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Insights Into Fetal and Neonatal Development Through Analysis of Cell-Free RNA in Body Fluids

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Abstract

The use of cell-free nucleic acids in the circulation of pregnant women for noninvasive prenatal diagnosis is arguably one of the hottest current topics in prenatal medicine. Between 1997 and the present era this field has gone from basic research to clinical application for diagnosis of fetal gender and Rhesus D status. Over the next few years it is likely that noninvasive prenatal diagnosis for Down syndrome will also be possible. Here we summarize current and future clinical applications of analyzing cell-free fetal DNA and RNA in both maternal and neonatal body fluids, including maternal plasma, serum, whole blood, amniotic fluid, and neonatal saliva. We describe methods to evaluate normal and abnormal fetal and neonatal development using gene expression microarrays. We also discuss the ways in which differentially-regulated gene lists can advance knowledge of both fetal and neonatal biology, as well as suggest novel possibilities for fetal and neonatal treatment.

Keywords

noninvasive prenatal diagnosis; cell-free fetal nucleic acids; gene expression; amniotic fluid; neonatal saliva

The use of cell-free nucleic acids in the circulation of pregnant women for noninvasive prenatal diagnosis is arguably one of the hottest current topics in prenatal medicine. Between 1997¹ and the present era this field has gone from basic research to clinical application for the noninvasive prenatal diagnosis of fetal gender and Rhesus D status. Over the next few years it is possible that noninvasive prenatal diagnosis for Down syndrome will also be accomplished through measurement of chromosome 21 DNA sequences that originate in the fetus but circulate in maternal plasma. In this review article we summarize current and future clinical applications of analyzing cell-free fetal DNA and RNA in both maternal and neonatal body fluids, including maternal plasma, serum, whole blood, amniotic fluid, and neonatal saliva. We describe methods to evaluate normal and abnormal fetal and neonatal development using gene expression microarrays. We also discuss the ways in which differentially-regulated gene lists can advance knowledge of both fetal and neonatal biology, as well as suggest novel possibilities for fetal and neonatal treatment.

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Cell-free Nucleic Acids in the Blood of Pregnant Women

DNA

There is already an extensive body of literature on cell-free fetal DNA in maternal serum and plasma (reviewed in 2). Essential facts include the following: both fetal and maternal cell-free DNA are detectable in the maternal circulation during gestation, fetal DNA can be detected as early as 32 days of gestation in maternal plasma, and fetal cell-free DNA is easily extracted and quantified using real-time polymerase chain reaction (PCR) amplification techniques. It is known that cell-free DNA is fragmented and that the fetal DNA fragments are considerably smaller than the corresponding maternal DNA fragments. In addition, it is believed that fetal DNA circulates in nucleosomes that likely derive from ruptured apoptotic bodies. The concentration of cell-free fetal DNA increases in maternal blood as gestation advances. Levels increase in the first trimester by approximately 21% per week, plateau in the second trimester, and increase sharply prior to delivery. Cell-free fetal DNA is rapidly cleared in the maternal circulation with a 16–30 minute half life in the healthy pregnant woman.³ This rapid turnover implies that fetal DNA is continuously liberated into the maternal circulation, and provides a real-time picture of fetal DNA production and clearance. Multiple studies have shown that if the proper centrifugation techniques are used, cell-free fetal DNA is virtually undetectable within hours of delivery. Therefore, cell-free fetal DNA is considered to be a biomarker of the current pregnancy. In contrast, intact fetal cells circulate in the maternal blood and reside in maternal organs for decades post partum.⁴

Measurement of cell-free DNA levels in the setting of fetal or maternal complications may ultimately contribute to noninvasive diagnosis. For example, women who are carrying fetuses with Down syndrome have approximately twice as much cell-free DNA present in their circulation. Furthermore, increased levels of circulating fetal DNA are associated with a number of pathological conditions including hemolytic anemia, elevated liver enzymes, low platelets (HELLP) syndrome, pre-eclampsia, and placental abnormalities such as placental accreta. The clearance of fetal cell-free nucleic acids is delayed in women with preeclampsia.

