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RESEARCH ARTICLE

Ancestry and genetic associations with bronchopulmonary dysplasia in preterm infants

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Torgerson DG, Ballard PL, Keller RL, Oh SS, Huntsman S, Hu D, Eng C, Burchard EG, Ballard RA; TOLSURF Study Group. Ancestry and genetic associations with bronchopulmonary dysplasia in preterm infants. *Am J Physiol Lung Cell Mol Physiol* 315: L858–L869, 2018. First published August 16, 2018; doi:10.1152/ajplung.00073.2018.—Bronchopulmonary dysplasia in premature infants is a common and often severe lung disease with long-term sequelae. A genetic component is suspected but not fully defined. We performed an ancestry and genome-wide association study to identify variants, genes, and pathways associated with survival without bronchopulmonary dysplasia in 387 high-risk infants treated with inhaled nitric oxide in the Trial of Late Surfactant study. Global African genetic ancestry was associated with increased survival without bronchopulmonary dysplasia among infants of maternal self-reported Hispanic white race/ethnicity [odds ratio (OR) = 4.5, $P = 0.01$]. Admixture mapping found suggestive outcome associations with local African ancestry at chromosome bands 18q21 and 10q22 among infants of maternal self-reported African-American race/ethnicity. For all infants, the top individual variant identified was within the intron of *NBL1*, which is expressed in midtrimester lung and is an antagonist of bone morphogenetic proteins (rs372271081, OR = 0.17, $P = 7.4 \times 10^{-7}$). The protective allele of this variant was significantly associated with lower nitric oxide metabolites in the urine of non-Hispanic white infants ($P = 0.006$), supporting a role in the racial differential response to nitric oxide. Interrogating genes upregulated in bronchopulmonary dysplasia lungs indicated association with variants in *CCL18*, a cytokine associated with fibrosis and interstitial lung disease, and pathway analyses implicated variation in genes involved in immune/inflammatory processes in response to infection and mechanical ventilation. Our results suggest that genetic variation related to lung development, drug metabolism, and immune response contribute to individual and racial/ethnic differences in respiratory outcomes following inhaled nitric oxide treatment of high-risk premature infants.

bronchopulmonary dysplasia; drug response; genetic ancestry; genome-wide association study; preterm infants

INTRODUCTION

Bronchopulmonary dysplasia (BPD) of premature infants is currently characterized by continuing requirement for supplemental oxygen and/or respiratory support at 36 wk postmenstrual age (PMA). BPD is the most common form of chronic lung disease in infants born prematurely and is associated with long-term respiratory morbidity, neurodevelopmental abnormalities, and death (33). The pathogenesis of BPD includes lung immaturity, with reduced pulmonary surfactant and low antioxidant and immune defenses, as well as exposure to insults of hyperoxia, barotrauma from ventilator support, and infections that damage lung epithelium and elicit inflammation. Sequelae of this injury are arrested lung development, fibrosis, and altered airway reactivity (7, 18, 24, 31, 33, 37, 47).

Therapeutic options for the prevention and treatment of BPD are limited and have not substantially affected the incidence of disease (reviewed in Refs. 26, 28). For example, vitamin A treatment evokes a modest reduction of BPD but is not in general use, and caffeine reduces oxygen use and is routinely used for prevention of apnea. Postnatal dexamethasone therapy improves respiratory status acutely and decreases the incidence of BPD. However, longer courses of this therapy are associated with neurodevelopmental abnormalities. Inhaled nitric oxide (iNO) is used off-label in preterm infants to prevent BPD, but the general efficacy of the drug has been brought into question (19).

The majority of studies evaluating the effectiveness of iNO have been performed in individuals with predominantly European ancestry (5). However, in the entire cohort of the Trial of Late Surfactant (TOLSURF; Ref. 60) and in a recent individual participant data meta-analysis across selected iNO trials (6), the incidence of BPD was significantly lower following treatment with iNO in infants of mothers who self-report as black/African-American ethnicity compared with those who self-report as non-Hispanic white. Coupled to observed differences in levels of urinary NO metabolites in black/African-American vs. non-Hispanic white infants (8), these results suggest that response to iNO in terms of preventing BPD varies between racial/ethnic groups.

