UTILIZATION OF CELL-FREE DNA SCREENING FOR SINGLE GENE DISORDERS SEEN MORE COMMONLY IN OFFSPRING OF OLDER FATHERS

BY

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ABSTRACT OF THE THESIS

Utilization of Cell-Free DNA Screening for Single Gene Disorders Seen More Commonly in Offspring of Older Fathers

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As novel technologies readily become available for patients, we sought to determine the uptake rates as well as any barriers, to cell-free DNA screening for single gene disorders with advanced paternal age as a primary indication for which the technology was offered. This novel technology uses cell-free DNA in maternal serum to screen for de novo, single-gene, autosomal dominant mutations associated with advanced paternal age. This small pilot study retrospectively examined one year of data (June 1, 2017 – June 30, 2018) at a regional perinatal center. Of the 1,843 genetic counseling encounters, we examined records for 51 patients that were offered this novel screening technology due to advanced paternal age in the male reproductive partner. We found that 17/51 (33%) patients utilized this screen. To better understand the potential barriers for the uptake of this screen, we assessed gravity and parity status, maternal age, paternal age, history of infertility, gestational age at the time screening was offered, presence of the father of the fetus at the appointment, and if the patient had invasive testing. Through multivariant analysis, our study revealed two statistically significant factors associated with technology uptake rates. These two factors were patients whose partner was present at the genetic counseling session when the screen was offered, and patients who had

invasive testing. Paternal age-related risks are an important discussion point in reproductive genetic counseling. Novel technologies that assess these risks have to be carefully studied before they are implemented into prenatal practice.

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Introduction:

Advanced paternal age (APA) is defined by the American College of Medical Genetics (ACMG) as men conceiving at age 40 and older. It has been linked to an increased risk for infertility, miscarriages, autism, schizophrenia, childhood cancers, chromosome aberrations, copy number variants, birth defects and de novo, gain-of-function, dominant, single gene disorders (Brandt *et al*. 2019, Goriley, 2012). APA as an indication for reproductive genetic counseling has received little attention when compared to advanced maternal age. In 1996, ACMG published the first set of guidelines for genetic counseling regarding advanced paternal age. In 2008, ACMG updated this statement based on new publications investigating reproductive impact of APA. Despite the ACMG proposing the age of 40-years as APA, the American College of Obstetrics and Gynecology (ACOG), the National Society of Genetic Counselors (NSGC) and the International Society of Prenatal Diagnosis (ISPD) have not established an age cut-off to define APA. In February 2019 ACOG published a practice advisory which reported that cell-free DNA screening for single gene disorders, which can be offered to screen for de novo conditions associated with APA, is not recommended in pregnancy given the limited data on the accuracy. NSGC and ISPD do not have a formal position statement regarding APA. However, when searching advanced paternal age on ISPD website, a literature review by Brandt et al, referenced throughout this paper, is populated. The lack of uniform guidelines from professional organizations and lack of technology to sufficiently address APA risks are likely contributing to lack of awareness regarding the reproductive risks associated with APA.

The understanding of advanced paternal age has been evolving over time. In 1912 Willheim Weinberg studied sporadic cases of achondroplasia and noted a higher incidence of the disease in last-born children. In 1955 Lionel Penrose followed-up Weinberg's speculation, reporting that the birth order was not the significant factor but increasing paternal age was the contributing factor. Further recognizing the risks associated with advanced paternal age in 1987, Risch et al. analyzed the distribution of parental ages for spontaneous cases of different autosomal dominant disorders. To make an observed/expected ration, Risch et al. compared parental age profiles to the age distribution in the general population for a few disorders including Apert syndrome, Crouzon syndrome, Pfeiffer syndrome and Achondroplasia. His study found that while some diseases had a small-linear or no relationship with both maternal and paternal ages, other disorders had an exponential increasing rate with advancing paternal age. In 2012, Goriely and Wilkie utilized sperm studies and found a significant correlation of certain point mutations with increasing paternal age. Specifically, they found that these mutations have a selective advantage due to the mutant protein, and therefore clonal expansion of the mutation leads to more sperm carrying the mutation over time. Importantly, they reported a triad of features for the now coined "paternal age effect" disorders stating that these disorders have a gender bias, a paternal age effect, and a high germline mutation rate. More recently, in 2017, Baylor Genetics launched the first non-invasive, multi-gene sequencing screen, "Pre-seek" that targets some of the more common autosomal dominant conditions associated with APA.

