birth remained elevated to a similar degree (aRR 2.6; 95%CI 1.9-3.7; aRD 10.0%; 95%CI 4.8-15.3). The risk of other adverse perinatal outcomes was also higher, including the risk of chromosomal abnormalities other than trisomies 21 and 18: aRR 81.1 (95%CI 69.4-94.8) and aRD 34.0% (95%CI 29.2-38.8). Pregnancies with double-positive results were also less likely to result in a live birth, even when excluding all diagnosed chromosomal abnormalities; and at increased risk of adverse perinatal outcomes for those resulting in a live birth.

Conclusion

Although a rare occurrence, double-positive multiple marker screening results are associated with an increased risk of preterm birth and other adverse perinatal outcomes, even when excluding all identified chromosomal abnormalities. These results should be taken into consideration when discussing further investigations with patients.

4.3 Introduction

Prenatal screening for trisomies 21 and 18 has been offered to pregnant individuals since the 1980s in the form of multiple marker screening.[1] Although the advent of cell-free DNA (cfDNA) screening technologies has considerably changed many prenatal screening programs, most still rely on multiple marker screening as the main screening modality and offer cfDNA screening in a contingent funding model or as a private-pay option.[2–4] In Ontario, Canada, for the great majority of screened pregnancies, the modality used has been multiple marker screening.[5,6]

The results of multiple marker screening generally yield either 1) a screen-positive result for trisomy 21 or for trisomy 18, in which case follow-up investigations are offered to either further define the risk of aneuploidy (through cfDNA screening) or confirm/refute the positive result through diagnostic cytogenetic testing; or 2) a screen-negative result, for which no follow-up investigations are indicated. However in some instances, the result is unexpectedly reported as positive for both trisomies 21 and 18 at once, referred to as a 'double-positive' result; in some of these cases, diagnostic testing confirms neither of these trisomies.[7] The patterns of biochemical marker levels that confer a positive result are different for the two trisomies, and at times in opposition (e.g., human chorionic gonadotropin is increased in pregnancies with trisomy 21 but very low in pregnancies with trisomy 18),[8] thus a double-positive result is both rare and difficult to interpret.

In a report of 32 pregnancies with double-positive results in 2002, 20 had no reported chromosomal abnormality, yet only 5 resulted in a live birth without obstetric or perinatal complications.[9] A subsequent study compared pregnancy outcomes between 33 patients with a double-positive result for which a prenatal diagnosis of trisomies 21 and 18 had been excluded and 66 age-matched pregnancies with screen-negative results, finding an increased risk of adverse outcomes including spontaneous abortion and lower gestational age at delivery for the double-positive group.[7] This study was limited by its small sample size and potential for selection bias resulting from restriction of the study to pregnancies in which prenatal cytogenetic investigations had been conducted. Nevertheless, the finding that a double-positive

result may indicate a higher risk of preterm birth is particularly concerning given the clinical importance of preterm birth for perinatal and child health outcomes.[10]

Although the occurrence of double-positive results appears to be rare (prevalence 0.3% - 0.4%), the paucity of evidence on this type of result hinders patient counseling.[7,11] The present study aimed to investigate the relationship between a double-positive multiple marker screening result and preterm birth, as well as other adverse obstetrical and perinatal outcomes, on a population level.

4.4 Methods

Study design & data sources

We conducted a population-based retrospective cohort study using data from the Better Outcomes Registry & Network, the prescribed perinatal registry in Ontario, Canada. Data from all multiple marker screening, cfDNA screening, and cytogenetic tests performed in Ontario are collected in the registry, which also captures pregnancy, obstetrical, and perinatal outcomes. All pregnancy outcomes beyond 20 weeks' gestation are captured in the registry, while outcomes for pregnancies lasting less than 20 weeks' gestation are captured less systematically. Data from the Canadian Institute for Health Information Hospital Discharge Abstract Database were used to supplement the capture of pregnancy outcome, preeclampsia, and neonatal intensive care admission (see supplementary Table 4.1).[12]

Setting and study population

In Ontario, multiple marker screening is offered as the first-tier screen; modalities have varied over time and are described in supplementary Table 4.2. cfDNA screening is universally covered under the province's publicly funded health care for those meeting specific eligibility criteria, including a positive multiple marker screen. Individuals may also choose to self-pay for cfDNA screening if the eligibility criteria are not met.[13,14]

All singleton pregnancies with an estimated date of delivery from September 1, 2016, to March 31, 2021, and a multiple marker screening result were included in the study (Figure 4.1).

Study exposure

The double-positive group was defined as pregnancies with a multiple marker screening result that was positive for both trisomies 21 and 18 *and* where we considered the result to be **false positive** due to: (i) a cytogenetic testing result, prenatally or postnatally, that excluded a diagnosis of trisomies 21 and 18; or (ii) a cfDNA screening result that indicated low risk of these trisomies. The low-risk cfDNA screening result was considered sufficient evidence to rule out trisomies 21 and 18 given its negative predictive value of >99.99%, (95%CI 99.97-100.00).[6]

Pregnancies with double-positive results as defined here were compared to pregnancies with a negative multiple marker screening result; pregnancies with a diagnosis of full, partial or mosaic trisomy 21 or 18 were excluded (Figure 4.1).

Study outcome

The primary outcome for this study was preterm birth, defined as a live birth before 37 weeks' gestation.[15] Among pregnancies resulting in a live birth, secondary outcomes included small for gestational age (<10th percentile), cesarean section, diagnosis of preeclampsia, and admission to the neonatal intensive care unit for more than 12 hours.[16]

Additional secondary outcomes were included for all pregnancies: chromosomal abnormalities other than trisomies 21 and 18, and a composite of pregnancy loss, stillbirth, or termination.

Chromosomal abnormalities were identified through cytogenetic testing including rapid aneuploidy detection techniques, karyotype, and microarray tests. Because not all pregnancies receive cytogenetic investigations, additional sources of information were used to ascertain whether pregnancies without such testing could reasonably be defined as not having a chromosomal abnormality. This was achieved based on cfDNA screening results and the findings of the clinical examination at birth recorded in the registry.

Statistical analysis

The study population was described using means and standard deviations for continuous variables and frequencies and proportions for categorical variables.

We computed the cumulative incidence of all study outcomes for both exposure groups and used modified Poisson regression models with robust variance estimation to generate risk ratios and risk differences. All models were adjusted for potential confounders identified *a priori*: characteristics of the pregnant individual (age, pre-pregnancy weight, racial origin, insulin dependent diabetes mellitus, smoking) and conception through assisted reproductive technologies, all factors for which screening laboratories make adjustments and for which an association with adverse perinatal outcomes has been reported.[17,18]

To address missing covariate data (11.6% of the study population were missing data on at least one covariate), we used multiple imputations by chained equations with 20 imputed data sets (Supplementary tables 4.3 and 4.4).[19]

Sensitivity analyses

Given the potential association between double-positive results and chromosomal abnormalities other than trisomies 21 and 18, we conducted a subgroup analysis excluding all pregnancies with identified chromosomal abnormalities to evaluate the impact on our main results.

In the main analysis, only pregnancies with double-positive results in which a diagnosis of trisomies 21 and 18 had been ruled out by follow-up investigations were included. However, 20 of the pregnancies that were excluded from the analysis due to having a double-positive result but no follow-up investigations (i.e., no cytogenetic or cfDNA screening were performed) resulted in a live birth. To determine the impact of the exclusion of these pregnancies, we performed a sensitivity analysis in which these pregnancies were included as part of the double-positive group.

Complete case analyses including only pregnancies for which data on all covariates were available were also conducted as a further sensitivity analysis.

Some individuals in the study population may have had more than one pregnancy over the course of the study period. Due to the complexity of the model given the multiple imputations, we were unable to account for non-independence of such pregnancies. We performed a

sensitivity analysis using one of the imputed data sets to determine the impact of not accounting for this clustering in our main analysis (Supplementary Table 4.5).

We performed the analyses using SAS, version 9.4 (SAS Institute Inc, Cary, NC) and R, version 4.3.1 for the multiple imputations and regression models.

This study received approval by the research ethics board of the Children's Hospital of Eastern Ontario (protocol # 22/05PE) and the University of Ottawa (protocol # H-06-22-8269).

This study is reported using the RECORD guidelines.[20]

4.5 Results

From 634 146 singleton pregnancies recorded in the registry during the study period, 458 240 (72.3%) had multiple marker screening. After excluding 25 651 with a positive screen for trisomy 21, 917 for trisomy 18, and 2 132 where no screening report could be issued, 429 540 pregnant individuals remained, of whom 428 677 had a screen negative result and 863 (0.2%) had a double-positive result (Figure 4.1). Over two-thirds (67.1%) of pregnancies with double-positive results had cytogenetic testing; 49.4% had prenatal cytogenetic testing specifically (Figure 4.2). For pregnancies with screen negative results, 2.8% had cytogenetic testing at any time and 0.6% had prenatal cytogenetic testing specifically.

Among the 863 pregnancies with a double-positive results, a diagnosis of trisomy 21 or 18 was confirmed by cytogenetic testing in 326 (37.8%); these pregnancies were therefore excluded. An additional 46 (5.3%) were excluded due to a high-risk cfDNA screening result for trisomies 21 or 18, presuming a likely diagnosis of aneuploidy. An additional 117 pregnancies were excluded from the double-positive group as a diagnosis of trisomy 21 or 18 could not be excluded (uninformative cytogenetic testing, failed cfDNA screening, or no follow-up investigations performed).

After these exclusions, 374 pregnancies with double-positive results in which diagnoses of trisomies 21 and 18 were ruled out were included in the analytic sample, 203 of which resulted in a live birth (Figure 4.1). These pregnancies were compared with 428 466 pregnancies with a

screen negative result, after excluding 211 screen-negative pregnancies in which a full, partial or mosaic trisomies 21 or 18 was diagnosed; 411 937 of the included screen-negative pregnancies resulted in a live birth (Figure 4.1).

In the analytic sample, pregnant individuals in the group of 374 pregnancies with double-positive results were older than those in the group of 428 466 individuals with screen-negative results (mean age at estimated date of delivery of 35.5 years versus 31.2 years, respectively) and they were also less likely to be nulliparous (22.9% versus 46.4%; Table 4.1). In pregnancies with double-positive results, 44.1% (152/345) had a multiple marker screening result for which the fetal nuchal translucency measurement was below the threshold of 3.5 mm, compared to 99.96% (396 017/396 186) for pregnancies in the group with screen-negative results.

Analyses of primary and secondary outcomes

Outcomes among pregnancies resulting in a live birth

Among pregnancies resulting in a live birth, the incidence of preterm birth was higher among pregnancies with double-positive results (17.0%) compared to the screen-negative group (6.1%; Table 4.2). The adjusted risk ratio (aRR) for preterm birth in those with double-positive results versus screen negative results was 2.6 (95%CI 2.0-3.6) and the adjusted risk difference (aRD) was 10.5% (95%CI 5.4-15.7) (Table 4.2).

The incidence of all secondary outcomes for pregnancies resulting in a live birth was also higher in the double-positive group compared to the group with screen-negative results, with some variation in the magnitude of the increased aRRs and aRDs (cesarean delivery, admission to the neonatal intensive care unit, and small for gestational age, preeclampsia) (Table 4.2).

Outcomes among all pregnancies

In the analyses including all pregnancies, those in the group with double-positive results had an increased risk of chromosomal abnormalities other than trisomies 21 and 18 (34.4%) compared to the screen-negative group (0.4%), with an aRR of 81.1 (95%CI 69.4-94.8) and aRD of 34.0% (95%CI 29.2-38.8) (Table 4.2; detailed numbers for specific chromosomal abnormalities,

supplemental Table 4.6). The proportion of pregnancies ending in a composite of pregnancy loss, stillbirth or termination versus live birth was also higher in the group with double-positive results (20.7%) compared to screen-negative results (0.7%), with an aRR of 26.9 (95%CI 20.8-34.7; Table 4.2). Further, a higher proportion of pregnancies with double-positive results had no pregnancy outcome recorded (31.6%) compared to the screen-negative group (3.2%) (supplemental Table 4.7).

Sensitivity analyses

In a sensitivity analysis where all identified chromosomal abnormalities other than trisomies 21 and 18 were additionally excluded (Table 4.3), the risk of preterm birth was still increased in pregnancies with double-positive results, compared to pregnancies with screen negative results (aRR 2.6 (95%CI 1.9-3.7), aRD 10.0% (95%CI 4.8-15.3)). The results for the secondary outcomes also remained similar in magnitude.

The complete case analysis for preterm birth excluded 47 838 pregnancies (11.6%) due to missing data for one or more covariates; the estimates were similar in magnitude and direction, but less precise (supplementary Tables 4.3 and 4.8).

In our primary analysis, we excluded from the analytic sample 102 pregnancies with double-positive results but no follow-up investigations (Figure 4.2), as we relied on these confirmatory results to rule out trisomies 21 and 18. In 20 such pregnancies, there was a live birth recorded. When these pregnancies were included in the double-positive group, the results for preterm birth were very similar to the main analysis (aRD of 9.9%, 95%CI 5.0-14.8 and aRR of 2.5, 95%CI 1.9-3.4; supplementary Table 4.9).

Additionally, in the sensitivity analysis of a single imputed data set in which we accounted for clustering among individuals with more than one pregnancy in the study cohort, the results were virtually identical to the main analyses (supplementary Table 4.5).

Finally, because some methods of determining the estimated date of delivery of a pregnancy are more accurate (first trimester ultrasound) than others (second trimester ultrasound, last menstrual period), we reviewed this information for pregnancies based on multiple marker

screening results. The methods of estimated date of delivery determination were similar in the two groups, with 94.1% and 95.6% of pregnancies with double-positive results and screen-negative results using first trimester ultrasound, respectively. We reviewed the cases of preterm birth among pregnancies with double-positive results where the estimated date of delivery was not determined by first trimester ultrasound and calculated the association if all these pregnancies had been misclassified as having a preterm birth; the crude risk ratio would decrease from 2.8 to 2.5 (Supplementary Table 4.10).

4.6 Discussion

Principal findings

In this large population-based study, we found that double-positive multiple marker screening results are relatively rare, occurring in 0.2% of pregnancies, confirming a diagnosis of trisomy 21 or 18 in 37.8%, and indicating a high suspicion of aneuploidy in a further 5.3% of pregnancies with a high-risk cfDNA screening result. Among pregnancies with double-positive results in which cytogenetic testing or cfDNA screening ruled out a diagnosis of trisomy 21 or 18, we observed a substantial increase in the risk of preterm birth, compared to pregnancies with a screen-negative result. These pregnancies resulting in a live birth were also at increased risk of admission to the neonatal intensive care unit, small for gestational age, and delivery by cesarean section. These elevated risks remained similar in magnitude when pregnancies with any identified chromosomal abnormality were excluded from the analysis.

There was also an increased risk of chromosomal abnormalities other than trisomies 21 and 18 among pregnancies with a double-positive result, and an elevated risk of a composite of pregnancy loss, stillbirth, or termination.

Results in the context of what is known

To our knowledge, only a descriptive case series and small comparative study exist on the topic of double-positive screening results.[7,9] Similar to our study, the analytic study found an increased risk of pregnancy loss and termination of pregnancy among 33 pregnancies with double-positive results, along with a lower gestational age at birth and lower birth weight.[7] The study was underpowered to identify associations with the other outcomes investigated, including preterm birth, low birth weight, and preeclampsia, whereas our study had a much larger sample. Further, the population included only pregnancies with normal prenatal diagnosis investigations on karyotype, which could represent a higher risk population if additional findings other than the screening result led them to have prenatal diagnosis.

Clinical and research implications

Given that many jurisdictions offer similar multiple marker screening modalities, the results of our study may be applicable to other settings. Our conclusions are broadly important to inform

clinicians and screening programs about the potential increased risk of adverse perinatal outcomes for this rare but clinically important finding, which could inform follow-up testing and monitoring of the pregnancy. Our findings indicate that a double-positive result may be indicative of chromosomal abnormalities other than trisomies 21 and 18, including some abnormalities that are not routinely identified by cfDNA screening; this may inform the type of follow-up investigations offered and clinical management following a double-positive result.

Strengths and limitations

Strengths

An important strength of this study is its population-based design, avoiding the potential for selection bias that may occur when only high-risk individuals who elected to have prenatal diagnosis are included. Our study also included a large sample size, enabling relatively precise estimates of risk for outcomes associated with this rare finding. For assessment of chromosomal abnormalities other than trisomies 21 and 18, we were able to investigate copy number variants, as cytogenetic testing results included microarray analyses. Finally, our thorough description of the study population (Figure 4.2) will serve as a useful reference to inform clinicians and programs regarding the type of follow-up investigations received by patients with this type of screening result.

Limitations

An important limitation of this study was that pregnancy outcomes were not available for all pregnancies that ended before reaching 20 weeks' gestation, as these are captured less systematically in the birth registry. Such pregnancies could represent early losses or terminations, which would most likely result in an underestimation of the association between double-positive multiple marker screening results and pregnancy outcomes given the higher proportion of pregnancies without an outcome recorded in the group with double-positive results. This would also apply to chromosomal abnormalities, as their incidence would be expected to be higher among pregnancy losses and terminations.

An additional limitation is possible misclassification of chromosomal abnormalities since not all pregnancies receive cytogenetic testing. We included a follow-up period of a minimum of three

months after birth to identify the results of postnatal cytogenetic testing, but for some conditions with more subtle clinical features that may not prompt cytogenetic investigations prior to three months of age, a cytogenetic diagnosis may not have yet been established. The misclassification could potentially be differential, due to surveillance bias in pregnancies with double-positive screening results, resulting in a potential overestimation of the risk of chromosomal abnormalities in pregnancies in this group.

4.7 Conclusions

This population-based cohort study provides robust evidence to support previous notions that, although rare, double-positive multiple marker screening results are associated with an increased risk of adverse perinatal outcomes compared to pregnancies with screen negative results. Pregnancies with double-positive results are at increased risk of preterm birth, which can contribute to adverse perinatal and child health outcomes. They are also at an increased risk of chromosomal abnormalities beyond trisomies 21 and 18 and are less likely to result in a live birth. These findings should be taken into consideration in patient counselling and program decisions regarding follow-up investigations and clinical management.

4.8 References

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Figure 4.1. Study Inclusions.

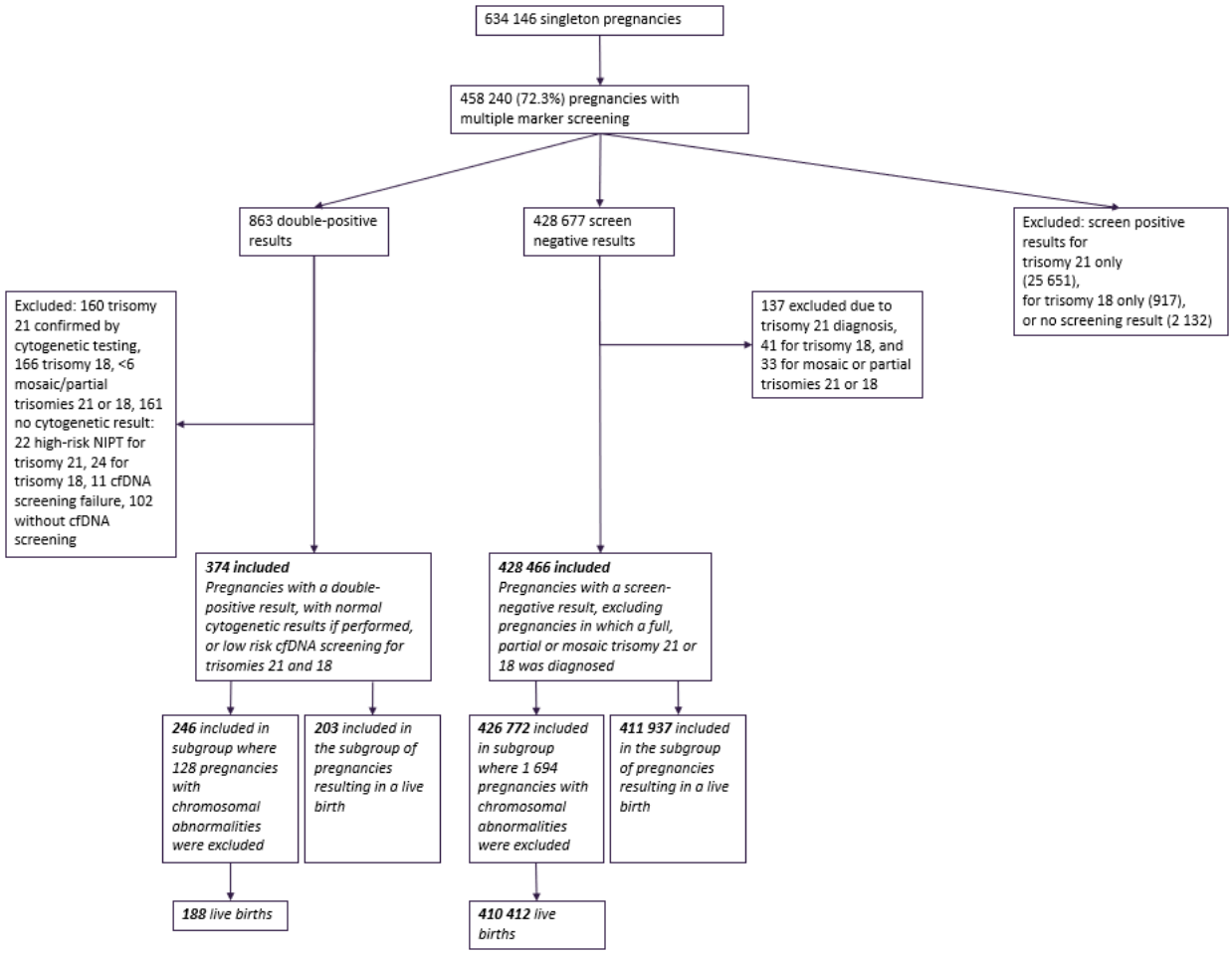


Table 4.1. Characteristics of the Study Population.

Characteristic	Pregnancies in the analytic sample[a]	
	Double-positive results	Screen-negative results
Total	**374**	**428 466**
Age of the pregnant individual at EDD, mean years (SD)	35.5 (5.2)	31.2 (4.7)
Gestational age at screening, mean days (SD)	87.0 (7.5)	88.9 (7.7)
Pre-pregnancy weight, mean kg (SD)	68.7 (15.8)	67.9 (17.1)
Parity, n (%)		
Nulliparous	55 (22.9)	190 997 (46.4)
Primiparous	91 (37.9)	146 318 (35.5)
Multiparous	94 (39.2)	74 531 (18.1)
Missing	*134*[b]	*16 620*
Racial origin, n (%)		
White	212 (57.5)	248 962 (58.8)
Asian	108 (29.3)	117 099 (27.7)
Black	24 (6.5)	30 135 (7.1)
Other	25 (6.8)	27 280 (6.4)
Missing	*<6*	*4 990*
Type of conception, n (%)		
Spontaneous	300 (98.4)	390 297 (96.3)
In vitro fertilization	<6 (S)	11 365 (2.8)
Other assisted reproductive technology	<6 (S)	3 558 (0.9)
Missing	*69*[b]	*23 246*
Smoking status, n (%)		
Nonsmoker	330 (93.2)	367 572 (91.0)
Smoker	24 (6.8)	36 350 (9.0)
Missing	*20*	*24 544*
Neighbourhood income quintile, n (%)		
First quintile	71 (19.6)	89 137 (21.6)
Second quintile	87 (24.0)	86 900 (21.0)
Third quintile	74 (20.4)	87 803 (21.3)
Fourth quintile	76 (20.9)	82 909 (20.1)
Fifth quintile	55 (15.2)	66 418 (16.1)
Missing	*11*	*15 299*
Nuchal translucency measurement, n (%)		
Measurement <3.5 mm	152 (44.1)	396 017 (99.96)
Measurement ≥3.5 mm	193 (55.9)	169 (0.04)
No measurement	*29*	*32 280*

EDD, estimated date of delivery; SD, standard deviation ; IVF, in vitro fertilization ; ART, assisted reproductive technology.

