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Non-invasive prenatal diagnosis for cystic fibrosis: Implementation, uptake, outcome and implications

Running title: Non-invasive prenatal diagnosis for cystic fibrosis

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Key words: non-invasive prenatal diagnosis, RMD, RHDO, cystic fibrosis, cell-free fetal DNA, monogenic disease

Abbreviations: Non-invasive prenatal diagnosis (NIPD); Cystic Fibrosis (CF), Relative haplotype dosage analysis (RHDO); circulating cell free DNA (cfDNA); single nucleotide polymorphism (SNP); sequential probability ratio testing (SPRT)

Human genes: CFTR

Abstract

Background

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Non-invasive prenatal diagnosis (NIPD) for monogenic disorders has a high uptake by families. Since 2013, our accredited public health service laboratory has offered NIPD for monogenic disorders, predominantly for *de novo* or paternally dominantly inherited mutations. Here we describe the extension of this service to include definitive NIPD for a recessive condition, cystic fibrosis (CF).

Methods

Definitive NIPD for CF was developed using next generation sequencing. Validation was performed on 13 cases from 10 families prior to implementation. All cases referred for CF NIPD were reviewed to determine turnaround times, genotyping results and pregnancy outcomes.

Results

Of 38 referrals, 36 received a result with a mean turnaround of 5.75 days (range 3-11 days). Nine cases were initially inconclusive, with three reported unaffected since the low risk paternal allele was inherited, and four cases where high risk paternal allele was inherited, receiving conclusive results following repeat testing. One case was inconclusive due to a paternal recombination around the mutation site and one case was uninformative due to no heterozygosity. Before 2016, three invasive referrals annually for CF were received compared with 38 for NIPD in the 24 months since offering a definitive NIPD service.

Conclusions

Timely, accurate NIPD for definitive prenatal diagnosis of CF is possible in a public health service laboratory. The method detects recombinations and the service is well-received as

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evidenced by the significant increase in referrals. The bioinformatic approach is gene agnostic and will be used to expand the range of conditions tested for.

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Prenatal diagnosis allows families to make informed choices, which may include preparing for the birth of an affected child, planning pre- or perinatal management or choosing termination of pregnancy. Until recently, this has required analysis of fetal genetic material obtained by invasive testing, amniocentesis or chorionic villus sampling, both associated with a small risk of miscarriage[1], thus posing a barrier to testing for some families[2]. The identification of circulating cell-free fetal DNA, representative of the whole fetal genome in the maternal plasma[3,4], has allowed the development of safer prenatal screening and diagnosis based on a maternal blood sample. While there has been widespread uptake of non-invasive testing for aneuploidy screening[5], there has been relatively little clinical implementation for non-invasive prenatal diagnosis (NIPD) of monogenic disorders, probably because there has been little commercial drive since the volume of testing is lower and many cases require labour-intensive test development on a by family basis.

Our accredited National Health Service Laboratory established a NIPD service for the diagnosis of monogenic disorders in 2011. Since the majority of circulating cell free DNA (cfDNA) is maternal in origin with the fetal fraction ranging from ~2% to 20% depending on gestation and other factors[6], this service was initially limited to the detection or exclusion of the paternal allele or those arising *de novo* for selected disorders such as achondroplasia and thanatophoric dysplasia[7-11] and a tailor-made service where familial-specific assays were developed[12].

Our NIPD service for cystic fibrosis (CF) was initially based on paternal mutation exclusion of ten common mutations found in the European population[12,13]. For parents to be eligible, they must be carriers of different mutations. Testing looks for the paternal mutation in the

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maternal cfDNA, which if absent means the fetus is unaffected but if present requires invasive testing to determine the inheritance of the maternal mutation and provide definitive diagnosis. However, since the most common CF causing mutation (*CFTR* p.(Phe508del)) is present in around 4% of Caucasians, an estimated 47% of carrier parents are ineligible because both carry this mutation. A survey of CF carrier parents showed that 56% would decline invasive diagnostic testing[13], but 94.9% of parents would choose to have NIPD for CF, including those who had previously declined invasive testing.

While detection or exclusion of an allele present in the fetus but not the mother is relatively straightforward, reliably determination of whether or not the fetus has inherited the maternal mutant allele to definitively diagnose X-linked or recessive conditions is challenging due to the high background of mutant allele in the mother's plasma. In this situation the relative amount of the maternal mutation must be measured rather than presence or absence of a mutation. Several approaches have been described including digital PCR and relative mutation dosage for β-thalassaemia[14] and sickle cell disorder[15], but these are not universal assays since separate assays are required for each mutation and to calculate the fetal fraction. As such neither are good options for routine clinical implementation for conditions with multiple mutations. In 2010, Lo *et al.*[4] described relative haplotype dosage analysis (RHDO) using whole genome next generation sequencing data as a universal assay inclusive of fetal fraction. More recently, RHDO has been applied to target enriched samples, which is more cost effective[16]. Because RHDO is a linkage based method, the same assay can be used for all mutations. RHDO of target-enriched next generation sequencing data has been described for a number of monogenic disorders[17-19].

