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Original Article

Routine cell-free DNA prenatal screening identifies pregnancies at high risk for cystic fibrosis that may benefit from fetal therapy

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ABSTRACT

Recent improvements in cell-free DNA technology have enabled non-invasive prenatal testing (NIPT) to screen for fetal single-gene autosomal recessive conditions from maternal blood as early as the first trimester. This technique can determine the fetal risk for cystic fibrosis (CF) with a single blood sample from a pregnant person without the need for a partner sample, which is required for traditional carrier screening.

A retrospective review of 100,106 consecutive general-risk pregnant patients who underwent CF carrier screening was completed. All positive CF carriers underwent cell-free DNA testing, which reported a risk of the fetus being affected with CF. Pregnancies with at least a 1 in 4 risk were classified as high risk. Results of confirmatory testing were solicited from all high-risk cases, and a random sample of 50 % of low-risk cases were used to compute test performance analytics.

The study cohort included 2,587 CF carriers and 20 cases with high-risk cell-free DNA results where the CF-affected status of the fetus/neonate was known, of which 13 were affected. All cases ($n = 8$) with a 9 in 10 cell-free DNA estimated risk were affected. The assay correctly identified all known affected fetuses as high risk (sensitivity of 100 %). Of the 13 affected, 12 cases had at least one *CFTR* variant eligible for CFTR modulator therapy. Additionally, 75 % of all cell-free DNA fetal risk results were returned before 18.5 weeks gestation, providing ample time for diagnostic testing and initiation of *in utero* treatment if indicated.

Carrier screening with reflex to cell-free DNA analysis provides a personalized fetal risk assessment and efficient turnaround times at an early gestational age, without the need for a partner sample for a general risk population. This screening method can precisely guide prenatal diagnostic testing to identify CF-affected fetuses that may benefit from *in utero* therapy.

1. Introduction

Approximately 1 in 30 individuals in the United States (US), Europe, United Kingdom (UK), and Australia are carriers of a disease-causing variant of the cystic fibrosis transmembrane conductance regulator (*CFTR*) and are at risk of having a child affected with cystic fibrosis (CF) [1,2]. However, most neonates with CF are diagnosed after birth and are born to individuals unaware of their carrier status [3]. Carrier screening recommendations vary by country. Routine general population screening, regardless of family history or ethnicity, is recommended in the US, Australia, and many European countries [2,4–6]. Carrier screening for CF often involves interrogating a limited number of known CF-causing variants in the *CFTR* gene (panel testing) or reporting *CFTR* variants following sequencing. Next-generation sequencing (NGS)-based carrier screening offers near full *CFTR* gene coverage, and is increasingly being used[1]. The most common method of carrier screening, which is referred to as traditional carrier screening, entails sequential screening which is first performed for the pregnant person, and if that individual is identified as a carrier the reproductive partner is then screened. Another less common method of traditional screening entails parallel carrier screening of both reproductive partners [7]. Regardless of the approach, when both partners are identified as carriers, there is a 1 in 4 chance for

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the fetus to be affected (Fig. 1a). However, multiple publications have demonstrated that fewer than 50 % of partners undergo carrier screening when a traditional screening method is utilized, even when it is recommended, resulting in incomplete fetal risk assessments for many pregnant carriers [8–11]. Furthermore, obstetricians report minimal comfort ordering carrier screening, explaining carrier results, and counseling patients about reproductive options, which may contribute to the barriers of completing traditional carrier screening [12,13].

