SHOTGUN NEXT-GENERATION SEQUENCING OF MATERNAL PLASMA: A METHOD FOR PRENATAL ANEUPLOIDY IDENTIFICATION

by

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PURPOSE: Every year, thousands of Americans pursue prenatal diagnosis of fetal aneuploidy though chorionic villus sampling (CVS) or amniocentesis. Because these procedures are invasive and carry an inherent risk for pregnancy loss, they are selectively offered to women who have an increased risk to have a child with a chromosome condition, such as aneuploidy. In order to identify pregnancies at an increased risk, several non-invasive screening methods have been developed. Although quite useful, these screening methods have limited accuracy and can only be completed during specific gestational age windows. Recent discovery of cell free fetal DNA in maternal circulation has created new and exciting possibilities for prenatal screening and non-invasive prenatal diagnosis. This research study explores shotgun next-generation sequencing of fetal DNA in maternal plasma as a method for non-invasive identification of fetal aneuploidy.

METHODS: We carried out shotgun next-generation sequencing on samples of maternal plasma DNA obtained in the first trimester of pregnancies with confirmed aneuploidy and control pregnancies. Three Trisomy 21 samples were compared to four control samples in order to identify any differences in the amount of chromosomal material.

RESULTS: We identified a statistically significant increase in chromosome 21 material in the cases of Trisomy 21 as compared to the control cases.

IMPLICATIONS: This research demonstrates that shotgun next-generation sequencing of maternal plasma DNA can successfully identify Trisomy 21, showing that it is possible to detect fetal aneuploidy using this noninvasive method. This technology could potentially be used as a method of noninvasive screening for fetal aneuploidy, which is likely to have improved accuracy over other screening methods. Development of a screening test with greater sensitivity and specificity could have significant public health implications. This would not only provide more accurate identification of pregnancies at an increased risk for aneuploidy, but it would also reduce the number of false positives. This in turn would reduce the number of pregnancies that are unnecessarily classified as "high risk", preventing avoidable parental anxiety and reducing the number of pregnancies that are put at unnecessary risk of invasive prenatal diagnostic procedures.

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1.0 INTRODUCTION

Chromosome conditions, such as aneuploidy, occur in approximately 20-50% of pregnancies [1-3]. It has been understood that the chance a woman will have a pregnancy with aneuploidy increases with her age [4-7]. Because of this, women are routinely offered screening tests that can help to identify fetuses at an increased risk for having a chromosome condition [8]. One such screening test is the First Trimester Screen, which combines blood work and ultrasound to adjust a women's age-related risk to a risk that is more personalized to her pregnancy [8]. The results of a "positive" screen result often lead to consideration of prenatal diagnosis options, such as Chorionic Villus Sampling or Amniocentesis. These procedures are invasive and carry an inherent risk for miscarriage. Although minimally invasive and low in cost, the First Trimester Screen has a sensitivity of approximately 85% and has a high false positive rate [8-10]. This may lead to unnecessary prenatal diagnostic procedures which, sequentially, would place these pregnancies at unnecessary risk. Recent efforts to improve methods for non-invasive aneuploidy detection have focused on the analysis of cell-free fetal DNA, which is detectable at low levels in the maternal plasma [11-13]. Various techniques for analyzing fetal DNA in maternal serum have been proposed, all with hopes of developing a new method for non-invasive identification of fetal aneuploidy.

In this project, we investigate next-generation sequencing of maternal plasma DNA as a method for non-invasive identification of fetal aneuploidy. This method has the potential to significantly improve sensitivity and specificity over existing prenatal screening methods. We carried out shotgun next-generation sequencing on samples of maternal plasma DNA obtained in the first trimester of pregnancies with confirmed aneuploidy and control pregnancies. Three Trisomy 21 samples were compared to four control samples in order to identify any differences in the amount of chromosomal material.

2.0 AIMS

The overall goal of this paper is to demonstrate the identification of fetal aneuploidy by shotgun next-generation sequencing of maternal serum. The data presented and discussed here provides a foundation for ongoing research by this group regarding non-invasive prenatal identification of fetal aneuploidy by shotgun next-generation sequencing.

3.0 BACKGROUND AND SIGNIFICANCE

3.1 ANEUPLOIDY

Aneuploidy is a type of chromosome condition in which there is an atypical number of chromosomes. [4] Typically, every cell in the human body has 23 pairs of chromosomes, or a total of 46 chromosomes. The first 22 pairs of chromosomes are numbered 1 through 22 and the last are the sex chromosomes, X and Y. One chromosome from each pair is inherited from our mother while the other is inherited from our father, giving the full set of 46 chromosomes. In the case of aneuploidy, there are extra or missing chromosomes in the cells of the body. Having extra or missing chromosomes can have severe effects on the health and function of the human body. Aneuploidy is thought to occur in 20-50% of conceptions, the majority of which are not compatible with life [1-3].