There are multiple pieces of evidence that suggest that most of the cell-free fetal DNA that is detected in maternal blood originates from the placenta. First, cell-free fetal DNA is detectable in the maternal circulation even before the placental circulation is established. Second, cell-free fetal DNA is detectable in the maternal circulation in anembryonic gestations. Interestingly, in cases of confined placental mosaicism, the DNA sequences that are detected in maternal blood reflect the placental karyotype. It is important to remember that feto-maternal DNA trafficking is bilateral, but the relative concentration of maternal DNA in the fetal circulation is significantly less than the relative concentration of fetal DNA in the maternal circulation (0.9% vs. 14.3%, respectively).⁵

Rhesus D genotyping—Currently, noninvasive prenatal diagnosis using cell-free nucleic acids in maternal blood is only offered clinically for fetal Rhesus D detection and gender determination. Antenatal Rhesus D diagnosis, in particular, is an important problem because 15% of Caucasian women, 3–5% of African women, and a very small percentage of Asian women are blood group Rhesus D negative. Molecular genetic studies have shown that most Rhesus D negative pregnant women have a deletion of both copies of their Rhesus D genes on chromosome 1. Thus, noninvasive prenatal diagnosis of Rhesus D testing works because the pregnant woman has no amplifiable Rhesus D gene sequence. If the fetus is Rhesus D positive, the PCR will amplify the D sequence in the cell-free fetal DNA and a positive amplification product will be detected. Such noninvasive diagnostics are clinically useful because no subsequent testing or therapeutic procedures are indicated if the fetus is Rhesus D negative. This prevents unnecessary administration of Rhesus D immunoglobulin.

In 2005 we summarized the results of existing publications describing large-scale clinical trials that had been performed in the United Kingdom, the Netherlands and France.⁶ These studies were shown to be highly accurate (sensitivities greater than 95%) in the detection of fetal Rhesus D sequence when the fetus was RhD positive. Although there were occasional cases of false negative diagnoses, these errors were mainly due to blood sampling very early in gestation or sub-optimal methods to extract and detect fetal DNA. The few cases of false positive diagnoses were due to genotypic variants (pseudogenes), mainly in African women. These were not due to a deletion of the Rhesus D sequence. The pseudogenes amplified an RhD-like sequence, but there was no Rhesus D protein made, so serology was negative in these individuals. Current genotyping protocols in molecular diagnostic laboratories take into account the potential presence of the pseudogene and do not amplify this region of the genome.

Non invasive prenatal diagnosis of Rhesus D is a clinical advance, improving prenatal care because it reduces the unnecessary use of D immunoglobulin to prevent immune hemolytic disease of the newborn. There have been a number of large scale clinical trials analyzing the accuracy of using cell-free fetal nucleic acids to determine fetal RhD status in maternal blood. The success of these published reports has translated into relatively routine prenatal care in the United Kingdom and the European Union. However, such testing has been slow to be incorporated into clinical care in the United States.

Fetal gender determination—Non invasive detection of fetal gender using DNA based techniques is useful for fetuses at risk for X-linked conditions. For example, as early as 8 weeks of gestation, maternal blood can be sampled for the presence of Y chromosome-derived sequences. If Y chromosome material is found, this strongly suggests that the fetus is male. If the pregnant woman is at risk for an X-linked condition such as hemophilia, and the fetus is male, this indicates that further invasive testing is necessary. However, if the fetus does not have evidence of a detectable Y chromosome, this suggests that the fetus is female. Therefore, an invasive procedure such as amniocentesis or chorionic villus biopsy, with its associated risk of miscarriage, can be avoided. Another indication for noninvasive prenatal diagnosis of fetal gender is if the fetus is at risk for congenital adrenal hyperplasia (CAH). Noninvasive determination of fetal gender may help in the decision whether or not to administer steroids antenatally. If a fetal Y chromosome sequence is detected, maternal steroids may not be indicated. If fetal ambiguous genitalia are observed on ultrasound examination during the late second to third trimester, a noninvasive diagnosis of fetal gender may help to determine whether or not the neonate will be postnatally treated as an over-masculinized female or an under-masculinized male.