Carrier screening with reflex to cell-free fetal DNA testing is available as an alternative to traditional carrier screening for CF and other common autosomal recessive conditions [14–17]. For this method a single sample of peripheral blood is taken from a pregnant individual, and a NGS-based carrier screening is performed on genomic DNA, just as in traditional carrier screening. When a disease-causing *CFTR* variant is identified, the test reflexes to fetal risk assessment via cell-free DNA analysis rather than partner carrier screening (Fig. 1b) [17]. *CFTR* gene sequencing of the cell-free DNA is used to determine the likelihood that the fetus is homozygous or compound heterozygous for disease-causing *CFTR* variants. This method can estimate a maximum fetal risk of 9 in 10 and a minimal fetal risk of 1 in 5000 for CF and can be performed as early as 9 weeks gestation. No partner sample is needed for this workflow, therefore it is unaffected by the challenges with paternal testing that can impact the traditional carrier screening method. Studies have demonstrated carrier screening with reflex to cell-free DNA analysis results in approximately 96 % of affected fetuses being identified as high risk, with greater than 99.9 % specificity for a general risk population [15,16].

Early diagnosis of CF is becoming increasingly important with the development of CFTR modulator therapies (CFTRm), which work to correct the malfunctioning protein, restore a functional chloride channel, and reduce disease symptoms in people with CF [18]. Although this treatment is currently approved only for postnatal therapy, a growing number of case reports describe administration of oral CFTRm medication to pregnant individuals to treat fetuses affected by CF where there has been resolution of meconium ileus and/or newborns with normal pancreatic enzymes, illustrating the potential for improved neonatal outcomes with prenatal therapy [19–23]. While *in utero* therapy is being sporadically deployed, there are no formal studies to determine its risk-benefit profile, nor are there yet treatment guidelines for therapy indications, timing, and dosage. Even with more experience and data on the *in utero* therapeutic use of CFTRm, one barrier to broader implementation of this potential therapeutic strategy is the need for early, accessible and reliable *in utero* identification of an affected fetus. This is particularly important for the general population, many of whom are unaware of their risk of having a child affected with CF.

The objective of this study is to explore the use of non-invasive prenatal testing (NIPT) via cell-free DNA analysis to identify fetuses with CF as high risk. This information could then be used to guide prenatal diagnostic testing and potential eligibility for CFTRm. To accomplish this, a retrospective chart review of 100,106 pregnant patients who underwent carrier screening at a clinical laboratory was conducted.

2. Methods

Carrier screening with reflex to cell-free DNA screening for CF is commercially available at a US-based laboratory. It is performed on genomic DNA using NGS to analyze all exons, exon-intron junctions, and select intronic regions of the *CFTR* gene. Identified *CFTR* variants are classified and reported according to American College of Medical Genetics and Genomics (ACMG) guidelines for carrier screening [24,25]. If a pregnant person is identified as a CF carrier, cell-free DNA analysis is performed on the plasma isolated from the same whole blood sample collected for carrier screening. Similar to carrier screening, this assay is NGS-based and includes coverage of all *CFTR* exons [16].

When assessing fetal risk for CF using cell-free DNA analysis, it is important to consider the possible zygosity of an affected fetus, which could be homozygous (inheritance of the same disease-causing variant from each parent) or compound heterozygous (inheritance of a different disease-causing variant from each parent). When genotyping an