Although both the intrauterine and postnatal environment play an important role in BPD, twin studies have estimated the heritability between 50 and 80% (13, 38), suggesting a genetic contribution as well (29). Genetic studies of BPD have identified several candidate genes and pathways through genome-wide association studies (GWAS; Refs. 4, 29, 43, 61) and exome sequencing (17, 39). However, none of the associations identified through GWAS has reached genome-wide significance, and replication of genetic associations has been prob-

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lematic. This may, in part, be due to low statistical power given the relatively small sample size of each study (<1,000 preterm infants) combined with the absence of a single genetic risk factor of large effect. Similarly, disease heterogeneity, including the potential for differences in the genetic architecture of BPD between racial/ethnic groups, and the specific definition of BPD used may reduce statistical power (4). However, pathway and gene set enrichment analyses have identified candidates with high biological plausibility (4, 39).

In this study, we performed a GWAS for survival without BPD in preterm infants in TOLSURF, which included infants of maternal self-reported African-American, Hispanic, and non-Hispanic white race/ethnicity who all received iNO. We examine the effects of genetic variation at the level of individual variants, genes, and genetic pathways and test the hypothesis that genetic ancestry at both the genomic and local scale is associated with survival without BPD in admixed populations.

METHODS

Study approval. Patient recruitment for the TOLSURF study was approved by the Institutional Review Boards at all participating sites, including the University of California, San Francisco.

Study subjects. TOLSURF was a masked, randomized, sham-controlled trial conducted in 25 US hospitals (https://clinicaltrials.gov/: NCT01022580). The study was designed to assess the effect of late doses of surfactant on BPD at 36 wk PMA in infants of 23–28 wk gestation who required intubation and mechanical ventilation between 7 and 14 days of age (9). A total of 511 infants were enrolled, and all received iNO (Ikaria, Hampton, NJ) according to the protocol followed in the Nitric Oxide (to Prevent) Chronic Lung Disease (NO CLD) trial (10). BPD was assessed at 36 wk PMA by physiological testing as described (10). There was no statistical difference in BPD incidence between control and surfactant-treated groups at 36 wk, and the 2 groups were combined for this genetic study. Some infants were coenrolled in the multicenter, observational Prematurity and Respiratory Outcomes Program (PROP; Ref. 52).

Genotyping and quality control. DNA was extracted from tracheal aspirate cells from 454 infants whose parents consented for DNA collection using cells from ≤5 tracheal aspirate collections per patient, obtained between *postnatal days 7* and *21*. DNA was isolated using an AutoGeneprep 965 instrument (AutoGen, Holliston, MA) by the manufacturer's recommended standard protocol for human body fluids. In some cases, where protein contamination was evident, DNA was reprecipitated using 3 volumes of 100% ethanol and 3 M ammonium acetate at a 3:1 ratio after incubation at −80°C overnight. Samples were initially quantified by NanoDrop (Thermo Fisher Scientific, Waltham, MA) to assess purity [absorbance at 260 and 280 nm (A260/280)] followed by analysis using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA) to assess DNA quantity more accurately. The values for DNA concentration (in ng/μl) ranged from 10 to 1,750, median 130; total DNA per patient (in ng) 130–4,200, median 1,600; A260/280, 1.32–1.91, median 1.77; 429 samples were of suitable quality and quantity for genotyping.

Genotyping was performed on the Affymetrix Axiom LAT 1 Array (World Array 4) that contained >800,000 single-nucleotide polymorphisms (SNPs) before quality control. SNPs were filtered based on call rates <95% and Hardy-Weinberg equilibrium P values $<10^{-6}$ using PLINK (53). Subjects were evaluated for call rates, consistency between genetic and reported sex, autosomal heterozygosity, and cryptic relatedness/genetic identity using identical by descent/identical by state estimates in PLINK (53). In the case of multiples, one individual was selected at random to be included in the study.

Statistical analysis. We inferred levels of African ancestry at both the genomic level (average across the genome) and the local level (at an individual locus). Genomic levels of African ancestry were evaluated using ADMIXTURE (3) in a quasisupervised analysis assuming three ancestral continental populations of origin ($k = 3$, African, European, and Native American). Windows were offset by a factor of 0.2, the cutoff for linkage was set to 0.1, and a constant recombination rate was set to 10^{-8}/bp. The proportion of global African ancestry was compared between cases (BPD/death) vs. controls (survival without BPD) using logistic regression within infants of maternal self-reported African-American race/ethnicity and those of Hispanic ethnicity, adjusting for gestational age, sex, birth weight, and multiple gestation (yes/no). Local ancestry was inferred using LAMP-LD (11) in infants with maternal self-reported African-American race/ethnicity using a two-population model. Unrelated individuals from the International Haplotype Map (HapMap) Project of African ancestry [Yoruba in Ibadan, Nigeria (YRI)] and European ancestry [Utah residents with Northern and Western European ancestry (CEU)] were used as a reference to estimate global and local African and European ancestry.

