DETECTION OF MICRODELETIONS BY NON-INVASIVE PRENATAL TESTING

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ABSTRACT

Background: Non-invasive prenatal testing (NIPT) is currently offered for the detection of Trisomy 21, 13, 18 and sex chromosome aneuploidy. The test is unique because it reaches nearly diagnostic levels of accuracy, otherwise achieved only by invasive procedures like chorionic villus sampling or amniocentesis, but requires only a sample of maternal blood. The NIPT technology continues to advance and a greater variety of genomic alterations can be detected. This research study describes the detection of two different fetal microdeletions using NIPT, which includes whole genome next-generation sequencing, and targeted region capture and sequencing methods.

Methods: Whole genome next-generation sequencing, and targeted region capture and sequencing methods, were used on samples of maternal plasma obtained from pregnancies with confirmed microdeletions. The DNA of these samples was compared to control DNA libraries to identify the fetal microdeletions.

Results: We were able to identify statistically significant differences between samples to detect fetal microdeletions on chromosome 12p12.1-p11.22 from maternal plasma samples. Identification of a fetal microdeletion on 5p15.33 from maternal plasma samples was achieved, but highlighted the difficulties in detection, and future challenges for NIPT.

Conclusion: Our research has demonstrated the ability to detect microdeletions by whole genome next-generation sequencing and targeted region capture and sequencing methods of NIPT. The findings indicate the ability of NIPT to detect a wide range of genomic alterations, which will impact prenatal care in the future if the technology improves. Development and expansion of NIPT has significant public health implications due to its high levels of accuracy as compared to current screening, and safety for the pregnancy as compared to current diagnostic testing options. NIPT could have major ethical implications, and could impact the role of prenatal genetic counselors and physicians.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0   INTRODUCTION

Pregnancy screening and testing options have been clinically available to women for many years. Different methods have been employed, including ultrasound imaging, blood tests for a variety of molecular markers, and invasive procedures used to analyze fetal chromosomes. As technology advances, new methods are being introduced to detect a variety of genetic conditions during pregnancy.

One of the newest clinical pregnancy screening tests is called non-invasive prenatal testing (NIPT). This technology screens for cases of fetal Trisomy 21, Trisomy 18, Trisomy 13, and sex chromosome aneuploidy. The test is unique because it reaches nearly diagnostic levels of accuracy otherwise only achieved by invasive procedures like chorionic villus sampling (CVS) or amniocentesis, but requires only a sample of maternal blood. As NIPT's technology advances, a variety of genetic alterations can be detected. This thesis describes two cases of microdeletions detected using NIPT, and describes the future of the technology, and the ethical, clinical, and professional implications of its use.

1.1   AIM

The goal of this paper is to demonstrate the ability to detect microdeletions by NIPT. The methodologies of NIPT procedures have advantages and disadvantages, which will be discussed

1

in order to establish a review and guide future optimization of methodology. There are public health, ethical, and genetic counseling implications with regard to NIPT's use in the clinical setting.

2.0   BACKGROUND AND SIGNFICANCE

2.1   GENETICS

Human cells contain 46 chromosomes, which serve as the body's guide for growth and development. The 46 chromosomes are composed of 22 pair of autosomes and one pair of sex chromosomes, discovered by Tjio and Levan in 1956 [1]. Chromosome pairs are formed by combining one maternal and one paternal copy of each chromosome during egg fertilization. Chromosomal abnormalities may occur in 20-50% of all pregnancies [2]. Aneuploidy describes the condition where there is an extra chromosome (trisomy), or a missing chromosome (monosomy), caused by an error in meiosis or mitosis. The most common cause of aneuploidy in pregnancy is nondisjunction during the first meiotic division in female gametes [3]. Nondisjunction occurs randomly, but rates have been shown to increase with maternal age. Trisomy is the most commonly identified aneuploidy, occurring in about 4% of all pregnancies, and 27% of miscarriages, with miscarriage occurring in 15% of recognized pregnancies [1, 4].

Trisomy 21, also called Down syndrome, is caused by an extra chromosome 21. The condition was first recognized by Lejeune in 1959 [1]. Trisomy 21 occurs sporadically in about 95% of cases, but at the age of 35, the chance for a fetus to have Trisomy 21 is 1/270 [5]. Down syndrome may also be caused by a Robertsonian translocation that becomes unbalanced, which accounts for about 5% of cases. Individuals can be carriers of Robertsonian translocations

without having a personal or family history of Down syndrome. The characteristic phenotype of Down syndrome includes developmental delay, mild to moderate intellectual disability, hypotonia, and characteristic facial features. Occasionally heart defects and gastrointestinal defects are identified.

Trisomy 13, or Patau syndrome, and Trisomy 18, or Edwards syndrome are two other trisomies that are commonly seen among women of advanced maternal age. Both of these conditions are more rare and severe than Down syndrome. Patau syndrome often includes severe intellectual disability, cleft lip and palate, and nervous system anomalies. Edward's syndrome can include severe intellectual disability, heart defects, and nervous system anomalies. In many cases, Patau and Edwards syndromes are not compatible with life, and many affected fetuses are spontaneously aborted, or die shortly after delivery.

Other common trisomies include sex chromosome aneuploidy. Included are monosomy X, or Turner syndrome, XXY or Klinefelter syndrome, XXX, and XYY, which range in phenotypic presentation. Turner syndrome is associated with short stature and lack of secondary sexual development. Klinefelter syndrome's phenotype includes infertility, tall stature, and learning difficulties. XXX and XYY both are associated with learning and behavioral difficulties [3].

Other structural chromosome changes may occur in pregnancy, regardless of maternal age. Deletions and duplications are missing or extra pieces of genetic material as large as an entire chromosome, or as small as a single base pair. Duplications of genetic material are typically less pathogenic, and have fewer phenotypic findings, while large deletions have a stronger effect on phenotype [1, 3]. Microdeletions and microduplications typically describe alterations too small to be detected by standard karyotype analysis. Microdeletion syndromes

can be found on many chromosomes, and they have extremely variable expression and range of phenotypic features depending on the genes involved. Deletions and microdeletions may occur when genetic material is rearranged, such as during an inversion or translocation, or spontaneously as a de novo mutation. Deletions and microdeletions can be inherited from an apparently unaffected parent before being expressed in a child, due to differences in disease penetrance, expression, genomic imprinting, other comorbid genetic changes, or for unknown reasons.

Deletions and duplications are not as common as aneuploidy, and their occurrence does not follow an age related trend in pregnancies. Currently, these genomic changes are not regularly screened or tested for in pregnancy. Since aneuploidy is more common, and the risk increases with maternal age, there are screening and diagnostic tests available to determine the fetal risk or status for the chromosome conditions described above.

2.2   CURRENT PRENATAL SCREENING AND DIAGNOSIS

2.2.1   Non-invasive Prenatal Screening Options

There are three non-invasive screening options for the assessment of fetal risk for a chromosome condition. Screening tests are used to assess risk or predict disease [6]. These screening tests include first trimester screening, multiple marker screening, and ultrasound imaging. Understanding the methodology, benefits, and limitations of these screening options is important for the comparison to NIPT.

First trimester screening and multiple marker screening consider maternal age and gestational age. Maternal age is the age of the patient's eggs, so caution must be used in cases of IVF pregnancies using donor eggs. Gestational age can be measured using last menstrual period (LMP), ultrasound measurement of crown rump length in the first trimester, biparietal diameter (BPD) in the second trimester, or physical exam dating. Dating of the pregnancy is crucial for accurate screening.

First trimester screening is a noninvasive pregnancy screening test that became widely used in the 1990s. It combines ultrasound and blood testing between 11 and 14 weeks gestation to determine the risk for Down syndrome and Trisomy 18. The nuchal translucency (NT) is assessed by ultrasound visualization. The best time to obtain an accurate measurement of the nuchal translucency is between 11 weeks and 13 weeks 6 days gestation [7]. When increased nuchal translucency is detected, one third of cases are found to have a chromosomal abnormality, of which, 75% have Trisomy 21 or Trisomy 18. Increased NT measurements have been associated with fetal cardiovascular and pulmonary defects, skeletal dysplasia, congenital infection, metabolic disorders, and hematologic disorders [7, 8]. The blood test measures the relative amounts of pregnancy-associated plasma protein A (PAPP-A) and free beta (or total) human chorionic gonadotropin (hCG). The amounts are reported as multiples of the median (MoM), which may vary by center [9]. In pregnancies where the fetus has Trisomy 21, hCG is increased and PAPP-A is decreased and for Trisomy 13 and 18, hCG and PAPP-A are decreased. Other chromosome conditions may be detected using first trimester screening information, but they are not regularly offered.

Ultrasound and blood measurements are combined to determine the risk for the pregnancy. Cut-off values have been determined: an increased risk for Down Syndrome is $\geq$

1/230, and an increased risk for Trisomy 18 is $\geq$ 1/100 [9]. First trimester screening detects 85-90% of cases of Down syndrome, with a false positive rate around 5% [5, 7, 10]. There are no risks to the pregnancy with this screening, but the results are not diagnostic, and only Down syndrome and Trisomy 18 are detected. For twin gestations, the NT is measured for each fetus, but the blood sample analysis is averaged between the two fetuses, thus is less accurate in predicting the likelihood of an affected fetus. In pregnancies with greater than two fetuses, NT measurements are the only reliable method of screening for chromosomal abnormalities [7].

Multiple marker screening, also called quadruple screening, was introduced in the 1980's and is a noninvasive pregnancy screening test done in the second trimester, between 15 and 20 weeks gestation [11]. Blood is analyzed for alpha-fetoprotein (AFP), total hCG, unconjugated estriol (uE3), and Inhibin-A. When Inhibin-A is not included, this screening is called Triple screen. These molecules are measured to determine a relative risk for an open neural tube defect (ONTD), Down syndrome, and Trisomy 18.

The pattern detected by multiple marker screening is used to determine the pregnancy risk. Low AFP, low uE3, high hCG, and high Inhibin-A indicate an increased risk for Down syndrome. Low levels of AFP, hCG, and uE3 indicate an increased risk for Trisomy 18. Triple and Quad screens adjust the risk based on maternal age and maternal weight. A screening assessment of $\geq$1/270 is considered increased risk for Down syndrome [9]. Second-trimester quadruple screening detects about 81% of cases of Down Syndrome, 60% of Trisomy 18, 80% of cases of spina bifida, 90% of cases of anencephaly, 80% of abdominal wall defects, and 60% of ONTDs in twin pregnancies with a 5% false positive rate [9, 12]. Screening for Trisomy 18 in a twin pregnancy is currently not possible. Occasionally uE3 is undetectable, which has been associated with fetal demise, IUGR, anencephaly, and genetic syndromes that are related to

cholesterol biosynthesis pathways including congenital adrenal hypoplasia, Smith-Lemli-Opitz syndrome, and steroid sulfatase deficiency [13].

Different screening algorithms have been proposed which define when to collect pregnancy information, how to synthesize the data, and when to report the results. Stepwise sequential screening gives the pregnancy risk results after each screening test. Contingent sequential screening allows further screening to be canceled if the first trimester screen is negative. Fully integrated screening gives a single result combining both first and second trimester screening [14]. Serum integrated screening also only gives a single result, but does not use NT measurement in its calculations [12]. Studies have found that fully integrated screening detects 94-96% of cases of Down syndrome, and integrated screening with serum detects 85-88% [12, 14, 15]. Integrated screening's major disadvantage is that it requires second trimester screening results, thus limiting access to CVS procedures. Sequential screening allows for earlier results, and combines the first and second trimester measurements into a final risk assessment.

The third method of non-invasive prenatal screening is ultrasound. There are three types of ultrasound examinations: standard or basic, limited, and specialized or detailed. Specialized ultrasounds are performed when there is a suspected fetal anomaly based on laboratory abnormalities, medical history, or family history. Specialized exams may include fetal Doppler ultrasonography, biophysical profile, amniotic fluid assessment, fetal echocardiography, or biometric measurements [16]. A detailed anatomy ultrasound is typically performed between 18 and 20 weeks gestation, and looks at the fetal head, face, neck, chest, heart, abdomen, spine, extremities, and sex. Anatomy ultrasound can detect 50% of cases of Down Syndrome, and

greater than 90% of cases of spina bifida and anencephaly [5, 16]. Overall, ultrasound examination detects 40% of fetal anomalies with variation between centers [16].

Ultrasound findings associated with Down syndrome include: increased NT, hyperechoic bowel, shortened humerus, echogenic intracardiac focus, shortened femur, and pyelectasis [17]. Echogenic intracardiac focus is one of the most common isolated markers in both healthy fetuses and those affected with Down syndrome. Ultrasound findings for Trisomy 18 can include IUGR, heart defects, strawberry shaped calvarium, clenched fists, rockerbottom feet, micrognathia, cleft lip or palate, omphalocele, diaphragmatic hernia, neural tube defect, cystic hygroma, polyhydramnios, and choroid plexus cysts [9]. Approximately 75% of fetuses with cystic hygromas by ultrasound have a chromosome abnormality, and of these, 95% of cases have Turner syndrome. When nuchal edema (or increased NT) is detected, one third of cases are found to have a chromosomal abnormality, of which, 75% have Trisomy 21 or Trisomy 18. Due to the limitations of ultrasound examination, it is not recommended as a primary method of screening for Down syndrome or other chromosome conditions.

2.2.2 Invasive Prenatal Diagnosis Options

There are two invasive diagnostic procedures available to women during pregnancy. These procedures include chorionic villus sampling (CVS) and amniocentesis which allow for the isolation of fetal cells. These cells can be directly analyzed or cultured in the laboratory for detection of genetic conditions by karyotyping, FISH analysis, or other molecular testing including microarray analysis. These methods are considered greater than 99% accurate. Previously, only women who were considered "high risk" were offered invasive procedures, but

now ACOG recommends that all women, regardless of age, be offered the option of invasive prenatal testing [14].

Chorionic villus sampling (CVS) is an invasive procedure performed between 10 and 12 weeks gestation that collects chorionic villi tissue, which is part of the placenta. Under ultrasound guidance, a catheter is used transvaginally, or a needle is inserted transabdominally to collect tissue samples. There is a risk of miscarriage with the procedure ranging from 1/200-1/1000, depending on the center where the procedure is performed, and the expertise of the individual performing the procedure. The risk for miscarriage extends approximately two weeks beyond the date of the procedure for CVS and amniocentesis. Rapid FISH results for chromosomes 21, 13, 18 and the sex chromosomes can be obtained 24-48 hours following the procedure. Karyotype results typically take 10-12 days following the procedure.

CVS is considered a more technically difficult procedure than amniocentesis, and 100-400 procedures need to be performed by a physician before the learning curve reaches a plateau, compared to 30 procedures for amniocentesis [18, 19]. The miscarriage rate was found to be increased in centers performing fewer than 136 procedures per year [19, 20]. Individuals undergoing CVS typically will not need to have an amniocentesis. One exception is in the case of placental mosaicism. Mosaicism can occur within a tissue, multiple tissues, or an entire organism, and depends when during development nondisjunction occurred. Mosaicism is detected in about 1% of CVS samples, and an amniocentesis is typically recommended to clarify the finding [1, 18].

Amniocentesis is an invasive procedure performed after 15 weeks gestation. A needle is inserted transabdominally to collect two tablespoons of amniotic fluid. The cells obtained from the sample, amniocytes, are shed by the fetus. FISH and karyotype results following

amniocentesis take the same period of time to be reported as the results from CVS. There is a risk of miscarriage with the procedure ranging from 1/200-1/1000 when completed before 22 weeks gestation, and a risk of preterm labor from 1/200-1/1000 after 22 weeks gestation [5, 21]. Other risks of the procedure may involve leakage of amniotic fluid, membrane rupture, bleeding, infection [22]. The risk for miscarriage was found to be increased in centers performing fewer than 45 procedures per year [19]. Diagnostic error may occur in cases of maternal cell contamination or low level mosaicism [18].

Samples obtained by CVS and amniocentesis are sent for standard karyotyping and FISH analysis. Karyotypes allow for the chromosomes to be visualized, counted, and analyzed for translocations, inversions, ring chromosomes, missing or extra chromosomes, and other structural changes, and can detect low level mosaicism that may be missed using other methods. FISH analysis uses fluorescently labeled probes that attach to specific chromosome regions, which can be counted to determine if the region is missing or replicated. Cytogenetic analysis typically detects changes at or above the resolution of 5 to 10Mb, and requires live cells to culture for analysis [23]. FISH and karyotype are considered the standard of care for prenatal diagnosis, but improvements in technology have given patients and physicians new options for obtaining a prenatal diagnosis. The most recent and debated methodology is microarray analysis.