[a]*Pregnancies with double-positive results include pregnancies with a double-positive multiple marker screening result where trisomies 21 and 18 were excluded by cytogenetic testing or a low-risk cfDNA screening result*

[a]*Pregnancies with screen-negative results include pregnancies with a screen negative multiple marker screening result in which pregnancies with a diagnosis of full, partial or mosaic trisomy 21 or 18 were excluded.*

[b]*Missing data for more than 10.0% of the pregnancies*

Figure 4.2. Use and results of cfDNA Screening and Cytogenetic Testing by Multiple Marker Screening (MMS) Result

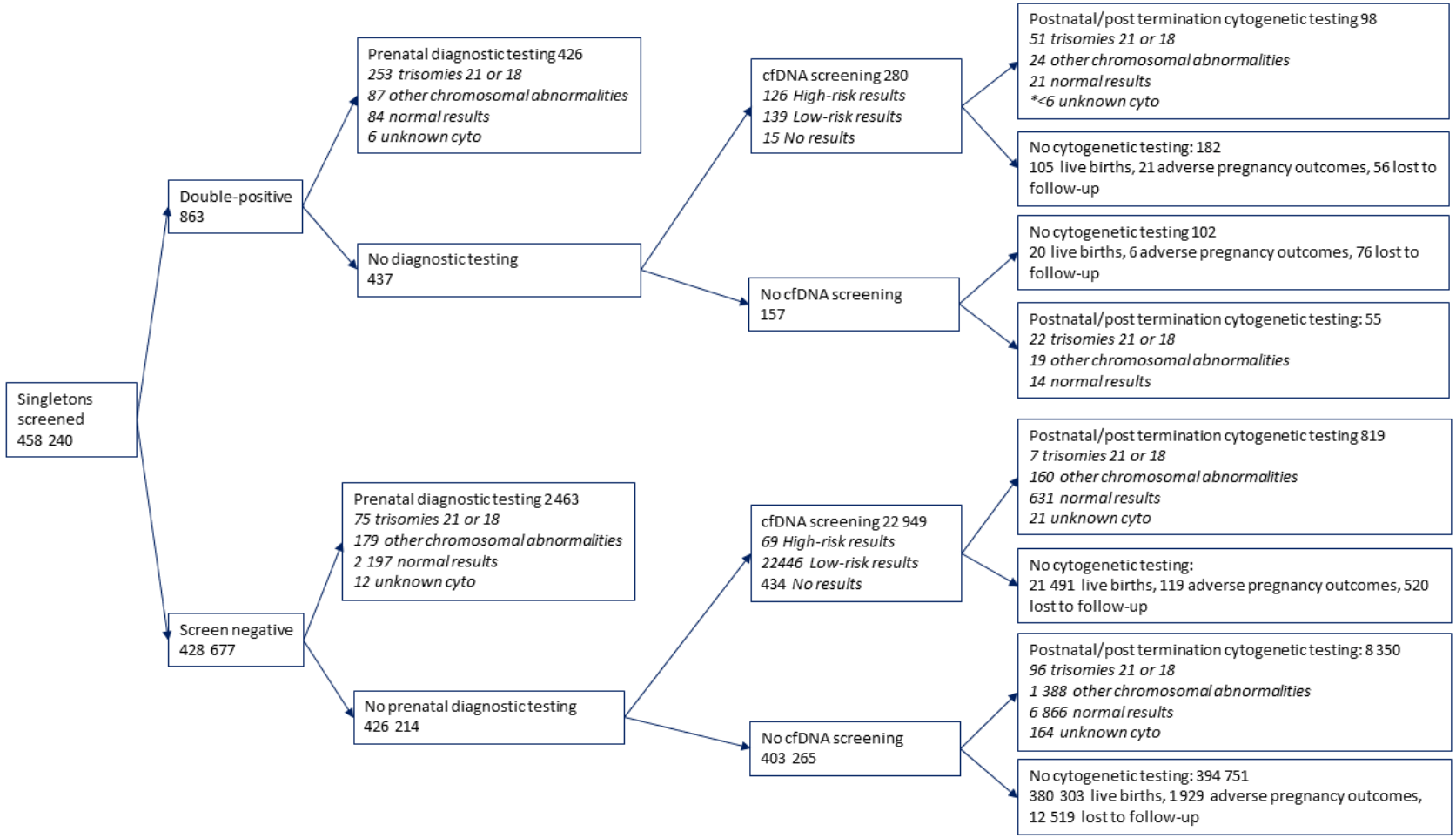


Table 4.2. Perinatal Outcomes in Pregnancies with Double-Positive Multiple Marker Screening Results.

Pregnancies resulting in a live birth	**Double-positive results[a] (n= 203)**	**Screen-negative results[b] (n= 411 937)**	**Crude model**		**Adjusted model**	
			RD, % (95% CI)	**RR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**
Perinatal Outcomes						
Preterm birth[c]	34/200 (17.0%)	25 084/410 936 (6.1%)	10.9 (5.7-16.1)	2.8 (2.0-3.8)	10.5 (5.4-15.7)	2.6 (2.0-3.6)
Small for gestational age[c]	39/198 (19.7%)	41 456/408 189 (10.2%)	9.5 (4.0-15.1)	1.9 (1.5-2.6)	10.5 (5.0-16.0)	2.1 (1.6-2.8)
Cesarean delivery[c]	84/200 (42.0%)	120 516/411 184 (29.3%)	12.7 (5.8-19.5)	1.4 (1.2-1.7)	6.8 (0.1-13.5)	1.2 (1.0-1.4)
Admission to NICU[c]	47/200 (23.5%)	40 609/410 284 (9.9%)	13.6 (7.8-19.5)	2.4 (1.8-3.1)	13.7 (7.9-19.6)	2.4 (1.9-3.1)
Obstetric Outcomes						
Preeclampsia[c]	19/200 (9.5%)	25 321/409 133 (6.2%)	3.3 (-0.8-7.4)	1.5 (1.0-2.4)	3.1 (-1.0-7.1)	1.5 (1.0-2.3)
All pregnancies	**Double-positive results[a] (n= 374)**	**Screen-negative results[b] (n= 428 466)**	**Crude model**		**Adjusted model**	
			RD, % (95% CI)	**RR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**
Chromosomal abnormality other than trisomies 21 or 18[d]	128/372 (34.4%)	1 694/388 244 (0.4%)	34.0 (29.1-38.8)	78.9 (68.0-91.5)	34.0 (29.2-38.8)	81.1 (69.4-94.8)
Composite of pregnancy loss, stillbirth, or termination[c]	53/256 (20.7%)	2 897/414 834 (0.7%)	20.0 (15.0-25.0)	29.6 (23.3-37.8)	19.9 (15.0-24.9)	26.9 (20.8-34.7)

RD, risk difference; CI, confidence interval; RR, risk ratio; aRD, adjusted risk difference; aRR, adjusted risk ratio

Notes: The detailed frequency table for perinatal outcomes by multiple marker screening result is available in the supplementary materials (Supplementary Table 7)

[a]Pregnancies with double-positive results include pregnancies with a double-positive multiple marker screening result where trisomies 21 and 18 were excluded by cytogenetic testing or a low-risk cfDNA screening result

[b]Pregnancies with screen-negative results include pregnancies with a screen negative multiple marker screening result in which pregnancies with a diagnosis of full, partial or mosaic trisomy 21 or 18 were excluded.

[c]Models adjusted for age, weight, racial origin of the pregnant individual, smoking, insulin dependent diabetes mellitus, and type of conception (spontaneous, in vitro fertilization or other assisted reproductive technology).

[d]Model adjusted for age of the pregnant individual, gestational age at screening, and type of conception (spontaneous, in vitro fertilization or other assisted reproductive technology).

Table 4.3. Perinatal Outcomes in Pregnancies with Double-Positive Multiple Marker Screening Results – Pregnancies with All Identified Chromosomal Abnormalities Excluded.

Pregnancies resulting in a live birth, chromosomal abnormalities excluded	**Double-positive results[a] (n=188)**	**Screen-negative results[b] (n= 410 412)**	**Crude model**		**Adjusted model[c]**	
			RD, % (95% CI)	**RR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**
Perinatal Outcomes						
Preterm birth	30/186 (16.1%)	24 836/409427 (6.1%)	10.5 (5.2-15.7)	2.8 (2.0-3.9)	10.0 (4.8-15.3)	2.6 (1.9-3.7)
Small for gestational age	33/184 (17.9%)	41 118/406 707 (10.1%)	9.0 (3.5-14.6)	2.0 (1.5-2.7)	9.5 (4.0-15.0)	2.1 (1.6-2.9)
Cesarean delivery	75/186 (40.3%)	119 976/409 675 (29.3%)	11.4 (4.3-18.4)	1.4 (1.2-1.7)	5.2 (-1.7-12.1)	1.1 (0.9-1.3)
Admission to NICU	42/186 (22.6%)	40 170/408 786 (9.8%)	13.6 (7.6-19.6)	2.5 (1.9-3.3)	13.5 (7.6-19.5)	2.5 (1.9-3.3)
Obstetric Outcomes						
Preeclampsia	17/187 (9.1%)	25 195/407 619 (6.2%)	3.0 (-1.2-7.1)	1.5 (0.9-2.3)	2.7 (-1.4-6.8)	1.4 (0.9-2.3)
All pregnancies, chromosomal abnormalities excluded	**Double-positive results[a] (n= 246)**	**Screen-negative results[b] (n= 426 772)**	**Crude model**		**Adjusted model[c]**	
			RD, % (95% CI)	**RR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**
Composite of pregnancy loss, stillbirth, or termination	18/206 (8.7%)	2 800/413 212 (0.7%)	8.5 (4.7-12.4)	40.3 (25.8-63.0)	8.5 (4.6-12.3)	30.8 (19.2-49.4)

RD, Risk Difference; CI, confidence interval; RR, Risk ratio; aRD, Adjusted Risk Difference; aRR, adjusted Risk Ratio

[a]Pregnancies with double-positive results include pregnancies with a double-positive multiple marker screening result where trisomies 21 and 18 were excluded by cytogenetic testing or a low-risk cfDNA screening result

[b]Pregnancies with screen-negative results include pregnancies with a screen negative multiple marker screening result in which pregnancies with a diagnosis of full, partial or mosaic trisomy 21 or 18 were excluded.

[c]Models adjusted for age, weight, racial origin of the pregnant individual, smoking, insulin dependent diabetes mellitus, and type of conception (spontaneous, in vitro fertilization or other assisted reproductive technology).

4.9 Supplementary materials

Supplementary Table 4.1. Description of Data Sources.

Better Outcomes Registry & Network (BORN) Ontario	Ontario's prescribed pregnancy, birth and childhood registry, the Better Outcomes Registry & Network (BORN Ontario) routinely collects information on a number of encounters that pregnant individuals and neonates have with the healthcare system. This includes prenatal screening from all laboratories performing the tests in Ontario, for multiple marker screening as well as cell-free DNA screening. The registry also collects information from all public cytogenetic testing laboratories. Pregnancy, obstetrical, and perinatal outcomes are also captured for all hospital births and home births attended to by midwifes. More information on the registry can be found on the BORN Ontario website: www.bornontario.ca and in the 2021 publication by Murphy et al. cited in this paper (PMID 34097034).

Canadian Institute for Health Information (CIHI) Discharge Abstract Database	CIHI's Discharge Abstract Database is a database collecting information for all hospital discharges, including deliveries and neonatal care.
	The Discharge Abstract Database is linked to the BORN registry on a yearly basis, and was used in this research project to supplement the following information:
	- Pregnancy outcome
	  ○ Fetal demise - ICD10 code : P95
	  ○ Spontaneous abortion – ICD10 codes: O03;P01.8
	  ○ Termination of Pregnancy – ICD10 codes: O04, O05, O06, P96.4
	- Preeclampsia – ICD10 codes: O14, O15

Supplementary Table 4.2. Description of Multiple Marker Screening Modalities.

Screening result	4-marker enhanced First Trimester Screening, n (%)	5-Marker enhanced First Trimester Screening, n (%)	First Trimester Screening, n (%)	Integrated Prenatal Screening, n (%)	Maternal Serum Screening, n (%)	Other modalities, n (%)	Total
Screen positive for trisomies 21 and 18	149 (0.2)	487 (0.2)	80 (0.4)	92 (0.1)	37 (0.1)	18 (0.2)	**863**
Screen positive for trisomy 21 only	4028 (4.8)	16091 (7.0)	1487 (6.9)	1804 (2.1)	1727 (5.8)	514 (6.3)	**25651**
Screen positive for trisomy 18 only	110 (0.1)	417 (0.2)	6 (0.0)	181 (0.2)	168 (0.6)	35 (0.4)	**917**
Screen Negative	79550 (94.9)	211319 (92.6)	20091 (92.7)	81987 (97.5)	28098 (93.6)	7632 (93.1)	**428677**
Total	**83837**	**228314**	**21664**	**84064**	**30030**	**8199**	**456108**

4-marker enhanced First Trimester Screening: Nuchal translucency, total or free beta human chorionic gonadotropin, alpha fetoprotein, pregnancy-associated plasma protein A. Screening cut-off of 1 in 350 at term for trisomy 21 and 1 in 200 for trisomy 18. **alpha fetoprotein is not used in the risk calculation for trisomy 18*

5-marker enhanced First Trimester Screening: Nuchal translucency, total or free beta human chorionic gonadotropin, alpha fetoprotein, pregnancy-associated plasma protein A, placental growth factor. Screening cut-off of 1 in 350 at term and 1 in 200 for trisomy 18. **alpha fetoprotein and placental growth factor are not used in the risk calculation for trisomy 18*

First Trimester Screening: Nuchal translucency, total or beta human chorionic gonadotropin, pregnancy-associated plasma protein A. Screening cut-off of 1 in 350 at term for trisomy 21 and 1 in 200 at term for trisomy 18.

Integrated Prenatal Screening: First trimester: nuchal translucency, pregnancy-associated plasma protein A Second trimester: alpha fetoprotein, total chorionic gonadotrophin, unconjugated estriol. Screening cut-off of 1 in 200 at term for trisomy 21 and trisomy 18.

Maternal Serum Screening (MSS-QUAD): alpha fetoprotein, total chorionic gonadotrophin, unconjugated estriol, inhibin A. Screening cut-off for trisomy 21 at term of 1 in 200 prior to April 2020, lowered to 1 in 350 at term after April 2020. Screening cut-off for trisomy 18 at term of 1 in 200.

Supplementary Table 4.3. Description of Missing Data.

All pregnancies	*Total number of pregnancies (those with no missing data for the respective outcome)*	*Number (%) of pregnancies with missing data on one or more covariates*	*Number of pregnancies included in the complete case analysis*
Chromosomal abnormality other than trisomies 21 or 18	388509	23154 (6.0%)	365355
Composite of pregnancy loss, stillbirth, or termination	414723	48596 (11.7%)	366127
Pregnancies with a recorded live birth			
Perinatal Outcomes			
Preterm birth	410638	47838 (11.6%)	362800
Small for gestational age	407887	47250 (11.6%)	360637
Cesarean delivery	410886	47850 (11.6%)	363036
Admission to NICU	410640	47839 (11.6%)	362801
Obstetric Outcomes			
Preeclampsia	408836	46421 (11.4%)	362415

Supplementary Table 4.4. Description of Multiple Imputation Methods.

We performed multiple imputations separately for each outcome of interest; only covariates were imputed (outcomes were not, but outcome information was used in the creation of imputed data sets) and performed 10 iterations with 20 imputed data sets to then pool the results presented in this paper. The variables below, along with the outcome of interest, were used to impute the missing data for covariates. The method of imputation was selected for individual types of covariates; polytomous logistic regressions for categorical variable with more than 2 levels, logistic regression for binary variables, level 2 logistic regression for the multilevel variable, and predictive mean matching for the continuous variables.

Multiple imputations were performed in R version 4.3.1, using the “mice” package.

Covariates	*Missing data*	*Method of imputation*
Type of conception	23529	Polytomous logistic regression
Smoking status	24602	Logistic regression
Insulin dependent diabetes mellitus	15618	Logistic regression
Racial origin	3687	Level 2 logistic regression
Gestational age	8313	Predictive mean matching
Age of pregnant individual	<6	Predictive mean matching
Weight of pregnant individual	22812	Predictive mean matching

Supplementary Table 4.5. Sensitivity Analysis Accounting for Clustering; for Individuals with More Than One Pregnancy Included in the Study Cohort.

All pregnancies	Adjusted model, main models with multiple imputations		Adjusted model, main models with multiple imputations – accounting for clustering on individuals	
	aRD, % (95% CI)	**aRR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**
Imputed data set 1	10.3 (5.2-15.4)	2.6 (2.0-3.6)	10.3 (7.7-12.9)	2.6 (2.0-3.6)
Imputed data set 2	10.3 (5.2-15.4)	2.6 (1.9-3.6)	10.3 (5.2-15.5)	2.6 (1.9-3.6)

Two imputed data set were selected a priori to compare the results of the regression models with and without generalized estimating equations to account for individuals with more than one pregnancy in the study cohort. This sensitivity analysis was performed, as we were unable to account for individuals with multiple pregnancies in the data set in our main analysis due to the complexity of the models.

Supplementary Table 4.6. Description of Chromosomal Abnormalities.

	Pregnancies in analytic sample	
	Double-positive results[a]	**Screen-negative results[b]**
	(n= 374)	**(n= 428 466)**
Pregnancies with cytogenetic testing	**248**	**11 585**
Normal results	119 (48.0)	9 694 (83.7)
Chromosomal abnormalities other than trisomies 21 or 18	128 (51.6)	1 694 (14.6)
Trisomy 13	*59*	*45*
Triploidy	*<6*	*13*
Monosomy X	*39*	*11*
Other sex chromosome aneuploidies	*<6*	*88*
Other	*24*	*1537*

[a]*Pregnancies with double-positive results include pregnancies with a double-positive multiple marker screening result where trisomies 21 and 18 were excluded by cytogenetic testing or a low-risk cfDNA screening result*

[b]*Pregnancies with screen-negative results include pregnancies with a screen negative multiple marker screening result in which pregnancies with a diagnosis of full, partial or mosaic trisomy 21 or 18 were excluded.*

A total of 198 cytogenetic tests had unknown results.

Supplementary Table 4.7. Perinatal Outcomes of Pregnancies by Multiple Marker Screening Result.

	Pregnancies in the analytic sample	
	Double-positive results[a]	**Screen-negative results[b]**
All pregnancies	**374**	**428 466**
Had cytogenetic testing, n	248	11 585
Normal, n (%)	119 (48.0)	9 694 (83.7)
Abnormal, n (%)	128 (51.6)	1 694 (14.6)
Pregnancy outcome, n (%)		
Live birth	203 (79.3)	411 937 (99.3)
Pregnancy loss	7 (2.7)	375 (0.1)
Stillbirth	14 (5.5)	1 574 (0.4)
Termination	32 (12.5)	948 (0.2)
No pregnancy outcome recorded	*118*	*13 632*
Among all live births	**203**	**411 937**
Preterm birth, n (%)		
Yes	34 (17.0)	25 084 (6.1)
No	166 (83.0)	385 852 (93.9)
Missing	*<6*	*1 001*
Small for gestational age, n (%)		
Yes	39 (19.7)	41 456 (10.2)
No	159 (80.3)	366 733 (89.8)
Missing	*<6*	*3 748*
Mode of delivery, n (%)		
Cesarean section	84 (42.0)	120 516 (29.3)
Vaginal	116 (58.0)	290 668 (70.7)
Missing	*<6*	*753*
Admission to NICU, n (%)		
Yes	47 (23.5)	40 609 (9.9)
No	153 (76.5)	369 675 (90.1)
Missing	*<6*	*1 653*
Preeclampsia, n (%)		
Yes	19 (9.5)	25 321 (6.2)
No	181 (90.5)	383 812 (93.8)
Missing	*<6*	*2 804*

NICU, Neonatal intensive care unit

[a]Pregnancies with double-positive results include pregnancies with a double-positive multiple marker screening result where trisomies 21 and 18 were excluded by cytogenetic testing or a low-risk cfDNA screening result

[b]Pregnancies with screen-negative results include pregnancies with a screen negative multiple marker screening result in which pregnancies with a diagnosis of full, partial or mosaic trisomy 21 or 18 were excluded.

Supplementary Table 4.8. Crude Models, Complete Case Analyses, and Main Analysis with Multiple Imputations.

All pregnancies	**Crude model**		**Adjusted model, complete case analysis**		**Adjusted model, main models with multiple imputations**	
	RD, % (95% CI)	**RR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**
Chromosomal abnormality other than trisomies 21 or 18[a]	34.0 (29.1-38.8)	78.9 (68.0-91.5)	31.8 (26.5-37.1)	78.7 (65.7-94.2)	34.0 (29.2-38.8)	81.1 (69.4-94.8)
Composite of pregnancy loss, stillbirth, or termination[b]	20.0 (15.0-25.0)	29.6 (23.3-37.8)	17.9 (12.7-23.1)	25.5 (19.0-34.2)	19.9 (15.0-24.9)	26.9 (20.8-34.7)
Pregnancies resulting in a live birth	**Crude model**		**Adjusted model, complete case analysis**		**Adjusted model, main models with multiple imputations**	
	RD, % (95% CI)	**RR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**
Perinatal Outcomes						
Preterm birth[b]	10.9 (5.7-16.1)	2.8 (2.0-3.8)	10.1 (4.6-15.6)	2.6 (1.9-3.7)	10.5 (5.4-15.7)	2.6 (2.0-3.6)
Small for gestational age[b]	9.5 (4.0-15.1)	1.9 (1.5-2.6)	9.0 (3.2-14.8)	2.0 (1.4-2.7)	10.5 (5.0-16.0)	2.1 (1.6-2.8)
Cesarean delivery[b]	12.7 (5.8-19.5)	1.4 (1.2-1.7)	14.8 (-2.9-32.6)	1.2 (1.0-1.4)	6.8 (0.1-13.5)	1.2 (1.0-1.4)
Admission to NICU[b]	14.5 (8.4-20.6)	2.3 (1.8-2.9)	15.8 (9.2-22.4)	2.4 (1.9-3.1)	14.7 (8.7-20.8)	2.3 (1.8-2.9)
Obstetric Outcomes						
Preeclampsia[b]	3.3 (-0.8-7.4)	1.5 (1.0-2.4)	3.5 (-0.9-7.9)	1.6 (1.0-2.5)	3.1 (-1.0-7.1)	1.5 (1.0-2.3)

RD, Risk Difference; CI, confidence interval; RR, Risk ratio; aRD, Adjusted Risk Difference; aRR, adjusted Risk Ratio; NICU, neonatal intensive care unit

[a]Model adjusted for age of the pregnant individual, gestational age at screening, and type of conception (spontaneous, in vitro fertilization or other assisted reproductive technology).

[b]Models adjusted for age, weight, racial origin of the pregnant individual, smoking, insulin dependent diabetes mellitus, and type of conception (spontaneous, in vitro fertilization or other assisted reproductive technology)

Supplementary Table 4.9. Sensitivity Analysis – Effect of Addition of Live Births Without Outcome Information on Trisomies 21 and 18 to the Group with Double-Positive Results.

			Crude model		Adjusted model, complete case analysis[a]		Adjusted model, with multiple imputations[a]	
Main model	**Screen-negative results** *Live births with screen negative results not identified as false negative* **(n= 411 937)**	**Double-positive results** *Pregnancies with double-positive results with normal cytogenetic testing or low-risk cfDNA screening for trisomies 21 and 18 with a recorded live birth* **(n= 203)**	**RD, % (95% CI)**	**RR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**
Preterm birth - main model	25 084	34	10.9 (5.7-16.1)	2.8 (2.0-3.8)	10.1 (4.6-15.6)	2.6 (1.9-3.7)	10.5 (5.4-15.7)	2.6 (2.0-3.6)
Sensitivity analysis	**Screen-negative results:** *Live births with screen negative results not identified as false negative* **(n= 411 937)**	**Double-positive results[b]** *Pregnancies with double-positive results with normal cytogenetic testing or low-risk cfDNA screening for trisomies 21 and 18, or with a recorded live birth* **(n= 223)**	**Crude model**		**Adjusted model, complete case analysis[a]**		**Adjusted model, with multiple imputations[a]**	
			RD, % (95% CI)	**RR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**	**aRD, % (95% CI)**	**aRR, (95% CI)**
Preterm birth - sensitivity analysis	25 084	36	10.3 (5.4-15.1)	2.7 (2.0-3.6)	9.6 (4.4-14.9)	2.5 (1.8-3.5)	9.9 (5.0-14.8)	2.5 (1.9-3.4)

RD, Risk Difference; CI, confidence interval; RR, Risk ratio; aRD, Adjusted Risk Difference; aRR, adjusted Risk Ratio

[a]Models adjusted for age, weight, racial origin of the pregnant individual, smoking, insulin dependent diabetes mellitus, and type of conception (spontaneous, in vitro fertilization or other assisted reproductive technology).