Imputation of genetic variation from the Phase 3 1000 Genomes Project was performed using the Michigan Imputation Server (21), including ~79 million variants across all populations. Variants were then filtered for imputation quality scores >0.3. Genetic association testing for survival without BPD was performed at both genotyped and imputed SNPs using logistic regression, adjusting for global genetic ancestry, gestational age, sex, birth weight, and multiple gestation. Analyses were performed within each racial/ethnic group using PLINK (53) and then combined in a meta-analysis using METAL (64). Gene-based statistics were calculated using versatile gene-based association study (VEGAS; Ref. 41) using genotyped SNPs and intersected with a set of genes previously identified as being upregulated in BPD-dysregulated lungs (14). Pathway and gene set analyses were performed using canonical pathways in Ingenuity Pathway Analysis (IPA; Ref. 36) and Protein Analysis THrough Evolutionary Relationships (PANTHER; Ref. 46) and Molecular Signature Database (MSigDB; Ref. 56) using Genomic Regions Enrichment of Annotations Tool (GREAT) version 3.0.0 (45). With the use of GREAT, we assigned a foreground of gene coordinates with an association $P > 0.05$ for survival without BPD and a background of all gene coordinates for which a gene-based statistic was calculated (from VEGAS; Ref. 41).

Admixture mapping for local African ancestry was performed in infants with maternal self-reported African-American race/ethnicity using logistic regression. Similar to association testing on individual variants, we performed association testing for the number of haplotypes of African ancestry at each genotyped SNP (homologous to association testing for the number of copies of the minor allele). Identical to our GWAS, we adjusted for global genetic ancestry, gestational age, sex, birth weight, and multiple gestation. To account for multiple testing, we estimated the independent number of tests using the coda package in R and applied a Bonferroni correction.

Measures of NO metabolites (NOx), including nitrate, nitrite, and nitrosylated compounds, were made from the urine of 62 infants included in the current genetic study collected between 6 and 65 days postnatal age, both before and following administration of iNO at 2–20 ppm as previously described (8). Briefly, urine was collected for 4–8 h, and NOx were assayed according to Ref. 50 and normalized to creatinine to adjust for renal excretory function. NOx were measured off iNO and at three different doses of iNO (2, 5, and 10–20 ppm). Genetic association testing at a single SNP was performed using linear regression to test for a correlation between genotype and values of NOx at a dose of 5 ppm. Values of NOx at 5 ppm were selected for analysis because they are highly correlated to levels at 2 ppm and more closely resemble a normal distribution compared with 10–20 ppm.

For selected genes of interest, mRNA expression levels were obtained from a previous study that performed RNA sequencing on three specimens of human fetal lung of 23 wk gestational age (Gene Expression Omnibus acc. no. GSE83888; Ref. 12).

Table 1. *Baseline characteristics of participants from the TOLSURF study included in the GWAS*

	Non-Hispanic White			African American			Hispanic White		
	BPD/Death	No BPD	*P* Value	BPD/Death	No BPD	*P* Value	BPD/Death	No BPD	*P* Value
n	136	41	N/A	82	51	N/A	28	14	N/A
Gestational age, wk	25.4 (1.3)	25.2 (1.2)	0.52	24.9 (1.0)	25.4 (1.0)	0.008	24.9 (1.3)	25.5 (0.95)	0.12
Birth weight, g	712 (182)	750 (165)	0.21	640 (147)	704 (145)	0.015	703 (155)	740 (210)	0.57
%Male	59.6	51.2	0.44	56.1	45.1	0.29	57.1	35.7	0.33
%Multiple gestation	15.4	22.0	0.46	9.76	9.80	1.0	3.57	14.3	0.53
RSS at entry	4.0 (2.1)	3.1 (1.4)	0.0008	4.0 (2.2)	2.7 (0.94)	<0.0001	3.8 (2.8)	3.3 (2.0)	0.49

Data are means with standard deviations in parentheses. *P* values represent comparisons using a Student's *t*-test for continuous measurements [gestational age, birth weight, and respiratory severity score (RSS) at entry] and a χ^2-test for categorical (%male and %multiple gestation). Demographics are shown by maternal self-reported racial/ethnic group. BPD, bronchopulmonary dysplasia; GWAS, genome-wide association study; N/A, not applicable; TOLSURF, Trial of Late Surfactant.