The risk for de novo mutations is the result of spermatogonial cells undergoing mitotic division every 16 days, spermatogenesis is prone to greater risk for DNA

fragmentation and ultimately higher frequencies of gain of function, deleterious point mutations with age (Humm, 2013). As men age, more of their sperm will carry spontaneous, gain of function mutations that are positively selected for (Goriely, 2012) and the resulting sperm from these mutations are viable for fertilization. The increased risk for these mutations has been found to lead to an increased rate for several disorders, with the same underlying molecular basis (Goriely, 2012). The PAE disorders result from dominantly acting point mutations which are in key developmental regulators that cluster within the growth receptor-RAS signaling pathway (Goriely, 2012). Typically, there is no family history of the genetic conditions associated with APA as these mutations occur in the sperm and are de novo. Specifically, these include Apert syndrome, achondroplasia, thanatophoric dysplasia, and Costello syndrome (Goriley 2012, Sigmn 2017, Urhoj et al. 2017, Bray 2015).

Advanced paternal age is a reproductive risk for many reasons, yet is has only recently been a focus of research. In turn, there is a lack of tests available for APA as more research is needed to focus on the cause of these risks. In comparison, advanced maternal age, defined as 35 and older at delivery has clearly been implicated in causing an increased rate of aneuploidy (Cedars 2015, Chiang et al. 2012, Allen et al. 2009). Unlike their female counterparts, men can continue to father children late into their lifespan (Sigman, 2017). For women, menopause signals the end of the reproductive cycle. Most perimenopausal women can only conceive with the aid of *in vitro* fertilization (Sigman, 2017). Despite the reproductive risks of APA, more research has focused on AMA. A google search on 02/15/2018 with “advanced paternal age” as the search term had 473,000 hits while “advanced maternal age” on the same date had

2,890,000 hits. On the same date a PubMed search with “advanced paternal age” as the search term had 306 hits while “advanced maternal age” had 2,481 hits (Ashkinadze, 2018). Just over one year later, a google search on 03/20/2019 with “advanced paternal age” as the search term had 9,140,000 hits while “advanced maternal age” on the same date had 58,700,000. On the same date a PubMed search with “advanced paternal age” as the search term had 442 hits while “advanced maternal age” had 3,538. Though more public education has become available, the information available for AMA far exceeds that of the information available for APA. The limited research on APA implies that it is less significant than AMA, yet we know there are many conditions in offspring as a result of APA (Ramasamy, 2015).

Prenatal diagnosis for aneuploidy is available for all pregnant women, regardless of their age. However, due to procedure related risks, it is not uncommon for women to choose a non-invasive assessment of aneuploidy instead. Recently, non-invasive screening for single-gene disorders has become available and is focused on paternal age effect de novo conditions. Baylor Genetics is the first laboratory to introduce maternal serum cell-free DNA screening called Pre-Seek™ for single-gene disorders that have been associated with APA. Natera is the lab that markets and sells the test and renamed it Vistara. It is likely that other labs will develop similar panels. Presently it is the only prenatal screen that analyzes specimens for paternal age effect disorders in a non-invasive manner (*Preseek™: Noninvasive Prenatal Sequencing Screen2018*). Specifically, this novel technology targets 30 genes for pathogenic and likely pathogenic mutations via next generation sequencing, with a minimum coverage of 200x (see figure 1). It requires a maternal peripheral blood sample collected after 9 weeks 0 days gestation, and either a

paternal peripheral blood or saliva sample. A minimum of 4.5% fetal fraction is required for trio testing (*Preseek™: Noninvasive Prenatal Sequencing Screen, 2018*). Given the sample requirements, if an egg donor was used to conceive, a specimen would be required from the egg donor. In addition, this screen cannot be used for multiples.