Microarray analysis is used to detect small imbalances in the chromosomes, called copy number variants (CNV), including deletions and duplications that are at least 1Kb in size [24]. The technology is considered standard of care, with karyotype analysis, in the pediatric setting as 12-15% of children with normal karyotype analysis have been found to have a genetic change by microarray [25]. Microarray analysis has only recently been used in the prenatal setting. Some

benefits of using microarray include decreased time to obtain results, as samples do not need to be cultured in the lab, and detection of clinically relevant deletions and duplications that would have been missed by karyotype analysis. One study found that microarray provided relevant information to an additional 1.7% of pregnancies tested due to advanced maternal age and positive screening results, and an additional 6.0% of pregnancies tested due to ultrasound findings. Microarray analysis could be used to diagnose stillborns, where genetic abnormalities are found in 6-13% of cases [23]. One benefit to using microarray on stillborns is that growing tissue is not required for analysis. One disadvantage is that 3.4% of microarray findings are considered variants of uncertain significance, of which, 72.3% were not easily classified as benign [25]. Other disadvantages of microarray testing in the prenatal realm include limited ability to detect triploidy, balanced translocations, marker chromosomes, low level mosaicism, and the lack of clinical findings to correlate to variants of uncertain significance. The cost of microarray analysis is higher and may not be proportional to the clinically useful information gained [24]. As the debate over the use of microarray analysis in the prenatal setting continues, new technologies are being introduced in the pediatric setting, including whole-exome and whole-genome sequencing. These technologies could be applied to the prenatal setting in the future, and may be able to detect an even greater number of genetic alterations than cytogenetic or microarray technologies. The benefits and disadvantages of these technologies will be discussed further, as they apply to the ethics and clinical challenges related to prenatal testing and diagnosis.

Amniocentesis and CVS are valuable options for genetic diagnosis during pregnancy, but the risk of miscarriage, physical discomfort, and anxiety of undergoing an invasive procedure are

deterrents for many women and families. The disadvantages of invasive diagnostic options highlight the potential benefits of non-invasive prenatal testing.

2.3   NON-INVASIVE PRENATAL TESTING

2.3.1   History of Non-invasive Prenatal Testing

Non-invasive prenatal testing originated from cancer research and tumor genetics. Researchers discovered tumor DNA in the plasma and serum of cancer patients' blood in 1996. In 1997, Lo et al. hypothesized that fetal DNA could be detected in a pregnant woman's blood. Lo et al. detected fetal Y chromosome DNA in 80% of maternal plasma and 70% of maternal serum from women carrying male fetuses, using PCR assays for the Y chromosome [26]. The average fetal DNA concentration in maternal plasma was 3.4% in the first trimester and 6.2% during the second and third trimesters [27]. Cell free fetal DNA had a half life of sixteen minutes, and was not detected in maternal circulation two hours after delivery [28].

The source of fetal DNA, and why there was an increase in concentration during the pregnancy was not determined until 2004. Gupta et al. found that fetal DNA and RNA were in a cell-free form in the maternal plasma, originating from the placenta. Gupta proposed that apoptotic release of syncytiotrophoblast membrane of the placenta explained the circulating fetal DNA that had been detected, and that preeclampsia was proposed to correlate with increased levels of cell-free fetal DNA [29]. Preterm delivery was associated with increased concentrations of cell-free fetal DNA in maternal circulation [30].

In 1999, fetal DNA in maternal circulation was used to determine fetal rhesus D (RhD) status. RhD status is significant for RhD-negative women carrying an RhD-positive fetus, as the mother's immune system may reject the antibodies produced by the fetus [31]. In the UK anti-D is given to all RhD-negative pregnant women, although 40% carry RhD-negative fetuses and do not require the prophylaxis. Previously, RhD status was detected by sampling fetal blood or amniocentesis. Determination of the fetal RhD status could prevent unnecessary medical interventions, and reduce exposure to hepatitis C and prion diseases by reducing anti-D injections [32].

Other novel uses of NIPT technology were discovered in the 2000's. In 2005, hemoglobinopathies were detected by NIPT, but methodologies were too complex for testing to be implemented clinically [33]. Fetal sex had been detected by NIPT, since male fetal DNA markers on the Y chromosome were used to measure fetal DNA concentration. Some groups proposed offering sex determination testing clinically for individuals at risk for X-linked genetic conditions [34]. Achondroplasia was detected using NIPT in 2007 [35, 36]. NIPT has the capability to include paternity testing, detection of single gene disorders, aneuploidy, microdeletions, and microduplications.

Tong et al. first proposed using NIPT to detect fetal aneuploidy. In 2006, the group used epigenetic allelic ratio analysis for chromosome 18. The ratios between fetal and maternal allele concentrations were calculated using *Maspin* sequences found on chromosome 18. The ratio was used to compare differences between euploid and Trisomy 18 fetuses [37]. Tong's discovery began the use of NIPT for fetal aneuploidy detection, now offered clinically for Trisomy 21, Trisomy 18, Trisomy 13, and sex chromosome aneuploidy.

2.3.2 Methods of Non-invasive Prenatal Testing

Several methods have been proposed in the development of NIPT. Researchers searched for ways to improve methods of fetal DNA detection by searching for reliable markers to quantify and distinguish fetal DNA from maternal DNA, and by testing new technology. Early NIPT only accurately tested male fetuses due to limitations of distinguishing fetal and maternal DNA. To study male and female fetuses, a universal marker for fetal DNA should be non-gender specific, applicable to all ethnicities and population groups, easily detectable, sufficiently cleared from maternal circulation following delivery, and unique to the fetal DNA (verses maternal DNA).

One of the first universal markers proposed was *Maspin*; a tumor suppressor gene on chromosome 18. *Maspin* is expressed and hypomethylated in placental tissue, but methylated in maternal blood [37]. The detection of methylation patterns was challenging due to bisulfate conversion and DNA degradation. A second epigenetic fetal DNA marker was *RASSF1A* on chromosome 3p21.3, which is hypermethylated in placental tissue and hypomethylated in maternal blood. *RASSF1A* methylation status was detected using methylation-sensitive restriction enzyme digests which were more reliable and sensitive than bisulfate based methods [38]. *HLCS* was discovered on chromosome 21, and is hypermethylated in placental tissue and hypomethylated in maternal blood [39]. Single nucleotide polymorphisms (SNPs) have also been used as markers. SNPs are benign base pair changes that occur once every 300 nucleotides, and make up approximately 90% of genetic variation in humans [40, 41]. There are approximately 10 million SNPs in the human genome, and SNP frequency relies upon the population of interest, as SNPs are more variable between different ethnicities and regional groups [28, 41]. One SNP used in NIPT is in the *PLAC4* gene, and is transcribed from

chromosome 21 only in placental cells [42]. Methylation pattern differences between fetal and maternal DNA can be distinguished using methylated DNA immunoprecipitation coupled with oligonucleotide array analysis. The advantages of this technique include genome wide screening, gender and SNP independence, and lack of DNA degradation, unlike bilsufite methods [43].

Polymerase chain reactions (PCR) and quantitative real-time PCR (q-RT PCR) were first used to identify and quantify fetal DNA in maternal blood, but challenges arose due to the variable concentration of cell-free fetal DNA. One strategy was to enrich samples, or increase the relative concentration of cell-free fetal DNA in blood. Size fractionation was proposed when Li et al. determined that fetal DNA sequences are smaller (< 300 base pairs) than circulating maternal DNA (> 500 base pairs) [33]. About 80% of cell free fetal DNA is less than 193 base pairs in length [28]. Size fractionation was challenging when maternal blood samples were held over a period of time, allowing maternal blood cell apoptosis which increased the ratio of maternal DNA fragments. Formaldehyde was used as an inhibitor of maternal blood cell apoptosis to keep the fraction of fetal DNA consistent [28]. Enrichment may also be achieved by targeting fetal specific genetic or epigenetic sequences as markers [43].

Several forms of technology have been used to develop NIPT. Li et al used mass spectrometry to analyze samples, which measured the mass of each DNA fragment to elucidate the sequence and detect allelic changes [28, 33]. Chiu et al. proposed massively parallel genomic sequencing and locus targeting, which measured the total maternal and fetal DNA for a specific chromosomal locus using digital PCR. Incremental changes in the ratio of chromosomes were used to detect trisomies [44].

One study considered two possible research-based approaches to NIPT in 2010: analysis of allelic ratios of SNPs in a fetal specific marker including analysis of circulating placental mRNA (RNA-SNP approach) or DNA-methylation markers (epigenetic allelic-ratio approach), with single molecule counting methods including digital PCR, microfluidics digital PCR, and massively parallel genomic sequencing [39, 45, 46]. High-throughput shotgun sequencing was used to detect aneuploidy by Fan et al. Shotgun sequencing did not rely on SNPs, and provided a uniform application of the technology. Variation in GC content of different chromosomes effected sequence counts between samples, and caused imprecise measurements when using sequencing methods [43, 47]. Chiu et al. described a way to identify chromosomal origin of each sequenced DNA molecule using massively parallel sequencing by comparing the nucleotide sequence with the reference human genome. In euploid fetuses, 1.35% of all fragmented DNA is contributed by chromosome 21 [48]. By comparing the genomic representation of specific chromosomes, like chromosome 21, the degree of deviation from the expected genomic representation could be determined and statistically analyzed. This method using sequencing-by-ligation showed less bias for chromosomes with low GC content [49, 50].

Different methods and analytical frameworks are being developed to accurately detect genetic alterations in cell-free fetal DNA. New developments focus on novel methods of enrichment, correcting for GC content of different chromosomes, decreasing costs, and optimizing technology. This thesis will describe some developments in the field of NIPT with regards to detection of fetal microdeletions.

2.3.3 Current NIPT Clinical Testing

There are currently four clinical laboratories offering NIPT; Verinata (Verifi), Sequenom (MaterniT21), Ariosa (Harmony), and Natera (Panorama). Each lab has specific methods, test sensitivity, and specificity. Magee-Womens Hospital in Pittsburgh, Pennsylvania offers NIPT to "high risk" pregnancies including advanced maternal age, abnormal serum screen result, personal or family history of genetic condition, and/or abnormal ultrasound. Testing is offered any time after ten weeks gestational age to women with singleton pregnancies. Before having NIPT, patients meet with a genetic counselor to discuss the technology, what is being tested for, the risks and benefits, costs, and how results will be reported. Follow up testing with CVS or amniocentesis is recommended if positive results are obtained. Currently, Magee-Womens Hospital offers NIPT through Verinata.

Verinata was one of the first three companies to publish validation studies. In April 2011, Sehnert et al. published a trial of their massively parallel DNA sequencing study to detect fetal Trisomy 21 and Trisomy 18. The research included 1014 patients from 13 clinics across the United States who were at least 18 years of age. There were 71 controls and 48 samples in the test set. Sequencing libraries were created with single-end reads of 36bp. Sequencing variation was controlled by calculating the normalized chromosome ratio of mapped sites for a given chromosome to counts observed on a predetermined chromosome. The mean and standard deviation for the training set libraries and the samples were used to calculate the normalized chromosome value (NCV), which is equivalent to a z-score for the ratios for each chromosome of interest. An NCV >4.0 was required to classify results as aneuploidy, and <2.5 to classify as unaffected. Trisomy 21 was accurately detected in the control and test sets (NCVs 5-14), Trisomy 18 in most cases (NCVs 3.3-22), and Trisomy 13 was not accurately detected (NCV's

"no call" through 4) [51]. Information was collected across the genome, so copy number variation and cases of mosaicism were detectable. In order for massively parallel sequencing methods to be efficient, algorithms must be able to account for random or systematic bias in sequencing by normalization. Compared to methods previously described by Chiu et al., which used sequence tag number on the chromosome of interest normalized by number of tags in the sequencing run, the previous study did not yield as precise data for detection of Trisomy 18 and Trisomy 13 as Sehnert's study. Sehnert et al. planned to improve methodology by developing sequencing run controls that measure baseline and can change based on sequencing behavior [51]. A validation study by Bianchi et al., called the MELISSA study, obtained samples from 60 medical centers in the United States. Recruitment was offered to pregnant women over the age of 18 who were between eight and 22 weeks gestational age, who met at least one criterion for high risk pregnancy. Five hundred thirty two singleton pregnancies were selected, and cases of abnormal fetal karyotypes were matched to cases with euploid fetal karyotypes. A total of 89 cases of Trisomy 21, 36 cases of Trisomy 18, 14 cases of Trisomy 13, and 16 cases of Monosomy X were analyzed using massively parallel sequencing. Cell free fetal DNA was quantified using two allele specific methods: one using nine short tandem repeat loci to compare intensity peaks (minor peaks represented fetal DNA), and a panel of 15 SNPs. Control libraries were composed of 110 unaffected samples, separate from samples recruited for the study. NCV calculations and thresholds for analysis were the same as the Sehnert study. The laboratory was blinded to the clinical information, so no adjustments were made for maternal BMI, smoking status, diabetes status, type of conception, prior pregnancies, prior aneuploidy, or gestational age. Results were as follows:

Table 1. Verinata's MELISSA Study Results

Massively Parallel Sequencing Results	Sensitivity	95% CI	Specificity	95% CI
Trisomy21 (n=493)	100.0% (89/89)	95.9-100.0	100.0% (404/404)	99.1-100.0
Trisomy18 (n=496)	97.2% (35/36)	85.5-99.9	100.0% (460/460)	99.2-100.0
Trisomy13 (n=499)	78.6% (11/14)	49.2-99.9	100.0% (485/485)	99.2-100.0
XX (n=433)	99.6% (232/233)	97.6-99.9	99.5% (199/200)	97.2-99.9
XY (n=433)	100.0% (184/184)	98.0-100.0	100.0% (249/249)	98.5-100.0
Monosomy X (n=433)	93.8% (15/16)	69.8-99.8	99.8% (416/417)	98.7-99.9
Adapted from Bianchi, D.W., et al., Obstet Gynecol, 2012. [52]				

NCV for Trisomy 21 ranged from 4-26 (one at four), Trisomy 18 NCV ranged from below 4 to 16 (three below four), and Trisomy 13 ranged from below four to approximately 18 (five below four) [52]. Findings confirmed that fetal aneuploidy including translocation trisomy, mosaicism, and complex variation can be detected with high sensitivity and specificity, and aneuploidy in one chromosome does not affect the ability of massively parallel sequencing to identify euploid status of other chromosomes [52]. Verinata posted on February 15, 2013 that twin pregnancy chromosome abnormalities were accurately detected, which may indicate the future expansion of testing population [53]. Currently Verinata's, Verifi offers testing for Trisomy 21, 13, and 18, and sex chromosome aneuploidy using a newly updated sequencing method. Verifi uses the SAFeR algorithm for statistical analysis and does not exclude patients due to ethnicity, BMI, or egg donor status. Patients must be at least 10 weeks gestational age and meet criteria for a high risk pregnancy. Results take eight to 10 days, and testing sensitivity and specificity are quoted as:

Table 2. Verinata's Verifi Testing

Chromosome	N	Sensitivity	95% CI	Specificity	95% CI
21	500	>99.9 (90/90)	96-100.0	99.8% (409/410)	98.7-100.0
18	501	97.4% (37/38)	86.2-99.9	99.6% (461/463)	98.5-100.0
13	501	87.5% (14/16)	61.7-98.5	>99.9% (485/485)	99.2-100.0
Monosomy X	508	95.0% (19/20)	75.1-99.9	99.0%	97.6-99.7
XX	508	97.6% (243/249)	94.8-99.1	99.2% (257/259)	97.2-99.9
XY	508	99.1% (243/249)	96.9-99.9	98.9% (276/279)	96.9-99.8
XXX,XXY,XYY		Limited data			
Adapted from Verinata's website [53]					