Most cases of aneuploidy are caused by nondisjunction of chromosomes in meiosis 1. When a pair of homologous chromosomes fails to separate and move to opposite poles during meiosis, it results in the uneven division of chromosomes between gametes. If one of these gametes were to be fertilized, it would result in fetal aneuploidy. Nondisjunction occurs spontaneously, but is more likely to occur in a woman's oocytes as she ages [4, 6]. It is well recognized that the risk to have a pregnancy with aneuploidy increases with maternal age [4-7].

3.1.1 Trisomy

Trisomy, which occurs when there are three copies of a chromosome, is the most common type of aneuploidy to result in live births [4]. Although trisomy has been reported in all chromosomes, most are lethal in early pregnancy [2, 3]. The most common types of trisomy seen in live births are Trisomy 21, Trisomy 18, and Trisomy 13 [4].

Trisomy 21, or Down syndrome, occurs when there are 3 copies of chromosome 21, rather than the typical 2 copies. Similarly, Trisomy 18 occurs when there is an extra copy of chromosome 18 and Trisomy 13 occurs when there is an extra copy of chromosome 13. Individuals with Down syndrome can have mild to moderate mental retardation, certain facial characteristics, and health problems, such as heart defects or intestinal problems [4]. Trisomy 18 and Trisomy 13 are not as common as Down syndrome, but are more severe. Babies born with these conditions have severe mental retardation, serious health problems, and typically do not survive very long after birth [4].

3.2 PRENATAL DIAGNOSIS OF ANEUPLOIDY

3.2.1 Non-invasive screening for fetal aneuploidy

Non-invasive screening tests are routinely done as a way to identify pregnancies at an increased risk for fetal aneuploidy, such as Trisomy 21. A variety of prenatal screening methods are

available throughout pregnancy, including First Trimester Screen, Maternal Serum Screening, Integrated Screening, and Ultrasound.

First Trimester Screening (FTS), which is done between 11 and 13 6/7 weeks of pregnancy, helps to identify pregnancies at an increased risk for Trisomy 21 and Trisomy 18 [8]. This test utilizes a combination of measurements obtained from blood work and ultrasound. Firstly, levels of two biochemical markers, called pregnancy-associated plasma protein A (PAPP-A) and human chorionic gonadotropin (hCG), are measured in the mother's blood [8]. Secondly, a measurement of the nuchal translucency (NT), the fluid filled area behind the fetal neck, is obtained by ultrasound [8]. These measurements are combined to adjust a woman's age-related-risk to a risk that is more specific to her pregnancy. It is estimated that the sensitivity of FTS is approximately 79-90% with a false positive rate of 5% [8-10].

Maternal Serum Screening (MSS) is done between 15-20 weeks of pregnancy and can help identify pregnancies with an increased risk for Trisomy 21 and Trisomy 18, as well as open neural tube defects (ONTDs) [8]. The most commonly practiced type of MSS is the Quad Screen, which measures levels of four biochemical markers in the mother's blood, called alphafetoprotein (AFP), human chorionic gonadotropin (hCG), unconjugated estriol (uE3), and inhibin-A [8]. This test detects approximately 75-80% of babies with Trisomy 21 [8, 14].

Integrated Screening is a screening test that combines information typically obtained for FTS and MSS. The Integrated Screen combines measurements of PAPP-A and NT in the first trimester along with measurements of AFP, hCG, uE3, and inhibin A in the second trimester [8]. The results of this test are not reported until the second trimester, after all of the necessary information has been collected and analyzed [8]. Although Integrated Screening takes the

longest to complete, it provides the highest sensitivity of all the screening methods, detecting approximately 93-96% of pregnancies with Trisomy 21 [15, 16].

Ultrasound can also be a useful screening tool for fetal aneuploidy. Ultrasounds done between 18-20 weeks can visualize certain features that are commonly seen in fetuses with chromosome conditions, such as heart defects, choroid plexus cysts, and other physical features [8, 17]. However, ultrasounds have a low sensitivity and specificity for detecting pregnancies with a chromosome condition and are not typically used as the primary screening tool for fetal aneuploidy [17, 18].