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Here our objective was to develop, validate and implement an accredited non-invasive NIPD service for the definitive diagnosis of CF using RHDO, regardless of parental genotype. This would extend our existing CF NIPD service based on paternal exclusion, which is only applicable to around 29.7% of CF carrier parents[13].

MATERIALS AND METHODS

Ethical Approval

The National Health Service Research Ethics Committee approved the collection of maternal and paternal blood samples for NIPD test development (01/0095). Subsequently, we received approval (Audit Registration number 1925) to audit the service delivered to date.

Study setting and patients

The study setting included parents with a pregnancy at risk of having CF because both parents were carriers. For the validation study, women undergoing invasive testing for CF were recruited as part of the Reliable Accurate Prenatal non-Invasive Diagnosis (RAPID) study (RP-PG-0707-10107) between 2009 and 2015. Eligibility included written informed consent from both parents to obtain blood and store parental germline DNA and maternal plasma, with the result of invasive testing for CF known. The clinical audit was conducted from 2016 to 2018 and included all pregnancies referred for NIPD for CF in this time period.

Test development

Sample collection

For the validation study, maternal plasma samples from 13 cases in 10 families with known prenatal outcomes were tested. Maternal blood was collected into EDTA or cell stabilising

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tubes and processed for storage at -80°C as previously described[15]. For the service samples, 20 ml of blood was collected from the mother after 9 weeks gestation (as determined by ultrasound scan) into EDTA or cell stabilising tubes and cfDNA was extracted once the samples were received in the laboratory. For all cases, genomic DNA from parental and affected or unaffected (non-carrier) proband was extracted. cfDNA was extracted from 2x4 ml of maternal plasma using the QIASymphony DSP/Virus Kit (Qiagen), eluting in 60 µl EB buffer.

Design of enrichment probes

1,748 biotinylated complementary ribonucleic acid probes (SureSelect Custom Enrichment; Agilent Technologies) were designed targeting the coding regions of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene (chr7:117,477,963-117,670,665 GRCh38) and including an additional 1 Mb flanking region upstream and downstream of *CFTR* gene. These probes targeted 225 heterogeneous single nucleotide polymorphisms (SNPs) in the captured region (range chr7:116,138,845-118,322,637).

Library preparation and sequencing

Parental and proband genomic DNA was prepared using the SureSelect XT Library Preparation kit. cfDNA libraries were prepared using the NEBNext®Ultra™II DNA Library Prep Kit (New England Biolabs) for CF, supplemented with the SureSelect Adapter Oligo Mix (Agilent Technologies) for adapter ligation and SureSelect Primer and SureSelect Reverse Primer (Agilent Technologies) for PCR. 16 cycles of PCR were used for cfDNA. Hybridisation, capture and indexing were performed as per SureSelect protocol, using 14 cycles of post-hybridisation PCR. Captured, indexed libraries were qualified using the Tapestation D1000 High Sensitivity Tape (Agilent Technologies) and quantified with QubitHS dsDNA assay (Life Technologies).

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Libraries from up to three CF families were pooled, with 20-60% of the total pool dedicated to each cfDNA library and 2.1-13.3% per genomic DNA sample dependent on the number of cases. The final pooled library was loaded at a concentration of 1.4-1.6 pmol plus 1% PhiX control and sequenced using 75-cycle paired-end reads with a NextSeq Mid Output 150cyclev2 reagents on an Illumina NextSeq500.

Data processing

FASTQ files were aligned using Burrows-Wheeler Aligner[20] and duplicate reads were removed. Variants were called with VarScan[21]. SNPs with read depth of at least 30 and separated by at least 200 bp from the adjacent SNP were included, as previously described[4]. Only single nucleotide variants were included. As initially described by Lo and colleagues[4], SNPs were split into the categories described in Table 1.

Fetal fraction

Fetal fraction was calculated from type1 SNPs (Table 1) as previously described[5]

Fetal fraction = (2 x Paternal read depth / (Maternal + Paternal read depth)) x 100%

Paternal allele inheritance

The presence or absence of SNPs linked to each of the paternal alleles was determined using type3 SNPs. Here, the fraction of the paternal-specific allele was calculated for each of the four potential allele combinations. The median fraction of each group was calculated and a Mann-Whitney U-test applied to the unaffected and affected counts to demonstrate significant difference between groups. In essence, a 0 fraction of a particular allele indicated absence of that allele; a fraction similar to the fetal fraction calculated using type1 SNPs indicated presence of that allele.