Genomics experts at Baylor selected the genes for this panel through a curation process which focused on selecting genes that lead to significant medical problems and have a high de novo incidence (Huseman, 2017*)*. In addition, the mutations being sequenced are positively selected for, gain-of-function mutations, also referred to as "selfish mutations" (Goriely 2012). Individually, the disorders associated with APA are rare, but the cumulative occurrence is comparable to the prevalence of Down Syndrome (1/600) (*Preseek™: Noninvasive Prenatal Sequencing Screen, 2018*). Some of the disorders screened by this panel have significant clinical impacts and are outside the realm of the more common non-invasive prenatal screening tests currently available for aneuploidy screening. The potential to screen for these conditions from a maternal blood sample may present the only opportunity for prenatal identification as many of these single gene disorders do not present clinically until later in pregnancy or after birth (*Preseek™: Noninvasive Prenatal Sequencing Screen, 2018*). Eighty-six percent (26/30) of the genes sequenced on this panel are associated with disorders that may present prenatally with ultrasound findings such as cardiac defects or increased nuchal translucency measures (see table 1). However, these findings are very non-specific.

Table 1. Genes included on the Baylor panel, their associated disorders and presence or absence of prenatal ultrasound findings.

Gene	Disorder	Prenatal ultrasound finding yes/no (finding)
JAG1	Alagille syndrome	Yes (cardiac)

CHD7	CHARGE syndrome	Yes (cardiac, IUGR, ear anomalies, among others)
NIPBL	Cornelia de Lange syndrome 1	Yes (Skeletal abnormalities of arms/hands, cardiac, IUGR, oligohydramnios among others)
SMC1A	Cornelia de Lange syndrome 2	Yes (See above)
SMC3	Cornelia de Lange syndrome 3	Yes (See above)
RAD21	Cornelia de Lange syndrome 4	Yes (See above)
HDAC8	Cornelia de Lange syndrome 5	Yes (See above)
CDKL5	Epileptic encephalopathy, early infantile, 2	No
SYNGAP1	Intellectual disability	No
MECP2	Rett syndrome	No
NSD1	Sotos syndrome 1	No
TSC1	Tuberous sclerosis 1	Yes (cardiac)
TSC2	Tuberous sclerosis 2	Yes (See above)
BRAF	Cardiofaciocutaneous syndrome 1	Yes (polyhydramnios, increased NT)
MAP2K1	Cardiofaciocutaneous syndrome 3	Yes (See above)
MAP2K2	Cardiofaciocutaneous syndrome 4	Yes (See above)
HRAS	Costello syndrome/Noonan syndrome	Yes (cardiac, increased NT)
PTPN11	Noonan syndrome 1/LEOPARD syndrome/cancers	Yes (cardiac, increased NT)
SOS1	Noonan syndrome 4	Yes (See above)
RAF1	Noonan syndrome 5/LEOPARD syndrome 2	Yes (See above)
NRAS	Noonan syndrome 6/cancers	Yes (See above)
RIT1	Noonan syndrome 8	Yes (See above)
SOS2	Noonan syndrome 9	Yes (See above)
SHOC2	Noonan syndrome-like disorders with loose anagen hair	Yes (See above)
CBL	Noonan syndrome-like disorder with or without juvenile myelomonocytic leukemia	Yes (See above)
KRAS	Noonan syndrome/cancers	Yes (See above)
FGFR2	Apert syndrome Crouzon syndrome, Jackson-Weiss syndrome, Pfeiffer syndrome types 1/2/3	Yes (syndactyly, craniosynostosis)
FGFR3	Achondroplasia, CATSHL syndrome, Crouzon syndrome with acanthosis nigricans, Hypochondroplasia, Muenke syndrome, Thanatophoric dysplasia types I,II	Yes (skeletal, brachycephaly)

COL1A1	Ehlers-Danlos syndrome classic & type VIIA, Osteogenesis imperfecta types I,II,III,IV	Yes (skeletal/fractures)
COL1A2	Ehlers-Danlos syndrome cardiac valvular form & type VIIB, Osteogenesis imperfecta types II,III,IV	Yes (See above)