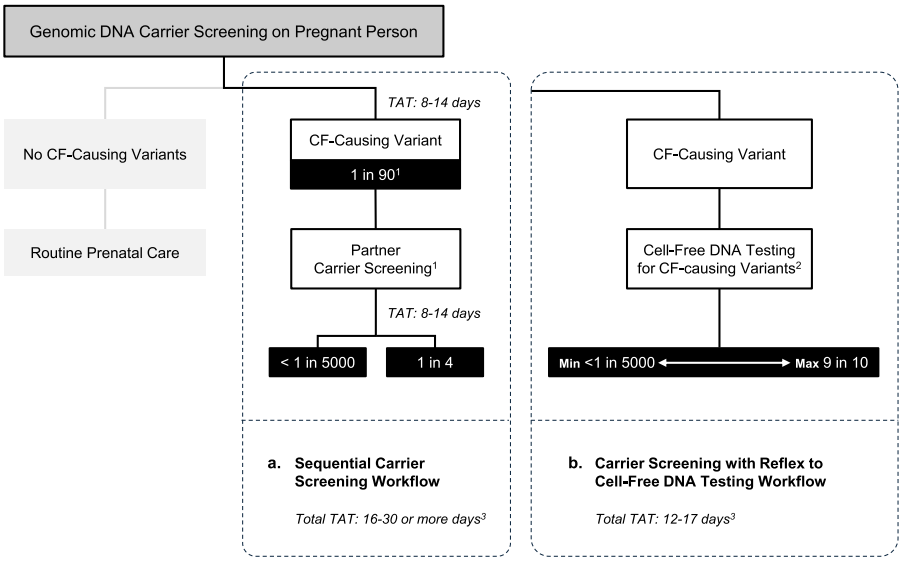

Fig. 1. The workflow for (a) traditional carrier screening with reflex to partner carrier screening compared to (b) carrier screening with reflex to cell-free DNA testing.

1. Partner carrier screening is completed <50 % of the time it is indicated, resulting in an incomplete risk assessment [11–15]. If partner carrier screening is incomplete, pregnancy risk to have an affected fetus is calculated with population carrier frequency. If partner carrier screening is completed, risk is binary of either 1 in 4 or residual risk after negative carrier screening in partner.

2. Completed on the same sample of blood collected for the initial carrier screening. Cell-free DNA testing returns a personalized fetal risk of CF that falls along a spectrum of <1 in 5000 to 9 in 10, based on the likelihood the fetus has two CF-causing variants.

3. Turn around time (TAT) of the traditional workflow is variable and dependent on the time required to contact the patient and arrange testing for the partner in workflow a. The cell-free DNA workflow is performed on the initial blood sample so there is a single TAT, as represented in workflow b.

individual using genomic DNA, variant status can be directly and qualitatively determined. However, when assessing fetal genotype using cell-free DNA, only a small fraction (usually less than 10 %) of the total cell-free DNA is fetal in origin; the remainder of the cell-free DNA is maternal in origin and arises from normal cellular turnover of the pregnant individual (maternal cell-free DNA). This presents a challenge in screening for an autosomal recessive condition, where the disease-cuasing variant is expected to be present in approximately half of the maternal cell-free DNA, eliminating the possibility of qualitative genotyping. Therefore, it is necessary to quantify the ratio of wildtype cell-free DNA versus variant cell-freeDNA to determine fetal zygosity. To determine this ratio and understand the precision of the measurement, the assay uses quantitative counting templates (QCTs)—synthetic molecules that amplify at the same rate as the cell-free DNA of interest—which allows quantification of the maternal disease-causing variant ratio relative to the fetal fraction (proportion of the cell-free DNA of fetal origin) to predict the expected ratio as a function of fetal zygosity [17]. Additionally, the assay analyzes the presence or absence of disease-causing variants distinct from the maternal variant to determine if the fetus is compound heterozygous. By combining these measurements with the *a priori* risk based on the carrier frequency of disease-causing variants, a personalized quantitative fetal risk for CF is calculated [17].