Maternal allele inheritance –RHDO analysis

RHDO was performed as previously described, with an odds ratio of 1200 in sequential probability ratio testing classification[4,16,18,22]. Briefly, sequential probability ratio testing[14]

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was applied to calculate the statistical significance of allelic imbalance for type4a and 4b SNPs, the hypothesis being that there is no imbalance between haplotype I (HapI) and II (HapII). HapI represented the allele that was present in the proband used for phasing and HapII the alternate allele; therefore, if an affected proband was included an overrepresentation of HapI 4a SNPs suggested inheritance of the affected allele by the fetus. For type4b SNPs, heterozygosity (an equal balance of HapI and HapII reads) indicated inheritance of the maternal mutation. This process is repeated in both 5' and 3' directions.

Audit of clinical service

Retrospective review of laboratory records was undertaken to determine all CF RHDO NIPD cases. Genotypes and turnaround times were ascertained. Pregnancy outcomes were determined from clinical records or contact with referring clinicians. Laboratory records were reviewed to determine the number of invasive test analyses performed for CF per annum since 2012.

RESULTS

Validation study

Cell free DNA from 13 cases at risk of CF from 10 families was tested (Table 2). Gestation ranged from 11 to 33 weeks. Fetal fraction was calculated using type1 SNPs for 9 families, with the exception of family 9, where type3 SNPs were used. Fetal fraction ranged from 6-19% (mean 13.2%) and we identified a mean of 161 SNPs per case. In 5 of 13 cases, the reference haplotype was derived from an invasive sample in the same pregnancy as the cfDNA with the remainder from a different pregnancy/living proband. Eleven of 13 cases were tested using an affected proband with the remaining 2 families using an unaffected (non-carrier) sibling. For the paternal allele, between 33 and 99 (mean 61.7) informative

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SNPs were used with a mean classification accuracy of 95% (range 85-100%). For the maternal allele, between 10 and 70 (mean 27) haplotype blocks were used with a mean classification accuracy of 94% (range 86-100%). All NIPD results aligned with the genetic testing of chorionic villus samples. In addition to ensuring there are maternal haplotype blocks / paternal SNPs on both sides of the mutation in order to identify recombination events, we used the data from the validation cases to set initial cut-off values for classification accuracy and number of blocks/paternal SNPs for determining acceptability of results in clinical service. Thus, we started with a lower cut-off of 10 maternal haplotype blocks because this was the lowest cut-off with 100% accuracy in the validation set. Since there was a trend observed that with an increasing number of blocks the classification accuracy decreased (Table 2 case 1 and 11 for examples), we set different acceptance criteria based on the number of blocks called. With increasing numbers in the service cohort, we had then reviewed the data and reduced the minimum number of blocks required to 8 since we had two cases with 8 blocks and 100% accuracy (online Supplementary Table 1, cases 13 and 15). Supplementary Table 2 shows our current classification criteria. It was more difficult to set cut-off values for the paternal SNPs because these were individual SNP calls rather than blocks and therefore PCR and sequencing artefacts could have a greater impact on classification accuracy. In addition, the type of class 3 SNP needed to be considered when making a judgement since it is the SNPs where the paternal contribution was observed that were the most important. If none of these were seen the outcome was inconclusive. The lowest number of SNPs observed in the validation cohort was 33 with a classification accuracy of 91% (Table 2, case 2). These values were initially used as our cut-off criteria but as the numbers increased in the service cohort we revised the minimum number of paternal SNPs required to 10 (Supplementary Table 1, case 16).

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Clinical service cases

Thirty-eight referrals for NIPD for CF were identified from 35 families (Supplementary Table 1). Sequencing metrics are shown in Supplementary table 3. Gestation ranged from 9 to 25 weeks. The mean reporting time was 5.6 calendar days. An example case (case 17) is shown in Figure 1. Of the 38 cases, 29 had fully conclusive reports issued from the initial test. Seven fetuses were predicted to be affected, six unaffected non-carriers, seven carriers of the maternal mutation and nine carriers of the paternal mutation. Nine cases were initially reported with at least one of the alleles being inconclusive when the classification accuracy fell below our accepted criteria (Table 3). For three of these cases (cases 14, 24 and 33), because the paternal allele was conclusive for the low risk haplotype, conclusive reports were issued predicting the fetus to be unaffected. In a further four cases (cases 12, 21, 34 and 36), repeat samples were requested, which gave conclusive results. These cases had relatively low fetal fraction (range 3 – 6.7%) resulting in a low number of maternal haplotype blocks and poor classification accuracy. The fetal fraction increased for all the repeat samples. For the remaining two inconclusive cases invasive testing was recommended. For Case 11, the high risk maternal allele was detected, but a likely recombination event was detected on the paternal allele (Figure 2). The recombination event occurred either within the gene or just 5' of the gene with the paternal mutation falling within the recombinant region. We were therefore unable to determine the inheritance of the paternal mutation. Subsequent invasive testing found the fetus to be affected. For case 32 there was a small number of type1 and type3 SNPs and a high level of type2 SNPs. None of the type1 and type3 SNPs were located 3' of the gene meaning we could not determine fetal fraction or the paternal inheritance 3' of the gene. Due to the risk of recombination events and the need for accurate fetal fraction for sequential probability ratio testing analysis, both were reported as inconclusive and invasive testing recommended. This result was thought likely to be caused by consanguinity.