[b]An additional 20 pregnancies for which no cytogenetic testing or cfDNA screening was performed, but a live was recorded, we added to the group with double-positive results

Supplementary Table 4.10. Method of Determination of Estimated Date of Delivery by Multiple Marker Screening Result.

Method of determination of EDD	**Double-positive results**	**Screen-negative results**	**Total**
First trimester ultrasound, n (%)	352 (94.1)	410824 (95.9)	**411176 (95.9)**
Second trimester ultrasound, n (%)	12 (3.2)	8501 (2.0)	**8513 (2.0)**
Last menstrual period, n (%)	10 (2.7)	9141 (2.1)	**9151 (2.1)**
Total	**374**	**428466**	**428840**

Chapter 5. Integrated Discussion

5.1 Introduction

A deeper understanding of the implications of unanticipated or unclear results in prenatal genetic screening is needed to inform policy decision makers and to ensure pregnant women and pregnant individuals can make informed decisions about screening and follow-up investigations with an understanding of what these results could mean to them. The aim of this doctoral thesis was to examine specific types of unanticipated or unclear prenatal screening results, on a population level, in association with important outcomes.

Previous studies that have attempted to answer the research questions posed here have often been subject to major limitations. For example, many studies in this field are small and are descriptive, lacking a reference group. Many are also subject to important selection biases due to their restriction to pregnancies identified as high risk. The studies presented in this dissertation are some of the first population-based analytic studies investigating these research questions, addressing several of the limitations in the literature.

In Chapter 1, important concepts of prenatal screening, perinatal outcomes, and uncertainty in screening were described. In that introductory chapter, examples were also presented that outlined the types of unanticipated or unclear findings that occur in clinical practice, for which limited or biased evidence is currently hindering patient counselling, providing a rationale for the thesis projects.

In Chapter 2, the first manuscript was presented, in which the association between nuchal translucency measurements and chromosomal abnormalities was investigated. This was followed, in Chapter 3, by a study evaluating pregnancy outcomes and adverse perinatal outcomes of pregnancies at all levels of nuchal translucency measurements. The third manuscript, focusing on outcomes of pregnancies with prenatal screening results that were positive for both trisomy 21 and trisomy 18 at once, referred to as ‘double-positive results’, was presented in Chapter 4.

This integrated discussion chapter: summarizes the findings of the three studies; provides comparisons with the existing literature to illustrate the key contributions of this research; outlines the implications for clinical practice and policy, as well as recommended future directions; and includes a summary of the strengths and limitations of this dissertation. This integrated discussion chapter was written following the guidelines developed by Lewis et al.[1]

The ultimate goal of this work was to provide evidence clarifying prenatal screening results that are characterized by uncertainty, to inform pregnant individuals and their providers about the implications of the screening tests offered, and to inform improved screening policy.

5.2 Summary of key findings

5.2.1 Summary of Article 1

Ultrasonographic Fetal Nuchal Translucency Measurements and Cytogenetic Outcomes

The first article, which was presented in Chapter 2, describes a retrospective cohort study in which we aimed to examine the association between all levels of nuchal translucency measurements and chromosomal abnormalities. Using data from a provincial population-based registry, we identified 414 268 singleton pregnancies for which a nuchal translucency measurement was performed in the context of multiple marker screening. Pregnancies with measurements under 2.0 mm were compared to the following groups: 2.0 to <2.5 mm, 2.5 to <3.0 mm, 3.0 to <5.0 mm, 5.0 to <6.5 mm and ≥6.5 mm. Using multivariable modified Poisson regression models with robust variance estimation and adjustment for gestational age, an increase in the risk of chromosomal abnormalities was identified with each increasing level of nuchal translucency measurement. Importantly, there was a clinically significant increased risk of chromosomal abnormalities in pregnancies with measurements below the widely used cutoff of 3.5 mm with an adjusted risk difference of 10.0% (95%CI 8.5-11.4) and adjusted risk ratio of 20.3 (95%CI 17.6-23.5) in the group with measurements between 3.0 to <3.5 mm, relative to the group with measurements <2.0 mm. In a subgroup analysis examining only the risk of chromosomal abnormalities that are not routinely screened by cfDNA screening in Ontario (chromosomal abnormalities other than trisomies 21, 18, 13 and sex chromosome aneuploidies) the risk was attenuated but still meaningful with an adjusted risk difference of 1.4% (95%CI 0.8-2.0) and adjusted risk ratio of 5.0 (3.5-7.2).

The findings of this study suggest that the widely used cutoff of 3.5 mm at which further investigations are offered to pregnant individuals may need to be revisited.

5.2.2 Summary of Article 2

Outcomes of pregnancies with varying levels of nuchal translucency measurements: a population-based retrospective study in Ontario, Canada

The main objective of the article presented in Chapter 3 was to further investigate the association between nuchal translucency measurements and pregnancy outcomes, overall, and within the subgroup in which pregnancies with identified chromosomal abnormalities had been excluded. A secondary objective was to study the association between nuchal translucency measurements and adverse perinatal outcomes among those resulting in a live birth.

The cohort of 414 268 singleton pregnancies with a valid nuchal translucency measurement was used to examine the risk of a composite of pregnancy loss, termination, stillbirth, and neonatal death in relation to nuchal translucency measurements. Through a modified Poisson regression model with robust variance estimation and adjustment for gestational age at screening and age of the pregnant individual, an increased risk of a composite of pregnancy loss, termination, stillbirth, and neonatal death was identified with every increasing level of nuchal translucency measurement (2.0 to <2.5 mm, 2.5 to <3.0 mm, 3.0 to <3.5 mm, 3.5 to <5.0 mm, 5.0 to <6.5 mm, and ≥6.5 mm) compared to pregnancies with measurements under 2.0 mm. Pregnancies with measurements between 3.5 mm and 5.0 mm had an adjusted risk difference of 11.2% (95%CI 9.0-13.5) and an adjusted risk ratio of 11.9 (95%CI 9.9-14.3) when compared to pregnancies with measurements under 2.0 mm. This association was also observed, though attenuated, in a subgroup analysis in which all pregnancies with identified chromosomal abnormalities were excluded, with an adjusted risk difference of 5.1% (95%CI 3.4-6.8) and an adjusted risk ratio of 6.4 (95%CI 4.8-8.5).

As a secondary objective, adverse perinatal outcomes were examined among those that resulted in a live birth. Pregnancies with very high nuchal translucency measurements were found to be at increased risk of being admitted to the neonatal intensive care unit and having a low APGAR score, and this also held true in the subgroup analysis in which pregnancies with identified chromosomal abnormalities were excluded.

The findings of this study provide evidence that pregnancies are at increased risk of pregnancy loss, termination, stillbirth, or neonatal death with increasing nuchal translucency measurements, even when no chromosomal abnormalities are identified. This evidence can be used for more comprehensive patient counselling at the time of the nuchal translucency ultrasound to provide general and more accurate estimates of potential pregnancy outcomes.

5.2.3 Summary of Article 3

Pregnancies with 'double-positive' multiple marker screening results: a population-based study in Ontario, Canada

When undergoing prenatal screening through multiple marker screening for trisomies 21 and 18, three types of results are anticipated: a screen negative result, a screen positive result for trisomy 21, or a screen positive result for trisomy 18. However, in some instances, the result is reported to be positive for both trisomy 21 and trisomy 18 at once, which is referred to as a 'double-positive' result. This type of result is unanticipated and difficult to explain, given that some of the biochemical markers used in the screening process follow opposite patterns in pregnancies with trisomy 21 compared to trisomy 18.

The aim of the third article, presented in Chapter 4, was to study the association between double-positive results and preterm birth, given its importance in the prediction of perinatal health. The study also aimed to investigate the association between double-positive results and chromosomal abnormalities beyond trisomies 21 and 18, pregnancy outcomes, and adverse perinatal outcomes among those that resulted in a live birth.

We conducted a population-based retrospective cohort study of 458 240 singleton pregnancies receiving multiple marker screening, in which the incidence of double-positive multiple marker screening results was reported at 0.2% (863). Of 374 pregnancies with double-positive multiple marker screening results in which a diagnosis of trisomy 21 and 18 had been excluded, 203 resulted in a live birth and were compared to pregnancies with screen negative results that resulted in live birth (411 937) in our primary analysis of risk of preterm birth. From a modified Poisson regression model with robust variance estimation, adjusting for age of the pregnant

individual, pre-pregnancy weight, racial origin, smoking, insulin dependent diabetes mellitus, and type of conception, the 'double positive' (but without trisomy 18 or 21) pregnancies were at increased risk of preterm birth, with an adjusted risk difference of 10.5% (95%CI 5.4-15.7) and adjusted risk ratio of 2.6 (95%CI 2.0-3.6). This association persisted when all identified chromosomal abnormalities were excluded from pregnancies in both groups: adjusted risk difference of 10.0% (95%CI 4.8-15.3), adjusted risk ratio of 2.6 (95%CI 1.9-3.7).

As secondary outcomes, we analyzed the full sample of 374 pregnancies with double-positive results in which trisomies 21 and 18 were ruled out, finding that these pregnancies were also at increased risk of chromosomal abnormalities other than trisomy 21 and 18 relative to pregnancies with screen negative results (428 466), with an adjusted risk difference of 34.0% (95%CI 29.2-38.8) and adjusted risk ratio of 81.1 (95%CI 69.4-94.8). Pregnancies with double-positive results were also less likely to result in a live birth, with an adjusted risk difference of 19.9% (95%CI 15.0-24.9) and adjusted risk ratio of 26.9 (95%CI 20.8-34.7) for a composite of pregnancy loss, termination or stillbirth when compared to pregnancies with a screen negative result.

Finally, for the pregnancies that did result in a live birth, those with double positive (versus screen negative) results were at higher risk of being admitted to the neonatal intensive care unit with an adjusted risk difference of 13.7% (95%CI 7.9-19.6) and adjusted risk ratio of 2.4 (95%CI 1.9-3.1).

This study confirms and reinforces the notion that pregnancies with double-positive results for trisomies 21 and 18 are at increased risk of adverse outcomes, providing valuable information for patient counselling.

5.3 Comparisons to existing literature and contributions

Nuchal translucency measurement and chromosomal abnormalities

The association between an increased nuchal translucency measurement and chromosomal abnormalities has been studied for decades, with the first study published by Nicolaides et al in 1992.[2] The measurement of the fetal nuchal translucency is used by many prenatal screening programs to screen for trisomies 21 and 18, although an increased measurement can also be indicative of other chromosomal abnormalities, single-gene conditions, and structural defects.[3] Many jurisdictions use a threshold of 3.5 mm to define pregnancies at increased risk for these conditions and offer follow-up investigations in the form of cfDNA screening or confirmatory cytogenetic testing, as 3.5 mm represents a measurement greater than the 99th percentile at all gestational ages at which the nuchal translucency can be measured.[4] Some previous studies have reported a clinically significant risk of chromosomal abnormalities associated with nuchal translucency measurements below the widely used cutoff of 3.5 mm,[5–7] leading some professional practice guidelines to recommend offering diagnostic testing with any measurement greater or equal to 3.0 mm.[8] Our study was able to contribute new evidence in this area in several important ways.

First, previous studies in this field have largely been descriptive in nature, and most have included only pregnancies with increased nuchal translucency measurements, without providing a reference group.[9–24] Although it was clear from these studies that pregnancies with an increased nuchal translucency measurement were at high risk of having a chromosomal abnormality, the magnitude of the risk was highly dependent on the cohort studied. Many of these studies were also subject to important selection bias, as they were conducted in tertiary care centres and included only pregnant individuals who opted to have prenatal diagnosis, resulting in a high-risk group that may not reflect the risk in the general population, limiting the applicability of the findings to the full population of screened pregnancies.[6,10,14–19,23–31] Given these important limitations, the case was made to conduct a population-based cohort study on this topic. This was possible due to the comprehensive data capture from Ontario's prescribed population-based registry, the Better Outcomes Registry & Network (BORN) Ontario. This

registry captures all prenatal screening and diagnostic tests performed in Ontario, including multiple marker screening results, cfDNA screening results, and cytogenetic investigations in the province, including tests performed prenatally, postnatally, as well as on samples obtained following pregnancy losses, stillbirths, or terminations.

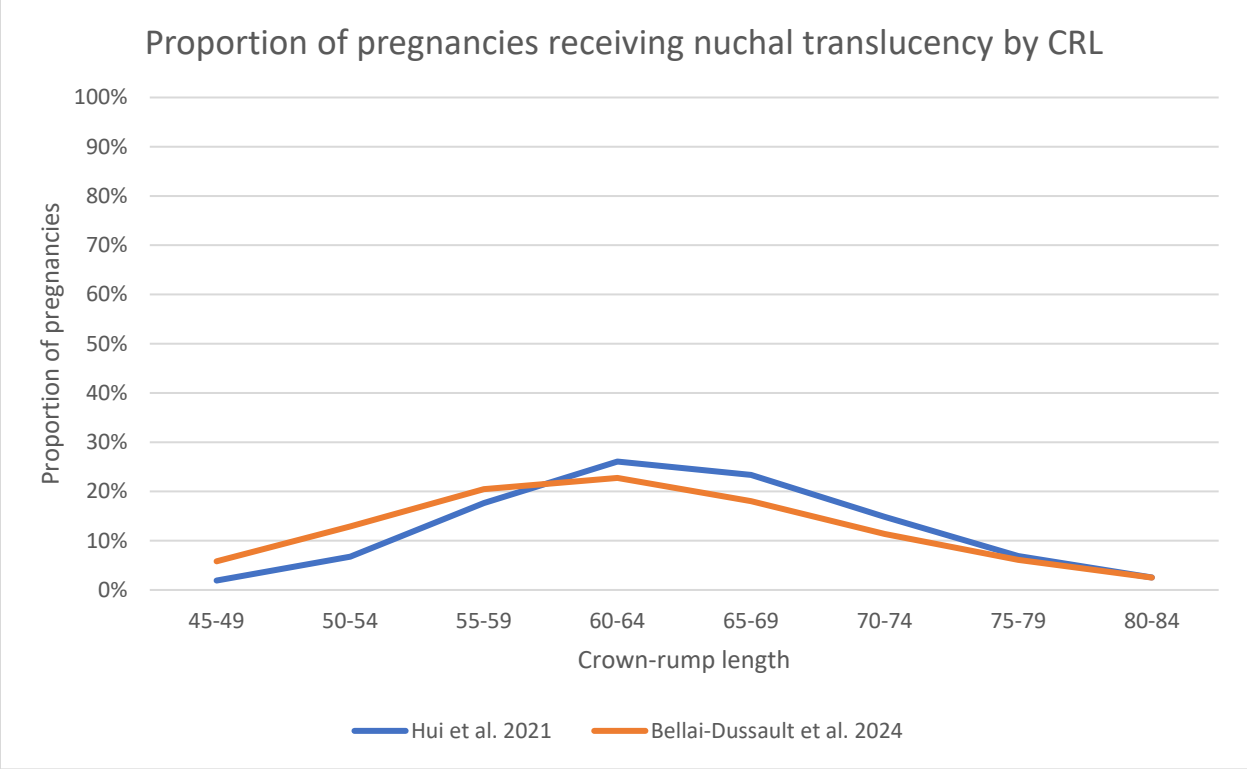
A second important contribution to the literature from our study of nuchal translucency and chromosomal abnormalities was the opportunity to estimate risk at various levels of nuchal translucency measurement rather than a single cutoff of 3.5 mm. Very few previous studies have reported on outcomes of pregnancies below 3.0 mm, and even fewer at all levels of nuchal translucency measurements.[6,32,33] While other perinatal registries, in Scandinavia for instance, may capture similar information about prenatal screening and follow-up investigations to the BORN Ontario registry, many are limited to smaller populations. In Ontario, annually over 145 000 births are recorded in the registry, and approximately 100 000 pregnancies receive prenatal genetic screening.[34–36] Thus accumulated data in Ontario provided a cohort large enough to enable comparisons among narrower bands of nuchal translucency measurement.

Finally, in order to be truly informative, studies on this topic need to have been performed fairly recently, as the follow-up testing options offered in the context of an increased nuchal translucency measurement have changed over time. For example, chromosomal microarray is now routinely offered in this context, and we were able to use microarray results in our study, where overall approximately 75% of pregnancies receiving cytogenetic testing received microarray, and up to 85% among those in which common aneuploidies had been excluded, while this testing option was not generally available previously. Those previous studies that have investigated the diagnostic yield of chromosomal microarray following the identification of an increased nuchal translucency measurement have also largely focused on higher risk pregnancies,[13,15,21–24,37] and thus were subject to the selection bias described earlier.

To our knowledge, there are only two population-based studies that have reported on the risk of chromosomal abnormalities at all nuchal translucency levels: 1) a study by Berger et al. conducted 2019 in California[38] and 2) an Australian study by Hui et al. published in 2021.[39] The

findings of the Californian study are quite comparable to the findings of our study; we report slightly higher risk of chromosomal abnormalities which would be expected given that our study has incorporated microarray testing while their study only included chromosomal abnormalities identified on karyotype. The study by Hui et al. reports a lower risk of chromosomal abnormalities: we hypothesize this may be in part due to the fact that their population is screened, on average, at a later gestational age than our study population, as expressed in Figure 5.1. Indeed, a nuchal translucency measurement of 3.0 mm in their population would be considered more normal as measured at a later gestational age than in our study population or in the study population from Berger et al. In our study and in the study by Berger et al., the total number of pregnancies with measurements <3.5 mm represents 99.5% of the study population, compared to 99.4% in the study by Hui et al. The proportion of pregnancies with nuchal translucency measurements between 3.0 and 3.5 mm varied by study, from 0.4% in our study to 0.6% and 0.7% in the studies by Berger et al. and Hui et al., respectively.

Figure 5.1. Distribution of Pregnancies by Crown-Rump Length.



Nuchal translucency measurement and pregnancy outcomes

An additional important topic of research for pregnancies with increased nuchal translucency measurements is the outcome of the pregnancy. Previous studies have generally reported that pregnancies with increased nuchal translucency measurements were less likely to result in a live birth.[11,40–52] One review article estimated that 70% of pregnancies with nuchal translucency measurements between 3.5 and 4.4 mm would be expected to result in a live birth without chromosomal abnormalities or major structural abnormalities, compared to only 15% of pregnancies with measurements greater than 6.5 mm.[117] However, the studies cited in the review, as many of the other studies on this topic,[10,48,50,54–59] generally focused on a specific subgroup of pregnancies in which prenatal diagnosis had been performed and was normal, rather than studying all pregnancies in which the nuchal translucency was measured.[43,45,60] As described in the previous section regarding studies of nuchal translucency and chromosomal abnormalities, many previous studies on this topic are also limited by a small sample size, with most studies cited here including fewer than 500 pregnancies with increased nuchal translucency measurements,[40,41,48,48,51,61–65] and many fewer than 100.[10,11,32,42,44,46,47,49,55,56,59,66,67] Studies restricted to pregnancies receiving prenatal diagnosis may also be affected by survival bias, where only pregnancies that survived to a point where prenatal diagnosis could be performed are included in the study, which could underestimate the risk of early pregnancy loss. Finally, also similar to the literature related to chromosomal abnormalities, few studies of nuchal translucency and pregnancy outcomes have focused on the pregnancies with lower levels of nuchal translucency measurements (<3.5 mm).[32,44,45,47,51,68]

Given these limitations to the evidence on this topic, our population-based cohort study using registry data from a large province and including all screened pregnancies at all nuchal translucency levels provided valuable new information. This is particularly important given that, based on the evidence from these prior studies, some authors have advocated for parental reassurance when the increased nuchal translucency measurement is followed by normal cytogenetic investigations, and anatomical ultrasound.[54] While this information is likely to be useful once the patient has undergone all follow-up investigations, it does not provide risk estimates for the variety of potential outcomes that could be discussed with the patient at the

time of the nuchal translucency ultrasound, to offer anticipatory guidance in advance of follow-up testing.

Nuchal translucency measurement and adverse perinatal outcomes

To our knowledge, very few studies have focused on the association of nuchal translucency measurements with adverse perinatal outcomes among pregnancies that resulted in a live birth. Some studies have reported that pregnancies with increased nuchal translucency measurements were at increased risk of overall adverse perinatal outcomes, including preterm birth,[51,57,69–71] fetal growth restriction,[51,65] low birth weight,[51,72] and preeclampsia.[51] An additional study reported that nuchal translucency measurements were correlated with needing to be admitted to the neonatal intensive care unit and obtaining a lower APGAR score.[72] By including adverse perinatal outcomes as a secondary objective of our study, we have brought additional evidence to contribute to the understanding of the association between nuchal translucency measurements and important perinatal outcomes that represent predictors of health in infancy and childhood.

Double-positive multiple marker screening results

The evidence currently available in the literature regarding double-positive multiple marker screening results (results that are positive for both trisomy 21 and trisomy 18 at once) is extremely scarce. This could largely be due to the rarity of this type of unanticipated or unclear result. Only one analytic study exists on this topic to our knowledge, published by Yee et al. in the United States in 2013.[73] This study reported that 0.3% of pregnancies screened in the first trimester using beta human chorionic gonadotropin (bhCG), Pregnancy-associated plasma protein A (PAPP-A) and nuchal translucency obtained a double-positive result. A group of 33 pregnancies with double-positive results in which prenatal diagnosis had been performed and a euploid karyotype was obtained was compared to 66 pregnancies with screen-negative results matched on age of the pregnant individual.[73] The study reported that pregnancies with double-

positive results were less likely to result in a live birth, had an increased risk of abnormal ultrasound findings, and had an earlier gestational age at birth and lower birth weight when compared to age-matched pregnancies with screen-negative results.[73] The study also reported a higher incidence of other adverse outcomes, namely preterm birth (14.3% in double-positive compared to 6.2% in screen-negative), low birth weight (10.7% compared to 4.6%), 5 minute APGAR score under 7 (3.6% compared to 3.1%), preeclampsia (10.7% compared to 7.7%), and neonatal length of stay (mean of 5.08 days compared to 3.32), among pregnancies with double-positive results.[73] However, the study was underpowered to identify statistically significant associations.[73]

A letter to the editor from Summers et al., in Ontario, Canada was also published in 2002, providing a description of 32 pregnancies with double-positive multiple marker screening results on second trimester multiple marker screening including serum alpha-fetoprotein (AFP), unconjugated estriol (uE3) and human chorionic gonadotrophin (hCG).[74] Only five (5/32 15.6%) pregnancies were reported to have an uneventful live birth. A total of 12 (12/32 37.5%) pregnancies were diagnosed with chromosomal abnormalities, 10 with trisomy 18, and two with an unspecified chromosomal abnormality. An additional 10 (10/32 31.3%) were reported to result in a spontaneous loss, termination, or intrauterine death, and finally five (5/32 15.6%) resulted in a live birth with complications.[74]

The paucity of evidence causes challenges when providing counselling to pregnant individuals with this type of result. The availability of population-level data thus presented an important opportunity to contribute unique and high-quality evidence regarding the outcomes of pregnancies with double-positive results. Our study described the outcomes of 863 pregnancies with double-positive multiple marker screening results and focused on adverse perinatal outcomes in a subgroup of 374 pregnancies in which trisomies 21 and 18 had been excluded, and in a smaller subgroup of 246 pregnancies in which all diagnosed chromosomal abnormalities had been excluded. Further, our study included more recent results, making the findings more generalizable to current screening practice., For instance the letter by Summers et al. focused on second trimester triple marker serum screening, a screening modality which is no longer in use in many jurisdictions, often having been replaced with first trimester screening

or with quadruple screening in the second trimester. Additionally, our study reflects more current practice in which chromosomal microarray is offered in the context of prenatal diagnosis, allowing us to identify additional chromosomal abnormalities that would not have been identifiable in the study conducted by Yee et al. through karyotype.