3.2.2 Invasive prenatal diagnosis of fetal aneuploidy.

Prenatal diagnosis of fetal aneuploidy is done by testing fetal cells, which can be obtained by two different procedures: chorionic villus sampling (CVS) or amniocentesis. These procedures are invasive and carry a risk for pregnancy loss, which is estimated to be between 0.1%-1.0% [19-22]. Thus, prenatal diagnosis is not routinely offered during pregnancy. Traditionally, women are offered prenatal diagnosis if they are 35 years or older at the time of delivery or if they are identified as "high risk" based on a screening test.

CVS is done between 10-13 weeks gestation and can diagnose chromosome conditions with about 99.9% accuracy [8]. The procedure, which can be done transcervically or transabdominally, involves collecting a sample of the placenta. The placental cells are grown in culture and then analyzed with traditional cytogenetic methods, such as fluorescent *in situ* hybridization (FISH) and karyotyping, to identify any chromosome conditions [8].

Like CVS, amniocentesis can diagnose chromosome conditions with about 99.9% accuracy. This test, which is done any time after the 16th week of pregnancy, involves the transabdominal collection of a sample of amniotic fluid [8]. The cells in this fluid, called amniocytes, are grown in culture and then analyzed with traditional cytogenetic methods to identify any numerical or structural changes in the chromosomes. [8]

3.3 NEW DIRECTION: NON-INVASIVE PRENATAL DIAGNOSIS

3.3.1 Cell free fetal DNA in maternal plasma.

Cell free fetal DNA was first discovered in maternal circulation in 1997 [11], creating new and exciting possibilities for non-invasive prenatal diagnosis (NIPD). Studies have estimated that the fetal DNA represents approximately 5-10% of the cell free DNA present in maternal plasma [12, 13].

Following the discovery of cell free fetal DNA in maternal circulation, efforts were initially focused on detection and measurement of paternally derived fetal DNA, like the Y chromosome, because it is easily distinguishable from maternal DNA [23, 24]. Developments in this research have begun to take place in clinical practice. For example, cell free fetal DNA may now be analyzed as a non-invasive method for fetal sex determination, a test often used as a screening step for X-linked disorders [23, 24]. Cell free fetal DNA has also been clinically used to verify fetal Rhesus D blood group status in Rh negative mothers, which can determine the

necessity of Rhogam [25-27]. More recently, analysis of cell free fetal DNA has correctly diagnosed paternally inherited thalassemia and achondroplasia [28, 29].

3.3.2 Proposed methods of NIPD using cell free fetal DNA.

Since the discovery of cell free fetal DNA in maternal circulation, multiple methods for NIPD of fetal aneuploidy have been explored. The most significant obstacle preventing progress towards this goal is the fact that maternally inherited fetal alleles are identical in sequence to their endogenous maternal counterparts. Therefore, recent efforts in the field have focused on the use of functional genomic screens to identify potential diagnostic biomarkers on a neuploid chromosomes. This strategy relies on the observation that the maternal DNA present in the plasma mostly originates from maternal leukocytes while fetal DNA in the plasma originates from the trophoblast or placenta [30-33]. Studies have identified differences in gene expression or DNA methylation patterns between placental cells and maternal leukocytes, which can be useful in differentiating between maternal and fetal DNA [34-36]. These unique differences between fetal DNA and maternal DNA can be used for selective amplification of heterozygous fetal polymorphisms, whose allelic ratios are then quantified for the diagnosis or exclusion of aneuploidy [36-38].

Although these methods are promising, they are technologically complex and face practical limitations. Firstly, these methods require independent identification of each potential biomarker, which must then be extensively validated. Secondly, methods requiring allelic ratio determination at biallelic loci only work if the fetus is heterozygous for the analyzed SNPs.

Also, these approaches may be confounded by potential individual differences in allelic-specific DNA methylation of mRNA expression.

3.3.3 Shotgun next-generation sequencing.

Shotgun next-generation sequencing has recently been explored as a method for NIPD using cell free fetal DNA [39-42]. This method utilizes random sequencing of fetal and maternal DNA fragments that freely circulate in maternal plasma. This "shotgun sequencing" creates sequence tags of at least 36 base pairs, each representing a small sequence of cell free DNA fragments. More than 10 million sequence tags are produced, which are then computationally aligned to their chromosomal origin in the human genome. Perfectly aligned matching tags are then quantified according to their chromosome-specific or region-specific location in the genome. This makes it possible to identify small alterations in inter-chromosomal ratios which may be due to fetal aneuploidy. This concept is outlined in Figure 1 [39].