Although early fetal gender identification is warranted, in some single-gene disorders, the availability of fetal gender testing directly to consumers via the internet (www.tellmepinkorblue.com) suggests that this technology is also used for fetal sex selection, with termination of fetuses that do not have the desired gender. Several professional organizations in the United States have stated that noninvasive fetal gender testing should only be offered for medical indications, such as families at risk for fetuses with X-linked conditions and other single gene disorders.

In collaboration with the Center for Genetics and Public Policy at Johns Hopkins University, we performed a meta-analysis of over 135 published papers on noninvasive fetal gender determination. We demonstrated that academic and clinical laboratory improvement amendment (CLIA) certified laboratories have a 92–97% sensitivity of detection of Y chromosomal DNA sequences if the fetus is truly male. The accuracy of noninvasive prenatal diagnostic testing is related to the efficiency of cell-free DNA extraction from maternal plasma and serum. If the techniques to extract DNA are sub-optimal, there may be no fetal DNA present in the reaction, which results in a false negative diagnosis of a male fetus. There have been a

few false positive cases in the literature, but these have been mainly due to contamination with male DNA from laboratory workers and/or inadequate centrifugation speed (which perhaps retains circulating intact male cells from prior pregnancies).

Trisomy 21—Noninvasive prenatal diagnosis of trisomy 21 has long been considered the “holy grail” of prenatal diagnosis. The main challenge in noninvasive detection of Down syndrome is that the majority of circulating cell-free DNA in maternal plasma originates from the mother. Only approximately 5–10% of the DNA is from the fetus. From the 1990s onward, there have been multiple approaches developed to detect the small incremental amount of DNA present from the extra chromosome 21 if the fetus has Down syndrome. Due to space limitations, these cannot be described in detail here, but the reader is directed to reference 7. The most current and exciting approach involves the use of massively parallel (also known as “high throughput”, “shotgun”, and “next generation”) DNA sequencing methodologies to detect extra copies of chromosome 21 DNA fragments in the maternal circulation. The feasibility of this technique was shown in 2008 by two different groups, one at Stanford University in the United States and one at The Chinese University of Hong Kong.^{8,9} Massively parallel DNA sequencing technology involves large-scale random sequencing of small fragments of total circulating DNA (a mixture of fetal and maternal DNA); those fragments are then mapped to their chromosomes of origin. If the fetus has an extra copy of chromosome 21, there should be a small but measurable over-representation of all of the sequences (maternal + fetal) that derive from chromosome 21. An overview of the approach to quantify cell-free DNA is as follows: a maternal blood sample is obtained and fetal cell-free DNA is isolated from the plasma. This cell-free fetal DNA is then applied to a solid support to facilitate high throughput DNA sequencing. The reads from the DNA fragments in maternal plasma are aligned to the chromosomes of interest and the relative amounts of chromosome 21 present are compared to normal controls. The technique is extremely sensitive, but costly in terms of time, equipment, and reagents.

There are many advantages to high throughput sequencing. First, the entire process can be automated and multiple samples can be analyzed simultaneously. Second, because the test DNA is bound to a solid support, such as an array, thousands of sequencing reactions can occur in parallel. The disadvantages of this approach are that the technique requires state-of-the-art sequencing equipment, bioinformatics expertise to interpret the data, and significant reagent costs per sample. However, because of the dramatic decrease in the overall costs of sequencing the human genome, it is anticipated that in the future, noninvasive prenatal testing for Down syndrome using maternal blood may reach a price point where it can be offered clinically. A number of clinical trials are currently ongoing; these are mainly being performed by different biotechnology companies. Table 1 shows how sequencing of maternal blood for fetal trisomy 21 compares to current clinical techniques of screening for Down syndrome using nuchal translucency measurement and serum analytes.

RNA

The fact that circulating fetal mRNA sequences can be detected in maternal blood was somewhat unexpected due to the presence of ribonucleases in maternal plasma and the known instability of RNA in tissue. In 2000, Poon and colleagues first showed that fetal *ZFY* mRNA sequences could be detected in the plasma of pregnant women carrying male fetuses.¹⁰ mRNA in maternal blood is apparently stable; this is thought to be due to the fact that it circulates within apoptotic bodies and is consequently protected from degradation.