A data warehouse is maintained as part of the quality assurance (QA) program of the clinical laboratory performing this test. For this analysis, the data warehouse was first queried for a consecutive set of 100,106 pregnant patients who underwent clinical carrier screening and met eligibility criteria at the time of the study. Eligible cases included singleton pregnancies at or beyond 10 weeks gestation (since the collection of these data the assay has been validated at 9 weeks gestation) and not conceived via an egg donor or carried by a gestational surrogate. Cell-free DNA analysis was completed for all eligible CF carriers identified in this cohort, regardless of *a priori* risk or specific CFTR2 or CFTR-France *CFTR* variant classification. However, for this study, which focused on the ability to identify fetuses potentially eligible for CFTRm in a general-risk population, certain cases were excluded from the analysis. Specifically, individuals who were part of a known high-risk couple (*a priori* risk $\geq$1 in 4) or who themselves had two *CFTR* variants were excluded. Additionally, only fetuses predicted to have one or more CF-causing variants, as defined by CFTR2 and/or CFTR-France, were included. Variants associated with CFTR-related disorder phenotype and/or classified as variant of varying clinical consequence (VVCC) were excluded from this study cohort [25,26]. If a *CFTR* variant was not classified by either database, the variant was reviewed in ClinVar and included in the study cohort if determined to result in loss of function (LOF; e.g. nonsense, frameshift, canonical splice) *and* interpreted as "pathogenic" or "likely pathogenic" (P/LP) by the reporting laboratory. The identified *CFTR* variants were compared to the list of variants amenable to CFTRm per the US Food and Drug Administration as of December 2024 to determine the proportion of potentially eligible fetuses [27]. Population characteristics were calculated prior to application of exclusion criteria to best describe real-world users of this assay. The complete cohort is presented in supplementary data. The study was approved by the WCG institutional review board (IRB).

Results were converted to binary classifications for analysis of test characteristics. Pregnancies in individuals determined to be carriers of a CF-causing *CFTR* variant and whose cell-free DNA analysis calculated a risk of a CF-affected pregnancy greater than or equal to 1 in 4 were designated high risk. All other pregnancies among CF carriers were designated low risk. Affected fetal/neonatal CF status was solicited from all high-risk pregnancies, and from a random sampling of 50 % of the low-risk pregnancies. CF-affected status was determined through a parental report of *CFTR* molecular testing (prenatal or postnatal) and/or the results of newborn screening (NBS). NBS for CF is performed on all babies born in the US, is highly sensitive, and results in follow-up molecular and other diagnostic testing in infants who have a positive NBS [28]. All pregnancies were at least 4 weeks post estimated due date at the time of contact to ensure NBS was complete. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) with Clopper-Pearson 95 % confidence intervals were computed using the binary classification of the cell-free DNA results for cases with known CF outcomes. End-to-end clinical performance of CF carrier screening with reflex to cell-free DNA was calculated according to methods described in a prior publication [16]. A Brier score was calculated by taking the mean squared difference between the predicted risk and the observed outcome to assess the performance of the cell-free DNA individual numerical fetal risk results. A lower Brier score indicates a higher degree of calibration and accuracy in the predictions. Statistical analysis was completed in R Studio [29].

3. Results

Among the 100,106 consecutive carrier screening samples from pregnant patients, submitted to a single clinical laboratory, 99.7 % produced an informative result (negative carrier or quantitative cell-free DNA fetal risk assessment). The median turnaround time (days from the laboratory's receipt of the specimen to reporting of results) was 8.7 days, and the median gestational age (GA) when results were reported was 15.7 weeks. Compared to the US general population, the cohort was enriched for Black and Hispanic individuals [30], likely reflecting the regional demographics of clinical sites using this test (Table 1 [30]).

Among 100,106 pregnant individuals, 3621 were identified to be CF carriers (carrier frequency of 3.6 %). The median test turnaround time for identified CF carriers was 13.7 days from receipt of the original sample (12.7–17.7), and on average results were returned when the patient was less than 16 weeks GA (Table 2). A total of 212 cases were excluded due to less than minimum GA, more than one gestation, pregnancy conceived via an egg donor, or no-call/quality control failure. There were 32 cases (0.8 % of CF carriers) which resulted in a final no-call (where a fetal cell-free DNA risk could not be returned).