A second company, Sequenom, offers NIPT through MaterniT21. Sequenom was the first company to offer NIPT clinically, in October of 2011 [54]. Ehrich et al. published a pilot study of multiplexed massively parallel shotgun sequencing for detection of Trisomy 21 in 480 blood plasma samples from women with a high risk pregnancy. Four libraries of DNA fragments were created and were analyzed together (multiplexed). Each 36pb DNA fragment yielded 5 million copies. Statistical analysis was modeled off of Chiu et al.'s previous work. Of the samples collected, 40 Trisomy 21 cases were correctly identified. The differences in this study compared to previous studies were that they used custom purified enzymes in the library generation process, a GAIIx sequencer with the CASAVA version 1.6 software, and indexing primers for library amplification to allow for multiplexing. These alterations led to cost savings, increased sequence reads per lane, and more sequence reads per sample, totaling four times higher throughput for four times lower cost. Overall, the study produced 100% sensitivity and >98% specificity [48]. Then Chiu et al. published a validation study of NIPT for Trisomy 21 detection. Samples were obtained from 753 women with high risk pregnancies in Hong Kong, the Netherlands, and the UK. Samples were analyzed by multiplex massively parallel sequencing using 8-plex and 2-plex protocols, where the 2-plex protocol analyzed seven times more plasma DNA molecules. The Z-score statistic was calculated with a cut off set at greater

than three to represent aneuploidy, representing the 99.9[th] percentile of the reference sample. An average of 300,000 reads was obtained per sample for the 8-plex sequencing analysis, and 2.3 million for the 2-plex sequencing analysis. The 8-plex sequencing protocol was not as accurate, as some non-trisomy 21 were above the z-score cut off of three and some Trisomy 21 cases fell below the cut off. The 2-plex protocol had fewer inaccuracies [55]. The study also explained that if fetal DNA concentration is approximately 10%, the percentage of chromosome 21 is 1.05 times higher in a Trisomy 21 pregnancy than a euploid pregnancy. To detect this change, the coefficient of variation of the test would need to be $\leq 0.83\%$. The 8-plex protocol was greater than 0.83%, while the 2-plex protocol was less than 0.83%, explaining the superior ability to detect Trisomy 21 in samples with low fetal DNA concentrations. From the study, they concluded that NIPT for Trisomy 21 is best performed as a screening test for high risk pregnancies, and in individuals who are considering CVS and amniocentesis [55]. Palomaki et al. also performed a validation study of NIPT for detection of Trisomy 21 in 4664 high risk pregnancies. The study determined that the detection rate was 98.6% with a false positive rate of 0.2% [56]. In 2012, Sequenom moved toward validation studies of other chromosomal trisomies, like Trisomy 13 and Trisomy 18. Palomaki et al. studied 62 pregnancies with Trisomy 18 and 12 with Trisomy 13 to test the ability of the current technology to accurately identify these aneuploidies. The total failure rate for testing (due to low fetal concentration and sample quality) was 0.9%. Detection rate for Trisomy 18 cases was 100% with a false positive rate of 0.3%. For Trisomy 13, the detection rate was 91.7% with a false positive rate of 0.9%. Repeat masking, which normalizes the GC content, was used to analyze cases of Trisomy 13 and 18, and showed no change in detection or false positive rates. Overall, the study showed that Trisomy 21, 13, and 18 can be accurately detected using NIPT [57]. Canick et al. studied the use of NIPT

for multiple pregnancies. They studied samples from a cohort of 4664 high risk pregnancies, which included 25 twin pregnancies (17 euploid, seven Trisomy 21, and one Trisomy 13), and two euploid triplet pregnancies. The GC content bias was statistically corrected and the results showed that Trisomy 21 was correctly identified in all twin pregnancies with Down syndrome, and one Trisomy 13 case was correctly identified. Multiple pregnancies contributed 35% more fetal DNA than singleton pregnancies, which is expected due to greater placental mass. Differences in fetal-maternal ratio of cell free fetal DNA arise when twins are discordant for chromosomal aneuploidy, and technical difficulties arise if the fetal DNA concentrations fall between four and six percent. At the conclusion of the study, it was determined that definitive assessments of sensitivity of detecting trisomies could not be completed, and assessment of twins individually is not possible [58]. Currently, the clinical test, MaterniT21 reports the following for testing sensitivity and specificity:

Table 3. Sequenom's MaterniT21 Testing

Study	Chromosome	N	Sensitivity	Specificity
Ehrich et al. 2011	21	450	>99% (39/39)	99.7%
Palomaki et al. 2011	21	1696	99.1% (210/212)	99.9%
Palomaki et al. 2012	18	1988	>99.9% (59/59)	99.6%
	13		91.7% (11/12)	99.7%
Canick et al. 2012	21 and 13	2015	>99.9% (8/8)	
ASHG Poster 2012	X and Y	2107	99.4%	
Adapted from Sequenom's website [59]				

Ariosa is the third clinical NIPT lab, whose test is Harmony. The lab's first publication was by Sparks et al. in January of 2012. The study collected samples from singleton 289 pregnancies and sought to develop NIPT for the detection of Trisomy 21 and Trisomy 18 using DANSR (digital analysis of selected regions) by highly multiplexed assay. The goal was to make the NIPT process more efficient by generating fewer unused sequence reads. DANSR analysis is completed by using 384 loci on chromosome 18 and chromosome 21. The loci are

unique to the chromosomes of interest and do not coincide with known polymorphisms or copy number variants. There are three oligonucleotides per loci to amplify the regions of interest.

After sequencing the regions, the median count per locus was scaled to 1000, and then log transformed to account for bias among chromosomes, loci, and samples to create a linear statistical model. Data was used to calculate a z-test of proportions, and the separation distance was calculated as the distance between the z statistic value of the upper 95th percentile of average-risk samples and the lower 5th percentile in the affected samples. Analysis of chromosome 21 showed a separation distance of 4.2 with 204,000 reads, 5.2 with 410,000 reads, and 5.4 with 620,000 reads. For chromosome 18, analysis showed a separation distance of 4.9 with 204,000 reads, 5.2 distance with 410,000 reads, and 5.2 with 620,000 reads. With chromosome 18, sequence reads above 410,000 did not provide any greater level of detection [60]. Several months following the publication of the DANSR model, Sparks et al. published the FORTE model for optimized detection. The FORTE model analyses polymorphic and nonpolymorphic loci to optimize trisomy evaluation. The study recruited 250 euploid pregnancies, 72 cases of Trisomy 21, and 16 cases of Trisomy 18 pregnancies, with aneuploidy status blinded from the lab. Like the last study by Sparks et al., chromosome 18 and 21 had nonpolymorphic loci each, but there were an additional 192 SNP containing loci on chromosomes one through 12. SNPs were used to determine fetal fraction. All olgionucleotides were pooled, and run as a multiplex assay. The FORTE algorithm estimates risks of aneuploidy using an odds ratio. The major differences when using FORTE analysis are that 96 samples in a single lane can be analyzed using the observed variance within and between samples rather than estimating variance upon information from a previously analyzed data set. FORTE also responds to fetal fraction for euploid and aneuploid cases, and it incorporates age-related risks

for aneuploidy. All cases of fetal Trisomy 21 and 18 were correctly identified using these methods [61].

Ashoor et al. studied the detection of Trisomy 21 and 18 by chromosome-selective sequencing of cell free fetal DNA during the first trimester. Samples were obtained from 300 euploid pregnancies, 50 pregnancies with Trisomy 21, and 50 pregnancies with Trisomy 18, at gestational age 11-13 weeks, prior to having CVS. Trisomy 21 detection sensitivity was 100%, and Trisomy 18 sensitivity was 98%, both with 100% specificity [62]. Norton et al. performed a large scale validation study, deemed the Non-Invasive Chromosome Evaluation (NICE) study. The validation study collected 81 cases of Trisomy 21, 38 cases of Trisomy 18, 2888 "normal" cases and 73 "other" samples from three countries. The study determined Trisomy 21 sensitivity to be 100%, with specificity of 99.9%, and for Trisomy 18 a sensitivity of 97%, with 99.9% specificity. The testing had a 4.6% failure rate, where the fetal DNA concentration was too low, or the assay did not work properly. The study determined that fetal fraction did not vary with subject's race, ethnicity, maternal age, or trisomy type, or gestation age between 10-22 weeks [63]. Studies were completed by Ashoor et al. and Brar et al. regarding factors that influence fetal fraction of cell-free DNA in maternal plasma. Brar studied the concentration of cell-free fetal DNA in maternal plasma in relation to the a priori risk for fetal trisomy, as used in the FORTE method to determine baseline risk evaluation. The study determined that there is no statistically significant association between fetal karyotype, crown-rump length, NT measurement, screening results, or maternal characteristics and the concentration of cell free fetal DNA in circulation [64]. Ashoor et al. studied the concentration of cell-free fetal DNA in maternal plasma at 11-13 weeks gestational age in relation to maternal and fetal characteristics. Approximately 2000 pregnant women were recruited for the study that were between 11 and 13

weeks gestational age. They found that fetal fraction decreases with increased maternal weight and Afro-Caribbean racial origin, and increases with increased levels of hCG and PAPP-A, and with increased fetal crown-rump length, fetal Trisomy 21, and maternal smoking [65]. These associations with cell-free fetal DNA concentration may be important factors when statistically correcting for sample variability.

Harmony currently does not require a high risk pregnancy classification in order to perform NIPT, and they do not offer sex chromosome aneuploidy testing. The detection rates for clinical NIPT through Harmony are listed below:

Table 4. Ariosa's Harmony Testing

Chromosome Aneuploidy	Detection Rate	False Positive Rate
Trisomy 21	>99% (214/214)	<0.1%
Trisomy 18	>98% (103/105)	<0.1%
Trisomy 13	80% (8/10)	<0.1%
Adapted from Ariosa's website [66]		

The final laboratory offering NIPT is Natera, whose test is named Panorama. The proof of concept study was published in 2012, which included 166 samples (11 Trisomy 21, three Trisomy 18, two Trisomy 13, two 45,X, and two 47,XXY). Most aneuploidy samples were drawn after invasive testing, and karyotype results were not blinded by laboratory researchers. The method described, called Parental Support, requires parental genotypes, data from the Hapmap Database, and the number of sequence reads associated with the relevant alleles at 11,000 SNP loci. The data are used to create billions of possible monosomic, disomic, and trisomic fetal genotypes at these loci, and Bayesian statistics are used to determine the relatively likelihood of each hypothesis. Probability distributions are calculated for the observed allele distributions compared to expected allele distributions to determine the most likely scenario. Statistical calculations are completed by the NATUS statistical algorithm. A data quality test is

also performed to assess DNA quality. In this study, 21 samples did not pass the DNA quality test (12.6%), and sensitivity and specificity for all euploid and aneuploid samples was 100% [67]. They currently are not requiring high risk pregnancy classification, and will collect samples as early as nine weeks gestational age.

3.0   MATERIALS AND METHODS

3.1   DATA COLLECTION

3.1.1   Patient Population

Patient recruitment occurred at Magee-Womens Hospital in Pittsburgh, Pennsylvania.   The patient population was composed of pregnant women over the age of 18, who were between 10 and 22 weeks gestation.   Patients were given the opportunity to participate in the research if they were considered among the "high risk" pregnancy population.   The definition of "high risk" population includes advanced maternal age, abnormal serum screen result, personal or family history of genetic condition, and/or abnormal ultrasound [68].   Patients were undergoing routine first trimester screening, or electing prenatal diagnosis by CVS or amniocentesis.   Several "low risk" pregnancies were also recruited by inviting patients who were scheduled for routine first trimester screening and did not meet "high risk" pregnancy criteria.   This pool of "low risk" patients served as negative controls [69].

3.1.2   Participant Recruitment and Informed Consent

All patients who elected to participate in the research study were recruited and gave informed consent at the Center for Medical Genetics at Magee-Womens Hospital in Pittsburgh,

Pennsylvania under IRB protocol number PRO07090033. Recruitment was accomplished by genetic counselors and genetic counseling interns during genetic counseling appointments or during brief consults. Patients who were eligible to participate in this research were selected from genetic counseling and first trimester screening schedules, available on Magee's Genetic Information System (GIS).

Information regarding research participation was provided and informed consent was obtained at the end of genetic counseling sessions, prior to first trimester screening or prenatal diagnostic procedures. For patients not participating in genetic counseling sessions, genetic counseling interns (employed as a patient recruiter by the research laboratory) met with patients to provide information and obtain informed consent prior to blood collection for first trimester screening.

Informed consent consisted of a discussion of research study background, specific aims, participation requirements, risks of participation, rights of the participant, and sample handling. Patients were given the opportunity to review the consent form (Appendix A), discuss questions and concerns, and provide signature, printed name, and date on the last page of the consent form. Genetic counselors or genetic counseling interns, who served as recruiters, completed the last page of the consent form by providing their signature, printed name, and date. The completed consent form was copied and retained in the patient's chart at Magee-Womens Hospital. The original completed consent form is filed in a locked cabinet at the research laboratory. No samples were obtained without the consent of a patient.

3.1.3 Sample Collection and Tracking

After providing informed consent, and completing consent paperwork, research participants were given a prescription for blood work. The phlebotomy lab at Magee-Womens Hospital obtained approximately 36 milliliters of blood from each patient (six purple top tubes). Blood samples were collected and transported to the research lab within two hours of blood draw for processing.

Sample collection and transportation to the research lab were documented in the patients GIS entry in the Reference Lab tab. All samples were de-identified upon entering the lab. A bar code was given to each sample, which was used to track the blood sample, DNA, sequencing library, and results. The bar code also provides patient clinical and demographic information, which includes prenatal diagnostic test results and pregnancy and birth outcome.

3.2   SAMPLE PROCESSING

3.2.1 Separation of Plasma from Whole Blood

Whole blood was centrifuged for 13 minutes at 1600xg at 4ºC, with acceleration and deceleration set to 3. Aliquots of 1 ml of plasma were pipetted into 1.5 ml microcentrifuge tubes and spun at 16000xg for 10 minutes at 4ºC to form a pellet. 900 µL of suspension from each tube were transferred to a clean 1.5 ml microcentrifuge tube. Plasma aliquots were stored at -80ºC [69].

3.2.2 DNA Extraction from Plasma

Plasma samples were used to extract DNA using the QIAamp DNA Blood Mini Kit and specified reagents. One frozen plasma sample was thawed to room temperature and divided into two tubes. 40 µL of Qiagen Protease was added to each tube and mixed by inverting the tube five times. 400 µL of Buffer AL was added to each tube, and the sample was mixed by vortexing for 15 seconds. Samples were incubated at 56ºC for 10 minutes. 400 µL of 100% ethanol was added to each tube and tubes were mixed by vortexing for 15 seconds. 600 µL of the mixture was applied to the spin column and spun at 6000xg for one minute. Elution of the sample may be repeated in order to pass the entire plasma sample through the column. The column was washed by adding 500 µL Buffer AW1 and spun for 1 minute at 6000xg. 500 µl of Buffer AW2 was added to the column and centrifuged at max speed for 3 minutes. Residual ethanol was removed by placing the column in a clean collection tube and centrifuging for 1 minute at maximum speed. To elute the DNA, the column was placed in a 1.5 mL tube and 75 µL of RNase/DNase free water was added to the column. The column was incubated at room temperature for 5 minutes and then centrifuged for 1 minute at 6000xg.

3.2.3 Real Time PCR Analysis of SRY and Bglobin

Primers and probe sequences for the real time PCR reaction were obtained from Maron, et al.

SRY: Forward primer 5' – TCCTCAAAAGAAACCGTGCAT – 3'; Reverse primer 5' – AGATTAATGGTTGCTAAGGACTGGAT – 3'; Probe 5' – FAM – CACCAGCAGTAACTCCCCACAACCTCTTT – TAMRA – 3'.

B-globin: Forward primer 5'– GTGCACCTGACTCCTGAGGAGA – 3'; Reverse primer 5' – CCTTGATACCAACCTGCCCAG – 3'; Probe 5' – FAM – AAGGTGAACGTCCATGAAGTTGGTGG – TAMRA – 3'.

B-globin is a ubiquitous housekeeping gene and was run concurrently with SRY to ensure that DNA was present in each sample, irrespective of fetal gender. In order to estimate DNA concentration in the plasma DNA, standard curve DNA was run simultaneously with the plasma DNA. The standard curve DNA was prepared using commercially available DNA with known concentrations. The range of values for the standard curve was 6.4 pg/5 µL to 20,000 pg/5 µL. Each real time PCR reaction consisted of: 12.5 µL 2x TaqMan Universal PCR Master Mix, 1.25 µL 10 µM forward primer, 1.25 µL 10 µM reverse primer, and 0.0625 µL 100 µM probe. 10 µL plasma DNA, 5 µL standards, or 10 µL water (to serve as a negative control) were added to the appropriate wells. Each plasma DNA sample and the negative control were run in triplicate. The standard curve DNA was run in duplicate. The thermal cycling conditions were initial denaturation step of 95°C for 10 min, followed by 50 cycles of 95°C for 15 sec and 60°C for 1 min. The real time PCR reactions were done using the 7900HT Sequence Detection [69].