RESULTS

Following quality control, our study included a total of 795,465 genotyped SNPs and 387 unrelated infants; demographics by respiratory outcome are shown in Table 1 for 271 infants who died or had a diagnosis of BPD and 116 survivors without BPD. Overall, mean values for gestational age and birth weight were approximately 25 wk and 700 g, respectively, for this group of infants who still required intubation and ventilation between 7 and 14 days of age, representing a cohort at high risk for BPD. Within infants of maternal self-reported non-Hispanic white ethnicity (white), infants with BPD/death had a significantly higher respiratory severity score on study entry compared with survivors without BPD but had no significant difference in gestational age, birth weight, sex, and multiple gestations. Within infants of maternal self-reported black/African-American ethnicity (black/AA), infants with BPD/death had significantly lower gestational age, lower birth weight, and higher respiratory severity score compared with no BPD. These differences for infants with/without BPD are consistent with the known influence of immaturity and severity of early lung disease on BPD. No significant differences in clinical characteristics were observed between the two groups of maternal self-reported white Hispanic ethnicity (white Hispanic).

Global ancestry and admixture mapping. Individual proportions of African ancestry across the entire genome were consistent with expectations given maternal self-reported race/ethnicity (Fig. 1, *A* and *B*). Specifically, black/AA infants had

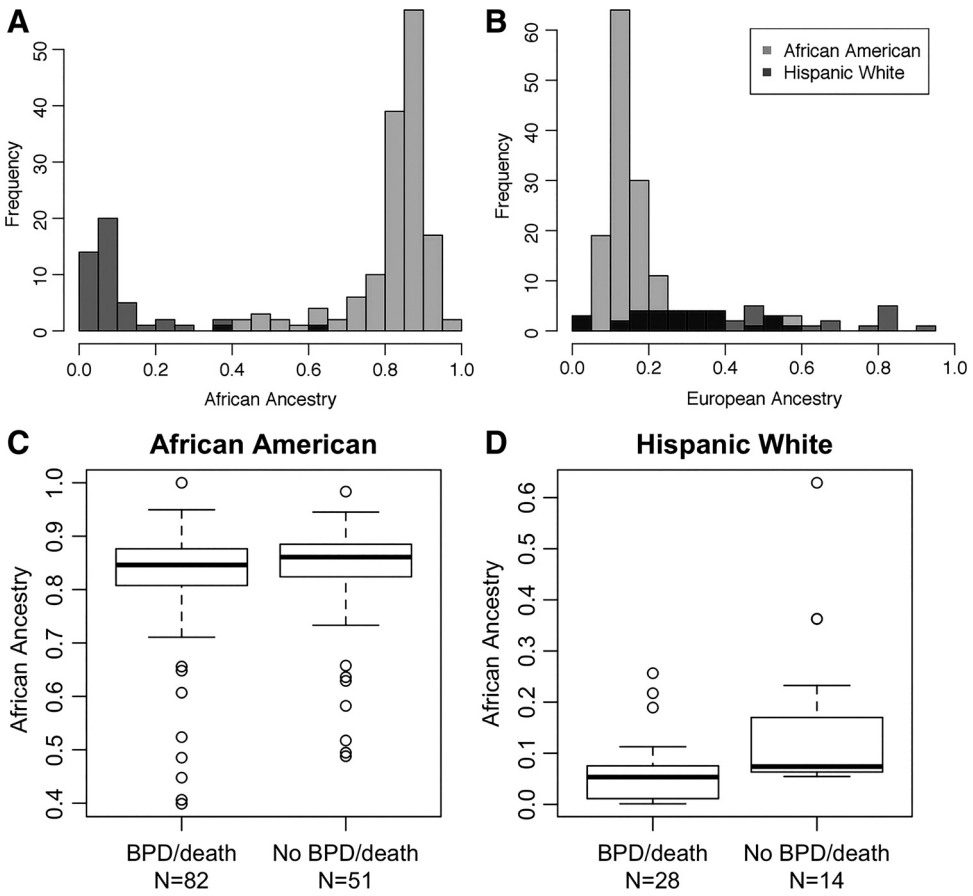

Fig. 1. Global ancestry proportions and survival without bronchopulmonary dysplasia (BPD). Proportions shown are of global African (*A*) and European (*B*) ancestry in preterm infants participating in the Trial of Late Surfactant (TOLSURF) study by maternal self-reported race/ethnicity. Global ancestry was inferred using ADMIXTURE. Box plots compare global African ancestry and survival without BPD in infants of maternal self-reported black/African-American race/ethnicity (*C*; logistic regression: $P = 0.97$, $\beta = -0.015 \pm 0.37$) and Hispanic white race/ethnicity (*D*; $P = 0.01$, $\beta = -1.5 \pm 0.60$).