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Fetal fraction was calculated using type1 SNPs for 33 families. In five families there were more type3 SNPs so these were used instead. Fetal fraction ranged from 3.8% to 19.8% (mean 10.0%). We identified a mean of 192 informative SNPs per case. For the service cases, all probands were affected siblings. For the results issued as conclusive for the paternal allele, between 10 and 121 (mean 58) informative SNPs were used with a mean classification accuracy of 96.3% (range 90-100%). For those conclusive for the maternal allele, between 8 and 122 (mean 35) haplotype blocks were used with a mean classification accuracy of 94.8% (range 82-100%). These statistics for the service samples closely matched that of the validation cases. (Supplementary Table 4)

Outcomes were requested when pregnancies completed. All outcomes that have been received to date are correct with two outcomes being confirmed by genetic testing postnatally. The most recent service case pregnancies are ongoing so outcome data is not available.

Discussion

Here we developed an assay for definitive NIPD of CF, regardless of mutation type, which includes an automated pipeline for speedy data analysis to expedite return of results in a busy public health service laboratory. In the first two years of service we have had 38 referrals, delivering results within 5.6 calendar days (range 3–11 days) to inform pregnancy management without need for invasive testing. This included 19 families where parents were carriers of the same mutation and so would not have been eligible for paternal mutation exclusion[1] and a further 10 where inheritance of the paternal mutation would necessitate invasive testing to determine inheritance of the maternal allele. In nearly half of our cases one

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or both parents carried the common p.(Phe508del) mutation, and it could be argued that alternative technologies such as droplet digital PCR could be used. However, because droplet digital PCR will only detect one mutation at a time it cannot be used as a universal assay and would require development of additional assays in more than half of our cases. In addition, the RHDO method will be more robust due to the use of multiple SNPs or haplotype blocks rather than just the one mutation site and is therefore much less likely to generate incorrect results.

The main limitation of the haplotyping method is that samples for both parents and a proband (either an affected proband or an unaffected non-carrier child) are required to determine phase and allow genotyping using cfDNA in any new pregnancy. One solution might be to look at methods of expanding the range of family members that could be used for testing to include carrier siblings where the parents carry two different mutations and then grandparents plus siblings of a parent. However, recent reports using microfluidics-based linked-read sequencing technology suggest that haplotypes can be deduced directly without the need for a proband or relative. These approaches have been demonstrated in a small series but currently the cost would prohibit use clinically in a public sector genetics laboratory[23,24]. Another limitation of using a haplotype method is that recombination events can impede analysis. Our assay is able to detect such events but invasive testing is required if the mutation of interest falls within the recombinant region (Figure 2). Our results show that a fetal fraction >4% is required to make a diagnosis for the maternal allele since all four samples with levels below this yielded inconclusive results. All inconclusive results were at fetal fractions ≤6.7%, although the other cases with fetal fractions in this range gave conclusive results (Supplementary Table 1). The cause of the low fetal fraction is unclear but could be due to high maternal body mass index. Unfortunately, we did not have access to maternal weight.

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One potential factor may be maternal cell lysis prior to separation of plasma since all samples with low fetal fraction were collected into EDTA tubes. However, all were centrifuged within 25 hours of blood draw and handled according to our standard procedures. Other samples with higher fetal fraction were collected under similar conditions. However, we were unable to control temperature and sample storage prior to receiving the samples, which could also be a factor. Our current practice is to accept samples in EDTA tubes providing they are received and centrifuged within 48 h of blood draw. If transit times are longer we ask that cell stabilising tubes be used to prevent excessive maternal cell lysis in transit[25].

Another limitation of this approach is with twin/multiple pregnancies. In theory, this approach could be used for confirmed monozygotic twins however this approach would need validation prior to clinical implementation. This approach is not suitable for dizygotic twins due to the potential for different haplotypes. This approach also needs to be considered in cases of vanishing twins. Thus, it is essential that all patients referred for testing have had an ultrasound scan prior to blood draw.