Baylor conducted a validation study for their PreSeek™ panel. They analyzed 76 samples from pregnant women with or without a clinical or family history related to the conditions on the panel. The women ranged from 10-40 weeks gestations and the fetal fraction ranged from 4.5-30%. They labeled cell-free DNA molecules prior to PCR. Using molecular barcodes, they were able to distinguish true DNA changes from artifacts. One hundred unique genome-wide SNPs that were paternally inherited were assessed in cell-free DNA, and they assisted in determining the estimated fetal fraction and helped to accurately identify DNA changes in cell-free plasma DNA. Cell-free DNA was extracted from the maternal sample. The paternal sample was used for estimating the fetal fraction, variant classification and quality control. Variants were identified and curated for classification. For the validation study Baylor defined true positives as either inherited paternal changes or de novo changes. A total of 554 true positive calls were detected from the 76 samples. In three pregnancies de novo pathogenic variants were identified and confirmed with either invasive testing or postnatal specimens. True negatives were defined using Human Genome version 19 as the reference DNA sequence defined in both parents. Of all 30 genes on the panel, eight million nucleotides were accurately detected. False positives occurred when a non-reference base was shown in the cell-free plasma DNA and both parents had the reference sequence. They found a total of 7 false positives when using capture based next generation sequencing (NGS), but when following up with amplicon-based NGS, 0 were confirmed. They found no false-negative

results. Overall, analytical sensitivity and specificity were defined at greater than 99% while analytical positive and negative predictive values were also defined as greater than 99%. For result reporting, Baylor follows the guidelines developed by both ACMG and the association for molecular pathology. These guidelines recommend the use of specific standard terminology when defining evidence-based variants in genes that are known to cause Mendelian disorders. (Richards et al, 2015). Explicitly, Baylor will report on any pathogenic or novel truncating variants that are detected in exons and within 10bp of the exon/intron boundary (*Medical Genetics Test Details: PreSeek™ non-invasive prenatal gene sequencing screen).* As with any abnormal screen, diagnostic testing for the specific mutation identified by the screen is recommended to determine if it is a true positive or false positive.

Irrespective of parental ages, average risk pregnancies with a normal karyotype have a 1.6% risk for copy number variants making fetal microarray analysis beneficial for all patients (Wapner, 2012). Both Awomolo et al and Khalifeh et al reported a decline in the uptake rates of diagnostic testing over the past ten years, inferring that low-risk cell-free DNA screening was sufficient reassuring patients. Novel screening technologies are becoming more successful with identifying high risk pregnancies, leading to a decreased incidence of unnecessary invasive procedures and a higher yield of abnormal results when invasive procedures are conducted (Awomolo, 2018). Unfortunately, given the limited data regarding the accuracy, positive and negative predictive values, cell-free DNA screening for single-gene disorders is not recommended by ACOG as per their recent practice advisory (ACOG Practice Advisory, 02/21/2019). Prenatal diagnosis is a growing field and patients are referred for prenatal genetic counseling for numerous

indications such as maternal age-related risk counseling, teratogen counseling, abnormal ultrasound or screening results, family history of birth defects or genetic conditions, carrier screening, infertility and history of multiple miscarriages. With the increasing recognition of risks associated with advanced paternal age, this will likely correlate with APA becoming another common indication for reproductive genetic counseling. With the advent of novel screening technologies, Baylor's cell-free DNA screen for single-gene disorders is the only screening tool on the market that attempts to screen for paternal age effect mutations.

As couples elect to have children at older ages, having a validated test which includes a high detection rate, low false positive rate, and high positive predictive value will continue to be important in the field of reproductive genetics. Over the last 15 years, couples have increasingly delayed childbearing until their 30's and 40's. In fact, from 2003-2013, the rate of women giving birth between 45-49 increased by 60% and men aged 45-49 having children increased by 16% (Cedars 2015). Assessing the barriers to utilization of novel screening technologies helps increase marketability, applicability, awareness and uptake. Once barriers are better-understood, genetic counselors can further aid patients to incorporate novel technology more appropriately and effectively. This data will continue to be important as trends suggest that more people will continue to bear children in the older age group. The question remains whether couples will elect to utilize this new technology and what the barriers are to utilization, if any. This study will examine both the uptake rates and the barriers to pursuing cell-free DNA screening for single gene disorders with APA as an indication. We hypothesize that women who

perceive their risk to be high and have invasive testing are information seekers and therefore are more likely to accept the novel screening tool.

Materials and Methods:

Participants:

This study has IRB approval from the Rutgers New Brunswick Health Sciences Institutional Review Board. We identified our study population from the genetic counseling patient logs and non-invasive prenatal testing clinical database.