5.4 Main points of integration

5.4.1 The need to understand the meaning of unanticipated and unclear results

The studies presented in this dissertation reviewed outcomes of pregnancies with unanticipated or unclear prenatal screening results. We found that a significant proportion of pregnancies with nuchal translucency measurements below the current clinical threshold of 3.5 mm still had a clinically significant increased risk of chromosomal abnormalities. Further, with increasing levels of nuchal translucency, there was an increased risk of a composite outcome of pregnancy loss, termination, stillbirth, or neonatal death, as well as adverse perinatal outcomes such as admission to the neonatal intensive care unit and low APGAR score among pregnancies resulting in a live birth. Pregnancies with double-positive multiple marker screening results that resulted in a live birth were at increased risk of preterm birth, even when identified chromosomal abnormalities were excluded. Pregnancies with double-positive results were also at increased risk of chromosomal abnormalities beyond trisomies 21 and 18, were less likely to result in a live birth, and for those that did, there was an increased risk of adverse perinatal outcomes.

It is striking that we identified increased risks, often strongly elevated, for most of the outcomes we investigated in association with these unanticipated and unclear results. We hypothesize that some other unanticipated or unclear results arising from prenatal screening programs may also indicate an increased risk of adverse perinatal outcomes. An additional example of currently unresolved uncertainty would be the implications of failed cfDNA screening results, reported to impact approximately 4.8% of pregnant individuals receiving cfDNA screening in Ontario.[127] Some studies have found that pregnancies with failed cfDNA screening results are at increased risk of chromosomal abnormalities,[76–81] and there is

conflicting evidence as to whether there is an increased risk of adverse perinatal outcomes.[82–91] Private companies are advertising directly to pregnant individuals that cfDNA screening will lead to peace of mind[92,93] and the possibility of obtaining a failed screen is seldom considered.[94] We are only starting to understand the clinical implications of these failed screens, a notable research gap. Irrespective of whether studies of failed cfDNA screening or other unanticipated or unclear prenatal screening results uncover an increased risk of adverse outcomes, this research would be valuable for informing decisions regarding the clinical management of pregnancies with such results.

Unanticipated or unclear results will always be part of prenatal screening programs and some level of uncertainty will always remain. This will especially be true of genetic screening programs, with everchanging technologies uncovering new findings for which the appropriate clinical management has not yet been established. However, it is important for screening programs to strive to reduce uncertainty to the extent possible and provide robust information to empower patients to make informed decisions. Understanding unanticipated or unclear results can also inform policy, so that screening programs can make decisions about the screening modalities and configurations of follow-up services offered. Prenatal screening programs also need to provide guidance to practitioners and their patients about appropriate clinical management of unanticipated or unclear results. Not addressing these important clinical situations could compromise trust in prenatal screening programs and may lead different practitioners and clinics to develop distinct approaches to management, resulting in inequities in the care received by pregnant individuals.

5.4.2 Defining uncertainty in prenatal screening

As part of understanding uncertainty associated with unanticipated or unclear results, it may be helpful to better describe the nature of the uncertainty in question. This can be challenging without a shared concept of what uncertainty means, and importantly, terminology used to define it.[95] To address this, Han et al. developed a taxonomy of uncertainty specific to health care.[95] This taxonomy helps define three important dimensions of uncertainty: source

(explaining the origin of the uncertainty, whether it be due to probability, ambiguity, or complexity), issue (the issues caused by the uncertainty, which can be scientific/data-centered, practical/system-centered, or personal/patient-centered), and locus (defining if the uncertainty exists for the patient, the clinician, or both). This taxonomy may be used when describing unanticipated or unclear results, to identify precisely where the uncertainty lies and inform efforts to reduce or manage it when possible.

In the context of unanticipated or unclear prenatal genetic screening results, the source of the uncertainty can be traced to 1) probability, where the outcome of the pregnancy is indeterminate, 2) ambiguity, due to a lack of information, or imprecise or even conflicting evidence on the outcome of pregnancies with these uncertain or unclear results, and 3) complexity of navigating the prenatal screening system, with different screening and diagnostic tests, the multiplicity of potential outcomes with a given screening result, and understanding complex genetic concepts.

The issues caused by the uncertainty pertaining to the unanticipated or unclear results described in this dissertation can mostly be defined as data-centered. Han et al. describe this data-centered or scientific uncertainty in terms of establishing or predicting a diagnosis, prognosis, causal explanation, or treatment recommendations.[95] In the context of the nuchal translucency measurement: the threshold for judging a result to be high risk is uncertain, with different publications and jurisdictions recommending different cutoffs; the prognosis can also be uncertain depending on the measurement, mainly due to the limited or biased evidence at all levels of nuchal translucency measurements; the causal explanation remains undefined although some theories about the pathophysiology have been hypothesized; and finally, the appropriate course of clinical management is uncertain, for instance, whether cfDNA screening should be offered above a certain threshold versus prenatal diagnosis through cytogenetic testing. This is also true for double-positive multiple marker screening results: the underlying reason for this type of result is difficult to explain given the expected patterns of biochemical markers, the course of pregnancies with such results is difficult to predict as it has not been extensively described, and management is not clear as there are no formal guidelines on the recommended clinical management of a double-positive multiple marker screening result.

This scientific uncertainty that surrounds unanticipated or unclear prenatal screening results can also lead to issues of practical or system-centered uncertainty. When prognosis and appropriate treatment are unclear due to a scarcity of robust evidence about such findings, this results in a lack of clarity regarding system implications, including resource implications associated with making changes to the constellation of screening and follow-up tests. The scientific uncertainty also contributes to personal or patient-centered uncertainty about the implications of continuing a pregnancy with an increased nuchal translucency measurement or of the future of a child with a diagnosis of a chromosomal abnormality identified in the context of these unanticipated or unclear screening results.

The locus of uncertainty associated with unanticipated and unclear prenatal screening results lies with both the clinician, who is faced with uncertainty in how best to provide counselling and clinical management; and the patient, who, is faced with uncertainty in decision-making about receiving follow-up testing and continuing a pregnancy.

Describing the source of the uncertainty, the issues it relates to, and where the uncertainty lies may inform priorities for generating robust evidence in order to reduce scientific uncertainty; and strategies for mobilizing the knowledge created to support policy, clinical, and personal decision-making about prenatal genetic screening.

5.4.3 Data needed to further reduce scientific uncertainty about unanticipated and unclear results

As described above, this dissertation relates most closely to scientific or data-centred issues related to the uncertainty associated with unanticipated and unclear screening results from prenatal screening, which can be mitigated with high quality data and appropriate epidemiological methods.

Although there are inherent data gaps in the field of prenatal genetic screening, namely that not all pregnancies will receive confirmatory testing and thus there is an ongoing risk of ascertainment bias, access to comprehensive data helped us to mitigate this risk and estimate

outcomes in the full screened population of pregnancies in all three studies. Further improving capture of and access to data regarding outcomes of interest would support future research to further reduce uncertainty. For instance, improving the completeness of data on pregnancy outcomes, mainly through enhancing data capture related to early losses and terminations where possible, would allow us to better estimate the risk of adverse pregnancy outcomes. In our first two studies, we identified an increasing proportion of pregnancies for which no pregnancy outcome had been recorded with increasing levels of nuchal translucency. Some of these "losses to follow-up" are expected in the BORN Ontario registry because pregnant individuals may have prenatal screening in Ontario and subsequently emigrate or deliver outside of Ontario. However, the increasing proportion of losses to follow-up with increasing levels of nuchal translucency points to the missing data on pregnancy outcome being informative. We have hypothesized that a higher proportion of these pregnancies with an unknown outcome would have ended in an early loss or termination, as only pregnancy outcomes beyond 20 weeks' gestation are systematically captured in the BORN registry, similar to other perinatal registries.[96] We addressed this limitation with sensitivity analyses, which indicated that our findings were robust against reasonable assumptions related to losses to follow-up, but more complete data on early losses and terminations would strengthen future studies of outcomes associated uncertain and unclear prenatal screening results.

An additional priority for future enhancement of outcome data in BORN Ontario and similar registries that would provide valuable information would be to extend the capture of genetic testing beyond cytogenetics to incorporate molecular testing of single-gene conditions, as well as longer term outcomes including neurodevelopmental outcomes. In practice, when pregnant individuals seek further investigations, they are often interested in and receive testing that can identify additional genetic conditions beyond chromosomal abnormalities. It is therefore important to expand this data capture where possible, to align the evidence with patient priorities and reflect current clinical practice.

An additional priority for perinatal registries such as BORN Ontario that would enhance their ability to support high-quality research about the meaning of unclear or unanticipated results is to improve the collection of data on diagnosed congenital anomalies, including structural

anomalies identified prenatally. For example, in the BORN Ontario registry, structural anomalies recorded at birth are submitted to the registry but pregnancies with such anomalies may not survive to a point where they can be recorded. Incorporating records of prenatal ultrasounds (routine first trimester ultrasound and the 18–22-week anatomy scan) within registries would expand the capacity to support studies of important pregnancy outcomes. This includes understanding the prevalence of congenital anomalies and answering important questions such as how well prenatal screening for congenital anomalies is performing with respect to timing, equity in access, and validity and utility of results, aligned with the World Health Organization recommendations for surveillance systems during pregnancy.[97]

5.4.4 Applying epidemiological methods to the field of clinical genetics

As already noted, much of the evidence to date on the association between unanticipated and unclear prenatal genetic screening results and adverse perinatal outcomes has been limited to descriptive studies. While this information has been helpful in clinical practice, genetic counselling based on limited evidence remains challenging, must be nuanced, and the important limitations of the evidence must be extensively discussed with patients. Therefore, alongside improved data capture, application of rigorous epidemiological methods is important to generate more robust evidence to reduce scientific uncertainty associated with unanticipated and unclear screening results.

In addition to small sample sizes, many studies in this field are subject to selection bias by only including a very select group of pregnancies. The studies presented in this dissertation had the clear strength of being population-based, which allowed us to investigate rare prenatal screening results in a large population and to reduce selection bias. We carefully considered the potential for residual selection bias by performing multiple sensitivity analyses to evaluate whether our findings were robust against assumptions we made.

5.5 Recommendations for future directions

Our results from the first study lead us to conclude that the 3.5 mm threshold for positive nuchal translucency screening results may need to be revisited. Some studies as well as clinical guidelines from professional societies such as the American College of Obstetrics and Gynecology with the Society for Maternal Fetal Medicine recommend implementing a cutoff of 3.0 mm for nuchal translucency, while others advocate for a gestational-age specific cutoff, such as 1.9 MoM, or the 95th percentile for instance.[6,8,39,98] Although the use of a gestational-age specific cutoff may provide a more accurate risk estimate, the use of a fixed measurement cutoff may be easier to implement in clinical practice.

Different studies have reported different risk estimates despite using the same threshold, and further, studies have reported different nomogram values of nuchal translucency measurements.[99,100] Some of the differences can be explained by practices related to the measurement of the nuchal translucency, with some jurisdictions or centres using the mean of three different measurements, and others using the highest of three measurements.[100] Other explanations for discrepancies could relate to the populations studied, as it has been hypothesized that, analogous to growth curves in early childhood, curves of nuchal translucency measurement by crown rump length may be population-specific and require their own nomogram.[100,101] Based on this information, it is likely that the best threshold of nuchal translucency measurement to be useful as an indication for an offer of further testing is not universal, but may be tailored to individual screening programs and determined based on the context of the screening program, its goals, and the population receiving screening.

In our future work, several options could be investigated to modify the threshold at which investigations for chromosomal abnormalities are offered. In Ontario, the nuchal translucency cutoff of 3.5 mm is used in two different ways: (i) to determine eligibility for publicly funded cfDNA screening; and (ii) to determine whether prenatal diagnosis should be offered. These two ways of using the nuchal translucency cutoff have different aims and implications.

Regarding the first of these purposes (i), when using the nuchal translucency measurement to determine **eligibility for publicly funded cfDNA screening**, the central question is about the

ability of the nuchal translucency threshold to identify the common aneuploidies identifiable by cfDNA screening (trisomies 21, 18, 13 and sex chromosome aneuploidies). Nuchal translucency in this instance needs to be considered in the context of multiple marker screening results, to determine the proportion of pregnancies currently already eligible for publicly funded cfDNA screening versus pregnancies that could become eligible in the case of a revised nuchal translucency cutoff. Using data from our cohort from Chapter 2, Table 5.1 presents the proportion of pregnancies with negative multiple marker screening results by nuchal translucency measurement. Among pregnancies with measurements between 2.5 and 3.0 mm, 73.8% have negative results and would therefore not be eligible for publicly funded cfDNA screening based on this indication. For pregnancies with measurements between 3.0 and 3.5 mm, 45.2% have negative multiple marker screening results. This provides an estimate of the proportion and number of additional pregnancies that would be eligible for cfDNA screening if eligibility were expanded to include a lower cutoff for nuchal translucency.

Table 5.1. Proportion of Pregnancies with Screen-Negative Results by Nuchal Translucency Measurement.

	Nuchal translucency measurement (in mm)							
	<2.0	**2.0-<2.5**	**2.5-<3.0**	**3.0-<3.5**	**3.5-<5.0**	**5.0-<6.5**	**>=6.5**	**Total**
All pregnancies	**359 807**	**43 219**	**7 474**	**1 789**	**1 088**	**404**	**487**	**414 268**
Screen-negative multiple marker screening result	343 914 (95.6%)	38 810 (89.8%)	5 519 (73.8%)	808 (45.2%)	132 (12.1%)	8 (2.0%)	21 (4.3%)	**389 212 (94.0%)**

In this same cohort from Chapter 2, among 5 519 pregnancies with screen-negative results and nuchal translucency measurements from 2.5 to 3.0 mm (Table 5.1), 11 pregnancies were ultimately found in our study to have a chromosomal abnormality that would have been identifiable on cfDNA screening (trisomies 21, 18, 13, or sex chromosome aneuploidies); most were identified after birth. There were no identified chromosomal abnormalities among 808

pregnancies with screen-negative multiple marker results and nuchal translucency the measurements from 3.0 to 3.5 mm. This suggests that a small number of pregnancies with chromosomal abnormalities that receive negative multiple marker screening results could be identified based on a nuchal translucency measurement from 2.5 to 3.5 mm if the cutoff for a positive nuchal translucency were revised. However, these estimates are subject to random error and when determining policy implications of such a change, there is a need to consider the screening system as a whole, for example, to understand whether multiple marker screening itself can (also or alternatively) be improved to reduce false negative results, and to understand how a changed nuchal translucency cutoff would alter subsequent care pathways. For example, pregnancies with chromosomal abnormalities may be identified through other pathways in the screening system, for instance publicly funded cfDNA screening following the identification of ultrasound findings suggestive of aneuploidy at 18-22 weeks gestation. Nevertheless, the preference would be to identify these pregnancies at an early gestation through either the nuchal translucency measurement or multiple marker screening, to facilitate reproductive choices and ensure appropriate care is being offered.

When it comes to the second purpose listed above (ii), identifying a **nuchal translucency measurement cutoff at which prenatal diagnosis should be offered**, the questions posed, and their implications are somewhat different. Prenatal diagnostic investigations have historically been offered when the probability of establishing a diagnosis in the pregnancy is higher than the procedure-related risk of pregnancy loss related to amniocentesis or chorionic villus sampling. Prenatal diagnosis continues to be important in the context of the availability of cfDNA screening, given that cfDNA screening remains a screening test and therefore a definitive diagnosis can only be established through cytogenetic testing following a prenatal diagnostic procedure or postnatally, and because it can be offered when there is an increased risk of a condition that can only be detected through tests performed in prenatal diagnosis, for instance microarray. These considerations are important when discussing the offer of prenatal diagnosis. While some pregnant individuals may want to have cfDNA screening following the identification of an increased nuchal translucency measurement, not all will, depending on risk, timing, and other factors. It is important to consider the impact of routinely offering cfDNA screening as a

first follow-up investigation in terms of potential delays in diagnosis due to screening failures, having to confirm a high-risk cfDNA screening result, and the role of microarray and considerations for conditions that cannot be detected by cfDNA screening. Thus, when considering changes to the nuchal translucency cutoff in the context of prenatal diagnostic testing, the implications for pregnant individuals and the system of care are complex. Policy decisions will require an in-depth understanding of potential impacts on the configuration of different follow-up testing modalities that may be offered and selected, with respect to timing, detection, and patient choice.

Based on the discussion above, the findings of our studies are important for prenatal screening programs around the world to consider when making decisions about nuchal translucency cutoffs, but such decisions will require some additional jurisdiction-specific discussions in the context of the full configuration of testing and care pathways available. In addition, future investigations should investigate both fixed measurement cutoffs and gestational-age specific cutoffs for nuchal translucency. Indeed, as described in section 5.3 and shown in Figure 5.1, if nuchal translucency is systematically measured at a later gestational age in one program compared with another, this will have an impact on the proportion of pregnancies with measurements above a non-gestational-age-specific cutoff and on the degree of risk for chromosomal and other conditions in those pregnancies. Gestational age-specific cutoffs would be more generalizable across programs with different screening practices although potentially more challenging to implement practically.

Overall, some of the strategies that may be promising but that need to be investigated include: 1) lowering the nuchal translucency cutoff to 3.0 mm to offer prenatal diagnosis through cytogenetic testing, 2) developing a cutoff that is gestational-age specific using percentile measurement for the corresponding gestational age or multiples of the median (MoM), and 3) adding a new eligibility criterion for publicly funded cfDNA screening under which all pregnancies with measurements greater or equal to 2.5 mm be offered cfDNA screening, regardless of the multiple marker screening result. Future research that focuses on the potential impact of these different strategies for the screening system are needed. Specifically,

there is an important need for translational research that could build on the findings of the first study presented in this dissertation, to fully estimate the downstream impacts, including costs and benefits of different thresholds and different screening modalities, using economic modeling that incorporates patient choice, and integrating assumptions about which care pathways may identify specific chromosomal abnormalities.

Additionally, to our knowledge, no comprehensive study has investigated the association between nuchal translucency measurement and all genetic conditions, including chromosomal abnormalities as well as single-gene conditions, structural defects identified prenatally and postnatally, and long-term outcomes. The piecemeal nature of the evidence available in the literature further complicates counselling, making it difficult to paint a full picture of potential outcomes with these individual studies. A study offering a comprehensive view of all potential outcomes associated with increased nuchal translucency measurements would be beneficial for clinical practice.

More generally, in the field of prenatal genetic screening, future research should seek to understand all types of screening results, including their implications for pregnancies in the screened population and for the screening system. In terms of currently unresolved uncertainties, as considered earlier (section 5.4.1), cfDNA screening failures are important to consider in future research. Indeed, there appears to be an increased risk of chromosomal abnormalities associated with screening failures, although few studies have addressed the meaning of a failed cfDNA result as their main objective, often simply stating the failure rate of cfDNA screening in the cohort studied, and reporting descriptively whether there were abnormal cytogenetic outcomes identified in these pregnancies.[82,102–108] Some studies have compared pregnancies with a failed cfDNA screen to those with a result on cfDNA screening, reporting the odds ratio for the association between these results and aneuploidies. However, the reference group used in these studies makes it difficult to interpret the results, as pregnancies with a result comprise those with both high-risk and low-risk results,[76,77] and the outcomes will vary depending on the population. Other studies have used a case-control design to report the differences in the proportion of screen failures in pregnancies with aneuploidies compared to euploid pregnancies.[78–81] Further, the evidence regarding the association between

screening failures and adverse obstetric outcomes is contradictory. A 2018 study reported an association between a failed screen and gestational diabetes and preeclampsia.[82] Other studies have focused on screens with a low fetal fraction, which is the most common contributing cause of a failed cfDNA screen, and have reported an association with adverse obstetrical outcomes,[82–88] while others have found no association.[89–91] A 2021 systematic review of the literature reported an association between low fetal fraction on cfDNA screening and hypertensive disorders of pregnancy, preterm birth, and fetal growth restriction. Importantly, in this review the authors noted the need for a large cohort study on this topic, as limited evidence was available in the literature.[109]

More research on this topic is therefore needed to inform current screening models incorporating cfDNA screening or considering its implementation. Prenatal screening programs considering a transition to universal cfDNA screening to replace a system based on multiple marker screening (alone or in a contingent model with cfDNA offered to those with positive results) may need to consider not only the high performance of cfDNA screening for identifying common aneuploidies,[110] but also the implications and management strategies for patients that receive a failed cfDNA screening result. Future work should focus on the likelihood of failed cfDNA screening, of obtaining a successful redraw, whether pregnancies with failed cfDNA screening followed by a low-risk result on redraw are at increased of adverse outcomes, and overall implications of such results for the patient, and for the prenatal screening system.

5.6 Implications for clinical practice and policy

Nuchal translucency ultrasound

The first two studies in this dissertation highlighted the importance of the nuchal translucency ultrasound in prenatal screening for chromosomal abnormalities but also for adverse perinatal outcomes. An additional argument for encouraging the continued incorporation of nuchal translucency ultrasound as part routine prenatal screening is that it is an important component of an early anatomy scan of the fetus during which fetal viability and well-being can be assessed

and major congenital anomalies incompatible with life such as anencephaly or holoprosencephaly can be identified.[111]

Counselling about the potential implications of an increased nuchal translucency measurement should take place prior to screening to allow the pregnant individual to make an informed decision about whether this type of screening is right for them and if they wish to participate in the screening process. Indeed, if the patient is choosing to screen for Down syndrome, but later finds out that the implications of the screening result go beyond the condition for which they provided consent, as our results indicate with the increased risk for other chromosomal abnormalities and other adverse perinatal outcomes, this unexpected result could be more distressing due to the fact that its possibility was not presented in advance. Conversely, if a patient declines screening for Down syndrome because they do not wish to receive this information during pregnancy, when in fact they would have requested screening for other chromosomal abnormalities and structural defects through nuchal translucency ultrasound, then this patient was not able to make an informed decision when declining screening. These situations highlight the importance of informed decision-making in prenatal screening, which can be psychologically beneficial through lower decisional conflict and higher decision satisfaction.[112]

The evidence generated by our studies on the association between nuchal translucency measurements and chromosomal abnormalities is also important when discussing potential investigations offered following the identification of the increased nuchal translucency, given that not all chromosomal abnormalities associated with an increased nuchal translucency measurement can be identified through the cfDNA screening platforms offered in Ontario and in many other jurisdictions (routine cfDNA screening is limited to trisomies 21, 18, 13, and sex chromosome aneuploidies). Although currently in Ontario patients are eligible for funded cfDNA screening when the nuchal translucency is measured at a minimum of 3.5 mm, counselling must include the notion that there is an increased risk of conditions that are not be identifiable on cfDNA screening.

Importantly, the findings of the studies on nuchal translucency measurement also describe the challenges and further emphasize the importance of quality assurance of nuchal translucency ultrasound.

Double-positive multiple marker screening results

The findings of our study of double-positive multiple marker screening results identified a range of risks associated with such results. This includes a higher risk of preterm birth and other adverse outcomes among such pregnancies that result in a live birth, with the potential for long-term health implications. In addition, given the high probability of identifying a chromosomal abnormality in pregnancies with double-positive results, our findings support offering prenatal diagnosis to pregnant individuals with such results. This information should be made widely available and proactively communicated to the genetics community so that patients have access to the appropriate information to guide their decision-making. Indeed, given the high probability of chromosomal abnormality, pregnant individuals may want to have prenatal diagnosis as a first intention rather than begin with cfDNA screening, which could cause a delay in care by either providing a high-risk result that will need to be confirmed through cytogenetic testing regardless, a screening failure that may require a redraw or subsequent prenatal diagnosis, or a low-risk result in which case the conditions not routinely screened by cfDNA screening platforms in Ontario and in many other jurisdictions (triploidy, other autosomal aneuploidies beyond trisomies 21 18 or 13, or copy number variants) would not be identified.