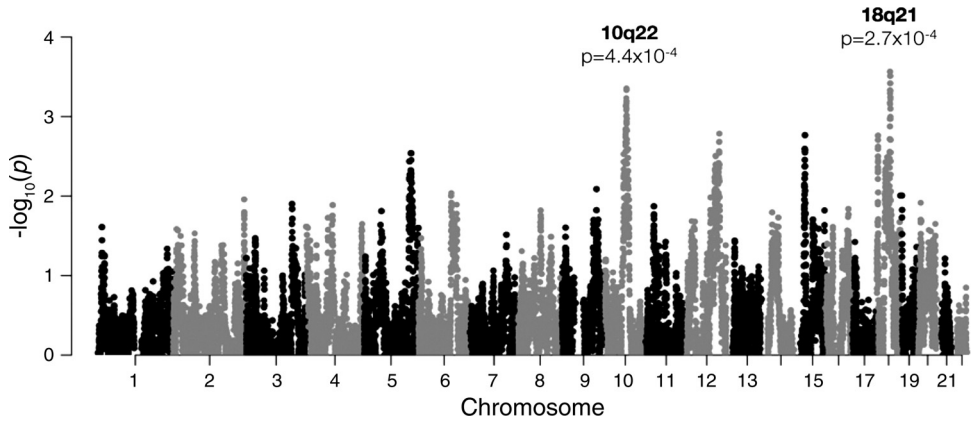

Fig. 2. Results of admixture mapping comparing local African ancestry and survival without bronchopulmonary dysplasia in 133 infants with maternal self-reported black/African-American race/ethnicity (82 cases, 51 controls). Top associations were observed at chromosome bands 10q21 (odds ratio = 0.17, $P = 4.4 \times 10^{-4}$) and 18q21 (odds ratio = 4.6, $P = 2.7 \times 10^{-4}$).

a higher degree of genomic African ancestry [median = 85% (range = 40–100%)] compared with white Hispanic infants [median = 6.3% (range = 1.2–63%)].

Global African ancestry was not significantly different between infants with BPD/death compared with those surviving without BPD for black/AA infants adjusting for gestational age, sex, birth weight, and multiple gestation ($\beta = -0.015$, SE = 0.37, $P = 0.97$; Fig. 1*C*). However, genomic African ancestry was protective for BPD/death in white Hispanic infants adjusting for the same covariates ($\beta = -1.5$, SE = 0.6, $P = 0.01$; Fig. 1*D*). Results were similar when all covariates were excluded.

With the use of logistic regression, African ancestry was further examined in black/AA infants at individual loci (i.e., local ancestry or admixture mapping) to evaluate differences in African ancestry at specific regions of the genome between cases and controls. The strongest associations with local ancestry were observed at chromosome band 10q21, where African ancestry was protective for BPD/death [$P = 4.4 \times 10^{-4}$, odds ratio (OR) = 0.17], and 18q21, where African ancestry was risky for BPD/death ($P = 2.7 \times 10^{-4}$, OR = 4.6; Fig. 2). The estimated number of independent ancestry blocks was determined to be 478, and thus neither of the admixture mapping peaks was statistically significant following Bonferroni correction ($\alpha = 1.0 \times 10^{-4}$).

GWAS and gene-based comparisons. Following genotype imputation, which infers additional variants for each infant using whole genome sequences from the 1000 Genomes Project, we tested the entire cohort for an association with survival without BPD. We examined associations at 8.8 million individual variants, adjusting for global genetic ancestry, gestational age, sex, birth weight, and multiple gestations. No individual variant was genome-wide significantly associated with BPD (all P values $>5 \times 10^{-8}$, Fig. 3). However, the top association was observed at a variant within the intron of *NBL1* (rs372271081; $P = 7.4 \times 10^{-7}$; Fig. 4*A*). The minor allele was protective for BPD (OR = 0.17) and showed a similar effect within each racial/ethnic group (Table 2). Local African ancestry at this locus was not significantly associated with BPD in black/AA infants ($P = 0.24$). *NBL1* and two additional genes within the same region (*CAPZB* and *MINOS1*) were expressed in fetal lung at 23 wk gestation (Fig. 4*B*). Furthermore, the minor allele at rs372271081 was significantly associated with decreased urinary NOx in non-Hispanic white infants but was not significant in black/AA or white Hispanic infants (Table 3, Fig. 5*A*). Notably, the protective allele for BPD at rs372271081 is at a somewhat higher frequency in populations with African ancestry (Fig. 5*B*).