3.2.4 System (Applied Biosystems).Plasma Sequencing

Plasma sequencing used the Illumina Truseq DNA sample prep kit, minus the gel purification step. The following oligos 5'-ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TC*T-3' and 5'-/5Phos/GAT CGG AAG AGC TCG TAT GCC GTC TTC TGC TTG – 3' were resuspended in TE and annealed in 1X T4 DNA Ligase Reaction Buffer (NEB) by heating at 95ºC for 5 minutes and then slowly cooled to room temperature for a final concentration of 36 µM annealed adaptor. Plasma DNA fragments were end repaired and then terminal A-residues

were added using the NEBNext End Repair and the NEBNext dA-tailing modules as per manufacturer's protocols. Following reaction cleanup using the MinElute Cleanup kit (Qiagen), DNA fragments were combined with 0.05 µM adaptor and 400U T4 DNA ligase (NEB) and incubated for 1 hour at 16ºC. After reaction cleanup with MinElute Cleanup kit, PCR was performed using the following primers: 5'- CAA GCA GAA GAC GGC ATA CGA GCT CTT CCG ATC*T -3' and 5'-AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC*T -3' and Phusion High-Fidelity DNA polymerase. PCR conditions were an initial denaturation (98ºC, 30s), 30 cycles of 98ºC for 10s, 65ºC for 30s, and 72ºC for 30s, with a final extension of 72ºC for 7 min. Following amplification, the PCR reaction was cleaned up using the MinElute PCR Purification kit (Qiagen) [69].

3.2.5   Preparation of Targeted Sequencing Libraries

Standard Illumina TruSeq protocols were used to create plasma DNA libraries. A 15 cycle PCR reaction was performed and 500 ng of the resulting product incubated Agilent with SureSelect biotinylated probes for 24 hours, according to the manufacturer's instructions. Baits spanning a region between chr12:22,455,568-30,651,389 (hg19) were designed for this purpose by Agilent. Targets were captured using Dynal MyOne Streptaviden T1 beads (Invitrogen) and a final library amplification of 12 cycles was carried out as described in the Illumina TruSeq protocol. Libraries were quantified using real time PCR and sequenced on a HiSeq2000 (Illumina) using 100bp paired-end reads[70].

3.2.6   Array Based Comparative Genomic Hybridization

Array based comparative genomic hybridization (aCGH) was performed using whole-genome 135K (SignatureChip Oligo Solution version 2.0, custom designed by Signature Genomic Laboratories, manufactured by Roche NimbleGen, Madison, WI, USA). Results were visualized using custom aCGH analysis software (Genoglyphix; Signature Genomic Laboratories). Fetal DNA was isolated from cultured amniocytes using PureGene DNA kit (Gentra) and tested for maternal cell contamination. Fetal DNA and normal male control DNA were differentially labeled with cyanine 3-dCTP and cyanine 5-dCTP (Perkin Elmer) using BioPrime labeling kit (Invitrogen), and hybridized onto an oligonucleotide array for 24 hours. Metaphase FISH analysis using 12p11.23 specific probe were performed on cultured amniocytes and in PHA stimulated peripheral blood samples obtained from the fetus and both parents [70].

4.0   CHROMOSOME 12 MICRODELETION

The following describes the proof on concept that was recently demonstrated for the detection of

a 4.2 Mb deletion on chromosome 12 using NIPT.

A family presented to Magee-Womens Hospital of UPMC at the Medical Genetics

department in 2011 due to a previous child with a 4.2 MB deletion on 12p12.1-p11.22.
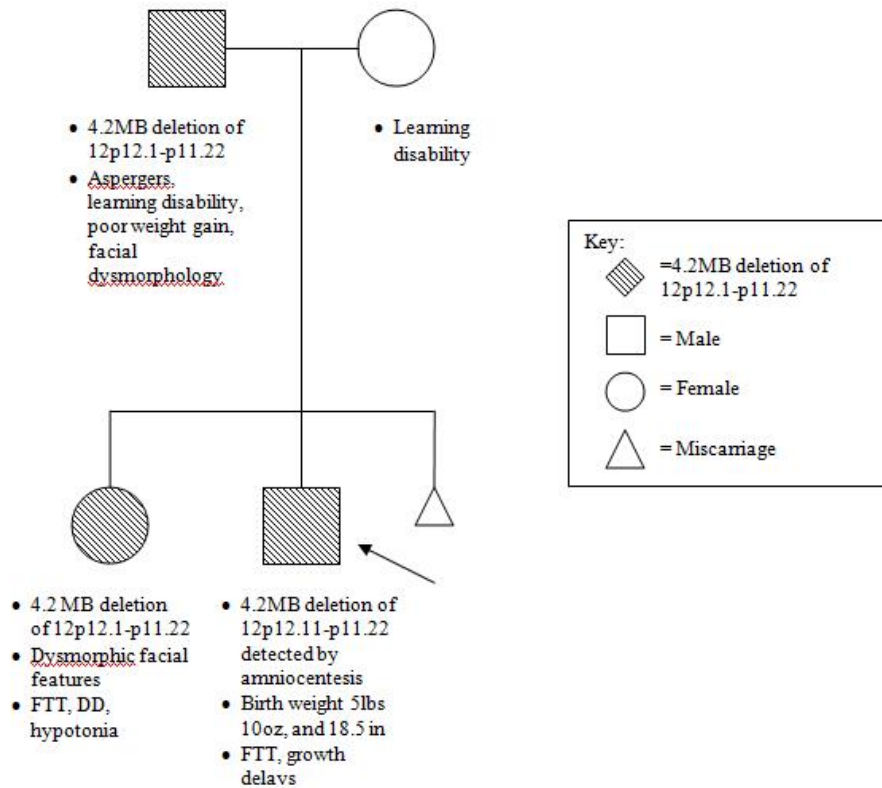
Figure 1. Chromosome 12 Pedigree

The previous child was born at 37 weeks gestational age, weighed five pounds, nine

ounces, and was 17.25 inches long. She was seen by the Genetics department at Children's

35

Hospital of Pittsburgh for dysmorphic facial features, failure to thrive, developmental delay, and low muscle tone at ten months of age. Testing was completed which revealed the deletion containing 16 OMIM genes. This deletion was determined to be paternally inherited. Genetic testing was performed for Prader-Willi syndrome and Fragile-X, which were negative. Metabolic and thyroid studies were within normal limits.

The family was expecting a second child, and came to the department for prenatal genetic counseling and testing. The mother was Gravida three, Para one, and had an amniocentesis at 21 weeks gestational age. A karyotype showed a normal male (46,XY). Array-CGH was performed on DNA extracted from the amniotic sample, and a single-copy loss of 12p12.1-p11.22, was detected and confirmed by FISH analysis. Microarray analysis also detected copy number changes on chromosomes 1q31.1 (161.6KB loss), 8p11.23 (92.1KB loss), and Xp22.33 (101.9KB gain). The child was delivered weighing five pounds, ten ounces, and was 18.5 inches long. The child has also been seen by Children's Hospital of Pittsburgh, Genetics department for failure to thrive and growth delays.

4.1   METHODS

Fetal genome equivalents from the maternal blood plasma sample were calculated as 5.7% by real time PCR. Plasma DNA was used as a substrate for single end read (35bp) Illumina DNA sequencing, which generated 243,340,714 reads. Of these reads, 75% could be mapped uniquely and perfectly to the latest version of human reference genome GRCh37. We selected 22 non-overlapping regions; about 4 million base pairs each, from the reference genome. Twenty of the regions were from chromosome 14 and two from chromosome 12. The two regions of

chromosome 12 chosen included the region of the deletion, and the region immediately upstream of the deletion. GC content and read density were calculated for each region. Read density is the median number of reads aligned to a moving window of width 50000bp in that region. Seven maternal plasma libraries were obtained which had both maternal and fetal diploid chromosomes 12 and 14, as reference libraries. The statistical methods used for this analysis have been previously published [70, 71].

4.2   RESULTS

The deletion sample was compared to these reference libraries using the 22 test regions to determine if the 12p deletion's region was diploid. The p-values were adjusted for family-wise error using Holm's method. In all seven comparisons, detection of a single copy fetal loss of the 12p region was determined in DNA from maternal plasma. No false positives were reported for the other 21 regions [70].

Whole genome DNA sequencing of maternal plasma DNA has potential for NIPT of fetal microdeletions and/or microduplications. One advantage to this approach is that such genomic changes can be detected without prior knowledge of the mutation or locus of interest. Due to the large amounts of sequence data required, and the cost of whole-genome next-generation sequencing procedure, it may be less likely to be implemented clinically. One way to reduce the amount of sequencing data required, thus reducing costs, would be to use genome capture and targeted sequencing.

The maternal plasma sample with a fetal 4.2Mb microdeletion, as described above, with three normal pregnancy maternal plasma samples, were subjected to targeted region capture of

an 8Mb sequence on chromosome 12p12.1-p11.22 followed by next generation DNA sequencing. The sample with the deletion (aka PL565) had approximately 45 million paired sequence reads, of which, 40 million could be aligned to the reference genome. The 8Mb sequence was divided into 19 non-overlapping regions of 400Kb each. Ten of these regions are located in the deleted region of interest, and nine lie outside the region and were used as references. For the PL565 sample, 1.3-1.9 million reads for completed for each region. The GCREM algorithm was used to test the PL565 library against three normal libraries. Significant p values were obtained for the ten regions in the deleted region of interest, and non-significant p values were obtained for the nine regions outside of the deleted region, suggesting that the targeted capture method with GCREM is capable of detecting deletions as small as 400Kb. The same result was obtained when testing a quarter of the PL565 library with GCREM, suggesting that about 300,000 reads per region is sufficient to detect fetal deletions and duplications in that region from a maternal plasma sample [70, 72]. See figure three for data analysis.

5.0 CHROMOSOME 5 MICRODELETION

The following describes the detection of a 319 Kb subtelomeric deletion using NIPT. A family presented to Magee-Womens Hospital of UPMC at the Medical Genetics department. The mother was Gravida eight, Para three, and was seen at 19.0 weeks gestational age due to an increased risk for Down syndrome (1:42) and open neural tube defect (1:160) by multiple marker screen.

Seizures in childhood, ID

5p15.33 deletion carrier

- 318.46KB deletion of 5p15.33
- Bilateral club feet, echogenic bowel, IUGR
- Birth weight: 4lbs 12oz, length: 43cm
- Jaundice, feeding difficulties, DD, bilateral parietal-temporal polymicrogyria, farsighted, hearing loss, brushfield spots

Key:
⬦ =318.46KB deletion of 5p15.33
☐ = Male
◯ = Female
△ = Miscarriage
◇ =Unknown gender
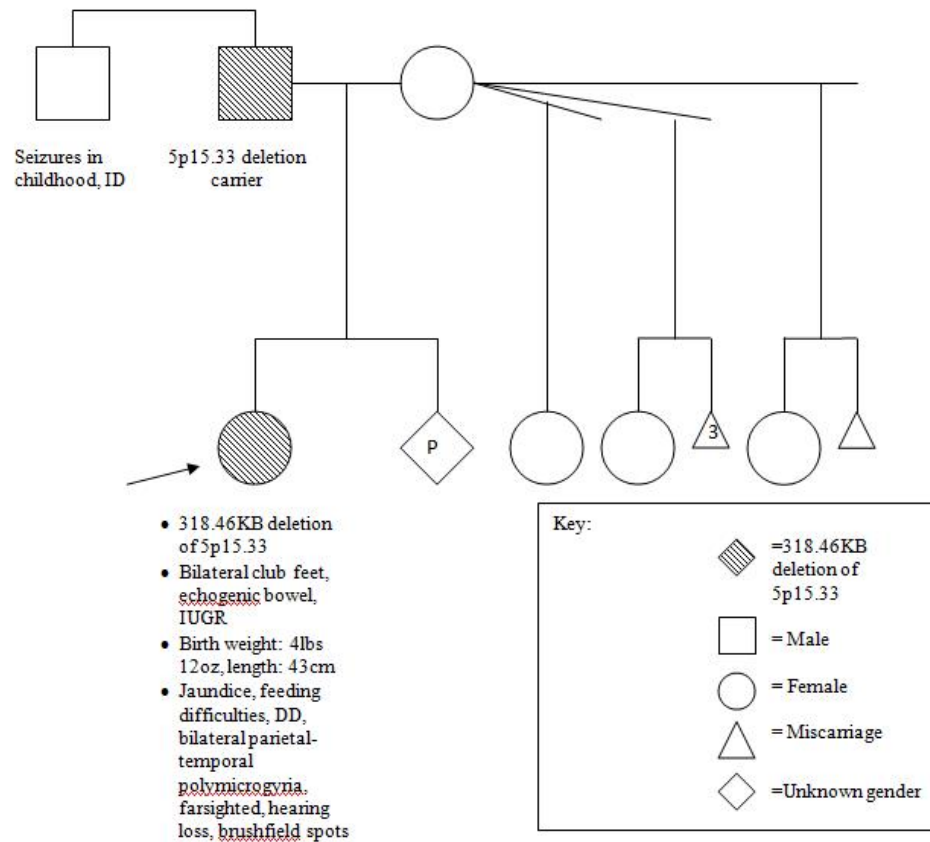
Figure 2. Chromosome 5 Pedigree

An amniocentesis was performed, and during ultrasound for the procedure, bilateral club feet and an echogenic bowel were noted. Karyotype and FISH analysis from the amniocentesis revealed 46,XX. Microarray analysis revealed a 318.46kb loss of 5p15.33; a variant of uncertain significance, whose region includes five OMIM genes. Cri-du-chat syndrome is found in the deleted region, but the critical region was not included. FISH studies determined the deletion was of paternal origin. The patient was recruited for the study during a follow up prenatal appointment. The child was born at 39 weeks gestational age, weighed four pounds, 12 ounces, with a length of 43 cm. She was seen by the Genetics department at Children's Hospital of Pittsburgh following reports of developmental delay, hypotonia, polymicrogyria and bilateral posterior white matter loss with distortion of the lateral ventricles on MRI, farsightedness, and some hearing loss. Physical exam during that consultation revealed Brushfield spots, and height, weight, and head circumference in the seventh percentile for growth. Due to the polymicrogyria, genetic testing for *GPR56* was pursued, and results are currently pending.

Currently the couple is pregnant, and came in for prenatal counseling and CVS at 11 weeks, one day gestational age, where mother and father were re-recruited for the research study, with the anticipation of sample collection from CVS, and correlation with FISH, Karyotype, and microarray testing results.

5.1   METHODS

The samples were statistically analyzed using the MINK derivative, GCREM, as previously published [70, 71].

5.2   RESULTS

Appendix two contains the analysis of the samples used to detect the fetal microdeletion. Figure four shows the analysis of the chromosome five region of interest with the DNA from maternal whole blood compared against plasma DNA control libraries. Figure five shows the analysis of the same region with DNA from maternal whole blood compared against placental DNA from CVS control libraries. Changing the control libraries altered the number and degree of outliers, with CVS control libraries providing a more precise data output. The result is unexpected, as a whole blood sample should be best controlled by another blood sample library, rather than placental tissue. Since we know the fetal microdeletion is paternally inherited, we do not expect to find any copy number variants in the region in the maternal sample.

Figure six shows the fetal sample from CVS/amniocentesis with the plasma DNA control library. Figure seven shows the fetal sample from CVS/amniocentesis with the control library of DNA from CVS. In both figures, the microdeletion is well visualized within the outlined region between 227979 and 530932. There is less variability in the amount of loss represented when using the CVS DNA control library.

Figure eight represents the analysis of cell free fetal DNA from maternal plasma with a plasma DNA control library. Figure nine shows the same sample with placental (CVS) DNA control library. Both control libraries allow for the detection of the microdeletion, but the placental DNA control show less variation surrounding the deletion, and neither cell free fetal DNA sample shows the microdeletion as clearly as the amniocentesis sample.