Our laboratory’s central hypothesis is that fetal messenger RNA in the maternal blood can give noninvasive real-time fetal functional developmental information about the fetus. A major

need exists for the detection of fetal functional abnormalities, especially in the setting of a structurally normal ultrasound examination.

In a prior study by our laboratory, we performed a comparative microarray analysis of gene sequences present in maternal and neonatal whole blood.¹¹ In this study, 157 potential fetal biomarkers were identified in third-trimester maternal whole blood. Using the Unigene database, we found that 6 gene sequences were expressed only by the embryo and 20 were expressed only by the fetus or neonate. Twenty seven of the 157 differentially-regulated genes related to neonatal development, and the majority (48%) of these were specifically involved in the developing nervous system. Surprisingly, 5 of the 157 fetal biomarkers detected related to fetal sensory perception in the vision or olfactory systems. Twenty-two of the 157 genes were involved in fetal physiology, such as natriuretic peptide receptor A (*NPR1*), which is believed to play a role in the normal diuresis of a neonate shortly after birth. Significantly, over 10% of the genes detected were involved in host defense against pathogens, suggesting that the newborn is preparing to fight infections *ex utero*.

Some key fetal genes of interest detected in the third trimester maternal whole blood included the following: neural retinal leucine zipper (important in visual perception), olfactory receptor family 7 (perception of smell), defensin alpha 4 (response to pathogen), CD19 (cellular defense), and placenta-specific 1 (placental development). In the Maron et al study¹¹, the majority of gene transcripts suggested that the fetus had ongoing developmental processes indicating that it was getting ready to leave its *in utero* environment by developing its visual pathways, immune system, sense of smell, and its nervous system. The information acquired in this study was relevant to the study subjects, who were normal healthy fetuses delivered by cesarean section at full term. This particular set of subjects was selected because of the need to coordinate antepartum, postpartum, and umbilical cord blood samples. We acknowledge that it is important to generate data from earlier time points in gestation. Furthermore, there is a need to compare healthy with abnormal fetuses.

The Maron et al. study¹¹ taught us that gene expression analysis presents an opportunity to study not only differentially-regulated genes, but also pathways and networks involved in the pathophysiology of specific fetal conditions. The gene lists allowed us to predict proteins that might be used in future prenatal diagnostic assays. An advantage of gene expression data is that it can be re-mined in future studies for comparison with different subjects or different pathologies. The initial phase of determining which fetal genes are detectable in the maternal circulation is unbiased and “discovery driven”, as opposed to “hypothesis-driven.” The use of gene-expression microarrays is expensive, but once we identify genes that play a key role in normal fetal development and/or in specific pathological conditions, we can then develop a multiplex reverse transcriptase (RT)-PCR assay that would be cost effective and rapid. We have already shown that it is technically feasible to amplify key fetal gene sequences from maternal whole blood in 10 pregnant women (Figure 1). Once we have identified important genes at each stage of pregnancy we can incorporate them into a panel for a survey of fetal well-being at different stages of gestation.

Cell-Free Nucleic Acids in Amniotic Fluid

For the past 9 years, we have focused many of our efforts on studying cell-free nucleic acids in amniotic fluid.¹² Amniotic fluid is an abundant source of both cell-free RNA and DNA. Amniotic fluid has an advantage over maternal blood in that it contains almost exclusively fetal cell-free nucleic acids. Unlike blood, there is little, if any, maternal nucleic acid contamination in amniotic fluid. Furthermore, within the amniotic cavity there appears to be no nucleic acids derived from the placenta. The fetal tissues represented in amniotic fluid are those that are either in direct contact with the amniotic fluid or drain into the amniotic fluid, such as the

oropharynx, lungs, GI tract, bladder, and skin. An advantage to using amniotic fluid is that there are large amounts of discarded clinical material available for research, along with corresponding medical record and fetal karyotype information. Amniotic fluid thus provides a unique opportunity to understand molecular pathophysiology in the living fetus.