We designed a retrospective cohort study to examine the uptake rates of cell-free DNA screening for single gene disorders when advanced paternal age was a factor. We evaluated test utilization trends in a prenatal genetic counseling population from June 2017 – June 2018. For the purpose of our study, we selected patients whose reproductive partner was at least 44 years old at the time of the genetic counseling session. In the clinical practice at the study location, it was decided that all patients who have a reproductive male partner age 44+ would be offered cell-free DNA screening for single gene disorders.

Inclusion criteria for the study included singleton gestation, no history of a co-twin demise or maternal malignancy or history of organ transplantation and no use of gamete donation. Patient information was recorded, including gravity and parity, maternal and paternal ages, history of infertility, gestational age at the time cell-free screening for single gene disorders was offered, presence or absence of the patient's spouse, and whether or not the patient opted for invasive testing during the pregnancy.

There was a total of 1,843 genetic counseling encounters during the one-year period. Of these 1,843 encounters, 59 were offered the novel screening technology. Only 51 out of the 59 were used for this data set. The other 8 patients were offered cell-free DNA screening for single gene disorders for indications other than advanced paternal age. Both a maternal and a paternal blood sample was required for the screen to be performed.

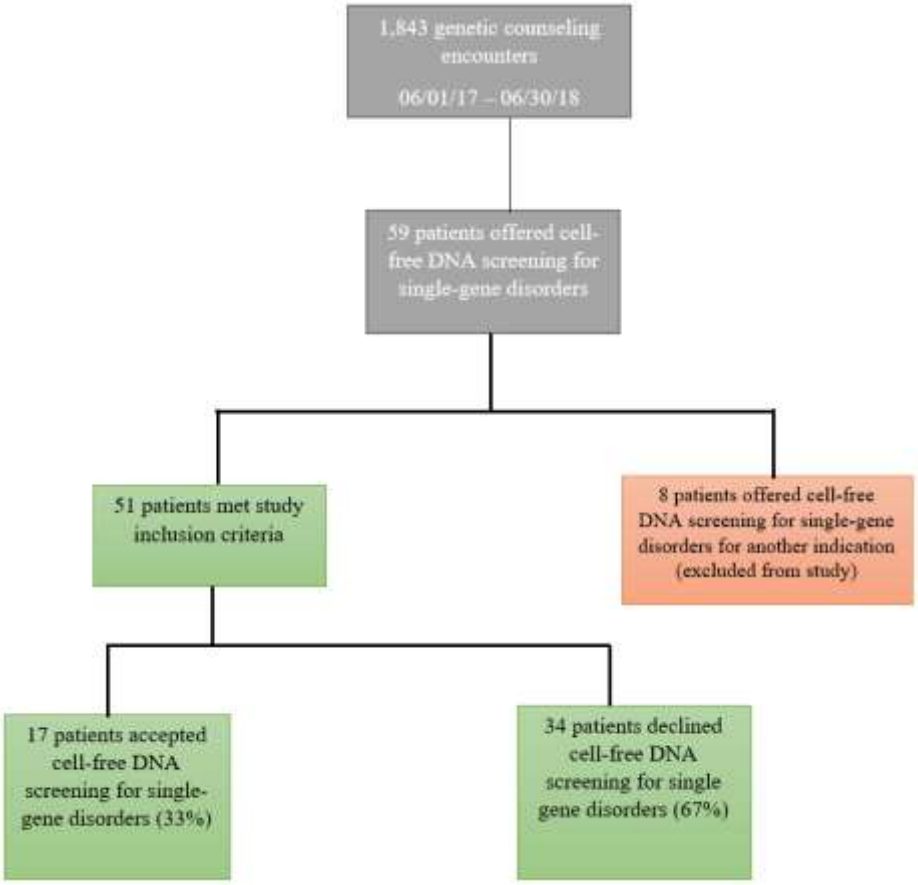
Statistical Analysis:

We compared all factors using both a univariate and multivariate chi-square analysis through Stata statistical software. We used logistic regression to assess significance of continuous variables. All associations were expressed as odds ratios with a 95% confidence interval.