Unlike other methods of analyzing cell free fetal DNA, shotgun next-generation sequencing does not rely on epigenetic differences and does not require pretest identification of biomarkers. The extensive work up that is necessary for other noninvasive methods is not required for shotgun next-generation sequencing because this method simply sequences a random portion of the cell free DNA in the maternal plasma [39]. Despite the randomness of the sequencing, the fraction that is sequenced is quite representative of the overall human genome and shotgun next-generation sequencing has been previously shown to be effective and accurate at identifying fetal aneuploidy [39-42].

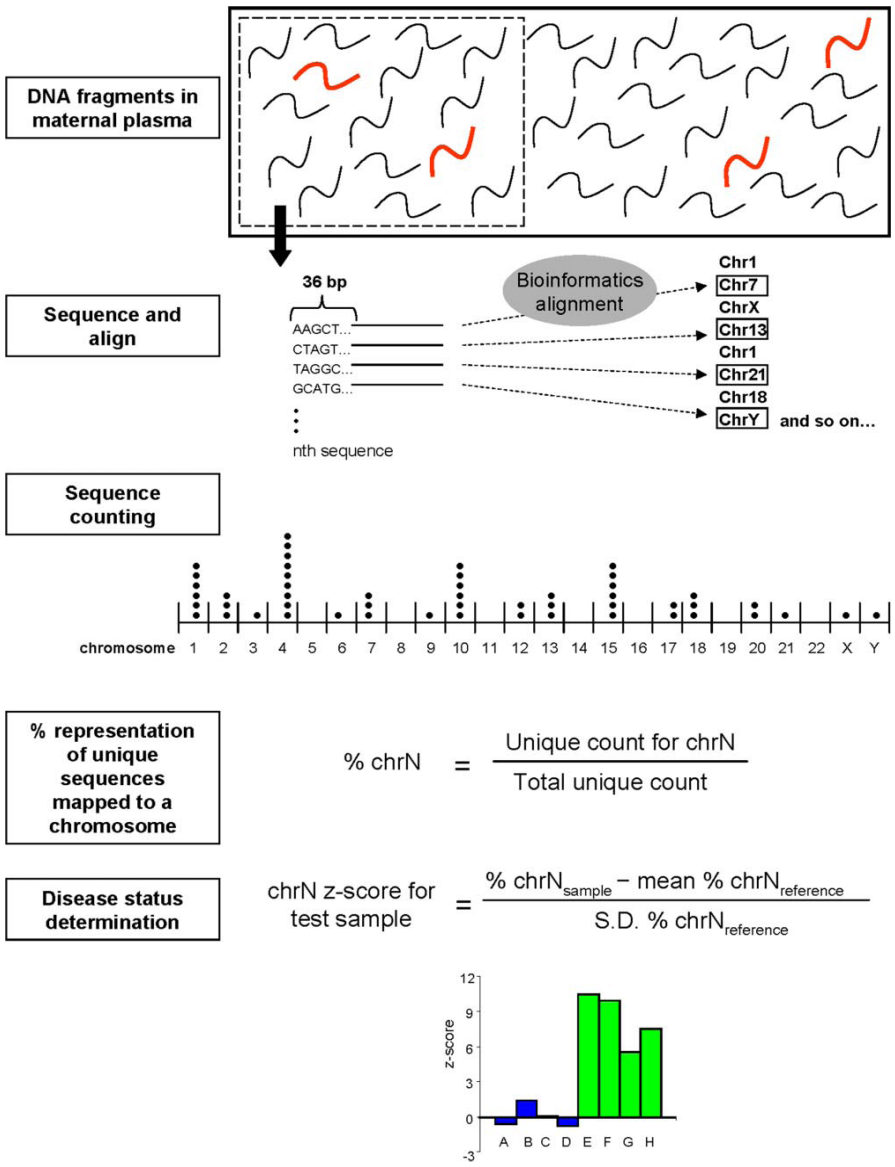

Figure 1: Schematic illustration of the procedural framework for using massively parallel genomic sequencing for the noninvasive prenatal detection of fetal chromosomal aneuploidy.
Fetal DNA (thick red fragments) circulates in maternal plasma as a minor population among a high background of maternal DNA (black fragments). A sample containing a representative profile of DNA molecules in maternal plasma is obtained. One end of each plasma DNA molecule was sequenced for a[pproximately 36 bp using the Solexa sequencingby-synthesis approach. The chromosomal origin of each 36-bp sequence was identified through mapping to the human reference genome by bioinformatics analysis. The number of unique sequences mapped to each chromosome was counted and then expressed as a percentage of all unique sequences generated for the sample, termed%chrN for chromosome N. *Z*-scores for each chromosome and each test sample were calculated using the formula shown. The *z*-score of a potentially aneuploid chromosome is expected to be higher for pregnancies with an aneuploid fetus (cases E–H shown in green) than for those with a euploid fetus (cases A–D shown in blue).