To increase statistical power, we combined the results of association testing of individual variants within known genes to create a whole gene-based statistic. No individual gene was significantly associated with BPD following Bonferroni correction for 17,670 tests (the number of genes tested, $\alpha = 2.8 \times 10^{-6}$; Table 4). However, by restricting our comparisons to 21 candidate genes for which expression is dysregulated in BPD lungs (14), variation in *CCL18* was significantly associated

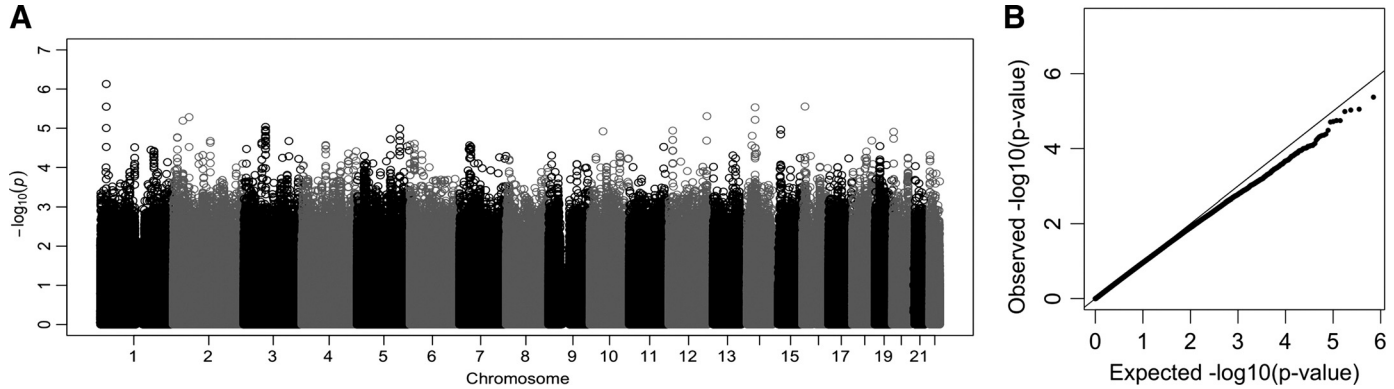

Fig. 3. Manhattan plot (*A*) and quantile-quantile plot (*B*) showing the results of a weighted meta-analysis for survival without bronchopulmonary dysplasia (BPD) across 3 maternal self-reported racial/ethnic groups, including non-Hispanic white (136 BPD/death infants, 41 no BPD), black/African American (82 BPD/death infants, 51 no BPD), and Hispanic white (28 BPD/death infants, 14 no BPD).