5.7 Summary of Strengths and Limitations

As described, the studies presented in this dissertation had several strengths compared to the evidence currently available in the literature. These strengths included the use of large population-based cohorts to reduce selection bias and to increase the generalizability of the findings to pregnant individuals undergoing prenatal genetic screening. We used appropriate

analytic methods and included several sensitivity analyses to evaluate the robustness of the findings against varying assumptions.

An inherent limitation in this research is that because not everyone has confirmatory testing, outcomes are not fully ascertained for all pregnancies receiving prenatal screening. To mitigate against this limitation, we used additional data sources available within the registry, such as cfDNA screening and clinical examination at birth as well as sensitivity analyses with varying assumptions for the pregnancies in which no confirmatory testing was recorded. Based on these methods however, subtle conditions that may not come to medical attention prenatally or in the first months of life may have been misclassified as not having a chromosomal abnormality. As these infants age, they may develop a different phenotype leading them to a diagnosis of a chromosomal abnormality. For instance, a diagnosis of Monosomy X or Klinefelter may only be identified in adulthood during investigations for infertility.[113,114] For this reason, the studies presented in this dissertation will be repeated in the future as more long-term data are gathered for this cohort.

An additional limitation is that not everyone has prenatal screening, and those pregnancies not receiving screening were therefore excluded from our studies. Reports of uptake of prenatal screening in Ontario have described that pregnant individuals opting out of prenatal screening tended to be younger, live in more rural areas of the province, and have a lower socioeconomic status.[115–117] While conditions associated with increased age of the pregnant individual may be less prevalent in the population excluded, the association between the prenatal screening result and the outcomes would not be expected to differ.

Finally, the studies presented in this dissertation were limited to the data currently collected by the registry, which limited the scope of outcomes that could be evaluated. We hope to pursue this work in future years and extend our analyses to additional important clinical outcomes if the registry were to expand its data collection.

5.8 Conclusions

The findings presented in this doctoral thesis demonstrate that unanticipated and unclear prenatal genetic screening results can be associated with an increased risk of adverse perinatal outcomes. These findings add important evidence to the field of prenatal genetic screening. The evidence generated can be used in patient counselling to facilitate informed decision making by reducing the gap in uncertainty for unanticipated or unclear prenatal screening results, and importantly, convey the possibility that unanticipated or unclear results can arise through screening. This evidence can also be used by policymakers to enhance the quality of the prenatal screening program and may inform efforts toward a unified approach to these unanticipated or unclear results. The findings also emphasize the importance of understanding the prenatal screening system as a whole, beyond the common or expected results. Screening can appear deceptively simple, but for a screening program to be successful and do more good than harm, all aspects of the program must be considered, including giving attention to the unanticipated or unclear results that will inevitably occur.[118]

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Appendix A. Research Ethics Board Approvals

CHEO REB
Letter of Approval

REB Protocol No: 22/03PE
ROMEO File No: 20221222
Principal Investigator: Ms. Kara Bellai-Dussault
Protocol Title: CHEOREB# 22/03PE - Cytogenetic and pregnancy outcomes following an increased nuchal translucency measurement

Protocol Status: Active

Approval Date: June 07, 2022
Approval Expiry Date: May 15, 2023

The CHEO REB has conducted a delegated review and determined that the conditions of approval have been satisfied for the above-named study. Approval is valid for the period indicated above. This research study is to be conducted by the investigator noted above. Annual renewals or study closures must be completed before the expiry date noted above.

REB members involved in the study do not participate in the review, deliberations, or decision.

Documents Approved:

Document Name	Comments	Version Date
Other Document	List of variables	2022/06/02
Protocol	Protocol	2022/06/06

Any modifications made to the study must be reviewed and approved by the REB prior to implementation, except when necessary to eliminate immediate danger or hazard(s) to study participants or when the change(s) involves administrative aspects of the study. Investigators must promptly alert the REB of any changes that increase the risk to participants or affect the safety of participants, all unanticipated and harmful events that occur, and new information that significantly impact the conduct of the study.

The CHEO REB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans (TCPS 2); the International Conference on Harmonization Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; and Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The CHEO REB is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP).

Please do not hesitate to contact the Research Ethics Office if you have any questions.

Best wishes for the successful conduct of your research.

Cécile Bensimon, MA, PhD
Chair, Research Ethics Board
Présidente, Comité d'éthique de la recherche

CHEO REB
Approval of Annual Renewal

REB Protocol No: 22/03PE
ROMEO File No: 20221222
Principal Investigator: Ms. Kara Bellai-Dussault
Protocol Title: CHEOREB# 22/03PE - Cytogenetic and pregnancy outcomes following an increased nuchal translucency measurement

Protocol Status: Active

Approval Date: April 17, 2023
Approval Expiry Date: May 15, 2024

The CHEO REB has conducted a delegated review and approved the renewal of the above-named study. Approval is valid for the period indicated above. Future annual renewals or study closures must be completed before the expiry date noted above.

The decision was ratified by the Full Board. REB members involved in the study do not participate in the review, deliberations, or decision.

Any modifications made to the study must be reviewed and approved by the REB prior to implementation, except when necessary to eliminate immediate danger or hazard(s) to study participants or when the change(s) involves administrative aspects of the study. Investigators must promptly alert the REB of any changes that increase the risk to participants or affect the safety of participants, all unanticipated and harmful events that occur, and new information that significantly impact the conduct of the study.

The CHEO REB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans (TCPS 2); the International Conference on Harmonization Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; and Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The CHEO REB is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP).

Please do not hesitate to contact the Research Ethics Office if you have any questions.

Best wishes with the successful completion of your research.

Cécile Bensimon, MA, PhD
Chair, Research Ethics Board
Présidente, Comité d'éthique de la recherche

Université d'Ottawa
Bureau d'éthique et d'intégrité de la recherche

University of Ottawa
Office of Research Ethics and Integrity

CERTIFICAT D'APPROBATION ÉTHIQUE | CERTIFICATE OF ETHICS APPROVAL

Numéro du dossier / Ethics File Number	H-06-22-8234
Titre du projet / Project Title	Cytogenetic and pregnancy outcomes following an increased nuchal translucency measurement
Type de projet / Project Type	Thèse de doctorat / Doctoral thesis
Statut du projet / Project Status	Renouvelé / Renewed
Date d'approbation (jj/mm/aaaa) / Approval Date (dd/mm/yyyy)	24/06/2022
Date d'expiration (jj/mm/aaaa) / Expiry Date (dd/mm/yyyy)	15/05/2024

Équipe de recherche / Research Team

Chercheur / Researcher	Affiliation	Role
Kara BELLAI-DUSSAULT	Département d'épidémiologie et santé publique / Department of Epidemiology and Public Health	Chercheur Principal / Principal Investigator
Elizabeth POTTER	Département d'épidémiologie et santé publique / Department of Epidemiology and Public Health	Superviseur / Supervisor
Julian LITTLE	Département d'épidémiologie et santé publique / Department of Epidemiology and Public Health	Co-superviseur / Co-supervisor

Conditions spéciales ou commentaires / Special conditions or comments

The uOttawa expiry date has been set in accordance with the one from the CHEO-REB.

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20/04/2023

Université d'Ottawa

Bureau d'éthique et d'intégrité de la recherche

University of Ottawa

Office of Research Ethics and Integrity

Le Comité d'éthique de la recherche (CÉR) de l'Université d'Ottawa, opérant conformément à l'*Énoncé de politique des Trois conseils* (2014) et toutes autres lois et tous règlements applicables, a examiné et approuvé la demande d'éthique du projet de recherche ci-nommé.

L'approbation est valide pour la durée indiquée plus haut et est sujette aux conditions énumérées dans la section intitulée "Conditions Spéciales ou Commentaires". Le formulaire « Renouvellement ou Fermeture de Projet » doit être complété quatre semaines avant la date d'échéance indiquée ci-haut afin de demander un renouvellement de cette approbation éthique ou afin de fermer le dossier.

Toutes modifications apportées au projet doivent être approuvées par le CÉR avant leur mise en place, sauf si le participant doit être retiré en raison d'un danger immédiat ou s'il s'agit d'un changement ayant trait à des éléments administratifs ou logistiques du projet. Les chercheurs doivent aviser le CÉR dans les plus brefs délais de tout changement pouvant augmenter le niveau de risque aux participants ou pouvant affecter considérablement le déroulement du projet, rapporter tout évènement imprévu ou indésirable et soumettre toute nouvelle information pouvant nuire à la conduite du projet ou à la sécurité des participants.

The University of Ottawa Research Ethics Board, which operates in accordance with the *Tri-Council Policy Statement* (2014) and other applicable laws and regulations, has examined and approved the ethics application for the above-named research project.

Ethics approval is valid for the period indicated above and is subject to the conditions listed in the section entitled "Special Conditions or Comments". The "Renewal/Project Closure" form must be completedfour weeks before the above-referenced expiry date to request a renewal of this ethics approval or closure of the file.

Any changes made to the project must be approved by the REB before being implemented, except when necessary to remove participants from immediate endangerment or when the modification(s) only pertain to administrative or logistical components of the project. Investigators must also promptly alert the REB of any changes that increase the risk to participant(s), any changes that considerably affect the conduct of the project, all unanticipated and harmful events that occur, and new information that may negatively affect the conduct of the project or the safety of the participant(s).

Coordonateur / COORDINATOR
Coordonnateur de l'éthique / Ethics Coordinator
Pour/For **Daniel LAGAREC** Président(e) du/ Chair of the **Comité d'éthique de la recherche en sciences de la santé et sciences / Health Sciences and Sciences Research Ethics Board**

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CHEO REB
Approval of Annual Renewal

REB Protocol No: 22/05PE
ROMEO File No: 20221237
Principal Investigator: Ms. Kara Bellai-Dussault
Protocol Title: CHEOREB# 22/05PE - Outcomes of pregnancies with a double-positive multiple marker screening result

Protocol Status: Active

Approval Date: May 18, 2023
Approval Expiry Date: June 15, 2024

The CHEO REB has conducted a delegated review and approved the renewal of the above-named study. Approval is valid for the period indicated above. Future annual renewals or study closures must be completed before the expiry date noted above.

The decision was ratified by the Full Board. REB members involved in the study do not participate in the review, deliberations, or decision.

Any modifications made to the study must be reviewed and approved by the REB prior to implementation, except when necessary to eliminate immediate danger or hazard(s) to study participants or when the change(s) involves administrative aspects of the study. Investigators must promptly alert the REB of any changes that increase the risk to participants or affect the safety of participants, all unanticipated and harmful events that occur, and new information that significantly impact the conduct of the study.

The CHEO REB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans (TCPS 2); the International Conference on Harmonization Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; and Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The CHEO REB is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP).

Please do not hesitate to contact the Research Ethics Office if you have any questions.

Best wishes with the successful completion of your research.

Cécile Bensimon, MA, PhD
Chair, Research Ethics Board
Présidente, Comité d'éthique de la recherche

CHEO REB
Letter of Approval

REB Protocol No: 22/05PE
ROMEO File No: 20221237
Principal Investigator: Ms. Kara Bellai-Dussault
Protocol Title: CHEOREB# 22/05PE - Outcomes of pregnancies with a double-positive multiple marker screening result

Protocol Status: Active

Approval Date: June 17, 2022
Approval Expiry Date: June 15, 2023

The CHEO REB has conducted a delegated review and determined that the conditions of approval have been satisfied for the above-named study. Approval is valid for the period indicated above. This research study is to be conducted by the investigator noted above. Annual renewals or study closures must be completed before the expiry date noted above.

REB members involved in the study do not participate in the review, deliberations, or decision.

Documents Approved:

Document Name	Comments	Version Date
Protocol	Double-positive MMS results_Kara_Bellai_ver_1.3_15Jun2022	2022/06/15
Other Document	2022-06-15 - Variable list_v1.1	2022/06/15

Any modifications made to the study must be reviewed and approved by the REB prior to implementation, except when necessary to eliminate immediate danger or hazard(s) to study participants or when the change(s) involves administrative aspects of the study. Investigators must promptly alert the REB of any changes that increase the risk to participants or affect the safety of participants, all unanticipated and harmful events that occur, and new information that significantly impact the conduct of the study.

The CHEO REB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans (TCPS 2); the International Conference on Harmonization Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; and Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The CHEO REB is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP).

Please do not hesitate to contact the Research Ethics Office if you have any questions.

Best wishes for the successful conduct of your research.

Cécile Bensimon, MA, PhD
Chair, Research Ethics Board
Présidente, Comité d'éthique de la recherche

28/11/2023

Université d'Ottawa
Bureau d'éthique et d'intégrité de la recherche

University of Ottawa
Office of Research Ethics and Integrity

CERTIFICAT D'APPROBATION ÉTHIQUE | CERTIFICATE OF ETHICS APPROVAL

Numéro du dossier / Ethics File Number	H-06-22-8269
Titre du projet / Project Title	Outcomes of pregnancies with a double-positive multiple marker screening result
Type de projet / Project Type	Thèse de doctorat / Doctoral thesis
Statut du projet / Project Status	Renouvelé / Renewed
Date d'approbation (jj/mm/aaaa) / Approval Date (dd/mm/yyyy)	27/06/2022
Date d'expiration (jj/mm/aaaa) / Expiry Date (dd/mm/yyyy)	15/06/2024

Équipe de recherche / Research Team

Chercheur / Researcher	Affiliation	Role
Kara BELLAI-DUSSAULT	Département d'épidémiologie et santé publique / Department of Epidemiology and Public Health	Chercheur Principal / Principal Investigator
Elizabeth POTTER	Département d'épidémiologie et santé publique / Department of Epidemiology and Public Health	Superviseur / Supervisor
Julian LITTLE	Département d'épidémiologie et santé publique / Department of Epidemiology and Public Health	Co-superviseur / Co-supervisor
Steven HAWKEN	Département d'épidémiologie et santé publique / Department of Epidemiology and Public Health	Collaborateur / Collaborator

Conditions spéciales ou commentaires / Special conditions or comments

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28/11/2023

Université d'Ottawa

Bureau d'éthique et d'intégrité de la recherche

University of Ottawa

Office of Research Ethics and Integrity

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L'approbation est valide pour la durée indiquée plus haut et est sujette aux conditions énumérées dans la section intitulée "Conditions Spéciales ou Commentaires". Le formulaire « Renouvellement ou Fermeture de Projet » doit être complété quatre semaines avant la date d'échéance indiquée ci-haut afin de demander un renouvellement de cette approbation éthique ou afin de fermer le dossier.

Toutes modifications apportées au projet doivent être approuvées par le CÉR avant leur mise en place, sauf si le participant doit être retiré en raison d'un danger immédiat ou s'il s'agit d'un changement ayant trait à des éléments administratifs ou logistiques du projet. Les chercheurs doivent aviser le CÉR dans les plus brefs délais de tout changement pouvant augmenter le niveau de risque aux participants ou pouvant affecter considérablement le déroulement du projet, rapporter tout évènement imprévu ou indésirable et soumettre toute nouvelle information pouvant nuire à la conduite du projet ou à la sécurité des participants.

The University of Ottawa Research Ethics Board, which operates in accordance with the *Tri-Council Policy Statement* (2014) and other applicable laws and regulations, has examined and approved the ethics application for the above-named research project.

Ethics approval is valid for the period indicated above and is subject to the conditions listed in the section entitled "Special Conditions or Comments". The "Renewal/Project Closure" form must be completedfour weeks before the above-referenced expiry date to request a renewal of this ethics approval or closure of the file.

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Coordonateur / COORDINATOR
Coordonnateur de l'éthique / Ethics Coordinator
Pour/For **Daniel LAGAREC** Président(e) du/ Chair of the **Comité d'éthique de la recherche en sciences de la santé et sciences / Health Sciences and Sciences Research Ethics Board**

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Appendix B. Confirmation of manuscript submissions

Manuscript 2

Manuscript submitted to Ultrasound in Obstetrics and Gynecology - UOG-2024-0152, Authors Copy

UOG Admin <onbehalfof@manuscriptcentral.com>
Fri 16/02/2024 4:38 PM

To: Kara Bellai Dussault [redacted]
Cc: Kara Bellai Dussault [redacted]; Deshayne Fell [redacted]; Julian Little [redacted]; Mark Walker [redacted]; Beth Potter [redacted]

Attention : courriel externe | external email

16-Feb-2024

Manuscript number: UOG-2024-0152

Dear Mrs Bellai Dussault

We are pleased to receive your manuscript entitled Outcomes of pregnancies with varying levels of nuchal translucency measurements: a population-based retrospective study in Ontario, Canada. by Bellai Dussault, Kara; Dougan, Shelley; Fell, Deshayne; Lavin Venegas, Carolina; Little, Julian; Meng, Lynn; Okun, Nan; Walker, Mark; Armour, Christine; Potter, Beth. We will shortly be assigning it to one of the Journal's Editors who will handle the peer review of the paper.

To track the progress of your manuscript through the editorial process using our web-based system, simply point your browser to:

https://mc.manuscriptcentral.com/uog

and log in using the following user ID and password:

(User ID):
(Password): https://mc.manuscriptcentral.com/uog?URL_MASK=32ea0d345e234da4ad406f19241a4ada

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If the single use link has expired, you can generate a single use password by entering your email address into the Password Help function on your site log in page: https://mc.manuscriptcentral.com/uog

Please remember in any future correspondence regarding this article to always include its manuscript ID number UOG-2024-0152.

This journal offers a number of license options, information about which is available here: https://authorservices.wiley.com/author-resources/Journal-Authors/licensing/index.html. All coauthors are required to confirm that they have the necessary rights to grant in the submission, including in light of each coauthor's funder policies. For example, if you or one of your coauthors received funding from a member of Coalition S, you may need to check which licenses you are able to sign.

Many thanks for submitting your manuscript

Yours sincerely

Sarah Hatcher
Managing Editor

Manuscript 3

AJOG Submission Confirmation

American Journal of Obstetrics & Gynecology <[redacted]>
Tue 4/2/2024 12:54 PM
To: Kara Bellai Dussault <[redacted]>
Attention : courriel externe | external email
"Pregnancies with 'double-positive' multiple marker screening results: a population-based study in Ontario, Canada"
https://www.editorialmanager.com/ajog/

Dear Ms Kara Bellai-Dussault:

This acknowledges the receipt of your submission cited above to The American Journal of Obstetrics & Gynecology.

Please Note:

1) If any item was omitted the submission will be returned.

2) It is the responsibility of the corresponding author to ensure:

* all authors have been consulted and approve of the submission.

* all appropriate Conflicts of Interest / Financial Disclosures / Funding for ALL authors has been included on the title page of the submission and in the online submission questions.

Thank you for submitting your research to the American Journal of Obstetrics & Gynecology for consideration.

Sincerely yours,

The Editors
The American Journal of Obstetrics & Gynecology (AJOG)
Editorial Manager: https://www.editorialmanager.com/AJOG

Editorial Office Contacts

Sandra Perrine
Managing Editor
email[redacted]

Donna Stroud
Managing Editor
email:[redacted]

Appendix C. Published version of manuscript 1.

JAMA Network Open

Original Investigation | Genetics and Genomics

Ultrasonographic Fetal Nuchal Translucency Measurements and Cytogenetic Outcomes

Kara Bellai-Dussault, MSc; Shelley D. Dougan, MSc; Deshayne B. Fell, PhD; Julian Little, PhD; Lynn Meng, MSc; Nan Okun, MD; Mark C. Walker, MD; Christine M. Armour, MD; Beth K. Potter, PhD

Abstract

IMPORTANCE Ultrasonographic measurement of fetal nuchal translucency is used in prenatal screening for trisomies 21 and 18 and other conditions. A cutoff of 3.5 mm or greater is commonly used to offer follow-up investigations, such as prenatal cell-free DNA (cfDNA) screening or cytogenetic testing. Recent studies showed a possible association with chromosomal anomalies for levels less than 3.5 mm, but extant evidence has limitations.

OBJECTIVE To evaluate the association between different nuchal translucency measurements and cytogenetic outcomes on a population level.

DESIGN, SETTING, AND PARTICIPANTS This population-based retrospective cohort study used data from the Better Outcomes Registry & Network, the perinatal registry for Ontario, Canada. All singleton pregnancies with an estimated date of delivery from September 1, 2016, to March 31, 2021, were included. Data were analyzed from March 17 to August 14, 2023.

EXPOSURES Nuchal translucency measurements were identified through multiple-marker screening results.

MAIN OUTCOMES AND MEASURES Chromosomal anomalies were identified through all Ontario laboratory-generated prenatal and postnatal cytogenetic tests. Cytogenetic testing results, supplemented with information from cfDNA screening and clinical examination at birth, were used to identify pregnancies without chromosomal anomalies. Multivariable modified Poisson regression with robust variance estimation and adjustment for gestational age was used to compare cytogenetic outcomes for pregnancies with varying nuchal translucency measurement categories and a reference group with nuchal translucency less than 2.0 mm.

RESULTS Of 414 268 pregnancies included in the study (mean [SD] maternal age at estimated delivery date, 31.5 [4.7] years), 359 807 (86.9%) had a nuchal translucency less than 2.0 mm; the prevalence of chromosomal anomalies in this group was 0.5%. An increased risk of chromosomal anomalies was associated with increasing nuchal translucency measurements, with an adjusted risk ratio (ARR) of 20.33 (95% CI, 17.58-23.52) and adjusted risk difference (ARD) of 9.94% (95% CI, 8.49%-11.39%) for pregnancies with measurements of 3.0 to less than 3.5 mm. The ARR was 4.97 (95% CI, 3.45-7.17) and the ARD was 1.40% (95% CI, 0.77%-2.04%) when restricted to chromosomal anomalies beyond the commonly screened aneuploidies (excluding trisomies 21, 18, and 13 and sex chromosome aneuploidies).

CONCLUSIONS AND RELEVANCE In this cohort study of 414 268 singleton pregnancies, those with nuchal translucency measurements less than 2.0 mm were at the lowest risk of chromosomal

(continued)

Key Points

Question Is there an association between nuchal translucency measurements less than 3.5 mm and chromosomal anomalies?

Findings In this population-based cohort study including 414 268 singleton pregnancies in Ontario, Canada, a significantly increased risk of chromosomal anomalies was associated with each increasing level of nuchal translucency measurement, compared with a reference group of pregnancies with nuchal translucencies less than 2.0 mm.

Meaning The findings of this cohort study suggest that pregnancies with nuchal translucency measurements greater than 2.0 mm are at increased risk of chromosomal anomalies, indicating that the widely used threshold of 3.5 mm may need to be reexamined.

+ Supplemental content

Author affiliations and article information are listed at the end of this article.

Abstract (continued)

anomalies. Risk increased with increasing measurements, including measurements less than 3.5 mm and anomalies not routinely screened by many prenatal genetic screening programs.

JAMA Network Open. 2024;7(3):e243689. doi:10.1001/jamanetworkopen.2024.3689

Introduction

Since the 1990s, ultrasonographic measurement of nuchal translucency—a collection of fluid behind the fetal neck[1]—has been included in prenatal genetic screening offered to pregnant individuals to identify trisomies 21 and 18, typically as a part of multiple-marker screening.[2,3] In addition to these aneuploidies, increased fetal nuchal translucency is associated with other chromosomal anomalies, single gene conditions, and structural defects.[4] The current practice in many jurisdictions, including Ontario, Canada, is to offer follow-up investigations when nuchal translucency is greater than or equal to 3.5 mm, which theoretically corresponds to the 99th percentile for all gestational ages.[5-9] These follow-up investigations may include prenatal cell-free DNA (cfDNA) screening and/or confirmatory diagnostic testing through cytogenetic analysis.

Many studies of the association between nuchal translucency measurement and chromosomal anomalies beyond trisomies 21 and 18 have been beset by methodological limitations such as selection bias, for example, by focusing on narrow or high-risk populations (eg, based at tertiary institutions)[10-14] or including only pregnant individuals who elected to have a prenatal diagnosis.[15-18] Studies have also been limited by failing to include a low nuchal translucency reference group, including a historical reference group only, having a small sample size, or including only prenatal cytogenetic testing results.[19-23] A small number of these studies[19,20,22] have provided preliminary evidence that pregnancies with nuchal translucency measurements that are elevated but still lower than 3.5 mm could also be at increased risk of clinically significant chromosomal anomalies. These findings require confirmation using robust methodological approaches in large, unselected samples with comprehensive follow-up to adequately assess the risk of chromosomal anomalies across the entire range of nuchal translucency measurements. In this study, we aimed to evaluate the association between all levels of nuchal translucency measurements and cytogenetic outcomes among pregnancies in Ontario, Canada, identified through a population-based provincial registry.