4.0 MATERIALS AND METHODS

4.1 DATA COLLECTION

4.1.1 Patient population.

The patient population consists of pregnant women over the age of 18 years who were seen in at Magee Women's Hospital in Pittsburgh, Pennsylvania between 10-13 weeks gestation. Two populations of patients were invited to participate in the research: 1) patients who were undergoing CVS or amniocentesis and 2) patients who were undergoing First Trimester Screening. Theoretically, patients who choose to pursue prenatal diagnosis through CVS or amniocentesis may do so because they have an increased risk for aneuploidy. Therefore, this population of patients may represent a "high risk" group and may be more likely to provide our laboratory with positive controls. Alternatively, those undergoing only First Trimester Screen may represent a "low risk" group and may be more likely to provide our laboratory with negative controls.

4.1.2 Participant recruitment and informed consent.

All participant recruitment and informed consent was obtained in the Center for Medical Genetics at Magee Women's Hospital in Pittsburgh, Pennsylvania under IRB protocol number PRO0709003. Patients were recruited by genetic counselors and genetic counseling interns either during a genetic counseling appointment or as a short consult.

For individuals undergoing genetic counseling, participation in this research was offered by the genetic counselor at the end of the appointment. For individuals having only First Trimester Screen and no counseling, participation was offered by a genetic counseling intern (who is employed as a patient recruiter by the laboratory) before the blood draw portion of the First Trimester Screen.

Informed consent involved a discussion of the background and aims of the research project as well as participation requirements, participant rights, and sample handling. After discussing the risks and benefits of participation in the research study, participants were given ample time to read the consent form (Appendix A), ask questions, and voice concerns. Consenting patients provided initials on each page of the consent form as well as a signature, printed name, and date on the last page. The individual obtaining informed consent also provided a signature, printed name, and date on the last page of the consent form.

A copy of each consent form is kept in the patient's chart at Magee Women's Hospital while the original copy is filed in a locked cabinet at the research laboratory. No sample was obtained without the consent of a patient.

4.1.3 Sample collection and tracking.

Following informed consent, participants were provided a script for blood work and were taken to the phlebotomy lab. Approximately 36 CC of blood was drawn from each patient, filling approximately 6 purple top tubes. Samples were then transferred to the research laboratory at Magee Women's Research Institute within 2 hours of collection for further processing.

Documentation of each blood draw and sample transfer to Magee Women's Research Institute for this research was recorded in the patients' Reference Lab on Magee's Genetic Information System (GIS). Upon entering the laboratory, all samples are de-identified and given a unique bar code, which will be used to track the patient's blood sample, plasma-derived DNA, sequencing library, and the resulting data. This unique bar code will also be linked to all available clinical and demographic information including diagnostic test results and birth outcome.

4.2 SAMPLE PROCESSING

4.2.1 Separation of plasma from whole blood.

Whole blood was centrifuged at 1600xg for 13 min at 4°C, setting acceleration and deceleration to 3. 1ml aliquots of plasma were pipetted into 1.5ml microcentrifuge tubes and centrifuged at 16000xg for 10 min at 4°C to pellet cellular debris. 900uL from each tube was pipette into a clean 1.5ml microcentrifuge tube. Plasma aliquots were stored at -80°C.

4.2.2 DNA extraction from plasma.

DNA was extracted from plasma using the QIAamp DNA Multi Kit and supplied reagents. Briefly, 1 vial of frozen plasma was thawed to room temperature and divided into two tubes. 40uL of Qiagen Protease was added to each tube and the sample was mixed by inverting 5 times. 400uL Buffer AL was added to each tube and the sample was vortexed for 15 sec. Samples were incubated at 56°C for 10 min. 400uL 100% ethanol was added to each tube and the tubes were vortexed for 15 sec. 600uL of mixture was applied to the spin column and centrifuged at 6000xg for 1 min, repeating this step as many times as necessary to get the entire plasma sample through the same column. The column was washed by adding 500uL Buffer AW and centrifuging for 1 min at 6000xg. The column was washed again by adding 500uL Buffer AW2 and centrifuging at maximum speed for 4 min. To remove residual ethanol, the column was placed in a clean collection tube and centrifuged at maximum speed for 2 min. To elute the DNA, the column was placed in a 1.5mL tube and 75uL 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.