Our laboratory has published a number of studies on cell-free nucleic acids in amniotic fluids, starting with the observation that large amounts of cell-free DNA are present in amniotic fluid (reviewed in 12). In 2004 our group showed that the cell-free DNA in amniotic fluid, although fragmented, could provide clinically useful information for comparative genomic hybridization (CGH) studies. This facilitated rapid screening of samples for aneuploidy and has already transitioned to clinical use in diagnostic laboratories. In 2005, our group showed that cell-free mRNA in amniotic fluid could be hybridized to gene expression arrays.¹³ The data demonstrated that fetal gene expression is dynamic and reflects developmental changes as gestation advances. An important practical corollary was that it was important to control for gestational age as well as clinical phenotype. In this initial study¹³ we specifically used cases with large volumes of amniotic fluid, such as *hydrops fetalis* and twin to twin transfusion syndrome (TTTS). Our results demonstrated that specific transcripts were differentially-regulated in certain pathologies, such as the up-regulation of aquaporin 1 in the amniotic fluid of the recipient twin in TTTS.

In subsequent studies we improved our techniques to be able to work with smaller amounts of amniotic fluid (5–30 mL). We also concentrated on the study of amniotic fluid from fetuses with common aneuploidies to better understand their pathophysiology *in utero*. We have used cell-free fetal RNA in amniotic fluid to perform comparative gene expression studies in fetuses with trisomies 18 and 21 to euploid fetuses. In the first study, we compared 7 second trimester cases of trisomy 21 to 7 singleton controls of the same gender matched for gestational age.¹⁴ Microarray analysis identified 414 probe sets (corresponding to 311 annotated genes) that were differentially-expressed between the trisomy 21 and the euploid samples. In trisomy 21, 54% of the genes were up-regulated and 46% of the genes were down-regulated. Somewhat surprisingly, only 5 of the differentially-regulated genes were physically located on chromosome 21. These corresponded to the genes *CLIC6* (a chloride intracellular channel protein), *ITGB2* (integrin beta chain beta 2), *RUNX1* (a transcription factor associated with hematopoiesis), *C21orf67*, and hypothetical protein *LOC642852*. A heat map analysis of the 409 differentially regulated probe sets not on chromosome 21 correctly grouped samples by karyotype (normal versus trisomy 21). The results therefore showed a consistent phenotypic pattern that was seen as early as the second trimester that was common to affected fetuses with trisomy 21. Subsequently we analyzed the list of differentially-regulated genes with a database known as DAVID (Database for Annotation, Visualization, and Integrated Discovery). This revealed that oxidative stress was the most significantly disrupted function in second trimester fetuses with Down syndrome.

Oxidative stress has been suggested to be the bridge between Down syndrome and Alzheimer's disease. Previously, it has been shown that oxidative stress response genes were over-represented in adult, but not fetal, brains from individuals with Down syndrome. Subsequently, genes related to oxidative stress and apoptosis have been identified in neural progenitor cell lines generated from the frontal cortex of second trimester fetuses with Down syndrome. Investigators have found biochemical evidence of oxidative stress in second trimester amniotic fluid from fetuses with Down syndrome fetuses using a marker of free radical catalyzed lipid peroxidation, isoprostane. These isoprostanes were elevated 9-fold in the Down syndrome fetuses compared to the normal fetuses.

Our laboratory has performed another study in which we compared 5 second trimester female cases of trisomy 18 to 6 euploid cases matched for gestational age. In this study, 356 probe