Cost of delivering an NIPD service warrants consideration since the RHDO pathway has been shown to be approximately £990 more expensive than the invasive testing care pathway for CF[26]. Furthermore, the cost of the service itself is likely to increase significantly if the increase in uptake of safer NIPD suggested by a review of parental opinions[2] translates to clinical practice. Our experience since offering NIPD for CF would suggest that this is likely to happen in reality because we have had 38 requests for RHDO NIPD for CF in two years, when we would usually only do three invasive prenatal tests per year. Notably, studies looking at reproductive decision making with CF indicate that while many parents report that they would use results to make a decision about termination of pregnancy, a large proportion

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are likely to use the knowledge to prepare for the birth of a baby affected with CF[13,27]. This highlights the ethical issues associated with directing resources to a test that would not change pregnancy management in state funded health systems, an issue raised by parents and health professionals[13,26]. Implementation strategies will need to give consideration to this issue, especially given that the parents may change their mind about the decision not to terminate the pregnancy once results are known[28] and there are psychological benefits afforded by early knowledge of whether the child is affected or not, even when termination would not be considered by the parents[29].

In conclusion, we have successfully expanded our NIPD service to include definitive diagnosis of CF. Our bioinformatics pipeline should be applicable to other recessive conditions and is now being applied to spinal muscular atrophy and congenital adrenal hyperplasia. This is a flexible and feasible approach for a routine diagnostic laboratory to implement. While the current need for a proband sample does place some restrictions on which families are eligible, it is likely that the assay could be performed without the proband sample in the near future, which would further increase the number of parents who could be offered NIPD. Since it is clear that parents prefer a non-invasive approach to the diagnosis of monogenic conditions wider implementation globally of NIPD would seem appropriate.

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Table 1 - Categorisation of single nucleotide polymorphism types based on parental inheritance and the information that can be interpreted from each type determined from the proband's genotype and the allelic inbalance.

SNP type	Subtype	Maternal genotype		Paternal genotype		Proband genotype		Information from genotype	Result interpretation
Type 1	A	A	A	B	B	A	B	Detection of paternal contribution but not specific allele	Fetal fraction
	B	B	B	A	A	B	A		
Type 2	A	A	A	A	A	A	A	Sequencing error rate	Quality control
	B	B	B	B	B	B	B		
Type 3	A	A	A	A	B	A	A	Detection of paternal specific allele	If B detected - discordant with proband
	B	A	A	B	A	A	B		If B detected - concordant with proband
	C	B	B	A	B	B	A		If A detected - concordant with proband
	D	B	B	B	A	B	B		If A detected - discordant with proband
Type 4a	1	A	B	A	A	A	A	Detection of maternal specific allele	SPRT call: 1) Homozygous for A - concordant with proband 2) Heterozygous – discordant with proband
	2	B	A	B	B	B	B		SPRT call: 1) Homozygous for B - concordant with proband 2) Heterozygous – discordant with proband
Type 4b	1	A	B	B	B	A	B		SPRT call: 1) Homozygous for B - discordant with proband 2) Heterozygous – concordant with proband
	2	B	A	A	A	B	A		SPRT call: 1) Homozygous for A - discordant with proband 2) Heterozygous – concordant with proband

SPRT, sequential probability ratio testing

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Table 2: Summary of NIPD results for the validation cohort.

Case	Paternal mutation	Maternal mutation	Gestation cfDNA sampled	Reference haplotype	cfDNA yield (ng)	Fetal fraction (%)	NIPD result (Paternal/ Maternal)	Confirmed result	Informative SNPs used	Haplotype blocks (mat only)	Paternal SNPs	Classification accuracy (mat/pat)	Mean sequenicng depth of SNPs used
1	p.Phe508del/N	p.Phe508del/N	12+3	Unaffected sibling	138	14	p.Phe508del/ p.Phe508del	Affected	174	16	99	86%/99%	563
2	p.Phe508del/N	p.Phe508del/N	12+1	Unaffected sibling	82	18	N/N	Unaffected	208	28	33	100% / 91%	353
3 $	p.Lys684Serfs/ N	p.Gly542*/N	11+2	Affected proband	191	19	N/p.Gly542*	Unaffected carrier maternal	136	34	60	100% / 95%	720
4 $	p.Lys684Serfs/ N	p.Gly542*/N	11+2	Affected proband	95	11	p.Lys684Serfs/ p.Gly542*	Affected	136	14	60	100% / 100%	619
5 $	p.Lys684Serfs/ N	p.Gly542*/N	11+4	Affected proband	45	16	p.Lys684Serfs/ p.Gly542*	Affected	136	28	60	100% / 100%	722
6	p.Phe508del/N	p.Phe508del/N	12+6	Affected proband	130	12	N/N	Unaffected	130	28	36	100% / 94%	401
7 ₸ (p1)	p.Phe508del/N	p.Phe508del/N	11+5	Affected proband 1	73	10	p.Phe508del/ p.Phe508del	Affected	117	10	58	100% / 95%	261
8 ₸ (p2)	p.Phe508del/N	p.Phe508del/N	11+5	Affected proband 2	73	10	p.Phe508del/ p.Phe508del	Affected	118	10	58	100% / 95%	262
9	p.Phe508del/N	p.Phe508del/N	12+0	Affected proband	125	9	N/ p.Phe508del	Unaffected Carrier	174	12	67	100% / 85%	442
10	p.Phe508del/N	p.Lys1177Serfs/ N	33+2	Affected proband	105	13	p.Phe508del/ p.Lys1177Serfs	Affected	200	22	83	91% / 95%	748
11	p.Leu732*/N	p.Phe508del/N	12+0	Affected proband	221	6	N/ p.Phe508del	Unaffected carrier (maternal)	158	25	44	86% / 95%	1183
12	p.Phe508del/N	p.Phe508del/N	11+5	Affected proband	80	23	N/ p.Phe508del	Unaffected Carrier	183	42	55	95% / 96%	413
13	p.Phe508del/N	p.Phe508del/N	12+1	Affected proband	116	11	p.Phe508del/ p.Phe508del	Affected	218	56	89	96% / 97%	585