4.2.3 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 an 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 6.4pg/5uL to 20,000pg/5uL. Each real time PCR reaction consisted of: 12.5uL 2x TaqMan Universal PCR Master Mix, 1.25uL 10uM forward primer, 1.25uL 10uM reverse primer, and 0.0625uL 100uM probe. 10uL plasma DNA, 5uL standards, or 10uL 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 System (Applied Biosystems).

4.2.4 Plasma sequencing.

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 95°C for 5 minutes and then slowly cooled to room temperature for a final concentration of 36μM 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 (NEB). Following reaction cleanup using the MinElute Cleanup kit (Qiagen), DNA fragments were then combined with 0.05uM adaptor and 400U T4 DNA ligase (NEB) and incubated for 1 hour at 16°C. 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 (NEB). PCR conditions were an initial denaturation (98°C 30s), 30 cycles of 98°C for 10s, 65°C for 30s and 72°C for 30s, with a final extension of 72°C for 7 min. Following amplification, the PCR reaction was cleaned up using the MinElute PCR Purification Kit (Qiagen).

4.3 DATA ANALYSIS

4.3.1 Analytical framework

The data analyzed and discussed in this paper is based on the comparison of three known Trisomy 21 cases against four normal samples. The data was gathered on the Illumina Genome Analyzer IIx and was analyzed using the statistical model proposed in a previous publication by our lab [40]. The data discussed in this paper was analyzed based on the null hypothesis that the amount of genetic material in a given chromosome is equal in the aneuploidy and control samples. This null hypothesis is individually applied to each chromosome. A p value of less

than 0.05 for a specific chromosome allows us to reject the null hypothesis and accept the alternative hypothesis that the amount of genetic material is not equal between the compared samples, indicating aneuploidy of that chromosome.

5.0 RESULTS

5.1.1 The Biobank and Sample Statistics.

Recruiting efforts for this research study are ongoing, with the goal of consenting 1350 pregnant women who are having CVS or Amniocentesis. Currently, a total of 592 patients have given their consent to participate in this study. Of those recruited, 183 participants have a prenatal diagnostic procedure and 409 had First Trimester Screening only (Table 1). Therefore, our Biobank currently holds 13.5% of our projected total inventory.

Table 1: Prenatal Testing Pursued by Participants

Testing Pursued by Participants	Number of Participants	Percentage of Participants
CVS	116	19.59%
Amniocentesis	67	11.32%
First Trimester Screen only	409	69.09%

Of the 592 samples collected, there are 16 known cases of aneuploidy (Table 2). The fetal karyotypes of these cases were confirmed by cytogenetic testing following CVS or amniocentesis.

Table 2: Known Aneuploidy Cases in Sample Population

Type of Aneuploidy	# of cases
Monosomy X (Turner Syndrome)	1
Trisomy 18 (Edwards syndrome)	3
Trisomy 13 (Patau syndrome)	1
Trisomy 14	1
Trisomy 21 (Down syndrome)	10

To ensure that our research produces quality data, it is important that we have an adequate collection of aneuploid samples in our Biobank. Currently, our Biobank contains a fraction of the aneuploid samples we anticipated. In order to increase our inventory of aneuploid samples, we hope to improve our recruitment of patients having CVS and amniocentesis. In theory, patients who elect these procedures may do so because they are at an increased risk to have a baby with a chromosome condition. Thus, the likelihood of collecting an aneuploid sample for our study is highest in this population.

5.1.2 Shotgun sequencing results.

Shotgun next-generation sequencing was done on three known cases of Trisomy 21 (samples pl96, pl125, and pl134) and four normal samples (pl124, pl132, pl137, and pl161). The amount of chromosomal material in each sample was quantified for chromosomes 1-22. The value

Table 3: P values of Trisomy 21 samples vs. normal samples at chromosomes 1-22.