Results

Of the 51 patients offered cell-free DNA screening for single-gene disorders, 17 accepted (33%). Of those 17 patients, 16 received a negative result. This means that no mutations were appreciated in the cell free DNA in the maternal serum. One patient did not receive results because the paternal sample was lost in transit and the partner opted not to return for a redraw. Ultimately, the female patient asked for the screen to be cancelled. The average turn-around-time for the panel was 16-days (median 14-days with a range of 8-41 days).

Figure 1. Break down of all the genetic counseling encounters from 06/01/17-06/30/18 to those patients who were offered, accepted and declined cell-free DNA screening for single gene disorders associated with APA.



In the univariate analysis we found that having at least one prior child and the presence of the spouse at the initial genetic counseling session were significant factors related to the utilization of cell-free DNA screening for single gene disorders with APA as an indication (see table 2). Specifically, those with a previous child were 4.4-fold (1/0.23) less likely to accept the panel. We also found that when the patient's partner was present at the genetic counseling session, the couple was over 7-fold more likely to accept the novel screening technology. No other factors were individually significant using the univariate model.

Table 2. Count (percent) and univariate relationship between subject characteristics and decision to utilize cell-free DNA screening for single gene disorders.

	Did not have cfDNA screen for	**Had cfDNA screen for**	**Univariate**

Characteristics	single gene disorders (n=34)	single gene disorders (n=17)	OR (95% CI) p-value)
Prior children n (%)	----	----	0.23 (0.05-0.93); p-value=0.02
Yes	22 (64.7%)	5 (29.4%)	
No	12 (35.3%)	12 (70.6%)	
Prior Miscarriages n (%)	----	----	0.78 (0.20-2.97); p-value=0.69
Yes	16 (47.1%)	7 (41.2%)	
No	18 (52.9%)	10 (58.8%)	
Prior infertility n (%)	----	----	0.62 (0.05-4.10); p-value=0.59
Yes	6 (17.6%)	2 (11.7%)	
No	28 (82.4%)	15 (88.2%)	
Trimester n (%)	----	----	1.68 (0.42-7.44) p-value=0.41
First	20 (58.8%)	12 (70.6%)	
Second	14 (41.2%)	5 (29.4%)	
Partner present when screen was offered n (%)	----	----	7.54 (1.60-46.8) p-value=.003
Yes	13 (38.2%)	14 (82.4%)	
No	21 (61.8%)	3 (17.7%)	
Invasive procedure (amnio/CVS) n (%)	----	----	3.18 (0.45-24.2) p-value=0.15
Yes	3 (8.82%)	4 (23.5%)	
No	31 (91.2%)	13 (76.5%)	
Mother's age Mean ± SD	38.6 ± 4.0	36.7 ± 3.6	0.88 (0.75-1.03); p-value=0.11*
Partner's age Mean ± SD	47.9 ± 4.0	48.8 ± 5.2	1.08 (0.93-1.25); p-value=0.29*

* For each one unit increase in age.

We then used a multivariate model to adjust for all factors together. This model accounted for thirty five percent of the factors we analyzed with respects to the utilization of cell-free DNA screening for single gene disorders (pseudo R^2=0.35). Two factors were independently significant, adjusting for all other variables in the model. When the spouse was present at the initial genetic counseling session, couples were just over 13-fold more likely to accept the panel (see table 3) as compared to 7-fold more likely in the univariate model. This model also showed that patients who had invasive testing (i.e., amniocentesis or CVS) were just over 12-fold more likely to accept the panel. No other variables were significant with the multivariate model. Though still not a significant factor, for every one unit increase in maternal age, patients were less likely to accept Vistara (OR=0.80; 95% CI=0.62--1.03; p-value=0.08). While also not significant in the multivariate model, the odds of having prior children lead to a patient being 6-fold less likely to have testing (OR=0.16; 95% CI=0.024--1.12); p-value=0.06).

Table 3. Multivariate logistic analysis between subject characteristics and decision to utilize cell-free DNA screening for single gene disorders

Characteristics	OR (95% CI); p-value
Prior children	0.16(0.024-1.12); 0.06
Prior Miscarriages	1.17(0.245-5.57); 0.85
Prior infertility	1.33(0.151-11.89); 0.79
Trimester	1.48(0.287-7.60); 0.64
Partner present when screen was offered	13.4(2.11-85.66); 0.006
Invasive procedure (amnio/CVS)	12.7(1.13-142.41); 0.04
Mother's age	*0.80(0.62-1.03);0.08
Partner's age	*0.98(0.81-1.18);0.84

* For each one unit increase in age.