The analysis for the entire cohort, regardless of *a priori* risk, i.e., known high-risk couples, and *CFTR* variant classification with respect to CF classification status is in Table S1 and S2. However, the focus of this study is the ability of the assay to identify affected CF fetuses that may be eligible for *in utero* therapy in a general population setting. The final study cohort included 2587 CF carriers who had a general population *a priori* risk and a *CFTR* variant (221 unique variants identified in this study) classified as CF-causing by CFTR2 and/or CFTR-FRance or classified as LOF and P/LP by ClinVar (Figure S1 and Appendix 1). Performance of this assay in a high-risk cohort has been previously published [16], therefore, as described in the methods, 154 cases who had more than one *CFTR* variant or were part of a known high-risk couple as well as 636 cases who were carriers of a *CFTR* variant classified by CFTR2 as VVCC or classified by ClinVar as VUS, benign, or not LoF who had clinical cell-free DNA analysis completed were excluded from the specific study cohort. A complete list of *CFTR* variants included and

Table 1
Patient demographics and test performance metrics of CF carrier screening with cell-free DNA analysis to estimate fetal risk.

Total Sample (n = 100,106)	N	%
Pregnant Person Race and Ethnicity		
Asian	2497	3.44 %
Black	15,466	21.28 %
Hispanic	20,511	28.22 %
White, non-Hispanic	28,583	39.32 %
Other	5628	7.74 %
Unknown	27,421	–
	Median	**Interquartile Range**
Gestational Age when Results were Returned (weeks)	15.7	14.8 16.9
Turn Around Time (days)	8.7	6.7 11.7

Table 2
Patient demographics and test performance of carrier screening with cell-free DNA analysis among CF carriers.

CF Carrier Sample ($n = 3621$)	US CF Carrier Frequency	N	Sample Carrier Frequency
Pregnant Person Race and Ethnicity			
Asian	2.52 %	61	2.44 %
Black	2.43 %	355	2.30 %
Hispanic	3.19 %	622	3.03 %
White, non-Hispanic	5.45 %	1459	5.10 %
Other	3.61 %	196	3.48 %
Unknown		928	3.38 %
	Median	**Interquartile Range**	
Gestational Age when Results were Returned (weeks)	15.1	13.7	18.1
Turn Around Time for carrier screening w/ cell-free DNA (days)	13.7	12.7	17.7
cell-free DNA Fetal Fraction	6.0 %	4.2 %	8.3 %

excluded in the final cohort is in Appendix 1. The assay performance characteristics were similar between the specific study cohort and the full cohort, with 100 % sensitivity and 57 % PPV obtained for the full cohort, in line with the mean calculated risk provided for the high-risk cases (3 in 5).

Out of the 2587 pregnancies in the specific study cohort, 40 were identified by cell-free DNA analysis to be high risk. Of these, 30 were predicted to be compound heterozygous and 10 were predicted to be homozygous. The 40 high-risk cases included 38 (95 %) where the fetus was predicted to have at least one *CFTR* variant responsive to CFTRm. In eight of these pregnancies only the paternal variant identified in the fetal cell-free DNA was CFTRm responsive. Of the high-risk cases, the mean calculated risk for the fetus to be affected with CF was 3 in 5 (range: 1 in 4 to 9 in 10).

Neonatal outcomes were solicited for the 40 high-risk cases and a 50 % random sampling of the low-risk cases. The CF-affected status was obtained for 20 of the 40 pregnancies identified as high-risk during pregnancy, of which 13 neonates were confirmed to have CF (positive predictive value [PPV]: 65 %; 95 % CI: 47 %–79 %). This included all 8 cases with a calculated 9 in 10 risk. All diagnoses in this sample were made postnatally (Table 3; full cohort in Table S1). Details for all 40 high-risk cases, including those without confirmed neonatal outcomes, are provided in Appendix 2.

An outcome was obtained for 335 low-risk neonates (median 1 in 2000; range: 1 in 5000 to 1 in 5). There were no false negatives identified in the study cohort or the full cohort (Table S2). There were five cases whose risk was greater than *a priori* but still less than 1 in 4 (range 1 in 5 to 1 in 43), and all were unaffected with CF. All neonates affected with CF were identified as high risk by cell-free DNA in pregnancy (sensitivity: 100 %, 95 %CI: 75 %−100 %; Table 4, Table S2 full cohort). The Brier score for all 357 pregnancies with a known CF neonatal outcome was <0.01, demonstrating that the calculated fetal risk from cell-free DNA analysis offers an accurate personalized positive predictive value for that pregnancy.