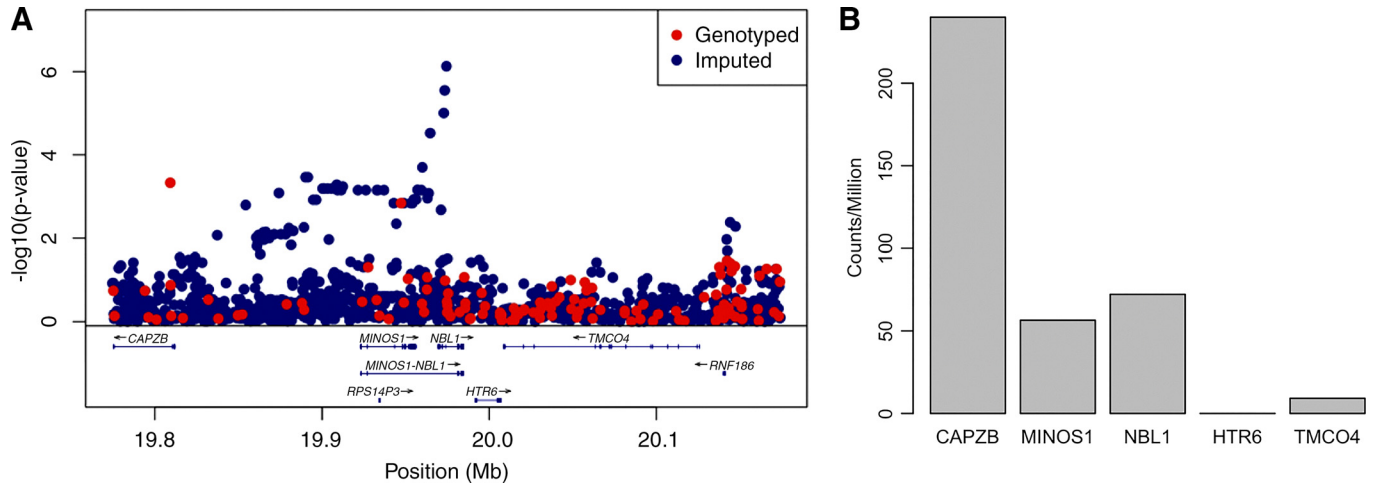

Fig. 4. *A*: LocusZoom plot of the region flanking the top association at rs372271081, an intronic variant of *NBL1*. Mb, megabase. *B*: expression of genes by RNA sequencing within this locus in fetal lung at 23 wk gestation.

with BPD after adjusting for a smaller number of tests in our hypothesis-driven approach ($P = 0.0011$). Local African ancestry at *CCL18* was not significantly associated with BPD in black/AA infants ($P = 0.76$), suggesting that any causal variants are at the same frequency between European and African continental populations and that our association is not confounded by population structure. This gene is expressed at a low level in 23-wk human fetal lung (0.31 ± 0.10 counts per million). None of the genes implicated through exome sequencing from Li et al. (39) were significantly associated with BPD in our study (minimum $P = 0.0018$, *ADCY8*), nor did we find any significant associations with 11 NO-related candidate genes with reported associations with human disease (Table 5; minimum $P = 0.18$, *KALRN*).

Pathway analysis. Pathway analysis can be a powerful means to identify an enrichment of genes with marginal signals of association on their own but which function in a similar biological pathway. Pathway analysis was performed using GREAT (45) on 1,024 genes with gene-based P values <0.05 compared with 17,640 genes as a background. A total of 5 pathways/gene sets were identified with a false discovery rate <0.05 from the PANTHER and MSigDB databases; this group contains 2 pathways related to cancers, 1 related to immune function, 1 related to methylation marks, and 1 implicated in experimental lung injury (Table 6). The pathway of highest statistical significance ($P = 5 \times 10^{-12}$) was "Genes within amplicon 1q21 identified in a copy number alterations study of 191 breast tumor samples." Eight of the eleven genes in this pathway are expressed in human fetal lung at 23 wk GA, and none is regulated by glucocorticoids, which enhance fetal lung maturity. Biological functions of potential relevance to lung development, injury, and repair for these genes include tyrosine kinase receptor signaling pathway (EFNA4, RUSC1, SHC1, ADAM15), developmental processes (EFNA4, RUSC1, ZBTB7B, PBXIP1, SHC1, ADAM15, PYGO2) including angiogenesis (SHC1), NF-κB signaling (RUSC1, ZBTB7B), and sex steroid receptor signaling (PBXIP1, SHC1).

Pathway analysis using IPA of 181 genes with gene-based P values <0.01 of 209 canonical pathways identified 2 with a significant enrichment of genes following a Bonferroni correction: agranulocyte adhesion and diapedesis ($P = 3.06 \times 10^{-5}$) and granulocyte adhesion and diapedesis ($P = 1.22 \times 10^{-4}$); genes in these 2 pathways are identical except for *MYL9* (Table 7). With the exception of *CLDN17*, all genes identified in these pathways are expressed in human fetal lung (12).

DISCUSSION

Unique aspects of our study are the patient population and rigorous assignment of BPD. All infants in TOLSURF were <28 wk gestation and were intubated at 7–14 days, representing infants with severe early respiratory failure and high risk for BPD as reflected by the occurrence of BPD/death in 68.5% of the total population (9). In addition, infants were enrolled from 25 different US sites, providing both racial/ethnic and geographic diversity. The diagnosis of BPD was assigned on a physiological basis using an oxygen/flow reduction challenge to establish a requirement for respiratory support. Thus it is

Table 2. *Results of tests of association at rs372271081 for survival without BPD using logistic regression and urinary NO metabolites following iNO treatment using linear regression*