Only informative SNPs that passed the quality control criteria were used for the analysis with the average sequencing depth calculated from only the informative SNPs. Classification accuracy for the maternal allele indicates the percentage of haplotype blocks that gave concordant results with the inherited allele determined by the majority call. Classification accuracy for the paternal allele indicated the percentage of type 3 SNPs giving concordant results with the inherited allele determined by the majority call. N= Normal. $ and ₸ = same family. ₸ family was tested using two affected probands (p1 & p2)

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Table 3 – Summary of initially inconclusive NIPD results for the service cohort

Case	Paternal mutation	Maternal mutation	Gestation cfDNA sampled	Reference haplotype	cfDNA yield (ng)	Fetal fraction (%)	NIPD result (Paternal/ Maternal)	Outcome (where known)	Informative SNPs used	Haplotype blocks (mat only)	Paternal SNPs	Classification accuracy (mat/pat)	Mean sequenicng depth of SNPs used	TAT (Calendar days)
11	p.Phe508del/N	p.Phe508del/N	10+3	Affected proband	66.7	10	Inconclusive# / p.Phe508del	Confirmed affected	176	48	27	100% / 59%#	646	8
12	p.Phe508del/N	p.Phe508del/N	10+4 Rpt 12+3	Affected proband	97.8 Rpt 125.4	3 Rpt 5	p.Phe508del / Inconclusive Rpt p.Phe508del / p.Phe508del	Miscarriage at 14 weeks	212 Rpt 209	6 Rpt 16	61 Rpt 80	67% /97% Rpt 88% / 99%	649 Rpt 1140	7 Rpt 3
14	p.Phe508del/N	p.Phe508del/N	9+4	Affected proband	178.5	3.8	N / Inconclusive	Unaffected (NBS-normal)	174	6	18	75% / 100%	608	3
21	p.Phe508del/N	p.Phe508del/N	9+5 Rpt 10+5	Affected proband	145.5 Rpt 131	6.7 Rpt 6.9	p.Phe508del / Inconclusive Rpt p.Phe508del / N	Unaffected (NBS-normal)	219 Rpt 219	18 Rpt 17	121 Rpt 121	67%/ 99% Rpt 88% / 100%	559 Rpt 600	4 Rpt 6
24	p.Phe508del/N	c.1585-1G>A/N	9+6	Affected proband	190.5	5.6	N / Inconclusive	Unaffected	181	7	65	86% / 97%	623	7
32	p.Phe508del/N	p.Phe508del/N	9+3	Affected proband	185.5	Undetermined	Inconclusive / Inconclusve	-	116	13	29	85% / 100%	452	3
33	p.Phe508del/N	c.4139del/N	9+1	Affected proband	102.2	4	N / Inconclusive	-	199	9	46	78% / 94%	422	9
34	c.1652G>A/N	p.Phe508del/N	11+5 Rpt 13	Affected proband	189 Rpt 174	6.2 **Rpt 7.4**	c.1652G>A /inconclusive Rpt c.1652G>A / p.Phe508del	-	222 Rpt 212	4 Rpt 13	83 Rpt 89	50% / 97% Rpt 85% / 94%	686 Rpt 476	3 Rpt 8
36	p.Phe508del/N	p.Phe508del/N	9+0 Rpt 10+4	Affected proband	140.5 Rpt 253	3 Rpt 10.2	Inconclusive / Inconclusive Rpt N / p.Phe508del	-	181 Rpt 182	2 Rpt 18	31 Rpt 33	100% / 95% Rpt 90% / 100%	438 Rpt 456	6 Rpt 3