Table 3
Pregnancies with cell-free DNA fetal risk ≥ 1 in 4 and resulting neonatal cystic fibrosis (CF) status in general-risk cases where at least one of the two predicted *CFTR* variants was classified by CFTR2 as CF-causing or classified by ClinVar as P/LP and LoF. Full cohort in Table S1.

cell-free DNA fetal risk	Affected with CF	Total High Risk	Positive Predictive Value
9 in 10	8	8	100 %
2 in 3 to 9 in 10	1	1	100 %
1 in 2	3	9	33 %
1 in 4 to < 1 in 2	1	2	50 %
Total	**13**	**20**	**65% (47 %−79 %)**

Table 4
Cell-free DNA fetal risk and neonate cystic fibrosis (CF) status and calculated assay performance analytics based on outcomes collected for the general-risk cases where at least one of the two predicted *CFTR* variants was classified by CFTR2 as CF-causing or classified by ClinVar as P/LP and LoF. Full cohort in Table S2.

	cell-free DNA fetal risk ≥ 1 in 4	cell-free DNA fetal risk < 1 in 4
Affected with CF	13	0
Unaffected with CF	7	335
	% (95 % CI)	
Sensitivity	100 % (75–100 %)	
Specificity	98 % (96 %−99 %)	
PPV	65 % (47 %−79 %)	
NPV	100 % (98–100 %)	
End-to-End Sensitivity	98.7 % (93.0–99.9 %)	
End-to-End Specificity	99.96 % (99.94–99.97 %)	

4. Discussion

The development of CFTRm has changed the natural history of CF, decreased the morbidity and mortality of CF, and increased fertility for individuals with CF [17]. *In utero* use of CFTRm to treat fetuses affected with CF shows great promise with earlier implementation potentially offering greater protection from disease complications early in life [22, 23]. At the same time, there are ethical considerations for *in utero* therapy and there is currently no consensus regarding indications, monitoring, measuring outcomes, and long term sequelae after *in utero* exposure.

In this study, cell-free DNA testing identified suspected maternally and paternally inherited *CFTR* variants, providing patients with a previously unknown reproductive risk for CF with a personalized risk assessment for the fetus to be affected with CF. On average, testing was completed in less than 2 weeks and prior to the 16th week of pregnancy. The test was highly accurate, providing a very high sensitivity and specificity, along with well-correlated risk estimates of the likelihood of an affected fetus. Comparatively, traditional carrier screening approaches may miss up to half of affected fetuses due to challenges with partner testing and misattributed paternity [8–12]. These missed cases may be disproportionately experienced by individuals with unplanned pregnancies and pregnancies in which a partner is unavailable or not involved in the pregnancy. Furthermore, traditional carrier screening provides an invariable maximum risk estimate of 1 in 4 whereas cell-free DNA analysis provides an individual fetal risk assessment which can better inform decisions around prenatal diagnostic testing. The traditional carrier screening method often takes longer to complete due to logistical challenges of multi-step screening and, therefore, may not allow for the same timely initiation of treatment [7].

Although outcomes were obtained for half of the high-risk cases and 25 % of low-risk cases, the positive predictive value was much higher than traditional carrier screening (65 % versus 25 %). There were no false negative cases, resulting in 100 % sensitivity for this cohort. The cell-free DNA assay covers all *CFTR* exons to identify pathogenic variants inherited from the reproductive partner and determines homozygous status for *CFTR* variants with a greater than 0.0001 allele frequency, including, but not limited, to p.Phe508del. The variant combinations not covered by the assay represent less than 1.5 % of all CF cases and the assay is designed to have an overall clinical sensitivity of >97 % [15]. In this study, the continuous measurement of personalized fetal risk was binned into "positive" or "negative" using a threshold of 1 in 4. This binning allows the calculation of sensitivity, specificity, NPV, and PPV of the test, measurements of screening tests familiar to clinicians. However, this diminishes the performance of a test that returns a