sets, corresponding to 256 annotated genes, were statistically significantly differentially expressed between trisomy 18 and the normal samples. Similar to the results of trisomy 21 study¹⁴, only 7 of the differentially-regulated genes were physically located on chromosome 18. In the trisomy 18 study, 65% of the genes were up-regulated and 35% were down-regulated. The heat map analyses again showed that there was a consistent molecular phenotype for the affected second trimester fetuses. We then compared the differentially-regulated genes in trisomy 18 to the differentially-regulated genes in trisomy 21. Only 6 genes were differentially-regulated in both trisomies. Two genes were down-regulated in both trisomies. These were *CYP2C9* (cytochrome P450, family 2, subfamily C, peptide 9) and *SLC25A33* (solute carrier, family 25, member 33). Four genes were up-regulated in both trisomies: *NKX1-1* (NK1 homeobox 1), *CCDC85B* (coiled-coil domain containing 85B), *TTL12* (tubulin tyrosine ligase-like family, member 12), and *ROCK1* (RHO-associated, coiled-coil containing protein kinase 1). A Pub Med™ search indicated that these genes have not been previously reported in association with either trisomy 18 or 21. Our discovery-driven research demonstrates that new genes can be identified that play a role in fetal development abnormalities. A good example of this is the *ROCK1* over-expression seen in trisomies 18 and 21. *ROCK1* is of particular interest because it is expressed in the endocardial cushion and heart valves during heart development.

Another advantage to using cell-free nucleic acids in amniotic fluid is that the gene expression information derived from living fetuses may be used to suggest new treatment approaches. We hypothesize that one can use the Connectivity map (www.BroadInstitute.org/Cmap), which is a publicly available reference collection of gene expression profiles derived from human cultured cells treated with bioactive small molecules along with pattern matching software to mine the data. The Connectivity map was named accordingly because it reveals “connections” between drugs, genes and diseases. Although the initial research was done in tumors, the elegance of the Connectivity map is that it uses gene expression as a common vocabulary. The Connectivity map allows one to enter a list of differentially-regulated genes. It then suggests small molecule compounds, generally drugs approved by the Food and Drug Administration (FDA) of the United States, to either mimic or reverse the phenotype of a particular cell line. For Down syndrome, the drugs suggested to “reverse” the phenotype include celastrol, dimethyloxalylglycine (DMOG), and scriptaid (a histone deacetylase inhibitor). Celastrol has already been considered as a potent antioxidant and anti-inflammatory drug for the treatment for Alzheimer’s disease. Currently, in our laboratory we are generating testable hypotheses to determine clinical value by co-culturing trisomy 21 cells with these compounds.

Cell-Free Nucleic Acids in Neonatal Saliva

Just as there is an important need to develop noninvasive biomarkers for prenatal diagnosis, there is a need to be able to monitor neonatal health and development. Saliva is a rich source of nucleic acids that is often underutilized as a body fluid. In previous work performed at Tufts by Maron et al.¹⁵, we determined if small volumes of neonatal saliva could provide novel information about neonatal development and gene expression. In this study¹⁵, 5 premature infants who were born between 28 and 32 weeks of gestation had serial salivary samples collected. The amount of saliva was minimal (between 10 and 100 microliters). The saliva was tested at 5 distinct time points during hospitalization to see if analysis of the salivary transcriptome could determine neonatal readiness to feed. The 5 time points were: prior to, introduction of, and advancement of enteral feeds, introduction of oral feeds, and achievement of full oral feeding. In this study each infant served as his or her own control. The results were significant in that they demonstrated that the small amount of saliva obtained provided an enormous amount of information on the individual infant. The development and function of the digestive system was significantly up-regulated over time. Genes of interest included *NPY2R*, which regulates food intake, *HCRTR2*, which is involved in the stimulation of food

intake, wakefulness and energy expenditure, *LALBA*, which enables the production of lactose by transferring galactose moieties to glucose, *LEPR*, which regulates adipose tissue mass through hypothalamic effects on satiety and energy, and *GALR3*, which regulates feeding behavior. In addition, an unexpected finding was that multiple genes related to nervous system development (n=407) were also highly represented in this data set. Interestingly, the trigeminal nerve was highlighted in the analysis. The trigeminal nerve has both sensory and motor components and innervates oral muscles involved in biting, chewing, and swallowing. Thus as the premature infants learned to orally feed, Maron et al.¹⁵ detected up-regulation of genes associated with the development, quantity, and survival of the trigeminal nerve. The salivary analysis also showed that genes associated with embryonic development (n=128) and hematopoiesis (n=122) were down-regulated over time. These genes included *RUNX1*, previously mentioned as being up-regulated in Down syndrome, *ETV6*, which is required for hematopoiesis and development of the vascular network, and *MAFB*, which plays an important role in lineage-specific hematopoiesis. Because of the wealth of information about the developing neonate that is present in saliva, it seems feasible that inexpensive point-of-care multiplex PCR assays could be developed to determine whether or not a premature infant is at risk for disease sequelae and subsequent developmental disorders.