	Trisomy 21 samples vs. Normal samples											
	Sample pl96 versus:				Sample pl125 versus:				Sample pl134 versus:			
Chromosome	pl124	pl132	pl137	pl161	pl124	pl132	pl137	pl161	pl124	pl132	pl137	pl161
1	1	1	1	1	1	1	1	1	1	1	1	1
2	1	1	1	1	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1	1	1	1
6	1	1	1	0.540655	1	1	1	0.739549	1	1	1	0.141622
7	1	1	1	1	1	1	1	1	1	1	1	1
8	1	1	1	1	1	1	1	1	1	1	1	1
9	0.756034	1	1	1	1	1	1	1	1	1	1	1
10	1	1	1	1	1	1	1	1	1	1	1	1
11	1	1	1	0.956444	1	1	1	1	0.296221	0.102155	1	1
12	1	1	1	1	1	1	1	1	1	1	1	1
13	1	1	1	1	0.865717	0.180907	1	1	1	1	1	1
14	1	1	1	1	1	1	1	1	1	1	1	1
15	1	1	1	1	1	1	1	1	1	1	1	1
16	1	1	1	1	1	1	1	1	1	1	1	1
17	1	1	1	1	1	1	1	1	1	1	1	1
18	1	1	1	1	1	1	1	1	1	0.563806	1	1
19	1	0.486549	1	1	1	0.929166	1	1	1	1	0.179956	1
20	1	0.327722	1	1	1	1	1	1	1	1	1	1
21	1.32E-13	2.93E-14	4.32E-12	3.72E-10	1.95E-14	1.95E-13	0	1.66E-13	1.31E-06	1.56E-05	1.52E-07	4.50E-08
22	1	1	1	1	0.310996	0.561283	1	1	1	1	1	1

obtained for each known Trisomy 21 sample were then compared to the values obtained for all four normal samples. A p value was calculated for each comparison at chromosomes 1-22. As shown in Table 3 (above), the p value for chromosome 21 was statistically significant in each example, indicating aneuploidy at this chromosome. Therefore, shotgun next-generation sequencing was able to correctly identify all three cases of Trisomy 21. These results show that this method has great promise as a noninvasive tool for identification of fetal aneuploidy.

6.0 DISCUSSION

6.1 PUBLIC HEALTH IMPLICATIONS

We demonstrated that shotgun next-generation sequencing of maternal plasma DNA can successfully identify Trisomy 21, showing that it is possible to detect fetal aneuploidy using this noninvasive method. This technology could potentially be used as a new method of noninvasive screening for fetal aneuploidy, which is likely to have improved accuracy over other screening methods. Development of a screening test with greater sensitivity and specificity could have significant public health implications. This would not only provide more accurate identification of pregnancies at an increased risk for aneuploidy, but it would also reduce the number of false positives. This in turn would reduce the number of pregnancies that are unnecessarily classified as "high risk", preventing avoidable parental anxiety and reducing the number of pregnancies that are put at unnecessary risk of invasive prenatal diagnostic procedures.

Furthermore, this research lends to world-wide efforts in developing a method for non-invasive prenatal diagnosis (NIPD) of fetal aneuploidy. Such a development could potentially eliminate the need for CVS and amniocentesis, allowing prenatal diagnosis of aneuploidy without the risk for pregnancy loss. Research groups around the world are racing to develop the first clinically applicable method of NIPD. Although countless methods have been explored, it

has been predicted that shotgun next-generation sequencing will become the dominant technology for NIPD [23].

6.2 FUTURE DIRECTION OF THIS RESEARCH

6.2.1 Direct extension of this project.

The data presented and discussed in this paper provides a foundation for ongoing research by this group. As we approach our recruitment goal, we will perform shotgun next-generation sequencing on samples of maternal plasma DNA obtained from large cohorts of confirmed aneuploidy and control pregnancies. Following this, our research group will use novel statistical analysis to determine the sensitivity and specificity of shotgun next-generation sequencing, which will then be compared to the sensitivity and specificity of First Trimester Screen. Based on the data discussed in this paper, we predict that shotgun next-generation sequencing will have high predictive powers and will identify fetal aneuploidy with greater accuracy than the First Trimester Screen.

Our research group also hopes to develop user-friendly software for non-invasive detection of aneuploidy. This software will use the data obtained from shotgun next-generation analysis as input and will use a graphical user interface implemented in Java. The final report will include test scores and the clinical interpretation of the test scores. Development of this software is an important step in the clinical application of shotgun next-generation sequencing for identification of fetal aneuploidy.

6.2.2 Future directions.

Our research group has also developed an interest in using shotgun next-generation sequencing as a method for prenatal diagnosis of monogenic diseases. We have recently begun to invite a small population of patients who have a personal or family history of a known or suspected genetic condition to participate in pilot study that would investigate this. Paternal samples may be collected along with maternal samples for this study. Maternal consent for this pilot study is included in the same consent form used for the project discussed in this paper (Appendix A). Fathers who are consenting have a different consent form, which is not attached to this document.

6.3 CONSIDERATIONS OF NONINVASIVE PRENATAL DIAGNSOIS

As noninvasive prenatal diagnosis (NIPD) works toward transition from "possible" to "clinically applicable", there are many factors that should be considered. Such a transition would change not only the way prenatal diagnosis is done, but also the way we approach prenatal diagnosis. NIPD could provoke drastic changes in the well-established clinical standards of Prenatal Care and would undoubtedly have effects on the way genetic counselors provide prenatal genetic counseling to these patients.