quantitative fetal risk by considering a risk of 1 in 5 and a risk of 1 in 5000 as equally negative and a risk of 1 in 3 and 9 and 10 as equally positive. In other study cohorts when binning is applied to continuous measurement, a lower cut-off for positive and negative may improve the test sensitivity (however in this dataset there were no affected cases with a risk lower than 1 in 4) at the expense of the specificity and vice versa. The sample included individuals from across the US and the CF carrier frequencies for each race and ethnicity were slightly higher than those of the general US population related to a bias of clinicians ordering the test for known carriers [1]. The performance of cell-free DNA may differ in a population with a different race and ethnicity breakdown, however, given both the genomic DNA carrier screening of the pregnant individual and the cell-free DNA analysis for fetal risk is NGS-based, it is not expected to differ greatly. Finally, this analysis focused on a specific cohort of CF carriers to investigate the performance of the assay for the detection of fetuses at risk for CF and potentially eligible for CFTRm. The analysis showing assay performance for the full cohort can be found in supplementary Tables 1 and 2 and reflects similar test performance to that demonstrated in prior studies of the assay which included all types of CF carriers. These data suggest that the performance of the assay is not impacted by variant type or *a priori* risk, as expected given that the assay utilizes NGS of the full *CFTR* gene [15,16].

Prenatal diagnostic testing decisions and/or use of *in utero* modulator therapy were not assessed in this cohort. However, other individuals with pregnancies identified by cell-free DNA screening to be high risk for CF reported they have pursued diagnostic testing and subsequent *in utero* CFTRm (internal data). Similar to all 40 of the high-risk cases and the 13 confirmed affected cases in this sample, these patients were unaware of their CF carrier status prior to cell-free DNA screening, demonstrating the power of this screening method for the general risk population to access therapy after diagnostic testing.

It remains unclear to what degree timing of CFTRm initiation for *in utero* therapy will affect clinical outcomes such as exocrine pancreatic sufficiency at birth or vas deferens development, although it seems likely that earlier initiation is associated with greater potential for clinical effect. Reports include variable success of treatment of hypoechoic bowel potentially impacted by gestational age of treatment initiation [19,21]. To realize the promise of *in utero* CF therapy, more formal studies are needed with consensus indications for treatment, optimal initiation time, uniform monitoring, and established end to end outcomes. These studies will only be fully comprehensive through the inclusion of the general population, in which high-risk pregnancies can effectively be identified through cell-free DNA screening and follow-up prenatal diagnostic testing.

5. Conclusion

In utero therapy for CF is on the horizon and an effective, efficient, and equitable screening method allows for access to this therapy. This paper demonstrates that carrier screening with cell-free DNA analysis has the potential to detect fetuses at high risk for CF in a general population cohort without the need for a paternal sample, predicting CFTR modulator therapy eligibility, and facilitating diagnostic testing and possible initiation of early (*in utero*) treatment. Furthermore, compared to traditional carrier screening, this workflow is predicted to identify more therapy-eligible fetuses with a shorter timeline to diagnosis and start of therapy, if indicated.

Author contributions

JW contributed to conceptualization, data curation, formal analysis, project administration, and original manuscript preparation. SR and DCB contributed to data curation, project administration, and review and editing of the final manuscript. RC contributed to data curation, and review and editing of the final manuscript. ATalati and MZ contributed to supervision, and review and editing of the final manuscript. ATrimble contributed to conceptualization, formal analysis, supervision, and review and editing of the final manuscript.

Declaration of competing interest

JW, SR, DCB, RC are employees of BillionToOne Inc., ATalati has received research funding for other research projects from BillionToOne. ATrimble and MZ have no conflicts to disclose.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2025.08.004.

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