Though having prior children was a significant factor in the univariate analysis making couples more likely to decline, it was not significant in the multivariate analysis which adjusted for all of the other factors (see table 4). Similarly, patients who had invasive testing during their pregnancy were found to be more likely to accept this novels screen in the multivariate analysis but not in the univariate analysis (see table 5). Having the spouse present when cell-free DNA screen for single gene disorders was offered made couples more likely to accept and was found to be a significant factor in both the univariate and multivariate models (See table 5).

Table 4. Factors evaluated that correlated with declining cell-free DNA screening for single gene disorders.

Significant factors	Univariant P-value;95% confidence interval	Multivariant P-value;95% confidence interval
Has prior children	0.02; (0.05-0.93)	Not significant with this model

Table 5. Factors evaluated that correlate with accepting cell-free DNA screening for single gene disorders.

Significant factors	Univariant P-value;95% confidence interval	Multivariant P-value;95% confidence interval
Partner present when screen was offered	0.003; (1.60-46.8)	0.006; (2.11-85.66)
Had invasive procedure	Not significant with this model	0.04; (1.13-142.41)

Discussion

This study focused on the utilization of cell-free DNA screening for single gene disorders with APA as an indication. Overall, 1/3 (33%) of the study cohort pursued non-invasive cell-free DNA screening for single gene disorders. It is possible that patients are unaware that there are risks associated with APA prior to seeing a genetic counselor. Perhaps patients would have felt better prepared to accept this novel technology if they knew about the reproductive risks associated with APA in advance of their appointment. Education of physicians is an important component so that patients can be made aware that APA poses a risk to their pregnancy prior to the patient seeing a genetic counselor. In addition, this novel screening tool is lacking clinical validation studies. Further-more, patients may be less likely to pay for a novel screen that in not validated and likely not covered by their insurance company. Though the cumulative rate of the disorders on the cell free DNA panel for single gene disorders is comparable to that of Down syndrome, individually these disorders are rare and unfamiliar to most patients.

This study demonstrated that patients were more likely to accept this novel screening tool under two circumstances based on a multivariate analysis. The circumstances included having the male partner present at the time the screen was offered and having an invasive procedure during their pregnancy. Cell-free DNA screening for single gene disorders targets conditions associated with advanced paternal age rather than maternal age. If the patient's partner was absent, it is plausible that the patient did not feel comfortable making the decision to test their pregnancy without input from her partner. It would be ideal for both parents to be present at the genetic counseling session. If present together, both can consider the risks they pose to the pregnancy, determine how they feel

about the risk, ask questions about the risks, become educated about screening options for these risks, and then make a joint decision about how they want to proceed. In addition, having both the patient and her partner present at the genetic counseling session would allow for the specimens for the screen to be collected that day, eliminating the need to schedule another appointment. We initially hypothesized that women who pursued invasive testing would be more likely to accept the novel screening tool because these women are information seekers. Our data supports this hypothesis. Presumably, if a patient is willing to pose even a small risk to their pregnancy through an invasive procedure (CVS/amniocentesis) to learn about a potential diagnosis for the fetus, they would also be willing to have screening that poses no risk and can yield more information for the diagnostic testing.

While not significant in the multivariate analysis (0.06), the presence of having prior children lead to couples in our study cohort to be 4.4 (1/0.23) times less likely accept the screening panel. In the univariate analysis, this was found to be a significant factor (p-value 0.02) making couples more likely to decline cell-free DNA screening for single gene disorders. It is possible that patients might do not appreciate the de novo nature of these conditions. Perhaps patients grow more confident in the possibility of a normal, healthy child after having children. In addition, this screening tool is new. Patients may not feel comfortable utilizing novel technology that was not offered in previous pregnancy. Further, it is possible that the patient's physician has not discussed this screen or even the risks of APA.