6.3.1 Clinical and Practical considerations.

Currently, the American Congress of Obstetrics and Gynecology (ACOG) states that all pregnant women should be offered First Trimester Screen, regardless of their age. ACOG also states that CVS and amniocentesis should be offered to women who are over the age of 35 or have an increased risk for aneuploidy based on screening results. It is interesting to consider what ACOG would recommend should noninvasive prenatal diagnosis (NIPD) become the new standard of fetal aneuploidy diagnosis. If NIPD reaches its intended potential, it would have all the prenatal diagnostic capabilities of traditional cytogenetic methods. This would completely eliminate the need for fetal cells, and would therefore eliminate the need for CVS and amniocentesis. One could then ask whether screening methods, such as First Trimester Screen, would be indicated. If these screening tests exist to identify who should be offered prenatal diagnosis in attempt to reduce the number of pregnancies placed at unnecessary risk for miscarriage, one could deduce that this type of screening would not be necessary for NIPD, as this procedure would not carry a risk for pregnancy loss. Therefore, NIPD could not only eliminate the need for CVS and amniocentesis, but it could also eliminate the need for noninvasive screening tests like the First Trimester Screen. Because ACOG states that all patients should be offered screening, becomes possible that all pregnant women could be offered NIPD. This type of change would undoubtedly have an effect on the way that genetic counselors provide prenatal counseling to patients.

6.3.2 Implications for Genetic Counseling

It is not uncommon for First Trimester Screen to be ordered by a physician with little to no pretest counseling. Only women who are over the age of 35 are routinely offered genetic counseling prior to screening, while most others only have counseling if their First Trimester Screen is screen positive. Regardless, genetic counseling is always provided before CVS and amniocentesis in order to thoroughly discuss the risks, benefits, and implications of prenatal diagnosis and to obtain informed consent. This process has thus far been effective and pragmatic.

However, a new plan for providing prenatal genetic counseling would be required if NIPD becomes clinically applicable. If NIPD becomes offered to all pregnant women, it is possible that the test could be ordered by a physician with little to no pretest counseling. Should a patient then have a positive result, counseling would only be provided post-test. Such a structure would impose upon a woman's reproductive rights and raise ethical issues concerning autonomy, informed consent, and the "right not to know".

Although NIPD does not carry a risk for pregnancy loss, the potential impact of a positive result is enough to warrant extensive pretest genetic counseling. It is essential that the patient be properly informed of NIPD and the results it could generate before having this test. Pretest counseling should include a discussion about the implications of aneuploidy, whether the patient would want to know this information, and what she would do with the information.

However, if NIPD becomes clinically available to all pregnant women, we could expect the uptake of this test to be significant. Genetic counseling is a relatively small field and many areas in the country do not have this service. Therefore, it may not be practically feasible to

provide sufficient pretest genetic counseling to every patient who pursues NIPD. Thus, the issues of "*When* and *how* do we counsel these patients?" may quickly become an issue of "*Who* will counsel them?" This situation would create a greater need for prenatal genetic counselors, which may not be financially achievable for some hospitals and clinics. Consequently, we may be faced with a situation in which patients are either briefly counseled by physicians or not counseled at all prior to NIPD of fetal aneuploidy.

6.4 CONCLUSION

The data presented in this paper demonstrates that shotgun next-generation sequencing can correctly identify Trisomy 21, showing that it is possible to identify fetal aneuploidy using this noninvasive method for cell free fetal DNA analysis. This technique has potential as a new noninvasive screening method for fetal aneuploidy, which is expected to have increased sensitivity and sensibility over existing screening methods. This technique also has potential as a method for noninvasive prenatal diagnosis (NIPD). Should shotgun next-generation sequencing of maternal plasma become clinically applicable as a screening or diagnostic test, its implementation would have effects on the well-established clinical standards of prenatal care. Careful consideration should be given to the potential impact of these noninvasive tests so that clinicians, genetic counselors, and other health care professionals can provide the highest standard of care possible.

APPENDIX

University of Pittsburgh

SCHOOL OF MEDICINE

Department of Obstetrics, Gynecology and Reproductive Sciences

CONSENT TO ACT AS A PARTICIPANT IN A RESEARCH STUDY

TITLE: Biological Analysis of Fetal Nucleic Acids in Maternal Plasma

Mother's Consent Form

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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 1350 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?

Screening Procedures:

None

Experimental Procedures:

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.

Monitoring/Follow-up Procedures:

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.

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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

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