Only informative SNPs that passed the quality control criteria were used for the analysis with the average sequencing depth calculated from only the informative SNPs. Classification accuracy for the maternal allele indicates the percentage of haplotype blocks that gave concordant results with the inherited allele determined by the majority call. Classification accuracy for the paternal allele indicated the percentage of type 3 SNPs giving concordant results with the inherited allele determined by the majority call. $ ₸ same family, different pregnancy; # inconclusive due to a recombination event (see figure 2); N=normal; NBS= Newborn screening test

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Figure Legends

Figure 1. Example case (Supplementary Table 1, case 17). Maternal mutation is p.(Phe508del) and the paternal mutation is p.(Trp1282*). These schematics are produced by the relative dosage analysis pipeline and provide a visual representation of where the different paternal SNPs or maternal haplotype blocks lie in the genomic region. The CFTR gene is shown as a black bar. Each green arrowhead represents a haplotype block. SNPs are sorted by the pipeline into 6 categories (Table 1). Haplotype I (HapI) is always the known genotype from the proband, with haplotype II (HapII) being used to determine the SNP type. a) Maternal haplotype blocks: When multiple SNPs are required to make a call it is termed a haplotype block and it is these that are used to interpret which of the maternal haplotypes have been inherited. Dark red= homozygous for HapI, light red = heterozygous 4a blocks, dark blue= heterozygous 4b blocks, light blue=homozygous for HapII. This schematic shows the fetus is homozygous HapI at type 4a haplotype blocks and heterozygous at type 4b blocks, so must have inherited the same maternal haplotype as the proband. b) Type 3 A/D SNP's– if the fetus has inherited a different paternal haplotype to the proband then a paternal contribution will be seen for these SNPs (purple dots when contribution seen, grey when absent). Type 3 B/C – These SNPs should be the opposite of the 3A/D SNPs: if the fetus has inherited the same paternal haplotype as the proband then a paternal contribution will be seen for these SNPs (turquoise dots when contribution seen, grey when absent). c) summary of results - in this case the fetus has inherited the high risk maternal allele with the low risk paternal allele. The genotype of the fetus is therefore p.(Phe508del)/N.

Figure 2. Paternal recombination event. These schematics are produced by the RHDO pipeline and provide a visual representation of where the different SNPs lie in the genomic region. The CFTR gene is shown as a black bar. Each green arrowhead represents a haplotype block. SNPs are sorted by the pipeline into 6 categories (Table 1), Haplotype I (HapI) is always the known genotype from the proband, with haplotype II (HapII) being used to determine the SNP type. a) Maternal haplotype blocks: When multiple SNPs are required to make a call it is termed a haplotype block and it is these that are used to interpret which of the maternal haplotypes have been inherited. Dark red= homozygous for HapI, light red = heterozygous 4a blocks, dark blue= heterozygous 4b blocks, light blue=homozygous for HapII. This schematic shows the fetus is homozygous HapI at type 4a haplotype blocks and heterozygous at type 4b blocks, so must have inherited the same maternal haplotype as the proband. b) Type 3 A/D SNP's– if the fetus has inherited a different paternal haplotype to the proband then a paternal contribution will be seen for these SNPs (purple dots when contribution seen, grey when absent). Type 3 B/C – These SNPs should be the opposite of the 3A/D SNPs: if the fetus has inherited the same paternal haplotype as the proband then a paternal contribution will be seen for these SNPs (turquoise dots when contribution seen, grey when absent). In this case there is a shift from one type of SNP to the other indicating a recombination event. c) Inconclusive result - Recombination event likely between Chr7: 116680679 and Chr7: 117229167. High risk haplotype at Chr7: 116680679, low risk at Chr7: 117229167, mutation at position Chr7: 117199609 in recombinant area

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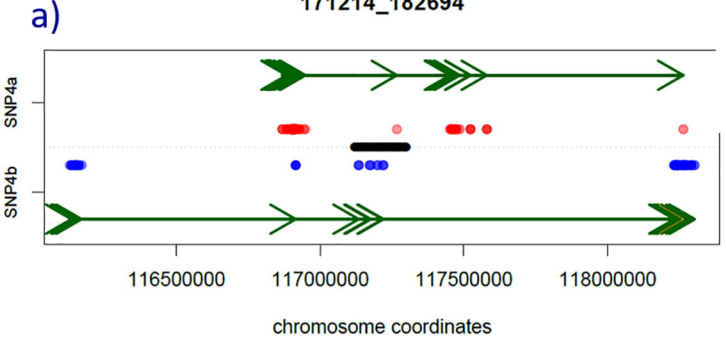
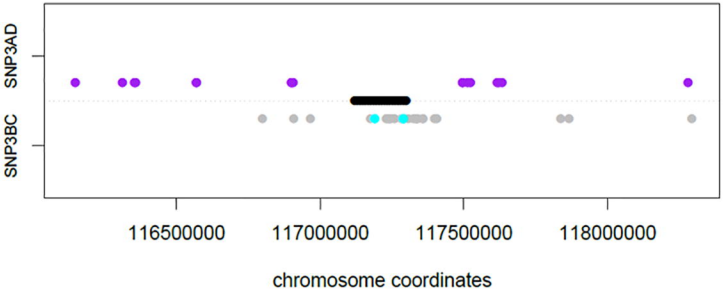
a)



b)