Methods

This study received approval from the research ethics boards of the Children's Hospital of Eastern Ontario and the University of Ottawa. The requirement of informed consent was waived owing to the use of deidentified patient data. All cell counts of less than 6 were suppressed to comply with the privacy requirements of the registry. The study followed the Reporting of Studies Conducted Using Observational Routinely-Collected Health Data (RECORD) reporting guideline.

Data Source

Better Outcomes Registry & Network (BORN) Ontario is a prescribed perinatal registry that collects data directly from all multiple-marker screening, cfDNA screening, and cytogenetics laboratories in Ontario.[24] Pregnancy and birth outcomes are also captured, including information on the clinical birth examination and linkage to discharge data from all hospitals through the discharge abstract database of the Canadian Institute for Health Information (CIHI). This enabled comprehensive ascertainment of pregnancy outcomes. Additional details on data sources are provided in eTable 1 in Supplement 1.

Setting and Study Population

Ontario offers a publicly funded screening program in which all pregnant individuals have access to multiple-marker screening in the first trimester, most often including a nuchal translucency measurement.[25] Cell-free DNA screening or cytogenetic testing is offered if the screen result is positive or as a first-tier screen if specific eligibility criteria are met.[26] Individuals may also self-pay for cfDNA screening.[26]

Nuchal translucencies are measured at crown-rump lengths of 45 to 84 mm by sonographers registered in Ontario's Nuchal Translucency Quality Assurance program.[27] This study included all singleton pregnancies in Ontario with a valid multiple-marker screening test including a nuchal translucency and with an estimated date of delivery (EDD) from September 1, 2016, to March 31, 2021 (eFigure 1 in Supplement 1). While ethnicity is recorded in the BORN database, we did not incorporate this information into our analysis as it was not expected to influence the associations studied.

Study Exposure

Nuchal translucency measurements for all pregnancies were identified from multiple-marker screening results. The reference group was defined as pregnancies with a measurement less than 2.0 mm, compared with pregnancies with the following categories of nuchal translucency measurements: 2.0 to less than 2.5 mm, 2.5 to less than 3.0 mm, 3.0 to less than 3.5 mm, 3.5 to less than 5.0 mm, 5.0 to less than 6.5 mm, and 6.5 mm or greater.

Study Outcome

Pregnancies with chromosomal anomalies were identified through cytogenetic testing results submitted by all Ontario cytogenetic laboratories to the BORN registry. The primary outcome was defined as any chromosomal anomaly identified on cytogenetic testing, during pregnancy or postnatally, including microarray analysis. As secondary outcomes, we stratified chromosomal anomalies by whether or not the condition is routinely tested through cfDNA screening in Ontario (trisomies 21, 18, and 13 and sex chromosome aneuploidies).

Because only a small number of pregnancies have cytogenetic investigations, we supplemented our outcome data with information from other sources (pregnancies with and without cytogenetic testing are described in eTable 12 in Supplement 1). Specifically, to identify pregnancies without chromosomal anomalies, we first used cytogenetic testing results if performed. If no cytogenetic testing results were available, we used cfDNA screening results, if performed; although a low-risk cfDNA screening result cannot be used clinically to exclude these conditions (trisomies 21, 18, and 13 and sex chromosome aneuploidies), for the purposes of this research, it was considered a reasonable proxy given its negative predictive value of greater than 99.9%.[28] Finally, for pregnancies with no cytogenetic testing and no cfDNA screening, we used results from the clinical examination at birth to exclude conditions typically clinically diagnosable at birth, relying on both BORN and CIHI data (eTable 2 in Supplement 1).

Statistical Analysis

Data were analyzed from March 17 to August 14, 2023. The study population was described using means (SDs) for continuous variables and frequencies and proportions for categorical variables. We used multivariable modified Poisson regression models with robust variance estimation and adjustment for gestational age at screening to compare the risk of chromosomal anomalies across pregnancies with varying categories of nuchal translucency measurements with the reference category (<2.0 mm).[29] This model also allowed us to account for clustering for individuals with more than 1 pregnancy within the study period.[30] Gestational age at screening was identified a priori as a potential confounder through directed acyclic graphs, as nuchal translucency measurements are on a continuum and will change with gestational age (eFigure 2 in Supplement 1). A post hoc analysis with

additional adjustment for age of the pregnant individual was also performed. Adjusted risk ratios (ARRs) and risk differences (ARDs) were reported with 95% CIs.

We conducted the following sensitivity analyses to evaluate the potential impact of incomplete or inaccurate ascertainment of the exposure and outcome and to address losses to follow-up. All analyses were performed using SAS, version 9.4 (SAS Institute Inc), and 2-tailed $P < .05$ was considered statistically significant.

Exposure Measurement Source

When a very high nuchal translucency measurement or cystic hygroma is identified, some pregnant individuals may not complete the multiple-marker screening process and, thus, may not be ascertained by the laboratories. We therefore performed a sensitivity analysis identifying pregnancies with increased nuchal translucency measurements through other sources available within the registry, including data obtained from consultations with genetics or maternal fetal medicine clinics and from documented clinical indications for testing obtained from cytogenetic laboratories (eTable 3 in Supplement 1).

Exposure Definition

Some studies rely on percentiles of the nuchal translucency measurement rather than absolute values. Therefore, we categorized nuchal translucency measurements as less than 90th, 90th to less than 95th, 95th to less than 99th, and 99th percentile or greater[10,21] in an additional sensitivity analysis (eTable 4 in Supplement 1).

Losses to Follow-Up

For some pregnancies, no outcome was recorded. These may reflect pregnancy losses or terminations in the absence of follow-up cfDNA screening or cytogenetic testing or pregnant individuals who had multiple-marker screening in Ontario but subsequently received care outside the province. Because of the unclear nature of the outcome for these pregnancies, we performed a sensitivity analysis in which we randomly classified the losses to follow-up to having twice the prevalence of chromosomal anomalies compared with pregnancies in the same category of nuchal translucency measurement for which an outcome was recorded or half the prevalence (eTable 5 in Supplement 1). A further sensitivity analysis included all pregnancies with varying assumptions of risk of chromosomal anomalies for those lost to follow-up based on the pregnancy outcome (eTable 6 in Supplement 1).

Outcome

Complete cytogenetic data for microarray testing was only available since January 2018. Therefore, we conducted an analysis restricted to pregnancies with EDD from September 1, 2018, to March 31, 2021 (eTable 7 in Supplement 1).

Time Period

An additional sensitivity analysis excluded pregnancies with an EDD from April 1, 2020, to March 31, 2021. This exclusion accounted for potential effects of the COVID-19 pandemic on prenatal care practices (eTable 8 in Supplement 1).

Results

From 643 146 singleton pregnancies in Ontario during the study period, 414 268 were eligible for the analysis (mean [SD] maternal age at EDD, 31.5 [4.7] years). Of these, 359 807 pregnancies (86.9%) had a nuchal translucency measurement less than 2.0 mm; 43 219 (10.4%), from 2.0 to less than 2.5 mm; 7474 (1.8%), from 2.5 to less than 3.0 mm; 1789 (0.4%), from 3.0 to less than 3.5 mm; 1088 (0.3%), from 3.5 to less than 5.0 mm; 404 (0.1%), from 5.0 to less than 6.5 mm; and 487 (0.1%), 6.5

mm or greater (**Table 1**). The mean (SD) maternal age at EDD increased across nuchal translucency categories, from 31.5 (4.7) years for pregnant individuals with nuchal translucency measurements less than 2.0 mm to 33.3 (5.4) years for those with measurements 6.5 mm or greater. We excluded 225 264 pregnancies without a valid multiple-marker screening test including a nuchal translucency measurement, 158 with no screening result report issued, and 3456 where the measurement was performed outside the gestational age range corresponding to the crown-rump length of 45 to 84 mm.

Figure 1 and **Figure 2** describe the uptake of follow-up investigations (cfDNA screening, cytogenetic testing) and pregnancy outcomes among pregnant individuals with nuchal translucency measurements less than 3.5 mm and 3.5 mm or greater, respectively. Of pregnancies with a nuchal translucency measurement 3.5 mm or greater, 1654 (83.6%) underwent follow-up investigations prenatally, compared with 44 849 (10.9%) of pregnancies with a measurement less than 3.5 mm. Among those with nuchal translucencies 3.5 mm or greater, 414 (20.9%) had both cfDNA screening and prenatal diagnosis with cytogenetic testing, compared with 1747 (0.4%) among pregnancies with a measurement less than 3.5 mm. Of chromosomal anomalies identified in pregnancies with measurements of 3.5 mm or greater, 496 (72.5%) were identified prenatally, compared with 947 (35.3%) for measurements less than 3.5 mm (eTable 9 in Supplement 1).

Among pregnancies with cytogenetic testing results (n = 15 755), the proportion with chromosomal anomalies increased across nuchal translucency measurement categories, from 1913 pregnancies (16.6%) with nuchal translucency less than 2.0 mm to 256 pregnancies (70.1%) with a measurement 6.5 mm or greater (**Table 2**). To ascertain potential chromosomal anomalies in pregnancies that did not receive cytogenetic testing, 38 041 (98.2%) received a low-risk cfDNA screening result (Table 2). Next, among pregnancies with no cfDNA screening performed, we identified 331 638 documented live births with no notable clinical findings reported (Table 2). These results were used to estimate the proportion of pregnancies in the study with chromosomal anomalies, increasing from 1913 (0.5%) in pregnancies with nuchal translucency less than 2.0 mm to 256 (52.6%) in pregnancies with nuchal translucency 6.5 mm or greater (Table 2).

Table 1. Characteristics of Study Population by Nuchal Translucency Measurement

Characteristic	All pregnancies with nuchal translucency measurement (N = 414 268)	Nuchal translucency measurement, mm						
		<2.0 (n = 359 807)	2.0 to <2.5 (n = 43 219)	2.5 to <3.0 (n = 7474)	3.0 to <3.5 (n = 1789)	3.5 to <5.0 (n = 1088)	5.0 to <6.5 (n = 404)	≥6.5 (n = 487)
Maternal age at EDD, mean (SD), y	31.5 (4.7)	31.5 (4.7)	31.6 (4.8)	31.9 (4.8)	32.2 (4.8)	32.8 (5.0)	33.0 (5.5)	33.3 (5.4)
Gestational age at screening, mean (SD), d	87.8 (3.3)	87.5 (3.3)	90.0 (2.6)	90.0 (3.0)	88.9 (3.4)	87.3 (3.6)	86.4 (3.6)	86.9 (3.1)
Crown rump length, mean (SD), mm	62.5 (8.3)	61.7 (8.1)	68.5 (7.5)	68.8 (8.2)	65.6 (8.9)	61.6 (9.0)	59.2 (8.7)	60.3 (7.5)
No. missing	199	134	53	10	<6	0	0	<6
Maternal weight, mean (SD), kg	68.0 (17.0)	67.9 (16.9)	68.5 (17.4)	68.3 (17.3)	68.1 (17.0)	67.2 (16.6)	68.8 (17.8)	67.0 (14.0)
No. missing	18 316	14 783	2009	448	209[a]	297[a]	227[a]	343[a]
Parity, No. (%)								
Nulliparous	183 587 (46.2)	161 994 (46.8)	17 689 (42.7)	2847 (40.3)	595 (37.5)	314 (39.4)	81 (46.0)	67 (45.6)
Primiparous	143 190 (36.0)	123 661 (35.7)	15 760 (38.0)	2739 (38.7)	614 (38.7)	320 (40.2)	56 (31.8)	40 (27.2)
Multiparous	70 967 (17.8)	60 848 (17.6)	8012 (19.3)	1487 (21.0)	378 (23.8)	163 (20.5)	39 (22.2)	40 (27.2)
No. missing	16 524	13 304	1758	401	202[a]	291[a]	228[a]	340[a]
Type of conception, No. (%)								
Spontaneous conception	374 873 (96.0)	326 450 (96.0)	38 712 (95.9)	6685 (95.9)	1568 (96.1)	876 (96.4)	275 (96.8)	307 (97.5)
IVF	12 025 (3.1)	10 429 (3.1)	1289 (3.2)	220 (3.2)	49 (3.0)	22 (2.4)	8 (2.8)	8 (2.5)
Other ART	3659 (0.9)	3188 (0.9)	378 (0.9)	66 (0.9)	15 (0.9)	11 (1.2)	<6 (NA)	0
No. missing	23 711	19 740	2840	503	157	179[a]	120[a]	172[a]

Abbreviations: ART, assisted reproductive technology; EDD, estimated date of delivery; IVF, in vitro fertilization; NA, not applicable.

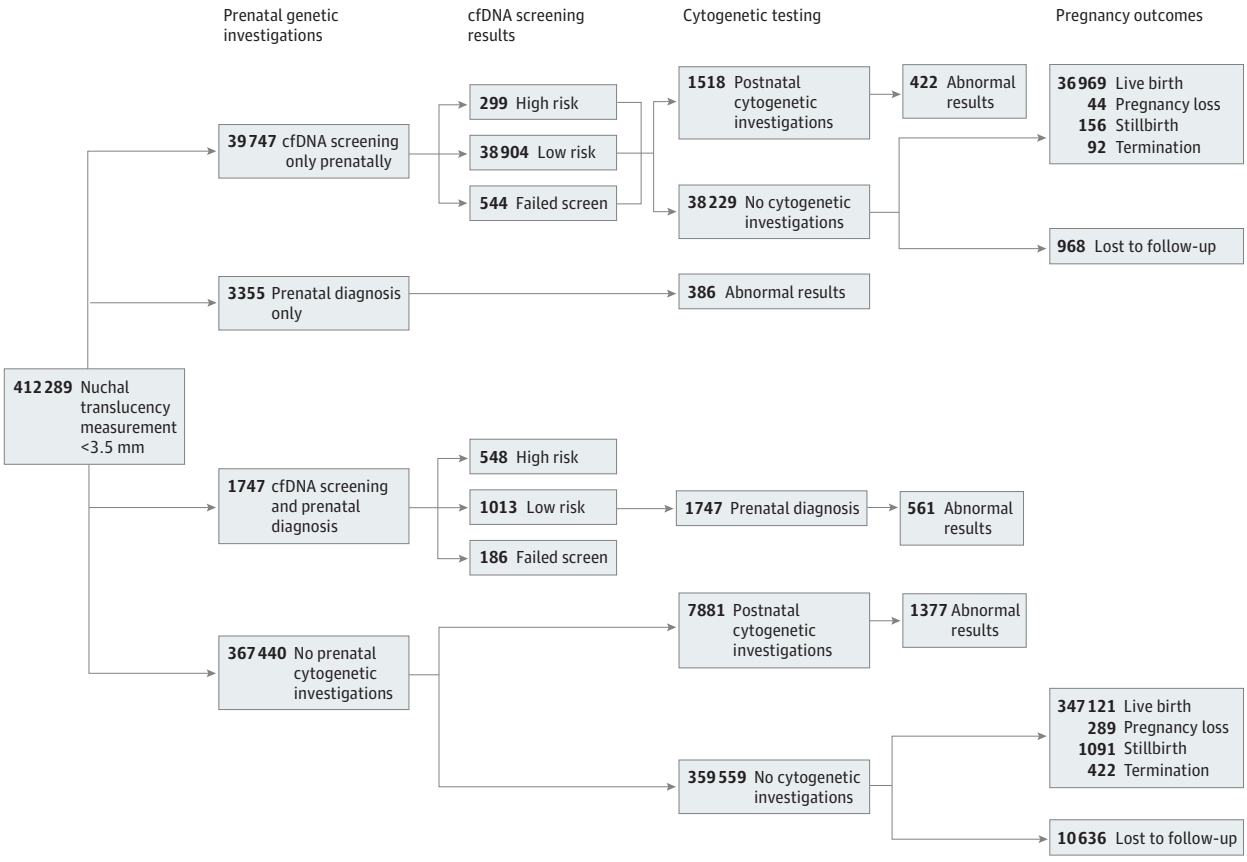
[a] Missing data for more than 10.0% of the pregnancies.

The risk of chromosomal anomalies increased with increasing nuchal translucency measurements (**Table 3**). The risk was markedly increased in pregnancies with nuchal translucency measurements from 3.0 to less than 3.5 mm relative to less than 2.0 mm (ARD, 9.94% [95% CI, 8.49%-11.39%]; ARR, 20.33 [95% CI, 17.58-23.52]).

The proportion of pregnancies excluded from the primary analysis due to an unknown outcome was associated with nuchal translucency category, from 9281 (2.6%) in the group with measurements less than 2.0 mm to 48 (9.9%) in the group with measurements 6.5 mm or greater (Table 2). We therefore conducted a sensitivity analysis randomly classifying the pregnancies lost to follow-up to have twice the prevalence of chromosomal anomalies compared with those for which an outcome was recorded within the given nuchal translucency measurement category, and results were mildly accentuated. The analysis assuming half the prevalence showed mildly attenuated results (eTable 5 in Supplement 1).

Table 3 further categorizes chromosomal anomalies into a group of conditions routinely screened by cfDNA screening (trisomies 21, 18, and 13 and sex chromosome aneuploidies) and a group of conditions beyond the cfDNA screening options consistently available in Ontario (other autosomal aneuploidies, triploidy, mosaic autosomal and sex chromosome aneuploidies, and copy number variants). The risk of chromosomal anomalies routinely screened by cfDNA screening increased with increasing nuchal translucency measurements: for the nuchal translucency category of 3.0 to less than 3.5 mm relative to less than 2.0 mm, the ARD was 8.62% (95% CI, 7.27%-9.96%) and the ARR was 52.15 (95% CI, 43.98-61.84). The risk also increased but with weaker magnitude for the subgroup with other chromosomal anomalies (detailed in eTable 11 in Supplement 1): for nuchal

Figure 1. Investigations Following an Ultrasonographic Nuchal Translucency Measurement Less Than 3.5 mm Under Current Practice



cfDNA indicates cell-free DNA.

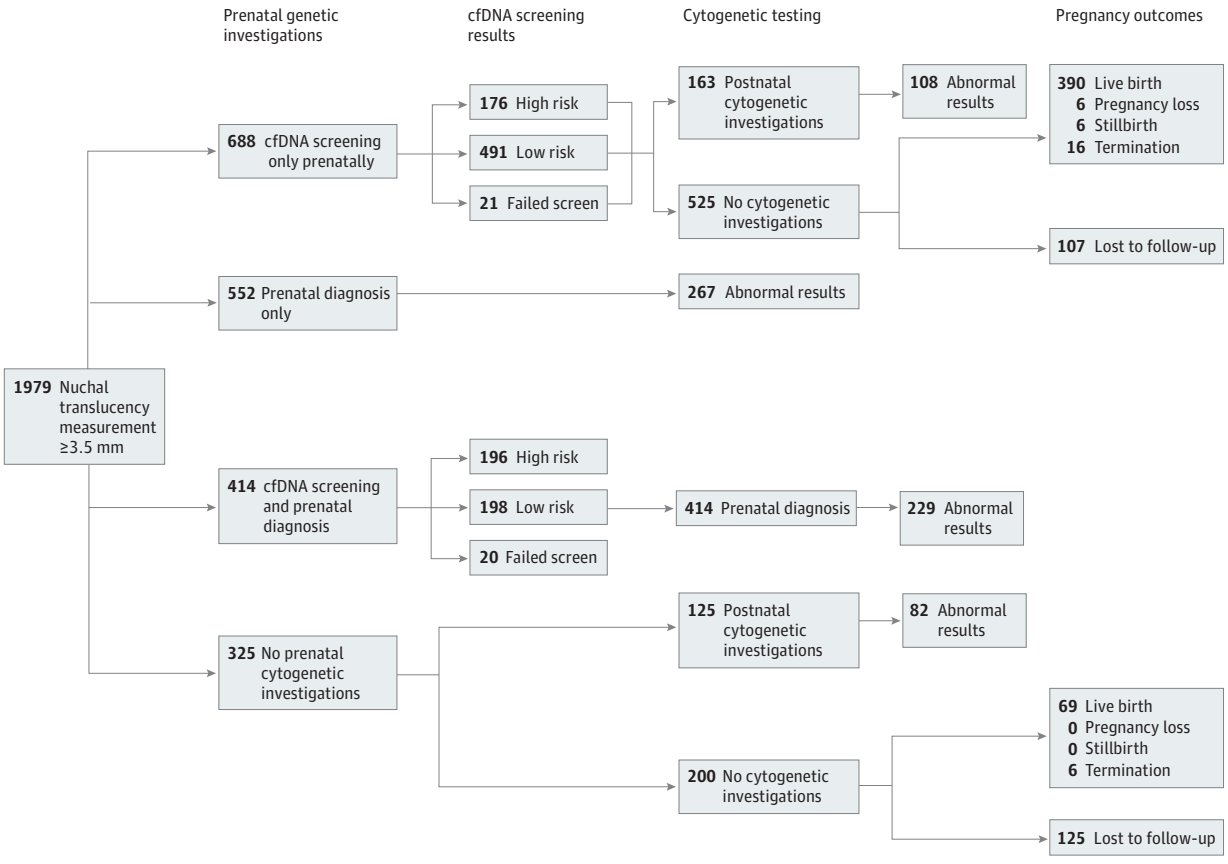
translucency category of 3.0 to less than 3.5 mm relative to less than 2.0 mm, the ARD was 1.40% (95% CI, 0.77%-2.04%) and the ARR was 4.97 (95% CI, 3.45-7.17).

All additional sensitivity analyses showed comparable findings to the primary analysis, including analyses incorporating pregnancies with nuchal translucency measurements identified by sources other than multiple-marker screening (eTable 3 in Supplement 1); analyses restricted to a timeline with complete capture of microarray data, with an EDD from September 1, 2018, to March 31, 2021 (eTable 7 in Supplement 1); analyses excluding pregnancies with an EDD from April 1, 2020, to March 31, 2021, to assess potential effects of the COVID-19 pandemic (eTable 8 in Supplement 1); and post hoc analyses additionally adjusting for age of the pregnant individual (eTable 10 in Supplement 1). Finally, for the pregnancies included in our study, the 99th percentile for nuchal translucency measurement was 2.8 mm, the 95th percentile was 2.2 mm, and the 90th percentile was 2.0 mm. When defining the exposure by nuchal translucency percentile, pregnancies with a measurement greater than the 99th percentile had a risk of any chromosomal anomaly 34.9 times greater than pregnancies with a measurement less than the 90th percentile (eTable 4 in Supplement 1).

Discussion

This population-based cohort study leveraged linked multiple-marker screening, cytogenetic testing, cfDNA screening, and birth registry data capturing pregnancy outcomes and findings from the clinical examination at birth to quantify the association of increased risk of chromosomal anomalies with increasing nuchal translucency measurement. We found a strongly increased risk of

Figure 2. Investigations Following an Ultrasonographic Nuchal Translucency Measurement 3.5 mm or Greater Under Current Practice



cfDNA indicates cell-free DNA.

chromosomal anomalies with increased nuchal translucency relative to values less than 2.0 mm, particularly for measurements of 3.0 mm or higher. The findings were consistent through several sensitivity analyses.

