[1]Department of Paediatrics, The Hospital for Sick Children, Toronto, Ontario, Canada
[2]Child Health Evaluative Sciences, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada
[3]Dalla Lana School of Public Health, University of Toronto, Toronto, Ontario, Canada
[4]Faculty of Medicine, Department of Pediatrics, Makassed Hospital, Al-Quds University, East Jerusalem, Palestine
[5]Department of Paediatrics, McGill University Health Centre, Montreal, Quebec, Canada

Correspondence
Maria Laura Avila, Division of Paediatric Haematology/Oncology, The Hospital for Sick Children, 555 University Av, Toronto, ON M5G 1X8, Canada.
Email: laura.avila@sickkids.ca

Deborah Levy and Leonardo R. Brandão are co-senior authors of this paper.

ORCID

Maria Laura Avila https://orcid.org/0000-0002-4340-9645
Nour Amiri https://orcid.org/0000-0001-6014-6157

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SUPPORTING INFORMATION

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Validation of single-gene noninvasive prenatal testing for sickle cell disease

To the Editor:

Screening all pregnancies for sickle cell disease (SCD), as recommended by the American College of Gynecologists,[1] is essential to enable informed decisions about diagnostic testing, clinical care, and expand available gene therapy treatment options.[2–4] However, traditional prenatal screening, in which both maternal and paternal DNA are required for carrier screening, has a low sensitivity of 42% due to insufficient paternal screening uptake in the United States.[5] Even when paternal screening is performed and the carrier result is positive, the maximum fetal disease risk is one in four.

Single-gene noninvasive prenatal testing (sgNIPT) is a promising new technology, able to achieve accurate prenatal results without requiring paternal screening. We previously reported the proof-of-principle development of a sequencing-based sgNIPT test for five conditions, including SCD, in 2019.[6] Single-gene NIPT analyzes cell-free DNA (cfDNA) from maternal plasma to provide a personalized fetal residual disease risk ranging from >9 in 10 to <1 in 20,000. The aim of this study was to build upon our previous work to validate the sgNIPT in clinical samples and identify high-risk SCD fetuses in a cohort of at-risk pregnancies.

This retrospective clinical study collected 77 maternal blood samples between October 2018 and December 2019 from pregnant patients at the Baylor College of Medicine or the University of Alabama at Birmingham who were known to have at least one pathogenic *HBB* allele. Newborn *HBB* genotype was determined by newborn screening chart review or genotyping of umbilical cord blood.

For sgNIPT processing, genomic DNA (gDNA) and cfDNA were extracted from the maternal blood sample. The SCD maternal carrier status was determined by next-generation sequencing of the gDNA. The cfDNA fraction was then sequenced to determine (1) fetal fraction, (2) molecular counts of cfDNA, (3) maternal variant fraction, and (4) variants that are not present in the maternal genotype (paternally inherited variants).[6] All patients start with an a priori risk calculated from the pregnant patient's carrier status, the highest subpopulation *HBB* carrier frequency in the United States (one in eight),[7] and the likelihood a fetus inherits two SCD alleles. A likelihood ratio is calculated through relative dosage analysis of the most abundant allele found in cfDNA, comparing the likelihood of inheriting one copy or two copies of the most abundant allele.[6] The likelihood ratio was used

(1) to calculate the genotype score and predict fetal *HBB* genotype (Figure 1A) and (2) to adjust the a priori risk and calculate a residual risk that the fetus is affected with SCD (Figure 1B). In a clinical report, the residual and associated risk categories (low risk, decreased risk, or high risk) would be provided.

Out of the 77 pregnancies in the cohort, maternal *HBB* genotypes included HbAS ($n = 59$), HbAC ($n = 13$), HbSS ($n = 4$), and HbCC ($n = 1$). The median fetal fraction was 9.3% (IQR = 5.8%–13.6%) for gestational ages ranging from 16.4 weeks to collection at delivery (Table S1). The fetal fraction was similar to a large clinical study that reported a median fetal fraction of 10.0% (IQR = 7.8%–13.0%) for gestational ages 11–13 weeks.[8] Therefore, this cohort is likely representative of the expected fetal fraction in the first trimester, when prenatal screening is most common.