Several questions are raised based on the difference in results observed by using plasma and CVS DNA control libraries. First, there are discrepancies in our records regarding the type of sample collected for analysis: either CVS or amniocytes, and if either was successfully

obtained. Secondly, would the sample type (amniocenteisis) discrepancy with the control library (CVS) significantly change the results? Depending on the sample collected, the control library used (CVS) may be inappropriate. Also, are there major differences in DNA sequence, variability, GC content, or other epigenetic factors between plasma and placental tissue DNA? Would these possible variations explain the differences in results, and are there ways to account for these variations in future studies? Next, are the other gains and losses noted by analysis true gains and losses, or are they artifacts of the procedure? This region on chromosome five does not currently have quality coverage by microarray probes. This lack of coverage may be due to highly repetitive sequences within this region, which lead to bias in analysis. Would this sufficiently explain the great variation observed in our analysis, or may there be other explanations? The variability in the results warrants further investigation. Laboratory techniques, analysis, and statistical methodologies may need to be improved in order to more accurately detect this type of microdeletion. Based on these results, expanding NIPT to detect microdeletions and microduplications in the clinical setting must be delayed until results can more accurately and reliably be determined.

6.0   DISCUSSION

6.1   PUBLIC HEALTH IMPLICATIONS

We demonstrated that some microdeletions can be accurately detected using NIPT, which implies the potential expansion of this technology in both research and clinical applications. Fetal microdeletions and duplications are currently detected by microarray analysis using invasive methods, including CVS and amniocentesis. Development of a non-invasive test to detect these small genetic changes has significant public health implications. It would provide a safe, non-invasive option, and with greater sensitivity and specificity than current screening options, potentially replacing current pregnancy screening options. The detection of aneuploidy using NIPT has impacted public health in the prenatal setting among high risk pregnancies, and further impacts will be made as technology improves.

High risk pregnancies could be reclassified following a negative result when testing for aneuploidy, microdeletions, and microduplications. Reclassifying pregnancies decreases patient anxiety, reduces unnecessary exposure to further screening and testing, and can limit invasive procedures and the associated risks of miscarriage. Saving patients the anxiety and time associated with current pregnancy screening options has the ability to save physicians time, but may cost hospitals and insurance companies' more money by consolidating routine screening

43

and testing procedures into one test. Implementation of NIPT in routine prenatal care has implications for reproductive and public health policies, not just in the United State, but globally.

This research describes technology that could be offered world-wide in an effort to improve the prenatal care of women. The NIPT technology could be useful in hospitals and medical practices that do not regularly perform invasive procedures. Centers with fewer procedures often have higher rates of complications, miscarriage, and preterm labor following CVS or amniocentesis, and NIPT could serve as a virtually risk-free alternative. Providing women and families with an option to gain information about their pregnancy empowers them, and provides reproductive autonomy and the ability to make informed decisions about pregnancy management. As technology improves and costs decrease, NIPT could be applied globally to aid in the detection of aneuploidy and other chromosomal alterations in pregnancy.

6.2   FUTURE DIRECTIONS OF THIS RESEARCH

The data and methodology presented in this paper provide a foundation for ongoing and future research by this group. As more samples are recruited for this study, validation studies for the detection of aneuploidy, microdeletions, microduplications, and other chromosomal alterations can be completed. These validation studies will help to identify and improve testing specificity and sensitivity. With the development and improvement of novel statistical analysis, our testing will provide greater predictive powers than first trimester screening for fetal aneuploidy. For the detection of microdeletions, NIPT may be a method to obtain results similar to microarray analysis. Validation studies of NIPT can determine if the levels of sensitivity and specificity of microarray analysis can be reached. Further studies should be completed to determine the

molecular differences between DNA samples derived from plasma and placenta. Differences between the two sources may have implications for analysis and statistical corrections.

Our research group hopes to expand the use of NIPT for detection of other fetal genetic conditions, including monogenic diseases. Other research applications of this technology may include paternity testing and carrier testing for the fetus. Our research group also continues to study the statistical analysis of samples in hopes to improve detection of genetic changes, and to correct for samples specific variability. As we continue to improve our methodology, it is possible that our group may be able to offer NIPT within Magee-Womens Hospital, thus reducing costs of shipping samples elsewhere, and providing revenue to the hospital.

6.3 TECHNICAL CONSIDERATIONS OF NON-INVASIVE PRENATAL TESTING

Non-invasive prenatal testing has expanded recently, and is offered clinically by several companies. Improvements are vital for the growth of NIPT, including more universal fetal specific markers, stronger statistical analysis, and molecular studies of sample differences.

The first area of technological improvement is the differentiation of fetal DNA from background maternal DNA by developing a universal fetal specific marker. Previous studies have used methylation specific and SNP specific markers to distinguish fetal DNA from maternal DNA. One disadvantage of using methylation specific markers is that the protocol used to differentiate methylation status may damage DNA, and make detection of small genomic alterations more challenging due to the degradation of the sample. SNP specific markers may not be advantageous due to the population and ethnic variances. Finding universal SNPs, to apply to all geographic populations would be advantageous for the distribution of this

technology. A universal marker that distinguishes fetal DNA from maternal DNA that is gender independent, widely applicable among diverse populations, and can be reliably detected without impacting DNA structure and integrity, is crucial for the improvement of NIPT.

The next area of technological improvement is the statistical correction for sample variation. Previous studies have found that GC content differs among each chromosome, and that very high or low GC content makes detection of genetic imbalances more difficult [49]. Appropriate statistical correction for this variation will improve testing specificity and sensitivity, and will make detection of a greater variety of genomic changes possible. Greater correction for individual variation between samples should be studied further to help optimize detection.

Finally, cell free fetal DNA was determined to originate from apoptotic cells associated with placental trophoblast cells, which are released into maternal circulation [28]. There are two considerations based on the source of DNA: first, the potential epigenetic changes to the fetal DNA due to the apoptotic process, and secondly the effects of confined placental mosaicism. First, further research must be completed on the differences in fetal DNA versus placental tissue, and the differences between healthy placental tissue and apoptotic placental tissue. Trophoblast cells rapidly divide to create the placenta, and they assist in the transportation of nutrients to the fetus. These biological roles and the potential for rapid growth and cell division of this tissue may have inherently different properties from other cellular DNA in the body. Epigenetic, structural, and chemical changes may occur in apoptotic tissue that have not been investigated thoroughly, and may impact the detection and reliable report of fetal genetic status. Secondly, confined placental mosaicism occurs in 1-3% of pregnancies [28]. Mosaicism is a concern when CVS is performed, as the tissue sample collected may be genetically different from the fetus. If

the cell-free fetal DNA analyzed originates from placental tissue that has a mosaic genetic abnormality, a false positive report may be generated, or the analysis of maternal plasma may give an intermediate representation of aneuploidy—thus resulting in an unreportable or ambiguous result. Further studies of confined placental mosaicism's effect on NIPT results must be completed to assure accurate results, specificity, and sensitivity.

6.4   ETHICAL CONSIDERATIONS OF NON-INVASIVE PRENATAL TESTING

6.4.1   Target Population

A current debate among many medical centers is: who is the most appropriate patient population for clinical use of NIPT, and when is the best time to offer testing? Currently, NIPT is recommended for women who are considered "high risk." The high risk category includes pregnancies of advanced maternal age, abnormal serum screen, personal or family history of aneuploidy, and/or abnormal ultrasound. The laboratories recommend the high risk patient population because they are the population used in validation studies.

ACOG stated that screening or invasive testing of pregnancy (CVS, amniocentesis) for fetal aneuploidy should be offered to women of any age, which is one source of debate. If NIPT expands beyond the detection of aneuploidy, would ACOG's statement apply? One contraindication for widespread use of NIPT is that is not considered a "standard of care" practice. Microdeletions and microduplications are not associated with maternal age, thus detection could occur in any age group. These genomic changes can occur sporadically, representing de novo changes, which may not be detected using traditional screening and testing

modalities. Would recommendations for expanded use of NIPT reflect the new policies and guidelines for prenatal use of microarray analysis? Clinicians in the obstetrical and genetic counseling fields must consider the current and future standard of care.

The standard of care is shaped by the medical and legal needs of patients and physicians. Historically, prenatal screening and testing have become "standard of care" options after review of literature, professional meetings, peer discussion, and the threat of liability [22]. If a microdeletion, duplication, or aneuploidy could have been detected prenatally, in a non-invasive, risk free form, would physicians and counselors be held liable if the test was not offered? If NIPT has higher specificity, sensitivity, and safety than other current options, should it be included as a standard of care option for pregnancies? Until further validations studies are performed on a wider population of pregnancies, NIPT is not expected to become standard of care for low risk pregnancies.

6.4.2 Result Reporting

NIPT's introduction has raised several ethical concerns including management of incidental findings, sex determination, findings that are unreportable or have unclear clinical significance, and detection of non paternity. Incidental findings and findings of unclear clinical significance are particularly relevant with potential improvements in technology that rival microarray analysis.

Incidental findings are identified genomic changes that were not described in the initial indication for testing. Incidental findings may include mutations that predispose to adult-onset conditions, conditions not explained by phenotype, and genetic changes that are associated with conditions not originally intended to be detected (findings other than trisomy when testing for

48

trisomy conditions). These findings may not be appropriate for the direction of care and decision making of a pregnancy. Typically, children are not offered genetic testing for adult-onset conditions so they can exercise their own autonomy in decision making as adults. Some have recommended limiting testing to well-defined genetic conditions, such as Trisomy 21, 13, and 18, which allow for greater patient education and informed consent, and decreases the possibility for the detection of incidental findings. Others argue that narrowing the scope of testing withholds potentially significant information and limits patient autonomy [6]. With the potential for NIPT to detect microdeletions, duplications, and other copy number variants, the scope of detection must be carefully defined. Increased scope is considered more ethically problematic for several reasons, including the impossibility of creating a list of conditions "worthy" of detection, variable expression of conditions, changes in treatment and information, and different perceptions of the severity of diseases [6]. Regardless of testing scope, informed consent must accurately describe the type of conditions potentially detected by NIPT.

Fetal sex determination is the second ethical concern. Detection of sex chromosomes and sex chromosome aneuploidy is offered clinically. The benefits of studying sex chromosomes in pregnancy are the detection of fetuses that may be affected by X-linked conditions, and sex chromosome aneuploidy syndromes such as Turner and Klinefelter syndromes. The perceived disadvantage of sex chromosome detection is fetal sex selection. Sex selection may occur when individuals want to "balance" their family, or for ethnic and cultural preferences for male or female children. Determining fetal sex by NIPT is considered a non-medical use of the technology, therefore the indication for testing is considered informational only [73]. Non-medical uses of screening or testing a pregnancy are typically not recommended or endorsed by medical professionals. While termination of a pregnancy for the sole reason of sex selection is

unlawful, and majority of physicians do not support such procedures, women are not required to give their reasons for seeking an abortion, thus fetal sex selection cannot be completely eradicated [74, 75]. Caution must be exercised when using NIPT for fetal sex determination. Emphasis must be placed on the indication for testing (aneuploidy), rather than associated findings, like fetal sex.

Unreportable results and detection of variants of uncertain clinical significance are concerns of NIPT expansion. Unreportable results occur when samples are of poor quality, have fetal fraction too low to utilize, or if there are lab errors or uncertain results. Improvements to technology may reduce the frequency of unreportable results. As NIPT improves to rival the level of detection of microarray, further information regarding uncertain variants must be obtained. For example, if a microdeletion was detected using NIPT which had unknown clinical significance, how would the pregnancy be managed? Without phenotypic correlations, it is more difficult to determine the role of the genetic change on development. Results of uncertain clinical significance place a greater burden upon the family, as decisions to terminate or continue the pregnancy are based on vague and untested information. Some women may feel guilt and worry regardless of their decisions to continue or terminate the pregnancy following the disclosure of test results [75]. A study was completed to assess women's reactions when receiving a positive result from prenatal microarray testing. Of the 23 women interviewed, most reported feeling that microarray testing was an offer too good to refuse since they were already undergoing invasive prenatal testing, and less was understood about the possible test results.

When a copy number variant was reported, women felt blindsided, since normal FISH and karyotype results were disclosed first. Other emotions included feeling overwhelmed, anxious, confused, and shocked. Confusion was reported when women received conflicting

information from counselors and physicians, which made pregnancy decision making more difficult. Women who had ultrasounds that revealed abnormalities tended to be less shocked by abnormal microarray results, which justified the findings, and pregnancy termination decisions. Many women considered abnormal microarray results "toxic knowledge," and wished they had not received the results. Bonding with the baby was impacted, as women continued to worry about their child's development, especially in cases of uncertain clinical significant findings. The study recommended that genetic counselors focus on social and moral values of patients, include discussion of the implications of positive test results prior to consent to testing, and continue to provide support after result disclosure [76]. Similar outcomes may occur in patients choosing NIPT, especially if the technology is capable of detecting small copy number variations. The concerns regarding variants of uncertain significance may be limited by reducing the scope of testing, as discussed above.

Another incidental finding, non paternity, describes the situation where the father of the fetus is biologically different from the social father. This can be detected when fetal DNA is compared to the paternal DNA. This occasionally occurs in cases where a genetic alteration has been detected in the fetus or child, and the mode of inheritance is being investigated. Requesting NIPT for paternity testing, like sex selection, is not considered medically indicated. It serves only as information, and is not typically recommended as part of prenatal care [73].

The ethical concerns relating to the reporting of results can be limited, or best anticipated, by discussing the scope and indication for testing, and by providing careful and thorough informed consent for NIPT prior to testing.

6.4.3 Implications of Testing

Due to the advantages of NIPT, more women could choose this testing over invasive procedures like CVS and amniocentesis. Perceived advantages for patients include less physical discomfort with a blood draw than an invasive procedure, increased accuracy over current screening options, earlier detection of genetic alterations in pregnancy, and potential for cost savings [22]. The shift in screening and testing could financially impact medical institutions, which regularly make profit from prenatal screening and testing. With changes to the standard of prenatal care, insurance companies may alter their coverage policies for NIPT, which will financially change the use of the testing by patients.

Some researchers have expressed concern that increased availability of genetic testing like NIPT, will increased the number of abortions. The culture surrounding individuals with disabilities may change if the prevalence of genetic conditions is decreased. If the culture becomes less accepting of individuals with disabilities, some have proposed that there would be increased societal pressure on women to undergo testing and to terminate effected pregnancies, possibly becoming a form of "passive eugenics." Increased societal pressure may lead to routinization of testing, negatively impact informed consent, and deemphasize voluntary participation [22, 77, 78]. Routinization of NIPT may occur regardless of societal pressure, as it may be perceived as "just another blood test." Another possible disadvantage of including NIPT in routine prenatal care is the "burden of choice." The burden of choice is when women and families feel overwhelmed by the options available to them for screening and testing, and they feel unsure about what to do with the information once it has been disclosed [6, 73]. Some cases of fetal aneuploidy or other genetic conditions detected early by NIPT may have naturally

aborted within the first trimester, and the information gained by early testing may have been considered "unnecessary" and served only to induce stress over pregnancy decisions [6, 79].

Conversely, NIPT could be beneficial for families. Anxiety may be alleviated with a negative result. Many families choose to undergo prenatal screening and testing to gain more information in preparation for caring for a child with a genetic condition, not just for termination of a pregnancy. When termination is considered, studies have found that the earlier in pregnancy the decision is made, the less psychologically challenging it is for families [77]. Although terminations rates may increase with the use of NIPT, there are safeguards in place against this trend, including the ability for women to obtain an abortion without a fetal genetic diagnosis, and couples facing the same decisions will not always choose the same course of action [78]. Early detection of a genetic condition can allow families to coordinate resources, support, and to gain information to help care for their child. Families are better prepared to overcome feelings of guilt, grief, and anxiety when prenatal testing is performed early. Early knowledge may allow families to form bonds with their future children rather than perpetuate feelings of ambiguity and uncertainty. Financial preparation can be an advantage for families who learn that their child is affected with a genetic condition. Planning for future medical care, education, and access to resources is beneficial to many families. Costs may extend beyond financial considerations, as the value of time spent away from family, jobs, and other children to care for a child with special needs, and the indirect costs of providing care, must be factored into the decision making process [22]. With the ability to determine the genetic status of the fetus early in pregnancy, families have time to consider these factors to make pregnancy decisions.

6.5 CLINICAL CONSIDERATIONS OF NON-INVASIVE PRENATAL TESTING

6.5.1 Clinical Need

There is a demonstrated need for a non-invasive option for individuals desiring information about the genetic status of their pregnancy. Couples must choose between a risk-free screening test that increases or decreases their risks with limited accuracy, or an invasive, physically uncomfortable, and potentially risky procedure to determine the exact genetic status of the pregnancy.