To our knowledge, this is the first population-based study assessing the risk of chromosomal anomalies across all levels of nuchal translucency measurements and incorporating information from antenatal as well as postnatal cytogenetic testing, cfDNA screening, pregnancy outcomes, and newborn clinical examinations. Indeed, most studies on this topic have focused on high-risk settings or have included only prenatal cytogenetic testing and, therefore, only represent a small proportion of pregnant individuals undergoing nuchal translucency ultrasonography.[11,13,14,16-18,21,31] The concern is that pregnant individuals who opted for cytogenetic testing in these studies may have had a higher risk of chromosomal anomalies, as additional findings beyond nuchal translucency measurement may have led them to have prenatal diagnostic testing; excluding pregnancies at lower risk for which outcomes are not available would, therefore, tend to overestimate the risk. This is particularly important when investigating nuchal translucency measurement values that would not independently trigger an offer of a follow-up investigation. As expected, we observed a substantial difference in the proportion of pregnant individuals who elected to have follow-up investigations prenatally in the group with nuchal translucency measurements less than 3.5 mm (10.9%) compared

Table 2. Chromosomal and Pregnancy Outcomes by Nuchal Translucency Measurement[a]

Outcome	All pregnancies	Nuchal translucency measurement, mm						
		<2.0	2.0 to <2.5	2.5 to <3.0	3.0 to <3.5	3.5 to <5.0	5.0 to <6.5	≥6.5
No. of cytogenetic testing results	15 755	11 552	1875	653	421	602	287	365
Unknown result	217 (1.4)	180 (1.6)	22 (1.2)	<6 (NA)	<6 (NA)	<6 (NA)	<6 (NA)	<6 (NA)
No chromosomal anomaly identified	12 106 (76.8)	9459 (81.9)	1397 (74.5)	450 (68.9)	241 (57.2)	341 (56.6)	112 (39.0)	106 (29.0)
Chromosomal anomaly	3432 (21.8)	1913 (16.6)	456 (24.3)	198 (30.3)	179 (42.5)	256 (42.5)	174 (60.6)	256 (70.1)
Identifiable on cfDNA screening[b,c]	1690 (49.2)	543 (28.4)	241 (52.9)	154 (77.8)	149 (83.2)	215 (84.0)	153 (87.9)	235 (91.8)
Not identifiable on cfDNA screening[b,d]	1742 (50.8)	1370 (71.6)	215 (47.1)	44 (22.2)	30 (16.8)	41 (16.0)	21 (12.1)	21 (8.2)
No. of cfDNA screening results for pregnancies without cytogenetic testing results	38 754	30 551	5310	1701	667	398	69	58
Unknown or uninformative result	495 (1.3)	404 (1.3)	58 (1.1)	14 (0.8)	<6 (NA)	8 (2.0)	<6 (NA)	<6 (NA)
Low-risk result	38 041 (98.2)	30 071 (98.4)	5234 (98.6)	1674 (98.4)	645 (96.7)	354 (88.9)	44 (63.8)	19 (32.8)
High-risk result	218 (0.6)	76 (0.2)	18 (0.3)	13 (0.8)	18 (2.7)	36 (9.0)	21 (30.4)	36 (62.1)
No. of pregnancy outcomes with neither cytogenetic testing nor cfDNA screening results	359 759	317 704	36 034	5120	701	88	48	64
Lost to follow-up	10 761 (3.0)	9281 (2.9)	1132 (3.1)	181 (3.5)	42 (6.0)	38 (43.2)	39 (81.3)	48 (75.0)
Live birth	347 190 (96.5)	306 838 (96.6)	34 727 (96.4)	4901 (95.7)	655 (93.4)	49 (55.7)	7 (14.6)	13 (20.3)
Clinical findings at birth[e]	15 552 (4.5)	13 919 (4.5)	1391 (4.0)	203 (4.1)	33 (5.0)	<6 (NA)	<6 (NA)	<6 (NA)
Pregnancy loss, termination, or stillbirth	1808 (0.5)	1585 (0.5)	175 (0.5)	38 (0.7)	<6 (NA)	<6 (NA)	<6 (NA)	<6 (NA)
No. of pregnancies with chromosomal anomalies	414 268	359 807	43 219	7474	1789	1088	404	487
No chromosomal anomaly identified[f]	382 478 (92.3)	332 945 (92.5)	40 046 (92.7)	6846 (91.6)	1522 (85.1)	771 (70.9)	179 (44.3)	169 (34.7)
Chromosomal anomaly[g]	3432 (0.8)	1913 (0.5)	456 (1.1)	198 (2.6)	179 (10.0)	256 (23.5)	174 (43.1)	256 (52.6)
Excluded	28 358 (6.8)	24 949 (6.9)	2717 (6.3)	430 (5.8)	88 (4.9)	61 (5.6)	51 (12.6)	62 (12.7)
Lost to follow-up	10 761 (2.6)	9281 (2.6)	1132 (2.6)	181 (2.4)	42 (2.3)	38 (3.5)	39 (9.7)	48 (9.9)
Excluded for other reason[h]	17 597 (4.2)	15 668 (4.4)	1585 (3.7)	249 (3.3)	46 (2.6)	23 (2.1)	12 (3.0)	14 (2.9)

Abbreviations: cfDNA, cell-free DNA; NA, not applicable.

[a] Unless otherwise indicated, data are expressed as No. (%) of patients.

[b] Includes those with choromosomal anomaly identified.

[c] Includes trisomies 21, 18, and 13 and sex chromosome aneuploidies.

[d] Includes all other chromosomal anomalies, including other autosomal aneuploidies, mosaic aneuploidies, copy number variants. A detailed list of all chromosomal anomalies is available in eTable 2 in Supplement 1.

[e] Includes any congenital structural anomaly identified among live births.

[f] Indicates normal cytogenetic results or no cytogenetic results but low risk cfDNA results or no follow-up testing results but documented live birth with no clinical findings on examination.

[g] Based on cytogenetic testing results.

[h] Includes pregnancy loss, stillbirth, termination, live birth with clinical findings, or a high-risk cfDNA screening result.

Table 3. Chromosomal Anomalies by Nuchal Translucency Measurement[a]

Nuchal translucency measurement, mm	Main analysis				Subgroup analysis, adjusted model[b]			
	Crude model		Adjusted model[b]		Conditions identifiable on cfDNA screening[c]		Conditions not identifiable on cfDNA screening[d]	
	RD (95% CI), %	RR (95% CI)	RD (95% CI), %	RR (95% CI)	RD (95% CI), %	RR (95% CI)	RD (95% CI), %	RR (95% CI)
<2.0	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]	1 [Reference]
2.0 to <2.5	0.55 (0.45-0.66)	1.97 (1.78-2.18)	0.61 (0.51-0.71)	2.39 (2.14-2.66)	0.45 (0.37-0.52)	4.21 (3.62-4.91)	0.16 (0.08-0.23)	1.48 (1.27-1.73)
2.5 to <3.0	2.24 (1.85-2.63)	4.92 (4.26-5.69)	2.26 (1.88-2.63)	5.93 (5.11-6.88)	2.01 (1.67-2.34)	14.76 (12.33-17.65)	0.27 (0.08-0.46)	1.82 (1.34-2.47)
3.0 to <3.5	9.95 (8.49-11.41)	18.42 (15.92-21.31)	9.94 (8.49-11.39)	20.33 (17.58-23.52)	8.62 (7.27-9.96)	52.15 (43.98-61.84)	1.40 (0.77-2.04)	4.97 (3.45-7.17)
3.5 to <5.0	24.36 (21.71-27.00)	43.63 (38.88-48.96)	24.31 (21.67-26.96)	42.94 (38.28-48.16)	21.10 (18.60-23.61)	107.31 (93.20-123.56)	3.50 (2.27-4.72)	10.15 (7.37-13.98)
5.0 to <6.5	48.72 (43.51-53.94)	86.28 (76.91-96.80)	48.68 (43.46-53.89)	80.85 (71.91-90.90)	43.97 (38.79-49.15)	208.01 (179.77-240.69)	5.15 (2.66-7.64)	14.17 (9.02-22.25)
≥6.5	59.66 (54.99-64.33)	105.44 (96.40-115.32)	59.64 (54.97-64.31)	101.88 (92.79-111.88)	55.79 (51.06-60.53)	276.14 (244.84-311.44)	4.30 (2.18-6.41)	12.11 (7.69-19.07)

Abbreviations: cfDNA, cell-free DNA; RD, risk difference; RR, risk ratio.

[a] Outcome determined by cytogenetic testing. Not having the outcome is determined by normal cytogenetic testing result if performed, normal cfDNA screen result if condition was tested, and by live birth without clinical findings if condition can be clinically diagnosed.

[b] Adjusted for gestational age at screening.

[c] Includes trisomies 21, 18, and 13 and sex chromosome aneuploidies.

[d] Includes all other chromosomal anomalies.

with the group with measurements of at least 3.5 mm (83.6%), illustrating the importance of including outcomes beyond cytogenetic results from prenatal diagnosis.

Our finding that nuchal translucency values below 3.5 mm, particularly those from 3.0 to less than 3.5 mm, are associated with chromosomal anomalies relative to values less than 2.0 mm, has important implications for prenatal genetic screening and counselling. Beyond the common aneuploidies (trisomies 21, 13, and 18 and sex chromosome aneuploidies) routinely identified by cfDNA screening in Ontario and included in many screening programs internationally,[32] we report that increased nuchal translucency measurements also yield an increased risk for other chromosome anomalies, although weaker. This has important policy implications given that in some jurisdictions, prenatal cfDNA screening is offered following the identification of an increased nuchal translucency measurement, whereas in others, cfDNA screening is part of first-tier prenatal screening, with or without accompanying nuchal translucency measurement. Screening programs should consider the value of nuchal translucency measurements when making decisions about whether to replace such measurements with cfDNA screening alone and may wish to reexamine the threshold of nuchal translucency at which diagnostic investigations are offered.[10,32] Further research, including economic modeling to estimate benefits and costs, is needed to inform the best options for policy and practice.[10,32]

The results of this study also have implications for the quality assurance of nuchal translucency: the 99th percentile for nuchal translucency measurement was well below the expected 3.5 mm, implying that using a cutoff of 3.5 mm to offer follow-up investigations may not be sufficient. While the increased risk of chromosomal anomalies in pregnancies with nuchal translucencies less than 3.5 mm could be partly due to chronic undermeasurement, it is unlikely that this would be the only factor. Indeed, some jurisdictions have adopted thresholds to offer follow-up investigations much lower than the 99th percentile (eg, 95th percentile in Finland, the Netherlands, Germany, and Switzerland).[33] These findings also highlight the importance of a robust quality assurance program to support nuchal translucency measurement, as chronic undermeasurement can reduce the screening sensitivity.[34-39]

Limitations

An inherent limitation of this study is that cytogenetic outcomes were not available for all pregnancies, as cytogenetic testing is only offered under specific clinical indications. We therefore included additional information from cfDNA screening and pregnancy outcomes recorded in the birth registry to maximize ascertainment of chromosomal anomalies. Moreover, sensitivity analyses with varying assumptions about outcome ascertainment yielded the same conclusions.

Additionally, some chromosomal anomalies may not have clinically significant features at birth, prompting postnatal cytogenetic investigations, such that an infant could possibly be misclassified as not having a chromosomal anomaly. Because of the difference in ascertainment of pregnancies with nuchal translucencies less than 3.5 mm and 3.5 mm or greater, it is possible that we overestimated the risk of chromosomal anomalies in pregnancies with measurements of 3.5 mm or greater. For this reason, our findings allow us to draw conclusions on the association between nuchal translucency measurements and chromosomal anomalies that have clear features at birth, whereas more careful consideration is needed for chromosomal anomalies for which a clear phenotype is not expected at birth. Additionally, single-gene conditions associated with increased nuchal translucency measurements such as RASopathies are not captured in the registry and were therefore not included.[40] Although there is evidence that some factors may influence the choice to have prenatal genetic screening (eg, maternal age, rural residence),[25,41] there is no reason to expect the association between the nuchal translucency measurement and chromosomal anomalies to differ in this population excluded from our study.

Conclusions

The findings of this population-based cohort study suggest that increased nuchal translucency measurements were associated with increased risk of chromosomal anomalies, even at values below the currently used standard threshold of 3.5 mm. These findings have important policy implications for setting the threshold nuchal translucency value such that further investigations may be offered to pregnant individuals and, in turn, contribute to determining the best approach to offering high-quality prenatal screening to pregnant individuals in Ontario and around the world.

ARTICLE INFORMATION

Accepted for Publication: January 11, 2024.

Published: March 26, 2024. doi:10.1001/jamanetworkopen.2024.3689

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Administrative, technical, or material support: Bellai-Dussault, Dougan, Walker, Armour, Potter.

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Conflict of Interest Disclosures: Ms Bellai-Dussault reported receiving grant funding from the Canadian Institute of Health Research (CIHR) during the conduct of the study. Dr Fell reported being employed by the University of Ottawa and having an academic appointment at the Children's Hospital of Eastern Ontario Research Institute during the conduct of the study; although she maintains those academic affiliations, she is now employed by Pfizer and works on an unrelated topic. Dr Little reported receiving grant funding from the Ontario Research Fund Genome Canada–CIHR outside the submitted work. Dr Okun reported serving as Comedical Director of Prenatal Screening Ontario for Better Outcomes Registry & Network Ontario during the conduct of the study. No other disclosures were reported.

Funding/Support: This study was supported by Frederick Banting and Charles Best Canada Graduate Scholarships Doctoral Award FBD-170866 from the CIHR (Ms Bellai-Dussault).

Role of the Funder/Sponsor: The CIHR had no role in the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Data Sharing Statement: See Supplement 2.

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SUPPLEMENT 1.

eTable 1. Description of Data Sources
eTable 2. Method of Ascertainment of Outcome
eFigure 1. Inclusion Flow
eFigure 2. Directed Acyclic Graph for Association Between Nuchal Translucency Measurement and Chromosomal Anomalies

Supplementary Online Content

Bellai-Dussault K, Dougan SD, Fell DB, et al. Ultrasonographic fetal nuchal translucency measurements and cytogenetic outcomes. *JAMA Netw Open*. 2024;7(3):e243689. doi:10.1001/jamanetworkopen.2024.3689

This supplementary material has been provided by the authors to give readers additional information about their work.

eTable 1. Description of Data Sources

Better Outcomes Registry & Network (BORN) Ontario	BORN Ontario is a prescribed registry for the province of Ontario, Canada, designed to capture various encounters the pregnant individual and child have with the healthcare system. The three laboratories that provide all multiple-marker screening in Ontario transfer records directly to BORN on a weekly basis, and include information on nuchal translucency measurements, biomarkers, and maternal clinical information. There are two cell-free DNA screening laboratories established in Ontario that provide publicly-funded screening, as well as privately paid screening and both contribute to the registry. All 9 cytogenetic laboratories in Ontario provide data to the registry through quarterly transfers and include all tests performed both prenatally as well as postnatally and on products of conception. The registry also captures all hospital births and home births with a midwife in the province. Additional information on BORN can be found in the 2021 publication by Murphy et al. as well as on the www.bornontario.ca website.

Canadian Institute for Health Information (CIHI)	The CIHI Discharge Abstract Database metadata (DAD) contains information from hospital discharges in Ontario, as well as other provinces and territories of Canada.
	Data from CIHI was used in this study to supplement information on pregnancy outcomes including congenital anomalies. More information on CIHI can be found at https://www.cihi.ca/en/discharge-abstract-database-metadata-dad.
	The following variables were supplemented using CIHI data:
	- Pregnancy outcome
	  ○ Fetal demise - ICD10 code : P95
	  ○ Spontaneous abortion – ICD10 codes: O03;P01.8
	  ○ Termination of Pregnancy – ICD10 codes: O04, O05, O06, P96.4
	  ○ Congenital anomalies – ICD10 codes from Q00 to Q99

eTable 2. Method of Ascertainment of Outcome

Chromosomal anomaly	Traditional cytogenetic testing[a]	Microarray testing	cfDNA screening[b]	Newborn clinical exam
Conditions formally screened by program				
trisomy 21 and 18	Tested	Tested	NPV >99.9[c]	Diagnosis may be excluded [d]
Secondary findings of the screening program				
trisomy 13	Tested	Tested	NPV >99.9 [c]	Diagnosis may be excluded [d]
other autosomal aneuploidies	Tested	Tested	Not tested	Diagnosis may be excluded [d]
mosaic autosomal aneuploidies	Tested	Can be ascertained, unless low-level[e]	Not tested	Cannot exclude diagnosis [f]
triploidy	Tested	Tested	Tested in only one cfDNA screening platform[b]	Diagnosis may be excluded [d]
monosomy x	Tested	Tested	May be tested (opt-in or automatically tested depending on platform) [b]	Cannot exclude diagnosis [f]
mosaic monosomy x	Tested	Can be ascertained, unless low-level[e]	Not tested	Cannot exclude diagnosis [f]
other sex chromosome aneuploidies	Tested	Tested	May be tested (opt-in or automatically tested depending on platform) [b]	Cannot exclude diagnosis [f]
mosaic other sex chromosome aneuploidies	Tested	Can ascertain mosaicism, unless low- level[e]	Not tested	Cannot exclude diagnosis [f]
22q11.2 deletion, Cri-du-Chat, Angelman/Prader-Willi, 1p36 deletion syndromes	Not routinely ascertained	Tested	May be tested (opt in, private pay), NPV unclear[b]	Cannot exclude diagnosis [f]
Other copy number variants	Large deletions or duplications only [g]	Tested	Not tested	Cannot exclude diagnosis [f]

NPV, Negative predictive value

[a] *Traditional cytogenetic testing refers to rapid aneuploidy detection techniques (fluorescence in situ hybridization (FISH) or quantitative fluorescence-PCR (QFPCR)), or karyotype.*

[b] *Two cfDNA screening laboratories are established in Ontario, one using Single Nucleotide Polymorphism (SNP) Based Analysis while the other performs Chromosome Specific Sequencing through their Digital Analysis of Selected Regions (DANSR) technology. At the time of the study, one laboratory automatically screened for sex chromosome aneuploidies, while the other allowed to opt in. Only the SNP-based technology can screen for triploidy. The option to self-paid for microdeletion syndromes was also available.*

[c] *Program Report, Prenatal Screening Ontario. Published online December 2021. Accessed July 19, 2023. https://www.bornontario.ca/en/pso/resources/Remediated-PDFs-2020/PSO-Program-Report---FINAL-Dec-8-2021.pdf*

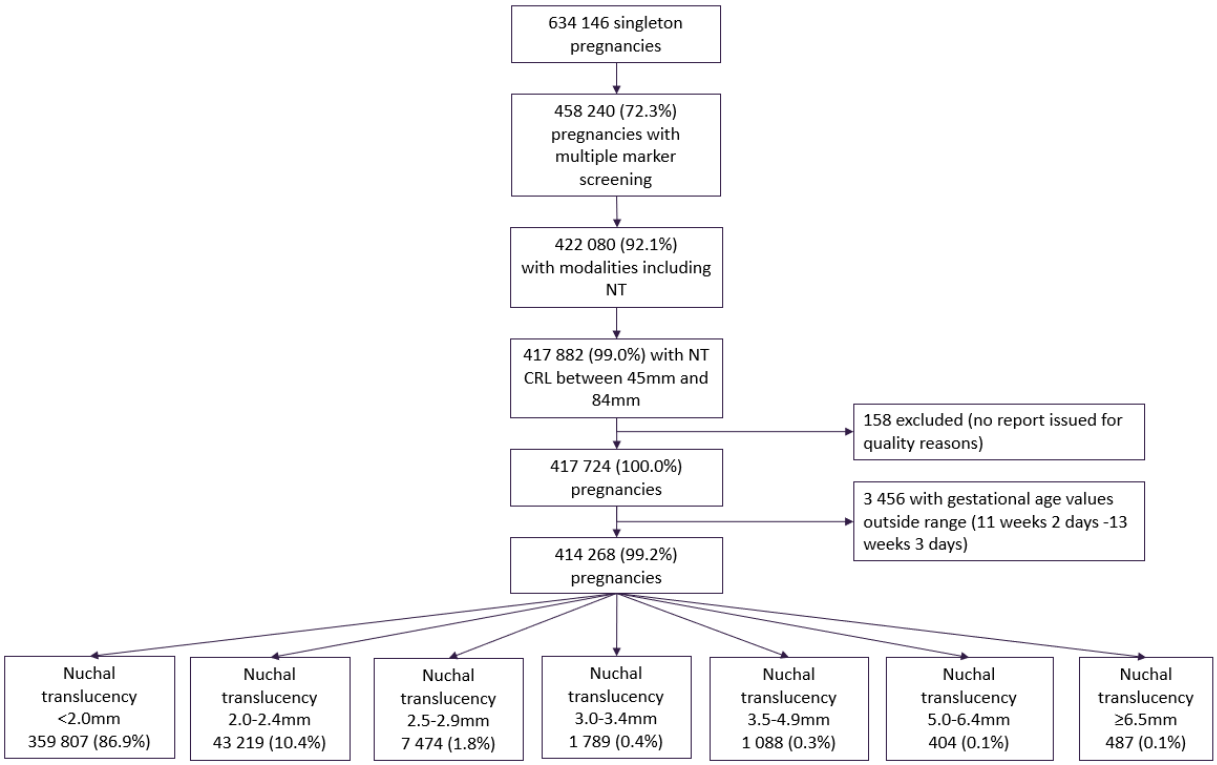
[d] *Recognizable clinical findings are expected at birth. Nussbaum R, McInnes R, Willard H. Thompson & Thompson Genetics in Medicine. Eight Edition. ELSEVIER; 2016.*

[e] *Armour CM, Dougan SD, Brock JA, et al. Practice guideline: joint CCMG-SOGC recommendations for the use of chromosomal microarray analysis for prenatal diagnosis and assessment of fetal loss in Canada. J Med Genet. 2018;55(4):215-221. Doi:10.1136/jmedgenet-2017-105013*

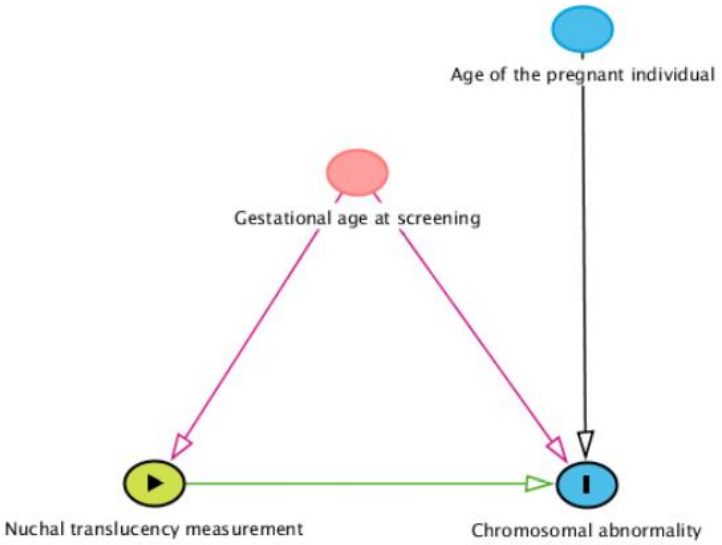
[f] *Clinical findings of this condition may not be easily and clearly identifiable at birth due to phenotype variability and syndromes that are less recognizable. Nussbaum R, McInnes R, Willard H. Thompson & Thompson Genetics in Medicine. Eight Edition. ELSEVIER; 2016.*

[g] *Microarray can detect smaller deletions and duplications not identifiable on karyotype: Wapner RJ, Martin CL, Levy B, et al. Chromosomal Microarray versus Karyotyping for Prenatal Diagnosis. N Engl J Med. 2012;367(23):2175-2184. doi:10.1056/NEJMoa1203382*

eFigure 1. Inclusion Flow



eFigure 2. Directed Acyclic Graph for Association Between Nuchal Translucency Measurement and Chromosomal Anomalies



Note: Adjustment for factors that incidentally would affect the nuchal translucency measurement and would also be related to chromosomal anomalies were made to the regression models; only gestational age met this criterion. A post-hoc analysis also included adjustment for age of the pregnant individual and is presented in Supplementary Table 10.

eTable 3. Regression Models Including Pregnancies Identified Through Other Data Sources Beyond Multiple-Marker Screening

Nuchal translucency measurement	***Any chromosomal anomaly***			
	Risk Difference % (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	Ref.		Ref.	
2.0-<2.5 mm	0.55	(0.45-0.66)	1.97	(1.78-2.18)
2.5-<3.0 mm	2.24	(1.85-2.63)	4.92	(4.26-5.68)
3.0-<3.5 mm	9.95	(8.49-11.41)	18.42	(15.92-21.31)
≥ 3.5 mm	35.83	(33.85-37.82)	63.72	(59.39-68.37)

Outcome determined by cytogenetic testing, not having the outcome is determined by normal cytogenetic testing if performed, normal cfDNA screen if condition was tested and by live birth without clinical findings if condition can be clinically diagnosed.

Crude model is presented, as pregnancies with nuchal translucency measurements identified outside the multiple-marker screening data set did not have information on gestational age at screening.