Summary

This paper has described and highlighted recent progress in three areas of interest to perinatologists and neonatologists: the current status of noninvasive prenatal diagnosis using cell-free nucleic acids in maternal blood, potential prenatal diagnosis of the future using cell-free messenger RNA in amniotic fluid to better understand molecular pathophysiology of the developing fetus, and the use of salivary genomics as a novel way of monitoring the premature neonate.

Noninvasive prenatal diagnosis is currently being used clinically to detect paternal alleles that are absent in the maternal genome, such as the Y chromosome or the Rhesus D gene. Whereas noninvasive prenatal diagnosis of trisomy 21 is not currently clinically available, the high throughput sequencing approach is accurate and will likely lead to a clinical test in the future. To be able to offer this on a wide-scale basis, the costs and the complexity of the bioinformatics analysis requirements need to decrease. Cell-free RNA in maternal blood can be used in a discovery-driven approach to identify key genes expressed in a normal fetus at different stages of gestation. This information can then be used to develop novel fetal “well-being” assays using real-time PCR amplification or other lower cost methods.

Comparative gene expression microarray analysis from amniotic fluid can provide novel information on normal and abnormal fetal developmental gene expression. Initial studies from our laboratory in normal and aneuploidy fetuses suggest that there are many complex effects, but that each aneuploidy has specific abnormalities. In addition, our data suggest that in aneuploid fetuses, the majority of dysregulated gene expression occurs in genes that map to chromosomes other than the one involved in the aneuploidy. Therefore, there are significant secondary adverse biological consequences that are in addition to the congenital anomalies observed in trisomies 18 and 21. These could serve as the target for new approaches to prenatal or postnatal treatment. For example, we hypothesize that the mental retardation seen in individuals with Down syndrome may be the result of exposure to prolonged oxidative stress.

In the last part of the paper, we have highlighted the innovative work from Dr. Jill Maron’s group that demonstrates that comparative genomic salivary analyses provide comprehensive, real-time information regarding nearly all organs and tissues in the developing preterm infant. This innovative and noninvasive technique represents a new approach to identify biomarkers in order to monitor health, disease and development in premature neonates. This discovery-

driven approach lends itself to both normal physiological processes (for example, readiness to feed), and diseases that are specific or more common in the neonate, such as necrotizing enterocolitis.

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Figure 1.

Multiplex reverse-transcriptase PCR amplification reactions performed on cell-free RNA in maternal whole blood obtained from 10 pregnant women at term. Amplification was performed courtesy of Fluidigm Corporation (South San Francisco, CA). Amplified sequences were previously identified as fetal in reference ¹¹. Each colored line within a box represents a different plasma sample. The majority of the samples amplify the sequence shown.

CAMP= cyclic adenosine monophosphate

NRL= neural retina leucine zipper

S100B= S100 calcium binding protein β

NRP1= neuropilin 1

ROBO4= roundabout homolog 4

Table 1

Noninvasive Prenatal Diagnosis of Trisomy 21: What is the Best Technique?

Current: Ultrasound/Analytes	Future: Cell-free Fetal DNA
<ul style="list-style-type: none"> - Already in clinical practice - Still in early stage clinical trials - Results validated in several Large-scale clinical trials - First trimester scan give additional information regarding heart disease, other anomalies, single gene disorders - Less expensive, required equipment widely available - Not diagnostic 	<ul style="list-style-type: none"> - Still in early stage clinical trials - Unclear if existing intellectual property will impede translation to practice - Sequencing equipment and bioinformatics analysis are expensive - Could be diagnostic (or an advanced screen)