Current screening tests, offered to all pregnant women, only detect Down syndrome and Trisomy 18, and in the case of multiple marker screening, open neural tube defects. The highest detection rates for Down syndrome are less than 90%, and results are risk estimates rather than diagnoses. The current risk-free, non-invasive options for pregnant women are limited, and NIPT would offer a way to detect genetic conditions with higher accuracy than the current screening methods.

For high-risk pregnancies, women may feel that they have limited options, and invasive procedures are necessary. Anxiety has been reported in nearly all women undergoing CVS or amniocentesis, and was most often due to concerns about possible procedure results. Women who felt ambivalent due to anxiety and fear may not be capable of giving informed consent. The source of these emotions may include: conflicts between invasive genetic testing and personal beliefs about the pregnancy, perceived value of being an educated health care consumer, maternal role of protector of the fetus, religious beliefs, and intellectual values [80]. Aside from anxiety and fear about the procedure, results, and risks, CVS and amniocentesis may be uncomfortable procedures. About 54% of patients undergoing CVS and amniocentesis described

them as "painful", with techniques to reduce pain, including subfreezing needles, local anesthesia, and massage, found to be ineffective [81]. The anxiety, fear, and discomfort associated with invasive procedures outline the need for safe, non-invasive prenatal options.

A study was completed which assessed pregnant women's interest in NIPT as a future option in their pregnancy care. Of the women interviewed, 72% showed interest in the technology, and 95% of women wanted to meet with a genetic counselor to discuss NIPT [82]. Other studies have shown that women place highest value on the safety of the fetus when considering prenatal testing and screening options. Women and physicians valued test safety, timing in pregnancy, accuracy, and the ability to learn about conditions in addition to Down syndrome when considering NIPT [83]. Women felt that NIPT gave them time to prepare mentally and physically for the possibility of an affected child, increased their sense of control over the pregnancy, normalized the pregnancy, enhanced the couple's decision making abilities, and provided peace of mind. Women who had NIPT preferred to be given information about the testing by specialists with knowledge of the testing and genetic conditions, such as genetic counselors, genetic nurses, or physicians [84]. Women and physicians have shown an interest in NIPT, but many clinical care centers are struggling to determine when and how to implement such technology into established schedules of prenatal screening and testing.

To determine when and how NIPT could be optimally used, studies have been completed prospectively to outline options for clinicians. First, implementation of NIPT depends on how the technology will be regulated in the prenatal setting. Three models have been described, including the governmental regulatory model, market model, and medical model. The governmental regulatory model describes a medical screening test that is required by the state or federal government to be offered or completed. The market model describes testing that can be

sold through advertising, often directly to patients. The physician's role is to provide services to consumers depending on what the patient wants, regardless of medical consensus. The medical model requires a medical indication for a test to be recommended, and the patient can choose to have the test completed. This is the method physicians have historically employed for testing specific diseases or conditions. The medical model also explains why prenatal determination of fetal sex is not performed—as this would indicate that sex or gender is a "disease," which it is not [85].

The current method of NIPT implementation is the medical model. The medical indication for NIPT is the increased risk of aneuploidy in high risk pregnancies. As NIPT detects genetic alterations beyond aneuploidy, the medical indication would need to be altered appropriately. The second most likely model for NIPT implementation is the market model. Other genetic testing has been offered in the direct-to-consumer setting, which poses risks to informed consent and proper explanation of results. If NIPT were offered in a market model setting, physicians would need to be educated and equipped to explain and interpret the results for patients. The market model of test implementation is not ideal, although it has potential to occur.

Based on the assumption that NIPT will occur under the medical model, the next question is where in the sequence of prenatal care does the testing fit? NIPT could replace current screening tests, be added to current screening tests, or between screening and invasive tests, or NIPT could replace invasive diagnostic tests [86, 87].

If NIPT replaces current screening, therefore eliminating first trimester screening and the second trimester multiple marker screening, there would be defined advantages and disadvantages. The first advantage is that NIPT could be completed earlier than traditional first

trimester screening at 10 weeks gestation. Earlier results would give women and families time to make decisions about their pregnancy management and care, and would give sufficient time to make decisions regarding invasive testing. Detection of aneuploidy and other chromosome abnormalities by NIPT would provide more information than the Trisomy 21 and 18 risks by first trimester screening, with considerably higher accuracy. If NIPT is offered for diagnosing Trisomy 21, there could potentially be 84% fewer invasive procedures, and an additional 7% of cases would be diagnosed prenatally [79].

The first disadvantage of replacing current screening with NIPT would be the loss of ultrasound imaging and nuchal translucency measurement. By replacing current pregnancy screening with NIPT, there would be no initial risk assessment (aside from age related risk). Other disadvantages include risks of routinization of testing, information over-load in the first trimester, burden of choice, and inability to detect increased risk for pre-eclampsia, intrauterine growth retardation, and fetal abnormalities [88]. Several authors have recommended ultrasound imaging or nuchal translucency measurement in addition to NIPT to determine the number of fetuses (singleton versus multiples), and ensure correct pregnancy dating [89]. The disadvantage of including ultrasound or NT measurement with NIPT would be the challenge of describing what each component detects, why they differ, and why both tests would be completed [79]. Since NIPT is still very new, many insurance companies do not provide coverage—and a stand-alone ultrasound to accompany NIPT may be problematic for institutional billing. If NIPT were to replace first trimester screening or multiple marker screening, would the patient population need to be altered, as current screening is offered to all pregnancies? Also, AFP measurement during the second trimester would need to be completed independently for open neural tube defect detection.

If NIPT is added to current screening, it would be an additional "advanced screening test" because it has only been validated in high-risk pregnancy populations [89]. NIPT would better detect aneuploidy (and potentially other genomic alterations) than first trimester screening, but an ultrasound would be done for first trimester screening. This method would allow for detection of other pregnancy complications by ultrasound. The disadvantage is that screening tests would be accompanied by more advanced screening tests—thus extending the time between results and limiting the options of pregnancy termination and invasive procedures. Some groups are concerned that offering NIPT with other pregnancy blood tests may cause it to become routinized, thus reducing the ability to obtain informed consent [87]. Physicians may be hesitant to order screening tests following other screening tests, rather than follow a positive screening test with a diagnostic test. An increased risk for Down syndrome detected on a first trimester screen, with positive NIPT, then confirmed by amniocentesis would not save time or money compared to completing a first trimester screen *or* NIPT *then* an invasive procedure.

If NIPT is added between screening and invasive testing, it would provide a more definitive risk to the pregnancy regarding aneuploidy following first trimester screening results. The advantage to adding NIPT between first trimester screening and multiple marker screening is the ability to determine pregnancy risk in a step-wise fashion [88]. The results could be confirmed by invasive tests if positive results are received. This method works so that first trimester screening can help differentiate pregnancies at low risk from those at an increased risk for chromosomal conditions prior to offering NIPT. This model is one of the most regularly used options, as it is specific to high risk populations, allows for diagnostic confirmation, and can be used as an option for women who do not desire invasive procedures to clarify their first trimester screening results. The disadvantage to this method is similar to adding NIPT to current

screening, in that it does not save considerable amounts of time or money, and a screening test is technically followed by another, more "advanced," screening test.

Non-invasive prenatal testing could one day replace invasive testing, if the ability to detect the vast majority of chromosomal alterations is achieved. At this point in time, the technology has only been validated in high-risk pregnancies for aneuploidy and sex chromosome detection. Further validation studies would need to be completed to determine if diagnostic levels can be reached to transition from "advanced screening" to "diagnostic testing." The clinical need has been demonstrated, as a non-invasive diagnostic option would be well received by patients and physicians, but until the technology is more robust, NIPT should not replace invasive diagnostic testing.

6.5.2 Implications for Genetic Counseling

Genetic counselors face challenges to understand and explain complex procedures and findings to clinicians and patients with the introduction of any new genetic technology. Non-invasive prenatal testing is no different, and the current and future challenges of incorporating the technology may reshape the field of prenatal genetic counseling.

The National Society of Genetic Counselors released a statement regarding NIPT in 2012. They stated, "The National Society of Genetic Counselors currently supports Noninvasive Prenatal Testing/Noninvasive Prenatal Diagnosis (NIPT/NIPD) as an option for patients whose pregnancies are considered to be at an increased risk for certain chromosome abnormalities. NSGC urges that NIPT/NIPD only be offered in the context of informed consent, education, and counseling by a qualified provider, such as a certified genetic counselor. Patients whose NIPT/NIPD results are abnormal, or who have other factors suggestive of a chromosome

abnormality, should receive genetic counseling and be given the option of standard confirmatory diagnostic testing" [90]. NSGC does not support NIPT in low risk pregnancies as a routine, first-tier screening test, and NIPT should be offered with pre and post-test genetic counseling in a non-directive fashion [91].

A consideration for genetic counselors with the implementation of NIPT is cultural awareness and competency. Prenatal care is deeply rooted in individual religious and cultural beliefs which must be respected. The discussion of termination must be done with careful attention to the needs and desires of the patient. Culture differences impact the implication of offering testing. One study reported that approximately 5% of pregnancies in Japan have prenatal genetic screening, which is considerably less than in the United States [92]. Presenting the wide variety and range of prenatal screening options to individuals not accustomed to such care may be interpreted by the patient in ways the counselor may not anticipate. Language differences may pose a challenge with the incorporation of new technology into prenatal care. Translation services must have an adequate understanding of the material and technology being discussed to accurately convey information to patients [78]. Counselors may wish to meet with the translator before the genetic counseling session to discuss what will be said and offered during the appointment, which may help to reduce misunderstandings. With careful understanding and cultural sensitivity, NIPT can be performed in the genetic counseling setting.

In clinical settings where a genetic counselor is unavailable, physicians and other clinicians may discuss and explain NIPT to patients. Studies have found that physicians involve women minimally in the decision making process of screening for Down syndrome. A study of 62 obstetric health care providers determined that 85% of respondents did not report a high level of knowledge about NIPT, although 29% were likely to offer the testing [93, 94]. The

discrepancy between physician knowledge and the implementation of NIPT in the clinical setting raises concerns over informed consent, misinformation, and patient autonomy.

Consent for NIPT needs to be thorough so that patients are aware of how the testing is performed and what information they could learn about their pregnancy. Discussions should include the limitations and benefits of NIPT, what conditions are detected, accuracy and reliability of the test, the procedure itself and risks associated with blood collection, timing of results and how they will be disclosed, and if there is a specific indication for the patient to qualify for NIPT [78]. Patients are encouraged to consider the possible outcomes of testing—if the result is positive for a chromosome condition, how will it change their pregnancy management, delivery plans, or lifestyles following birth of the baby? If the results are negative, will the family feel sufficiently comfortable with the results or will they still wish to pursue further testing? Discussion of testing options must be performed without force or pressure, so that the patient's decision can be made independently. Adequate pre-test counseling is vital to retain patient autonomy. Concerns arise when pre-test counseling is not performed, or is performed in a limited capacity. The risk of routinization occurs when blood is collected for NIPT along with blood for a panel of other pregnancy tests. In these situations, patients may not be aware of what is being tested for, or the implications of the results of NIPT. As the scope of NIPT increases to include small genomic alterations, like microdeletions, a generic consent may be implemented, where testing is explained, but possible outcomes and conditions that could be detected, cannot be described until results are returned; similar to consent for microarray analysis [6]. Families often do not grasp the variety and range of conditions that may be detected, and the differences in severity, penetrance, and implications of a positive test result are disadvantages of

generic consent. Families may realize after receiving the results that they did not want to know the information.

The concerns for patient autonomy and informed consent underscore the need for genetic counselors in the prenatal setting, and the importance for current genetic counselors to stay informed and educated regarding new prenatal testing options. The role for genetic counselors in the prenatal setting may shift away from discussion of less accurate screening tests, and risk-laden invasive procedures, and focus on newer, more accurate, and safer tests for their patients.

APPENDIX A: CONSENT TO ACT AS A PARTICIPANT IN A RESEARCH STUDY

CONSENT TO ACT AS A PARTICIPANT IN A RESEARCH STUDY

University of Pittsburgh
SCHOOL OF MEDICINE
Department of Obstetrics, Gynecology and Reproductive Sciences

<u>CONSENT TO ACT AS A PARTICIPANT IN A RESEARCH STUDY</u>

TITLE: Biological Analysis of Fetal Nucleic Acids in Maternal Plasma

Mother's Consent Form

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63

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SOURCE OF SUPPORT: Department of Obstetrics, Gynecology and Reproductive Sciences

Why is this research being done?

It is currently very difficult to test for diseases and abnormalities during pregnancy. Two common medical procedures that are used to test the health of the unborn baby are chorionic villus sampling (CVS) and amniocentesis. Both of these procedures carry a level of risk to the baby.

The study in which you are being asked to participate will explore new minimal risk methods for testing the health of unborn babies. These new methods use only a routine blood sample taken from the mother's arm. This blood sample is obtained in exactly the same way as any other routine blood sample. We will investigate whether blood samples obtained in this way can be used to evaluate the genetic disorders of unborn babies.

Who is being asked to take part in this research study?

We are inviting 1950 pregnant women (>18 years old) who are undergoing either chorionic villus sampling or amniocentesis to participate in this research. Also included in this study are women who are undergoing a first trimester blood test for fetal chromosomal abnormalities. We are also recruiting a small number (50) of partners of the above women so that paternal samples may also be collected in cases where a genetic anomaly in the family tree is known or suspected.

What procedures will be performed for research purposes?

<u>Screening Procedures:</u>

None

<u>Experimental Procedures:</u>

If you decide to take part in this research study, we will obtain a blood sample (about 8 teaspoonfuls) from a vein in your arm. This will require only a few minutes of your time. In addition, if you are having chorionic villus sampling or amniocentesis, we will save the cells that would normally be discarded afterwards and these will be analyzed in the same way as your blood sample in order to compare the results. If you are not undergoing chorionic villus sampling or CVS we may save a very small portion of your placenta or some blood from the umbilical cord. These are tissues that would otherwise be discarded after birth. This testing will allow the investigators to confirm that their analysis from your blood is a correct one. Approximately one month after your due date we may review your medical records to obtain general information about the pregnancy outcome.

The samples we obtain from you may be stored for an indefinite period of time prior to experimental analysis. If this is the case the sample will be stored in a freezer in the Principal Investigator's laboratory. This laboratory is in a security controlled building. Identifiers that link your sample to your medical records will be removed and your sample will be encoded such that only the Principal Investigator will know the details of the code. The details of the code will remain in a locked filing cabinet in the Principal Investigator's office, which itself is kept locked and is within a security controlled building.

No information obtained in this study will directly benefit you or your unborn child. Therefore, the results of the testing will not be communicated to study participants.

<u>Monitoring/Follow-up Procedures:</u>
None

What are the possible risks, side effects, and discomforts of this research study?
The risks of this procedure are no greater than those you would experience when undergoing a routine blood test. You may experience pain/discomfort from the needle insertion; slight bruising at the site, and there is a slight risk of infection and a very rare possibility of fainting. Because we will obtain information from your medical records there is a slight risk of breach of confidentiality. We will guard against this by removing any information that could be used to identify you and replacing this with a code. The only people with access to this code will be the principal investigator (Dr Peters) and co-investigators (Drs Emery, Hogge, Kolthoff, Rajkovic and Simhan). The details of the code will be kept in a locked filing cabinet in the principal investigators office, which is in a restricted access building.

Although we will not directly be performing paternity tests, it is possible that we will identify rare instances where the mother and father have genotypes that are not consistent with the genotype of the baby. This would indicate non-paternity. In these instances the results will remain confidential and will not be shared with the study participants.

What are possible benefits from taking part in this study?
You will receive no direct benefit from taking part in this research study. However, information learned from this study may contribute to better tests in the future to detect disorders of unborn babies.

What treatments or procedures are available if I decide not to take part in this research study?
The clinical treatments or procedures you undergo will not be altered in any way if you decide not to take part in this research study.

If I agree to take part in this research study, will I be told of any new risks that may be found during the course of the study?

You will be promptly notified if, during the conduct of this research study, any new information develops which may cause you to change your mind about continuing to participate.

Will my insurance provider or I be charged for the costs of any procedures performed as part of this research study?

Neither you, nor your insurance provider, will be charged for the costs of any of the procedures performed for the purpose of this research study (i.e., the Screening Procedures, Experimental Procedures, or Monitoring/Follow-up Procedures described above). You will be charged, in the standard manner, for any procedures performed for your routine medical care.