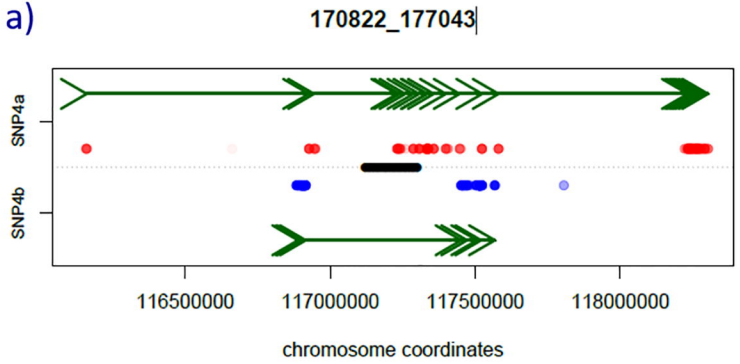
c)

Maternal haplotype				
	Homozygous.for.HapI	Heterozygous	Homozygous.for.HapII	Conclusion
Type_4a	34	0	0	Concordant with Proband
Type_4a_rev	34	0	0	Concordant with Proband
Type_4b	0	28	1	Concordant with Proband
Type_4b_rev	0	28	1	Concordant with Proband

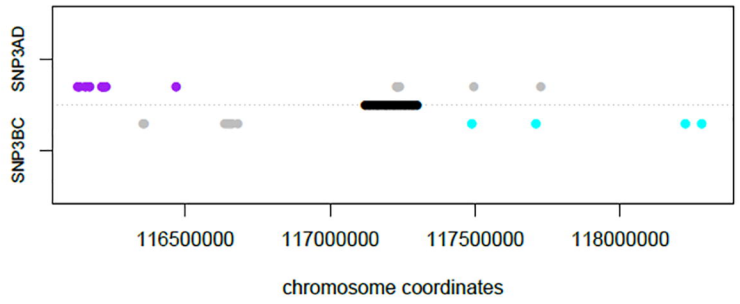
Paternal haplotype			
	Qualified.SNPs	Disqualified.SNPs	Conclusion
Type 3AD SNPs	17	0	Discordant to Proband
Type 3BC SNPs	3	25	NA

Proband	Affected
Maternal haplotype	Concordant with Proband
Paternal haplotype	Discordant to Proband
cffDNA result	Predict UNAFFECTED

a)



b)



c)

Type 3AD result:

CHROM	POS	REF	ALT	RD	AD	ff3AD	mother.geno	father.geno
chr7	116129088	C	T	567	24	0.0812183	0|0	0|1
chr7	116135123	G	A	585	39	0.125	0|0	0|1
chr7	116155979	A	C	171	7	0.0786517	0|0	0|1
chr7	116170195	T	A	32	572	0.1059603	1|1	1|0
chr7	116212511	T	C	451	22	0.0930233	0|0	0|1
chr7	116214642	T	C	49	912	0.1019771	1|1	1|0
chr7	116220532	G	A	52	615	0.155922	1|1	1|0
chr7	116226422	G	A	68	918	0.137931	1|1	1|0
chr7	116468914	G	A	676	37	0.1037868	0|0	0|1
chr7	117229167	G	A	0	673	0	1|1	1|0
chr7	117229537	T	A	0	363	0	1|1	1|0
chr7	117229837	G	T	0	286	0	1|1	1|0
chr7	117234537	A	G	0	691	0	1|1	1|0
chr7	117238445	T	G	1	459	0.0043478	1|1	1|0
chr7	117494841	T	A	64	0	0	0|0	0|1
chr7	117726480	C	A	0	647	0	1|1	1|0

Type 3BC result:

CHROM	POS	REF	ALT	RD	AD	ff3BC	mother.geno	father.geno
chr7	116354525	T	A	879	0	0	0|0	1|0
chr7	116358044	A	G	533	1	0.0037453	0|0	1|0
chr7	116635705	C	T	0	1211	0	1|1	0|1
chr7	116645880	T	C	0	274	0	1|1	0|1
chr7	116654201	A	G	1	508	0.0039293	1|1	0|1
chr7	116660803	A	G	0	1028	0	1|1	0|1
chr7	116680679	G	A	1019	0	0	0|0	1|0
chr7	117486274	G	T	492	27	0.1040462	0|0	1|0
chr7	117707293	G	T	69	925	0.138833	1|1	0|1
chr7	118222741	G	C	714	46	0.1210526	0|0	1|0
chr7	118280023	T	G	188	23	0.2180095	0|0	1|0