Though not found to be a significant factor, in the multivariate analysis we found that with every 1 unit increase in maternal age, patients were less likely to accept the cell-

free DNA screen for single gene disorders (p-values 0.08). Potentially our older patients who declined the screen have prior children and we already found that having prior children made patients more likely to decline the screen. These older patients may have utilized other screens in prior pregnancies which yielded false-positive results. If a patient received a false-positive result in the past, she may be less inclined to utilize a newer screening tool, especially one in which we do not yet know the positive and negative predictive values. Lastly, because the risk to have an affected child with any individual PAE disorder is relatively low, it is possible that patients were more concerned with other risks such as aneuploidy given maternal age. Very advanced maternal age risks outweigh the chance of a de novo, autosomal dominant disorder associated with APA. A larger study cohort may provide more power to this factor, making it significant.

The other factors considered were history of miscarriage, history of infertility, trimester and paternal ages. None of these factors were significant predictors for a patient to accept or decline this novel screening technology. Perhaps a larger cohort could prove these other parameters to be significant.

Due to the heightened awareness about the reproductive risks associated with AMA, as women age, they may be more likely to seek information about oocyte preservation. It is not clear whether the same considerations are raised for aging men who postpone childbearing. Banking sperm is less invasive and less costly than egg preservation. Thus, as more men become aware of the reproductive effects of advanced paternal age, they may pursue sperm banking to mitigate some of the reproductive risks in their future offspring. In the field of assisted reproductive technology, there are age cut-offs for both egg and sperm donations, and these precautionary measures make it

clear that older ages of both women as well as men pose reproductive risks. Therefore, as the growing trend of women banking their eggs continues, the dialogue needs to include men who post-pone child-bearing because the reproductive risks associated with advanced paternal age is continuing to have growing recognition.

This study provides evidence that some patients (17/51) have an interest in learning about the potential risk to their fetus given APA as a risk factor. The American College of Obstetricians and Gynecologist presently does not recommend this screening in pregnancy due to the insufficient data on accuracy as well as positive and negative predictive values. Cumulatively, the single-gene disorders on the Vistara panel occur as frequently as Down syndrome (*Noninvasive Prenatal Sequencing Screening*, 2018). Cell-free DNA screening is offered to women to assess the risk of their pregnancy being affected with Down syndrome (in addition to other aneuploidies). There are many published studies to validate cell free DNA screening for aneuploidy but none for single gene disorders. In turn, this may be why many couples decline this novel technology Perhaps, with more population based, blinded studies, cell-free DNA screening for single gene disorders will become more widely accepted both by medical professionals as well as patients. Therefore, more research is warranted to validate cell-free DNA screening for single gene disorders, with emphasis on APA.

Cost of this novel screening tool may also be a barrier for patients. As a comparison, when non-invasive prenatal screening for aneuploidy became well-validated, insurance companies began to cover this screening tool for women 35 and older. If more insurance companies agree to cover this screening for patients, cost would not be a potential barrier.

Given our findings, we conclude with a few counseling considerations. Patients should be advised to bring their partner with them to their genetic counseling appointment. Patients who are considering postponing childbearing should be counseled about the ability to bank sperm as well as eggs.

Limitations of this study include a small sample size. Expansion of the cohort would be the next step in obtaining a better representation of the utilization of cell-free DNA screening for single gene disorders. Another limitation of this study is the length of time. Future studies should aim to look at data across multiple years and try to understand if the awareness of APA risks increases over time and if an increase of awareness impacts the utilization of the novel screening technology. Future studies should also look at the socioeconomic status of patients accepting and declining cell-free DNA screening for single-gene disorders when advanced paternal age is a factor. When patients decline the novel screen, future studies should aim to document reasons why they opted to decline. As stated, many of the disorders screened for on the panel are associated with ultrasound findings. Futures studies can look to assess whether the presence of ultrasound findings in a pregnancy sways a patient's decisions to accept or decline cell-free DNA screening for single-gene disorder. In addition, collecting data on how much patients are paying for the screen would be useful information to gather.

Moreover, validation studies for cell-free DNA screening for single-gene disorders are needed. Physicians need to be aware of the risk associated with APA, and discuss these risks with their patients, even if only briefly mentioning the risks to them, prior to the patients genetic counseling session. Our study provides evidence that some

patients have interest in this novel screening tool, but given the trend of delaying reproduction in many populations, studies such as ours will become more important.

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