Single-gene NIPT returned a fetal *HBB* genotype prediction for 68 of the 77 pregnancies, with 9 undetermined. sgNIPT accurately distinguished heterozygous from homozygous fetuses (Figure 1A) with 100% sensitivity (90.8%–100%, 95% CI) and 96.5% specificity (82.2%–99.9%, 95% CI). The fetal genotype predictions were concordant with newborn genotypes in 67 out of 68 pregnancies (98.5%) (Tables S1 and 1). In the single discordant result, sgNIPT returned a fetal genotype prediction of HbAS when the newborn genotype was HbAA, both of which lead to low-risk results for sickle cell disease.

To calculate the residual risk, the data from sgNIPT assay and *HBB* genotype prediction are used to adjust the a priori risk (the a priori risk for an affected fetus for an African American patient is 1 in 32 when mother is heterozygous) to classify the fetus as "low risk," "decreased risk," or "high risk." Both the residual risk and risk classification would be included on the clinical report. sgNIPT returned a result for 75 of the 77 pregnancies with 2 no calls (2.6% no call rate). From these 75 pregnancies, sgNIPT correctly identified the two newborns affected with SCD (Figure 1B). Both high-risk sgNIPT results had a greater than 9 in 10 residual disease risk (Figure 1B). One case was a newborn affected with sickle cell anemia (genotype HbSS) whose mother who also had sickle cell anemia. The other case was a newborn affected with Hemoglobin SC disease (genotype HbSC) whose mother had an HbAC genotype. The fetal fraction of cfDNA used to determine the risk call for these two samples were 3.2% and 1.9%, respectively, highlighting the ability of sgNIPT to make informative calls even with low (<5%) fetal fraction.

Single-gene NIPT also correctly identified all unaffected fetuses (73 out of 75 pregnancies). Most low-risk calls had a fetal residual risk of 1 in 20,000; a few of the low-risk calls had a residual risk as high as 1 in 2,000 (Figure 1B). One sample was designated as "decreased risk" because carrier screening indicated that the mother was HbAS, and the paternal allele assay detected that the fetus inherited an HbC allele from the father. Dosage analysis on the maternal HbS allele