Nuchal translucency measurements categories were collapsed, as the additional data sources only provided whether the nuchal translucency measurement was 3.0-<3.5 mm or ≥ 3.5 mm

eTable 4. Regression Model With Varying Definition of Exposure by Nuchal Translucency Percentile

Nuchal translucency percentile	***Any chromosomal anomaly***							
	Crude model				*Adjusted model**			
	Risk Difference % (95% CI)		Risk Ratio (95% CI)		Risk Difference % (95% CI)		Risk Ratio (95% CI)	
<90th percentile	Ref.		Ref.		Ref.		Ref.	
90-95th percentile	0.38	(0.26-0.50)	1.67	(1.46-1.91)	0.44	(0.32-0.57)	2.12	(1.84-2.43)
95-99th percentile	1.12	(0.94-1.29)	2.96	(2.64-3.30)	1.15	(0.98-1.32)	3.82	(3.39-4.29)
≥ 99th percentile	17.75	(16.69-18.82)	32.08	(29.80-34.53)	17.73	(16.66-18.79)	34.90	(32.43-37.57)

Outcome determined by cytogenetic testing, not having the outcome is determined by normal cytogenetic testing if performed, normal cfDNA screen if condition was tested and by live birth without clinical findings if condition can be clinically diagnosed.
**Model adjusted for gestational age at screening*

eTable 5. Sensitivity Analysis for Losses to Follow-Up

A

Nuchal translucency measurement	Number of pregnancies for which an outcome was recorded	Prevalence of chromosomal anomaly among those for which an outcome was recorded	Number of pregnancies for which NO outcome was recorded	Number of pregnancies randomized to having a chromosomal anomaly. Assumption of half the prevalence of chromosomal anomalies compared to those for which an outcome is available.	Number of pregnancies randomized to having a chromosomal anomaly. Assumption of twice the prevalence of chromosomal anomalies compared to those for which an outcome is available.
	n (%)	n (%)	n (%)	n (%)	n (%)
< 2.0 mm	334858 (97.3)	1913 (0.6)	9281 (2.7)	26 (0.3)	103 (1.1)
2.0-<2.5 mm	40502 (97.3)	456 (1.1)	1132 (2.7)	6 (0.5)	25 (2.2)
2.5-<3.0 mm	7044 (97.5)	198 (2.7)	181 (2.5)	3 (1.4)	10 (5.5)
3.0-<3.5 mm	1701 (97.6)	179 (10.3)	42 (2.4)	3 (5.1)	9 (20.5)
3.5-<5.0 mm	1027 (96.4)	256 (24.0)	38 (3.6)	5 (12.0)	18 (48.1)
5.0-<6.5 mm	353 (90.1)	174 (44.4)	39 (9.9)	9 (22.2)	35 (88.8)
≥ 6.5 mm	425 (89.9)	256 (54.1)	48 (10.1)	13 (27.1)	48 (100.0)

Example

1913 (0.6%) of the 334 858 pregnancies with nuchal translucency measurement < 2.0 mm for which an outcome was recorded had a chromosomal anomaly. In this nuchal translucency category, 9281 (2.7%) pregnancies had no outcome recorded. In the sensitivity analyses, pregnancies lost to follow-up were randomized to having 1) half the prevalence of chromosomal anomalies (0.3% = 26 pregnancies randomly assigned to having a chromosomal anomaly) or 2) twice the prevalence of chromosomal anomalies (1.1% = 103 pregnancies randomly assigned to having a chromosomal anomaly).

B

Nuchal translucency measurement	*Main analysis*				*Sensitivity analysis***			
	Crude model		*Adjusted model**		*Adjusted model* Pregnancies lost to follow-up assumed to have half the prevalence of chromosomal anomalies*		*Adjusted model* Pregnancies lost to follow-up assumed to have twice the prevalence of chromosomal anomalies*	
	Risk Difference % (95% CI)	Risk Ratio (95% CI)	Risk Difference % (95% CI)	Risk Ratio (95% CI)	Risk Difference % (95% CI)	Risk Ratio (95% CI)	Risk Difference % (95% CI)	Risk Ratio (95% CI)
< 2.0 mm	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
2.0-<2.5 mm	0.55 (0.45-0.66)	1.97 (1.78-2.18)	0.61 (0.51-0.71)	2.39 (2.14-2.66)	0.60 (0.50-0.70)	2.38 (2.14-2.65)	0.63 (0.52-0.73)	2.37 (2.13-2.63)
2.5-<3.0 mm	2.24 (1.85-2.63)	4.92 (4.26-5.69)	2.26 (1.88-2.63)	5.93 (5.11-6.88)	2.22 (1.85-2.59)	5.91 (5.09-6.85)	2.31 (1.94-2.69)	5.87 (5.08-6.79)
3.0-<3.5 mm	9.95 (8.49-11.41)	18.42 (15.92-21.31)	9.94 (8.49-11.39)	20.33 (17.58-23.52)	9.81 (8.38-11.23)	20.30 (17.56-23.46)	10.19 (8.74-11.64)	20.21 (17.54-23.29)
3.5-<5.0 mm	24.36 (21.71-27.00)	43.63 (38.88-48.96)	24.31 (21.67-26.96)	42.94 (38.28-48.16)	23.90 (21.32-26.48)	42.82 (38.21-47.99)	25.10 (22.48-27.73)	43.27 (38.74-48.33)
5.0-<6.5 mm	48.72 (43.51-53.94)	86.28 (76.91-96.80)	48.68 (43.46-53.89)	80.85 (71.91-90.90)	46.07 (41.13-51.01)	76.76 (68.24-86.35)	52.68 (47.74-57.62)	84.55 (76.16-93.85)
≥ 6.5 mm	59.66 (54.99-64.33)	105.44 (96.40-115.32)	59.64 (54.97-64.31)	101.88 (92.79-111.88)	56.29 (51.81-60.76)	97.49 (88.76-107.10)	63.66 (59.33-68.00)	106.09 (97.57-115.35)

**Model adjusted for gestational age at screening*

***The sensitivity analyses randomly classified the losses to follow-up to having 1) half and 2) twice the prevalence of chromosomal anomalies compared to pregnancies in the same category of nuchal translucency measurement for which an outcome was recorded. (See supplementary Table 5A for details.)*

eTable 6. Regression Models Including All Pregnancies With Nuchal Translucency Measurements

Nuchal translucency measurement	***Any chromosomal anomaly***							
	Crude model				*Adjusted model**			
	Risk Difference % (95% CI)		Risk Ratio (95% CI)		Risk Difference % (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	Ref.		Ref.		Ref.		Ref.	
2.0-<2.5 mm	0.47	(0.32-0.63)	1.22	(1.15-1.30)	0.63	(0.47-0.79)	1.35	(1.26-1.44)
2.5-<3.0 mm	1.81	(1.37-2.25)	1.86	(1.66-2.08)	1.90	(1.47-2.34)	2.05	(1.82-2.30)
3.0-<3.5 mm	9.52	(8.04-11.01)	5.51	(4.84-6.28)	9.52	(8.04-10.99)	5.81	(5.10-6.61)
3.5-<5.0 mm	23.60	(21.00-26.21)	12.18	(10.98-13.51)	23.54	(20.94-26.13)	12.10	(10.92-13.42)
5.0-<6.5 mm	46.16	(41.28-51.03)	22.87	(20.62-25.36)	46.07	(41.20-50.94)	21.97	(19.80-24.37)
≥ 6.5 mm	55.95	(51.54-60.36)	27.51	(25.41-29.78)	55.91	(51.50-60.32)	27.01	(24.93-29.27)

Outcome determined by cytogenetic testing, not having the outcome is determined by normal cytogenetic testing if performed, normal cfDNA screen if condition was tested and by live birth without clinical findings if condition can be clinically diagnosed

If the outcome was not recorded, pregnancies were assumed to have the same risk of chromosomal anomalies as pregnancies with the same outcome, for which cytogenetic testing was performed. E.g., for the pregnancies that resulted in a pregnancy loss and had cytogenetic testing, 21.6% had a chromosomal anomaly, therefore 21.6% of pregnancies for which a pregnancy loss was recorded, but no cytogenetic testing was performed were randomized to have a chromosomal anomaly. The same process was followed for pregnancies for which cytogenetic outcomes were unavailable, but where a stillbirth, termination, live birth with clinical findings or loss to follow-up were recorded.

Pregnancies for which no outcome was recorded, but where a cfDNA screening result was high-risk, a positive predictive value of 90.0% was assumed and 90.0% of the pregnancies were randomized to have a chromosomal anomaly.

**Model adjusted for gestational age at screening.*

eTable 7. Regression Models Including Only Pregnancies With an Estimated Date of Delivery From September 1, 2018, to March 31, 2021

Nuchal translucency measurement	***Any chromosomal anomaly***							
	Crude model				*Adjusted model**			
	Risk Difference % (95% CI)		Risk Ratio (95% CI)		Risk Difference % (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	Ref.		Ref.		Ref.		Ref.	
2.0-<2.5 mm	0.52	(0.39-0.66)	1.98	(1.72-2.27)	0.58	(0.45-0.71)	2.41	(2.08-2.78)
2.5-<3.0 mm	2.20	(1.70-2.71)	5.12	(4.21-6.21)	2.22	(1.73-2.71)	6.19	(5.07-7.56)
3.0-<3.5 mm	10.56	(8.57-12.56)	20.74	(17.15-25.07)	10.56	(8.58-12.55)	22.99	(19.02-27.79)
3.5-<5.0 mm	24.85	(21.33-28.36)	47.42	(40.76-55.16)	24.80	(21.30-28.31)	46.94	(40.39-54.54)
5.0-<6.5 mm	50.91	(44.11-57.70)	96.10	(83.09-111.16)	50.87	(44.08-57.66)	89.97	(77.41-104.58)
≥ 6.5 mm	61.48	(55.56-67.40)	115.86	(103.43-129.77)	61.46	(55.53-67.38)	110.47	(97.98-124.55)

Indicates timeline with complete capture of microarray testing.Outcome determined by cytogenetic testing, not having the outcome is determined by normal cytogenetic testing if performed, normal cfDNA screen if condition was tested and by live birth without clinical findings if condition can be clinically diagnosed.

**Model adjusted for gestational age at screening*

eTable 8. Regression Models Excluding Pregnancies With an Estimated Date of Delivery From April 1, 2020, to March 31, 2021

Nuchal translucency measurement	***Any chromosomal anomaly***							
	Crude model				*Adjusted model**			
	Risk Difference % (95% CI)		Risk Ratio (95% CI)		Risk Difference % (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	Ref.		Ref.		Ref.		Ref.	
2.0-<2.5 mm	0.50	(0.38-0.62)	1.85	(1.64-2.08)	0.56	(0.44-0.68)	2.22	(1.96-2.51)
2.5-<3.0 mm	2.04	(1.61-2.46)	4.43	(3.74-5.25)	2.05	(1.64-2.47)	5.29	(4.44-6.30)
3.0-<3.5 mm	9.38	(7.78-10.99)	16.80	(14.20-19.88)	9.38	(7.78-10.97)	18.47	(15.61-21.85)
3.5-<5.0 mm	23.99	(20.99-27.00)	41.40	(36.28-47.25)	23.96	(20.95-26.96)	40.73	(35.70-46.47)
5.0-<6.5 mm	45.08	(38.95-51.20)	76.91	(66.65-88.74)	45.02	(38.90-51.15)	71.89	(62.22-83.05)
≥ 6.5 mm	59.28	(53.86-64.70)	100.83	(90.92-111.83)	59.26	(53.84-64.68)	97.93	(88.00-109.01)

Accounts for potential changes in practice during the COVID-19 pandemic. Outcome determined by cytogenetic testing, not having the outcome is determined by normal cytogenetic testing if performed, normal cfDNA screen if condition was tested and by live birth without clinical findings if condition can be clinically diagnosed

**Model adjusted for gestational age at screening*

eTable 9. Time of Cytogenetic Testing of Chromosomal Anomalies Identified by Nuchal Translucency Measurement

Time of cytogenetic testing for chromosomal anomalies identified	Nuchal translucency measurement (mm)							
	<2.0	2.0-<2.5	2.5-<3.0	3.0-<3.5	3.5-<5.0	5.0-<6.5	≥6.5	Total
Prenatal diagnosis, n (%)	490 (26.4)	204 (45.3)	119 (60.7)	134 (75.7)	196 (76.9)	135 (78.0)	165 (64.5)	1443 (42.9)
Postnatal cytogenetic testing, n (%)	1369 (73.6)	246 (54.7)	77 (39.3)	43 (24.3)	59 (23.1)	38 (22.0)	91 (35.5)	1923 (57.1)
Total	**1859**	**450**	**196**	**177**	**255**	**173**	**256**	**3366**

**For 66 cytogenetic tests performed, the timing of the test was unknown*

eTable 10. Post Hoc Analysis Including Additional Adjustment for Age of the Pregnant Individual at Estimated Date of Delivery

Nuchal translucency measurement	***Any chromosomal anomaly***							
	Crude model				*Model adjusted for gestational age at screening and age of the pregnant individual (continuous)**		*Model adjusted for gestational age at screening and age of the pregnant individual (categorical)***	
	Risk Difference % (95% CI)		Risk Ratio (95% CI)		Risk Ratio (95% CI)		Risk Difference % (95% CI)		Risk Ratio (95% CI)	
< 2.0 mm	Ref.		Ref.	Ref.	Ref.		Ref.		Ref.	Ref.
2.0-<2.5 mm	0.55	(0.45-0.66)	1.97	(1.78-2.18)	2.34	(2.10-2.61)	0.53	(0.43-0.63)	2.35	(2.11-2.61)
2.5-<3.0 mm	2.24	(1.85-2.63)	4.92	(4.26-5.69)	5.7	(4.92-6.61)	2.06	(1.70-2.42)	5.74	(4.96-6.66)
3.0-<3.5 mm	9.95	(8.49-11.41)	18.42	(15.92-21.31)	19.12	(16.57-22.06)	9.81	(8.37-11.25)	19.46	(16.85-22.47)
3.5-<5.0 mm	24.36	(21.71-27.00)	43.63	(38.88-48.96)	38.92	(34.80-43.53)	24.13	(21.49-26.76)	39.06	(34.86-43.76)
5.0-<6.5 mm	48.72	(43.51-53.94)	86.28	(76.91-96.80)	72.15	(64.30-80.96)	48.49	(43.28-53.69)	70.99	(63.09-79.87)
≥ 6.5 mm	59.66	(54.99-64.33)	105.44	(96.40-115.32)	87.96	(79.70-97.09)	59.47	(54.81-64.14)	87.23	(78.83-96.54)

**The model adjusted for age of the pregnant individuals using the continuous variable did not converge for the adjusted risk differences.*

***The age categories were defined as follows: (<25 years at estimated date of delivery, 25-<30 years , 30-<35 years, 35-<40 years, ≥40 years)*

eTable 11. Chromosomal and Pregnancy Outcomes by Nuchal Translucency Measurement: Detailed Results

Cytogenetic testing results	Pregnancies with nuchal translucency measurement and cytogenetic testing	Nuchal translucency measurement (mm)						
		<2.0	2.0-<2.5	2.5-<3.0	3.0-<3.5	3.5-<5.0	5.0-<6.5	≥6.5
Total pregnancies with cytogenetic testing, No.	**15,755**	**11,552**	**1,875**	**653**	**421**	**602**	**287**	**365**
Unknown results, No. (%)	**217 (1.4)**	**180 (1.6)**	**22 (1.2)**	**<6 (S)**	**<6 (S)**	**<6 (S)**	**<6 (S)**	**<6 (S)**
No chromosomal anomaly identified, No. (%)	**12106 (76.8)**	**9459 (81.9)**	**1397 (74.5)**	**450 (68.9)**	**241 (57.2)**	**341 (56.6)**	**112 (39.0)**	**106 (29.0)**
Chromosomal anomalies identified results, No. (%)	**3432 (21.8)**	**1913 (16.6)**	**456 (24.3)**	**198 (30.3)**	**179 (42.5)**	**256 (42.5)**	**174 (60.6)**	**256 (70.1)**
Trisomy 21	**1024 (29.8)**	299 (15.6)	180 (39.5)	127 (64.1)	117 (65.4)	151 (59.0)	74 (42.5)	76 (29.7)
Trisomy 18	**293 (8.5)**	110 (5.8)	20 (4.4)	11 (5.6)	13 (7.3)	32 (12.5)	50 (28.7)	57 (22.3)
Trisomy 13	**136 (4.0)**	39 (2.0)	23 (5.0)	10 (5.1)	10 (5.6)	25 (9.8)	16 (9.2)	13 (5.1)
Monosomy X	**114 (3.3)**	10 (0.5)	<6 (S)	0 (0.0)	<6 (S)	<6 (S)	11 (6.3)	83 (32.4)
Other sex chromosome aneuploidies	**123 (3.6)**	85 (4.4)	15 (3.3)	6 (3.0)	6 (3.4)	<6 (S)	<6 (S)	6 (2.3)
Triploidy	**61 (1.8)**	52 (2.7)	0 (0.0)	0 (0.0)	<6 (S)	<6 (S)	<6 (S)	<6 (S)
Mosaic or partial trisomies 21, 18 or 13	**59 (1.7)**	45 (2.4)	7 (1.5)	<6 (S)	<6 (S)	0 (0.0)	<6 (S)	0 (0.0)
Mosaic or partial sex chromosome aneuploidies	**154 (4.5)**	119 (6.2)	20 (4.4)	<6 (S)	<6 (S)	8 (3.1)	0 (0.0)	<6 (S)
22q11.2 microdeletion syndrome	**53 (1.5)**	40 (2.1)	<6 (S)	0 (0.0)	0 (0.0)	<6 (S)	0 (0.0)	0 (0.0)
Cri-du-Chat syndrome	**<6 (S)**	0 (0.0)	0 (0.0)	<6 (S)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Angelman or Prader-Willi syndrome	**12 (0.3)**	12 (0.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
1p36 deletion	**<6 (S)**	<6 (S)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Other autosomal aneuploidies	**<6 (S)**	<6 (S)	0 (0.0)	<6 (S)	<6 (S)	<6 (S)	0 (0.0)	0 (0.0)
Other mosaic autosomal aneuploidies	**12 (0.3)**	<6 (S)	<6 (S)	<6 (S)	<6 (S)	<6 (S)	0 (0.0)	<6 (S)
Other chromosomal anomaly	**1384 (40.3)**	1095 (57.2)	175 (38.4)	37 (18.7)	22 (12.3)	27 (10.5)	14 (8.0)	14 (5.5)
Pathogenic finding	***421***	*311*	*59*	*12*	*10*	*13*	*10*	*6*
Likely Pathogenic finding	***91***	*76*	*8*	*<6 (S)*	*<6 (S)*	*<6 (S)*	*<6 (S)*	*0*
Variant of Uncertain Significance	***717***	*598*	*85*	*21*	*<6 (S)*	*<6 (S)*	*<6 (S)*	*<6 (S)*
No interpretation available	***155***	*108*	*23*	*<6 (S)*	*6*	*7*	*<6 (S)*	*6*
Results from cfDNA screening for those without cytogenetic testing	**Pregnancies with nuchal translucency measurement and cfDNA (but no cytogenetic testing)**	**Nuchal translucency measurement (mm)**						
		<2.0	**2.0-<2.5**	**2.5-<3.0**	**3.0-<3.5**	**3.5-<5.0**	**5.0-<6.5**	**≥6.5**
Total pregnancies with cfDNA screening, No.	**38,754**	**30,551**	**5,310**	**1,701**	**667**	**398**	**69**	**58**
No results, No. (%)	**495 (1.3)**	404 (1.3)	58 (1.1)	14 (0.8)	<6 (S)	8 (2.0)	<6 (S)	<6 (S)
Low risk, No. (%)	**38041 (98.2)**	30071 (98.4)	5234 (98.6)	1674 (98.4)	645 (96.7)	354 (88.9)	44 (63.8)	19 (32.8)
High risk T21, No. (%)	**103 (0.3)**	18 (0.1)	15 (0.3)	12 (0.7)	16 (2.4)	21 (5.3)	9 (13.0)	12 (20.7)
High risk T18	**38 (0.1)**	13 (0.0)	0 (0.0)	<6 (S)	<6 (S)	7 (1.8)	7 (10.1)	9 (15.5)
High risk T13, No. (%)	**15 (0.0)**	6 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	<6 (S)	<6 (S)	<6 (S)
High risk monosomy X, No. (%)	**20 (0.1)**	6 (0.0)	<6 (S)	0 (0.0)	0 (0.0)	<6 (S)	0 (0.0)	11 (19.0)
High risk other SCA, No. (%)	**18 (0.0)**	16 (0.1)	0 (0.0)	0 (0.0)	0 (0.0)	<6 (S)	<6 (S)	0 (0.0)
High risk triploidy, No. (%)	**22 (0.1)**	16 (0.1)	<6 (S)	0 (0.0)	0 (0.0)	<6 (S)	<6 (S)	0 (0.0)
High risk microdeletion, No. (%)	**<6 (S)**	<6 (S)	0 (0.0)	0 (0.0)	<6 (S)	0 (0.0)	0 (0.0)	0 (0.0)

eTable 12. Characteristics of Pregnancies With and Without Cytogenetic Testing

Characteristic	Pregnancies with nuchal translucency measurement	Nuchal translucency measurement					
		<2.0 mm		2.0-<3.5 mm		≥3.5 mm	
		Did not have cytogenetic testing	Had cytogenetic testing	Did not have cytogenetic testing	Had cytogenetic testing	Did not have cytogenetic testing	Had cytogenetic testing
Total	**414,268**	**348,255**	**11,552**	**49,533**	**2,949**	**725**	**1,254**
Maternal age at EDD, years Mean (SD)	**31.5 (4.7)**	31.4 (4.7)	32.5 (5.1)	31.6 (4.8)	33.4 (5.2)	32.2 (5.4)	33.4 (5.1)
Gestational age at screening, days Mean (SD)	**87.8 (3.3)**	87.5 (3.3)	87.3 (3.3)	90 (2.7)	88.8 (3.1)	87.3 (3.7)	86.9 (3.5)
Crown Rump Length, mm Mean (SD)	**62.5 (8.3)**	61.7 (8.1)	61.2 (8.1)	68.6 (7.6)	65.4 (8.3)	61.4 (9.0)	60.4 (8.4)
Maternal weight, kg Mean (SD)	**68 (17.0)**	67.8 (16.9)	69.3 (18.5)	68.5 (17.3)	68.5 (18.2)	68.5 (17.4)	66.6 (15.6)
Parity, No. (%)							
Nulliparous	**183587 (46.2)**	157256 (46.8)	4738 (44.7)	20201 (42.4)	930 (37.7)	222 (45.5)	240 (38.0)
Primiparous	**143190 (36.0)**	119978 (35.7)	3683 (34.8)	18194 (38.2)	919 (37.2)	171 (35.0)	245 (38.8)
Multiparous	**70967 (17.8)**	58681 (17.5)	2167 (20.5)	9258 (19.4)	619 (25.1)	95 (19.5)	147 (23.3)
Missing	***16,524***	*12,340*	*964*	*1,880*	*481**	*237**	*622**
Conception, No. (%)							
Spontaneous conception	**374873 (96.0)**	316437 (96.0)	10013 (94.6)	44468 (95.9)	2497 (95.3)	546 (95.6)	912 (97.3)
IVF	**12025 (3.1)**	9970 (3.0)	459 (4.3)	1465 (3.2)	93 (3.5)	17 (3.0)	21 (2.2)
Other ART	**3659 (0.9)**	3075 (0.9)	113 (1.1)	429 (0.9)	30 (1.1)	8 (1.4)	<6 (S)
Missing	***23,711***	*18,773*	*967*	*3,171*	*329**	*154**	*317**
Smoking status, No. (%)							
Nonsmoker	**358781 (91.8)**	302150 (92.0)	9888 (91.4)	42473 (90.5)	2533 (91.0)	629 (92.2)	1108 (93.9)
Smoker	**32117 (8.2)**	26334 (8.0)	930 (8.6)	4477 (9.5)	251 (9.0)	53 (7.8)	72 (6.1)
Missing	**23,370**	19,771	734	2,583	165	43	74

**Missingness >10.0%*

SD, standard deviation; IVF, in vitro fertilization; ART, Assisted reproductive technology