Will I be paid if I take part in this research study?

There is no financial compensation associated with participation in this study.

Who will pay if I am injured as a result of taking part in this study?

University of Pittsburgh researchers and their associates who provide services at University of Pittsburgh Medical Center (UPMC) recognize the importance of your voluntary participation in their research studies. These individuals and their staffs will make reasonable efforts to minimize, control, and treat any injuries that may arise as a result of this research. If you believe that you are injured as a result of the research procedures being performed, please contact immediately the Principal Investigator or one of the co-investigators listed on the first page of this form.

Emergency medical treatment for injuries solely and directly related to your participation in this research study will be provided to you by the hospitals of UPMC. It is possible that UPMC may bill your insurance provider for the costs of this emergency treatment, but none of these costs will be charged directly to you. If your research-related injury requires medical care beyond this emergency treatment, you will be responsible for the costs of this follow-up care unless otherwise specifically stated below. There is no plan for monetary compensation. You do not, however, waive any legal rights by signing this form.

Who will know about my participation in this research study?

Any information about you obtained from this research will be kept as confidential (private) as possible. All records related to your involvement in this research study will be stored in a locked file cabinet. Your identity on these records will be indicated by a case number rather than by your name, and the information linking these case numbers with your identity will be kept separate from the research records. You will not be identified by name in any publication of the research results.

Will this research study involve the use or disclosure of my identifiable medical information?

This research study will involve the recording of current and/or future identifiable medical information from your hospital and/or other (e.g., physician office) records. The information that will be recorded will be limited to information concerning the outcome of your pregnancy.

Note: Research outcomes from this study will not be stored in your medical records.

Who will have access to identifiable information related to my participation in this research study?

In addition to the investigators listed on the first page of this authorization (consent) form and their research staff, the following individuals will or may have access to identifiable information (which may include your identifiable medical information) related to your participation in this research study:

Authorized representatives of the University of Pittsburgh Research Conduct and Compliance Office may review your identifiable research information (which may include your identifiable medical information) for the purpose of monitoring the appropriate conduct of this research study.

In unusual cases, the investigators may be required to release identifiable information (which may include your identifiable medical information) related to your participation in this research study in response to an order from a court of law. If the investigators learn that you or someone with whom you are involved is in serious danger or potential harm, they will need to inform, as required by Pennsylvania law, the appropriate agencies.

Authorized representatives of the UPMC hospitals or other affiliated health care providers may have access to identifiable information (which may include your identifiable medical information) related to your participation in this research study for the purpose of (1) fulfilling orders, made by the investigators, for hospital and health care services (e.g., laboratory tests, diagnostic procedures) associated with research study participation; (2) addressing correct payment for tests and procedures ordered by the investigators; and/or (3) for internal hospital operations (i.e. quality assurance).

For how long will the investigators be permitted to use and disclose identifiable information related to my participation in this research study?
The investigators may continue to use and disclose, for the purposes described above, identifiable information (which may include your identifiable medical information) related to your participation in this research study for a minimum of seven years after final reporting or publication of a project.

May I have access to my medical information that results from my participation in this research study?
In accordance with the UPMC Notices of Privacy Practices document that you have been provided, you are permitted access to information (including information resulting from your participation in this research study) contained within your medical records filed with your health care provider.

Is my participation in this research study voluntary?
Your participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above, is completely voluntary. (Note, however, that if you do not provide your consent for the use and disclosure of your identifiable information for the purposes described above, you will not be allowed to participate in the research study.) Whether or not you provide your consent for participation in this research study will have no effect on your current or future relationship with the University of Pittsburgh. Whether or not you provide your consent for participation in this research study will have no effect on your current or future medical care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

Your doctor (either Dr Emery, Hogge, Kolthoff, Rajovic or Simhan) is involved as a co-investigator in this research study. As both your doctor and a research investigator, s/he is interested both in your medical care and the conduct of this research study. Before agreeing to participate in this research study, or at any time during your study participation, you may discuss your care with another doctor who is not associated with this research study. You are not under any obligation to participate in any research study offered by your doctor.

May I withdraw, at a future date, my consent for participation in this research study?
You may withdraw, at any time, your consent for participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above. Note, however, that if you withdraw your consent for the use and disclosure of your identifiable medical record information for the purposes described above, you will also be withdrawn, in general, from further participation in this research study. Any identifiable research or medical information recorded for, or resulting from, your participation in this research study prior to the date that you formally withdrew your consent may continue to be used and disclosed by the investigators for the purposes described above. Should you withdraw your blood sample and chorionic villus/amniocentesis sample will be destroyed.

To formally withdraw your consent for participation in this research study you should provide a written and dated notice of this decision to the principal investigator of this research study at the address listed on the first page of this form.

Your decision to withdraw your consent for participation in this research study will have no effect on your current or future relationship with the University of Pittsburgh. Your decision to withdraw your consent for participation in this research study will have no effect on your current or future medical care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

If I agree to take part in this research study, can I be removed from the study without my consent?
It is possible but highly unlikely that you may be removed from the research study by the researchers.

****

VOLUNTARY CONSENT

The above information has been explained to me and all of my current questions have been answered. I understand that I am encouraged to ask questions about any aspect of this research study during the course of this study, and that such future questions will be answered by a qualified individual or by the investigator(s) listed on the first page of this consent document at the telephone number(s) given. I understand that I may always request that my questions, concerns or complaints be addressed by a listed investigator.

I understand that I may contact the Human Subjects Protection Advocate of the IRB Office, University of Pittsburgh (1-866-212-2668) to discuss problems, concerns, and questions; obtain information; offer input; or discuss situations in the event that the research team is unavailable.

By signing this form, I agree to participate in this research study. A copy of this consent form will be given to me.

_____
Participant's Signature

_____   _____
Printed Name of Participant   Date

CERTIFICATION of INFORMED CONSENT

I certify that I have explained the nature and purpose of this research study to the above-named individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual(s) have about this study have been answered, and we will always be available to address future questions as they arise." I further certify that no research component of this protocol was begun until after this consent form was signed

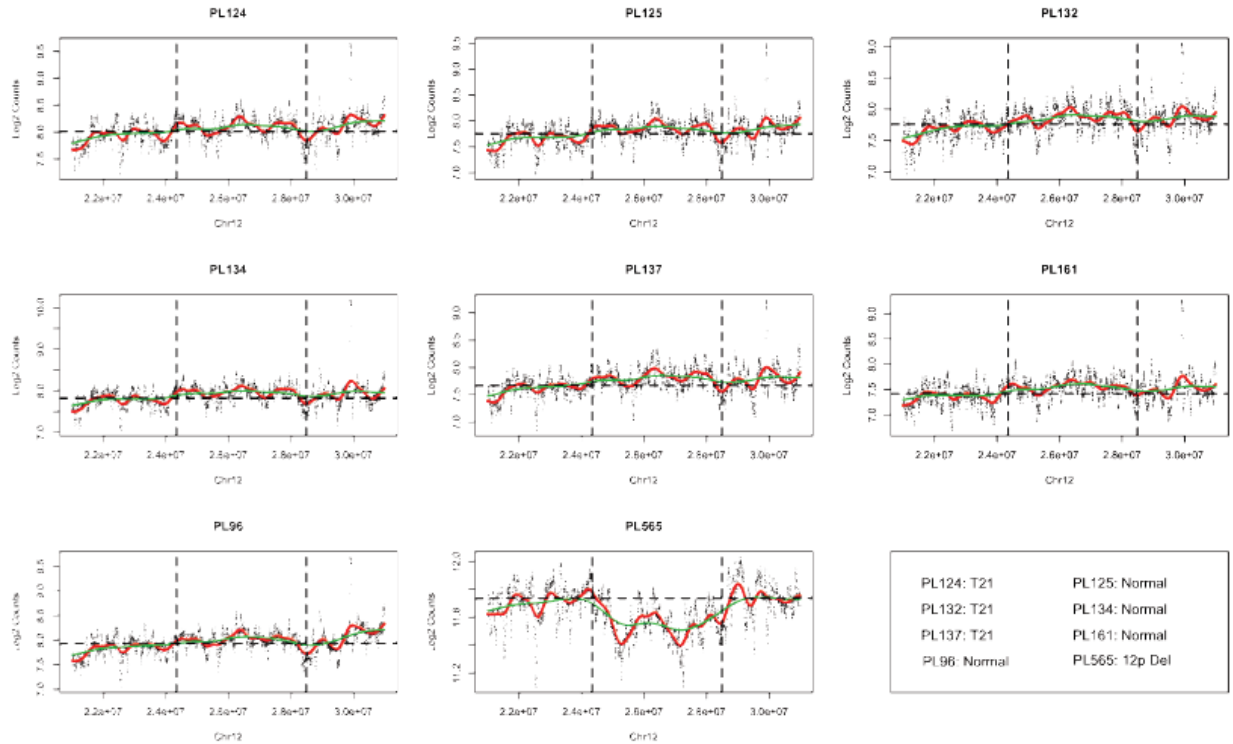_____   _____
Printed Name of Person Obtaining Consent   Role in Research Study

_____   _____
Signature of Person Obtaining Consent   Date
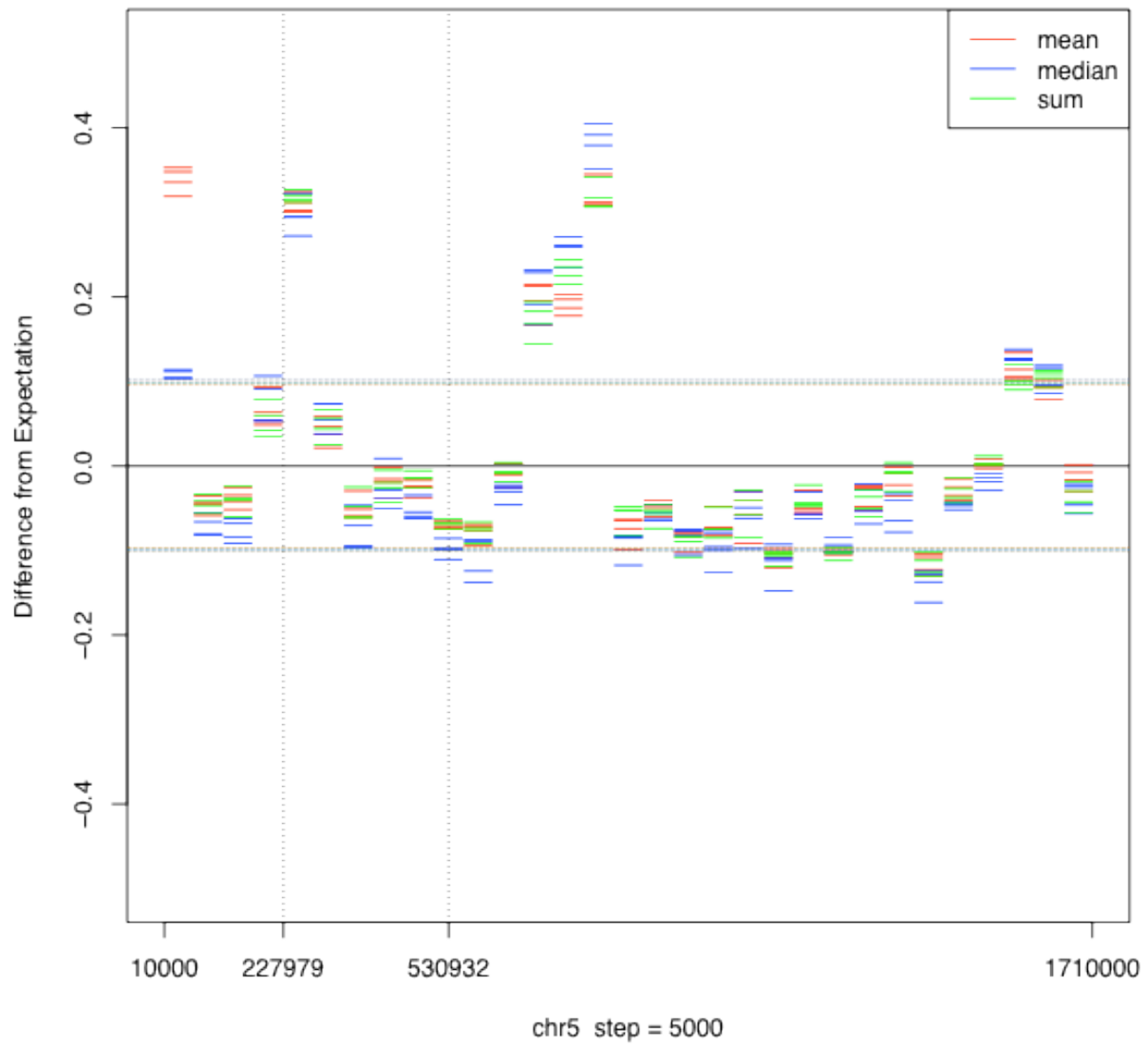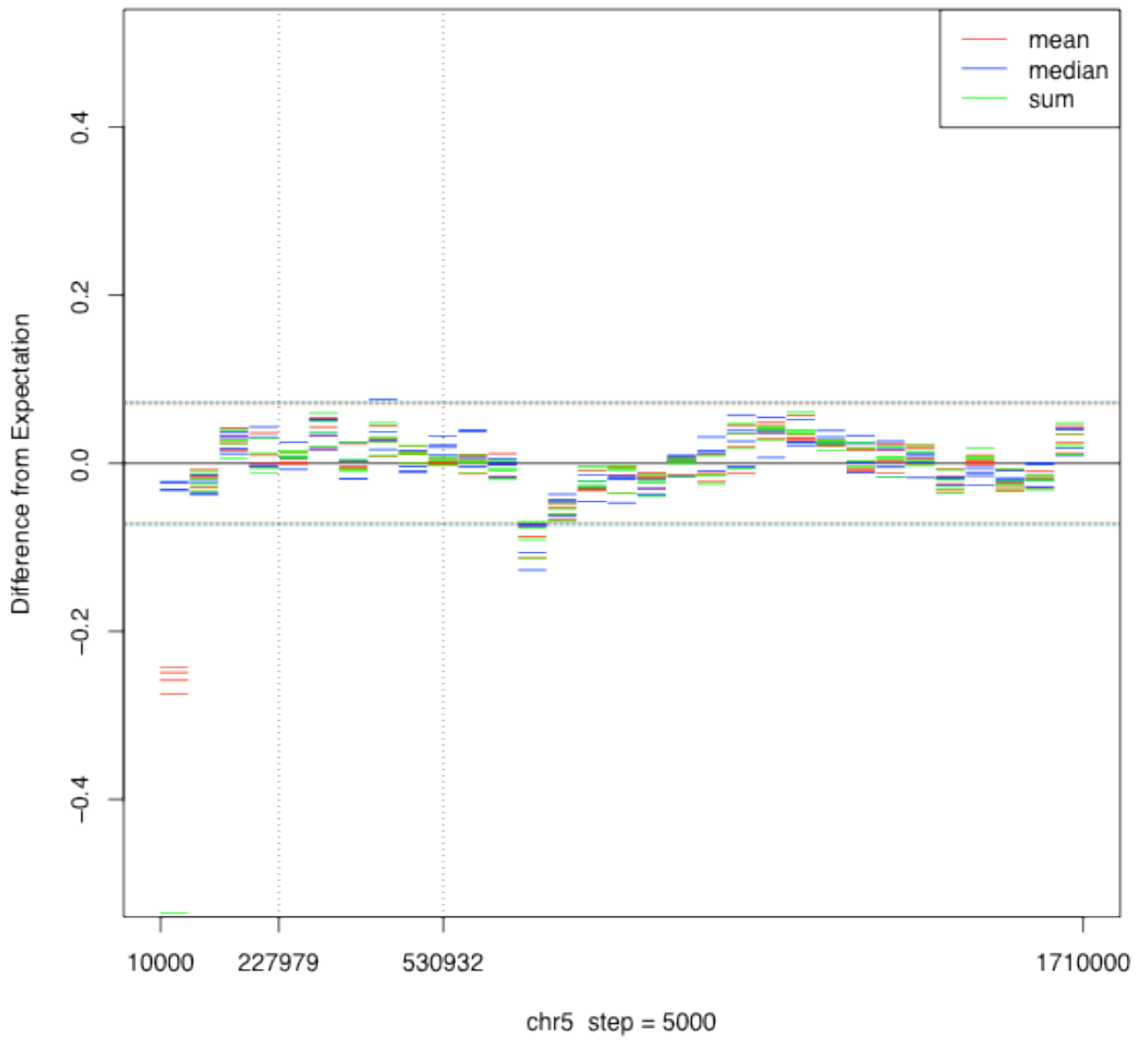
FIGURES