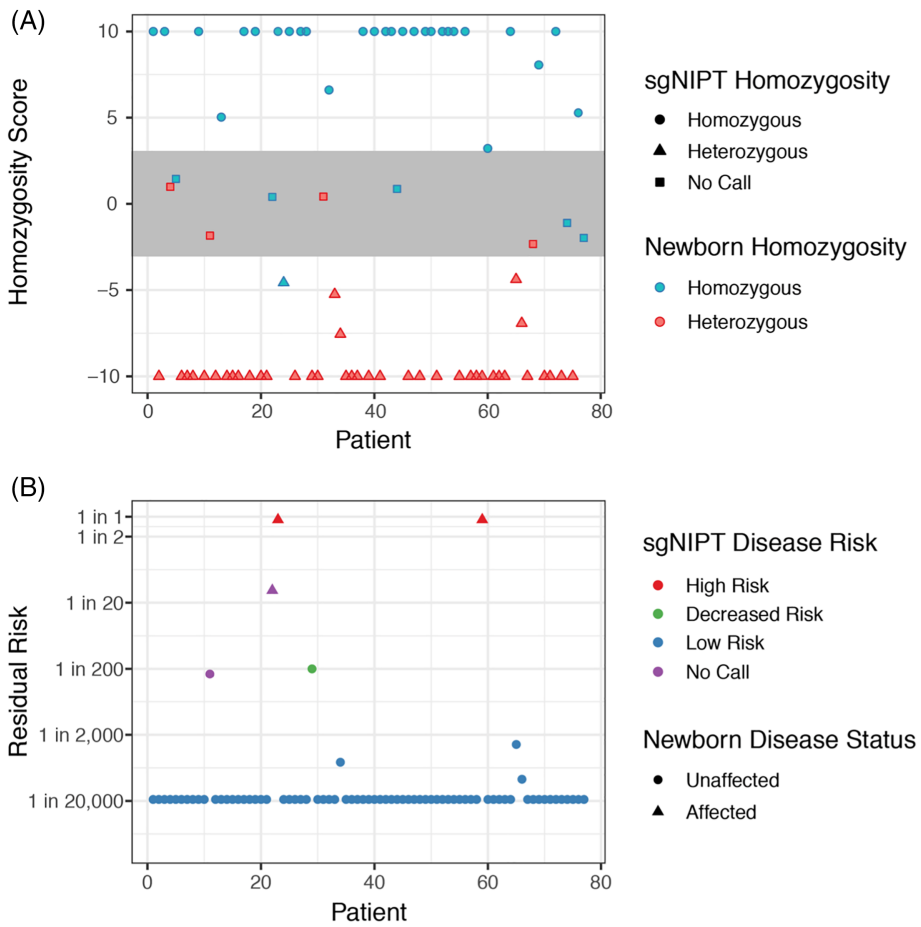

FIGURE 1 Single-gene noninvasive prenatal testing (NIPT) uses HBB genotype prediction to accurately determine fetal SCD risk. (A) Prediction of fetal homozygosity. Single-gene NIPT classified a sample as homozygous (HbAA or HbSS) when the homozygosity score was above 3, and as heterozygous (HbAC, HbAS, or HbSC) when the homozygosity score was below −3. Samples with scores more extreme than 10 (−10) were set to 10 (−10). Samples with scores between −3 and 3 had an undetermined genotype. Once homozygosity is determined, the genotype is determined by inspection of sequencing data. (B) Residual risk of fetal sickle cell disease after sgNIPT. The residual risk is calculated from the a priori risk derived from population carrier frequency (for African–Americans: 1 in 32 for all pregnancies when mother is heterozygous) and the likelihood ratio derived from sgNIPT

TABLE 1 Concordance between single-gene noninvasive prenatal testing (sgNIPT) and newborn results for *HBB* genotype

	Newborn *HBB* genotype				
	Normal	Traits		Disease	
NIPT *HBB* genotype	AA	AS	AC	SC	SS
AA	27	–	–	–	–
AS	1	31	–	–	–
AC	–	–	7	–	–
SC	–	–	–	1	–
SS	–	–	–	–	1

Notes: Newborn *HBB* genotype was determined by newborn screening chart review or genotyping of umbilical cord blood. The fetal genotype predictions were concordant with newborn genotypes in 67 out of 68 pregnancies (98.5%).

determined that the fetus was likely an unaffected carrier with genotype HbAC. Therefore, the residual risk was lowered from 1 in 2 to 1 in 200, which is the lower limit of risk reduction when a paternal pathogenic allele is detected.

An undetermined fetal *HBB* genotype or a risk classification no call occurs when the likelihood ratio falls between the internal high- and low-risk thresholds. This typically happens when there are an inadequate number of fetal molecules in cfDNA sample. In our study, nine genotypes were undetermined, but only two led to risk classification no calls. In the other seven samples, the data were consistent with the fetus inheriting at least one wild-type allele (genotypes HbAA or HbAS). Therefore, the sgNIPT could still accurately classify the fetus as low risk since neither genotype is associated with SCD. In a clinical setting, a risk classification no call would trigger a request for a second blood sample to re-run sgNIPT. The additional molecules can be used for data analysis to increase the likelihood of obtaining a reportable result.

The personalized fetal risk assessment reported by sgNIPT is more sensitive and precise than the current standard of care and allows parents and genetic counselors to make better-informed decisions regarding family and clinical planning. Furthermore, advanced detection of SCD allows the family and clinicians to prepare for cord blood collection to be used for gene-based therapies for an affected child, or an allogenic transplant for an affected sibling. There are multiple gene therapy strategies underway as part of Phases I, II, and III clinical trials that use gene insertion, fetal hemoglobin induction or editing of the sickle mutation to treat sickle cell disease.[2] These SCD-related gene therapies are expected to be in widespread clinical use in the near future, increasing the importance of widespread and informative prenatal screening for SCD.

Together, we have validated that SCD sgNIPT predicts fetal genotype with high sensitivity and specificity, which leads to an accurate determination of fetal SCD risk (Figure S1). Crucially, sgNIPT returned an informative fetal disease risk for 97.4% (75 out of 77) pregnancies compared to only 42% with traditional screening.[5] Furthermore, sgNIPT determined residual disease risks as high as >9 in 10, therefore, providing a more personalized risk assessment compared to traditional carrier screening. These results, combined with the unique workflow of reflex sgNIPT for carrier mothers without the need for a paternal sample, highlight that this screen should be considered for broad clinical adoption to promote efficient and accurate fetal risk assessment for SCD in pregnant patients.

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CONFLICT OF INTEREST

David S. Tsao, Oguzhan Atay, Brian P. Landry, Patrick P. Ye, Devon Chandler-Brown, Brian Alford, and Jennifer Hoskovec are employees of BillionToOne and hold stock or options to hold stock in the company. Vivien A. Sheehan received a grant from BillionToOne directly and as a subcontractor from NIH. Erik R. Westin, Akila Subramaniam, Kevin M. Pawlik, Spencer G. Kuper, Frederick D. Goldman, and Tim M. Townes report no conflict of interest.

AUTHOR CONTRIBUTIONS

Erik R. Westin, Akila Subramaniam, Kevin M. Pawlik, Spencer G. Kuper, Frederick D. Goldman, Vivien A. Sheehan, and Tim M. Townes performed patient recruitment and sample collection. David S. Tsao, Brian P. Landry, Patrick P. Ye, Devon Chandler-Brown, Brian Alford, Jennifer Hoskovec performed data analysis. Erik R. Westin, David S. Tsao, Jennifer Hoskovec, Tim M. Townes, and Vivien A. Sheehan wrote the manuscript. All authors discussed and commented on the manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

Erik R. Westin[1], David S. Tsao[2], Oguzhan Atay[2], Brian P. Landry[2], Patrick P. Ye[2], Devon Chandler-Brown[2], Brian Alford[2], Jennifer Hoskovec[2], Akila Subramaniam[3], Kevin M. Pawlik[4], Spencer G. Kuper[5], Frederick D. Goldman[1], Tim M. Townes[4], Vivien A. Sheehan[6]

[1]*Department of Pediatrics, University of Alabama at Birmingham, Birmingham, Alabama, USA*
[2]*BillionToOne Inc., Menlo Park, California, USA*
[3]*Department of Obstetrics and Gynecology, University of Alabama at Birmingham, Birmingham, Alabama, USA*
[4]*Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama, USA*
[5]*Tri-State Perinatology, Deaconess—The Women's Hospital, Newburgh, Indiana, USA*
[6]*Department of Pediatrics, Division of Hematology/Oncology, Baylor College of Medicine, Houston, Texas, USA*

Correspondence
Erik R. Westin, PhD, Department of Genetics, University of Alabama at Birmingham, Kaul 606, 720 20th St. South, Birmingham, AL 35294. USA.
Email: westine@uab.edu

Tim M. Townes and Vivien A. Sheehan contributed equally to this study.

ORCID

Erik R. Westin https://orcid.org/0000-0002-5771-8457
Brian P. Landry https://orcid.org/0000-0001-6570-6950
Patrick P. Ye https://orcid.org/0000-0003-1160-5016

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Salvage therapy with basiliximab and etanercept for severe steroid-refractory acute graft-versus-host disease

To the Editor:

Acute graft-versus-host disease (aGVHD) remains a significant complication of allogeneic hematopoietic cell transplantation (alloHCT). At best, only half of patients respond to initial corticosteroids, with limited long-term overall survival (OS) observed in steroid-refractory (SR) patients. Ruxolitinib (Rux) is approved for SRaGVHD; however, ~40% of patients do not respond by day 28 and only ~40% maintain response at day +56.[1] We read with interest three recent articles in the journal characterizing novel approaches for managing SR aGVHD. Zhao et al. prospectively combined Rux and etanercept in 64 patients with severe (grade 3–4) SRaGVHD, 86% of which had received haploidentical (haplo) alloHCT. An impressive overall response rate (ORR) of 87.5% was observed at day +28, of which 73.4% were complete responses (CR).[2] Most responses were observed within 7 days, more rapidly than observed with Rux alone.[1,2] Importantly, CMV infection occurred in 50%, and 39% experienced grade ≥3 infections. Two-year overall survival (OS) was 61.2%, and 15.7% relapsed. Patients in this study were younger (median 29 years)[2] than in the Rux registration trial (median 54 years),[1] which may have contributed to these favorable outcomes.

Liu et al. retrospectively assessed basiliximab as initial therapy for SRaGVHD in 230 patients, 90% of which had underwent haplo alloHCT, with a reported ORR of 78.7% (60.9% CR). However, severe aGVHD was less responsive (HR for ORR 0.50, 95% CI 0.32–0.76, $p = .001$). Response occurred quickly at a median of 12–15 days from basiliximab initiation. Basiliximab was initiated early, at a median of 5 days after GVHD diagnosis, at which time only 16.9% of patients had severe GVHD, which likely contributed to the high ORR. Long-