Figure 3. Chromosome 12 Microdeletion Analysis

Reproduced with permission from Peters, D.G., et al., *Noninvasive Prenatal Diagnosis of a Fetal Microdeletion Syndrome.* NEJM, 2011. **365**(19)., [72]. Copyright Massachusetts Medical Society
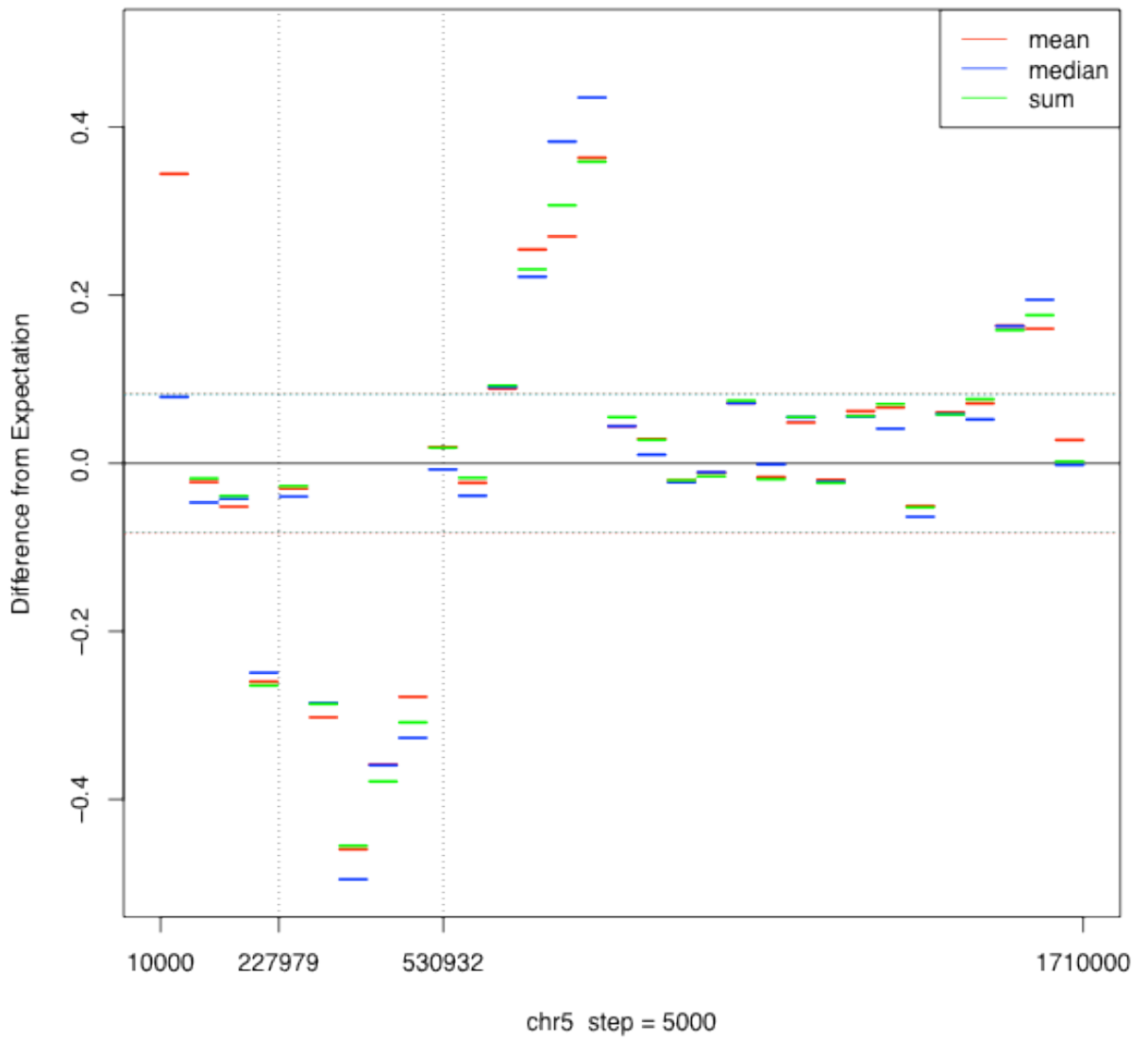
Figure 4. Chromosome 5: Maternal sample with plasma DNA control libraries

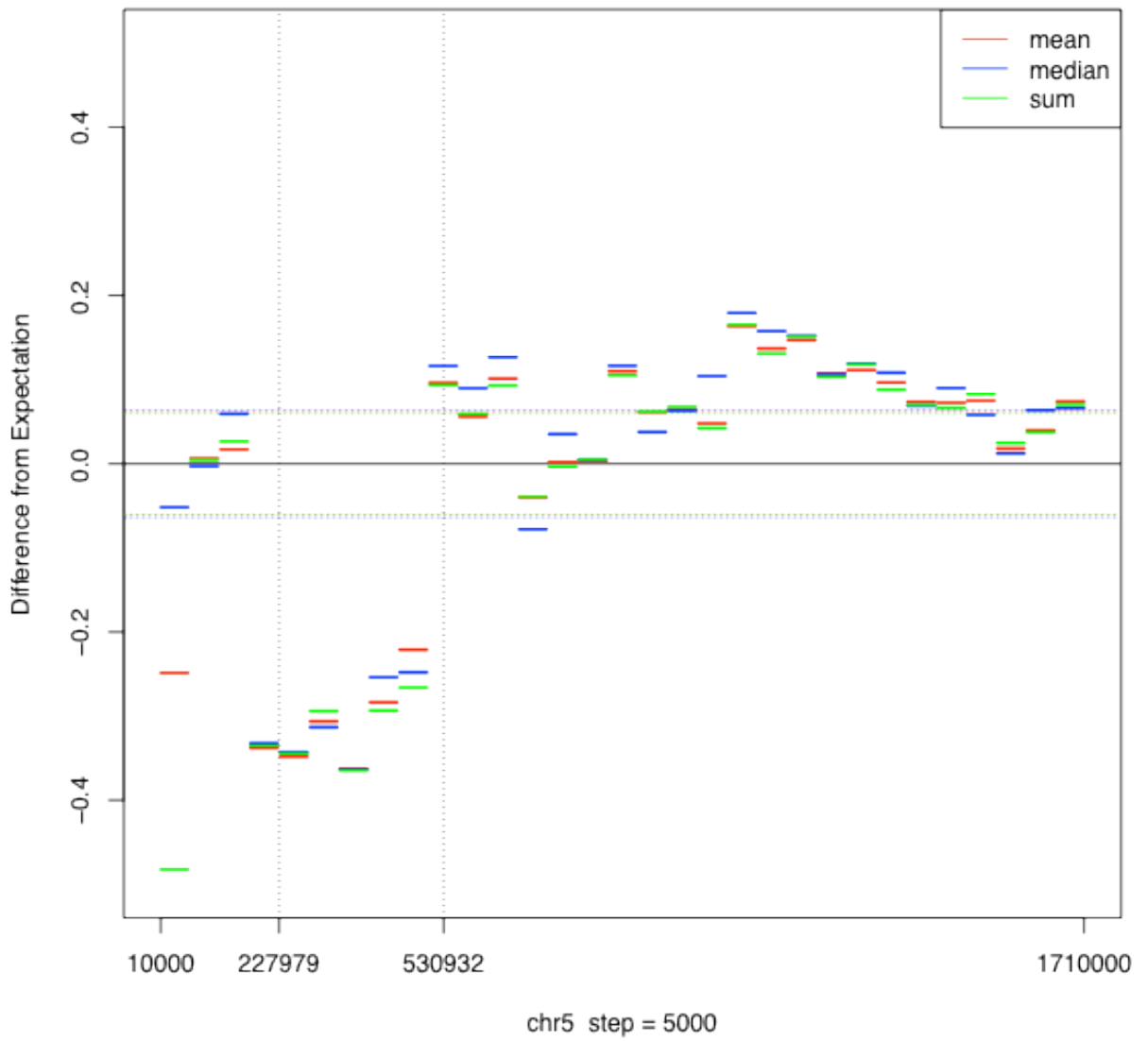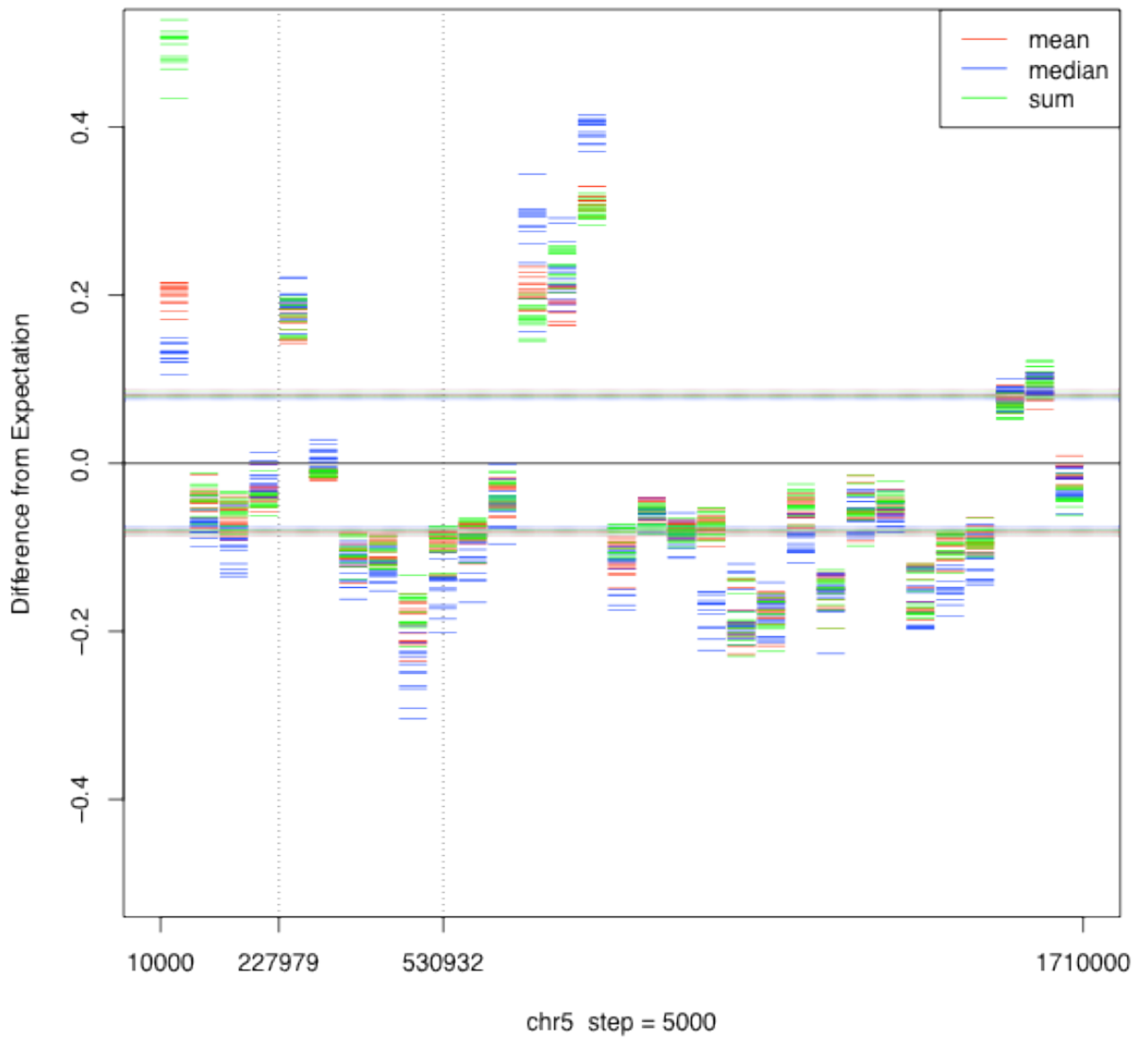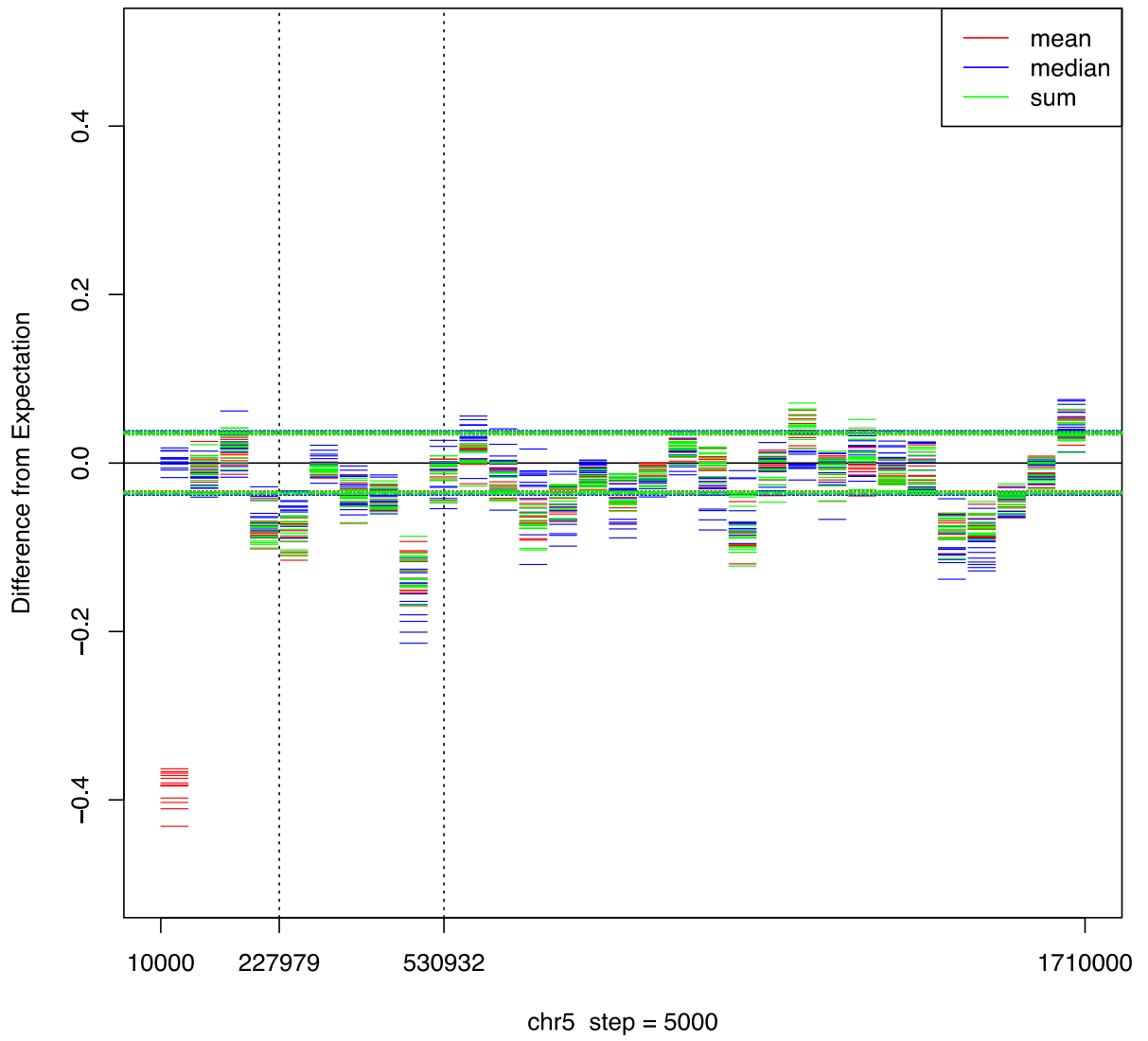**Figure 5. Chromosome 5: Maternal sample with placental DNA control libraries**

Figure 6. Chromosome 5: Fetal sample (by CVS) with plasma DNA control libraries

Figure 7. Chromosome 5: Fetal sample (by CVS) with placental DNA control libraries

Figure 8. Chromosome 5: Maternal plasma sample with plasma DNA control libraries

Figure 9. Chromosome 5: Maternal plasma sample with placental DNA control libraries

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