	Survival without BPD				Urinary NO Metabolites			
Population	Frequency in Cases, *n*	Frequency in Controls, *n*	Odds Ratio	*P* Value	*n*	β (SE)	95% CI	*P* Value
Non-Hispanic white	0.040 (136)	0.12 (41)	0.30	6.2×10^{-3}	26	−5.3 (1.7)	(−8.7, −1.9)	6.2×10^{-3}
African American	0.055 (82)	0.19 (51)	0.25	6.9×10^{-4}	23	0.74 (2.3)	(−3.8, 5.2)	0.75
Hispanic white	0.018 (28)	0.11 (14)	0.15	0.070	13	−1.9 (2.3)	(−6.5, 2.7)	0.45

Results are shown with respect to the minor allele (A), which trends as protective for bronchopulmonary dysplasia (BPD) in 3 populations and is significantly associated with lower urinary NO metabolites in infants of maternal non-Hispanic white race/ethnicity. β, Regression coefficient/effect size; CI, confidence interval; iNO, inhaled nitric oxide; SE, standard error.

Table 3. *Genetic variants associated with survival without BPD at $P < 10^{-6}$ in a meta-analysis across 3 racial/ethnic groups*

Chr	Position (hg19)	SNP	Allele	Annotation	NHW OR	AA OR	HW OR	Meta OR	Meta *P* Value
1	19974397	rs372271081	A	Intron, *NBL1*	0.19	0.10	0.17	0.17	7.42×10^{-7}
2	14648908	rs10193074	G	Intergenic	0.26	0.25	N/A	0.26	4.17×10^{-6}
2	33777089	2:33777089	C	Intron, *RASGRP3*	0.39	0.17	0.22	0.28	6.41×10^{-6}
2	54980799	2:54980799	G	Intron, *EML6*	0.33	0.78	0.44	0.40	5.20×10^{-6}
2	105035900	rs4851694	T	Intergenic	3.8	8.7	N/A	4.3	5.92×10^{-6}
2	105039687	rs2889323	C	Intergenic	3.8	8.7	N/A	4.3	5.92×10^{-6}
2	105091271	rs6543256	G	Intron, *LOC150568*	3.2	9.0	N/A	4.1	7.24×10^{-6}
3	74073182	rs1949931	G	Intergenic	0.38	0.082	0.44	0.39	9.25×10^{-6}
10	134044152	rs60417571	T	Intron, *STK32C*	0.19	N/A	0.27	0.21	3.06×10^{-6}
12	131048872	12:131048872	CTG	Intron, *RIMBP2*	0.44	0.11	0.41	0.39	4.91×10^{-6}
14	47459909	rs8016110	A	Intron, *MDGA2*	2.9	43	2.5	3.5	2.92×10^{-6}
16	8834085	rs75055007	A	Intron, *ABAT*	0.30	0.061	0.28	0.26	2.79×10^{-6}

For loci with multiple single-nucleotide polymorphisms (SNPs) at $P < 10^{-6}$, only a single SNP with the smallest *P* value is included in the table. AA, African American [82 bronchopulmonary dysplasia (BPD)/death infants, 51 no BPD]; Chr, chromosome; HW, Hispanic white (28 BPD/death infants, 14 no BPD); Meta, meta-analysis (total 246 BPD/death infants, 106 no BPD); N/A, not applicable; NHW, non-Hispanic white (136 BPD/death infants, 41 no BPD); OR, odds ratio.

possible that some of our findings may be restricted to extremely premature infants with severe early respiratory disease. Other characteristics of our cohort, which may limit generalization of some of our findings, are exposure to late surfactant treatment in approximately half of the infants and the use of iNO for 3 wk in all infants. Although surfactant therapy transiently improved respiratory status, it did not affect outcome at 36 wk PMA. iNO therapy likely influenced overall outcome in infants of self-identified black/African-American women but not non-Hispanic white women (6), and thus we examined NO metabolism as it relates to genetic associations with BPD in our study.

Higher genomic levels of African ancestry were associated with better respiratory outcome in iNO-treated infants with maternal self-reported Hispanic white race/ethnicity but not for infants with maternal self-reported black/African-American race/ethnicity. Although the protective effect of African genomic ancestry in white Hispanic infants requires independent replication, our results suggest that the protective effect of African ancestry may be saturated at lower levels of ancestry than are present in the majority of black/African-American infants in the study. In other words, there is an increase in the protective effect of African ancestry between the range of 1.2 and 63% (that observed in white Hispanic infants in the study) but not between the range of 40 and 100% (that observed in black/African-American infants in the study). This may reflect a polygenic basis for the beneficial effect of iNO whereby similar protection from BPD can be conferred by a variety of

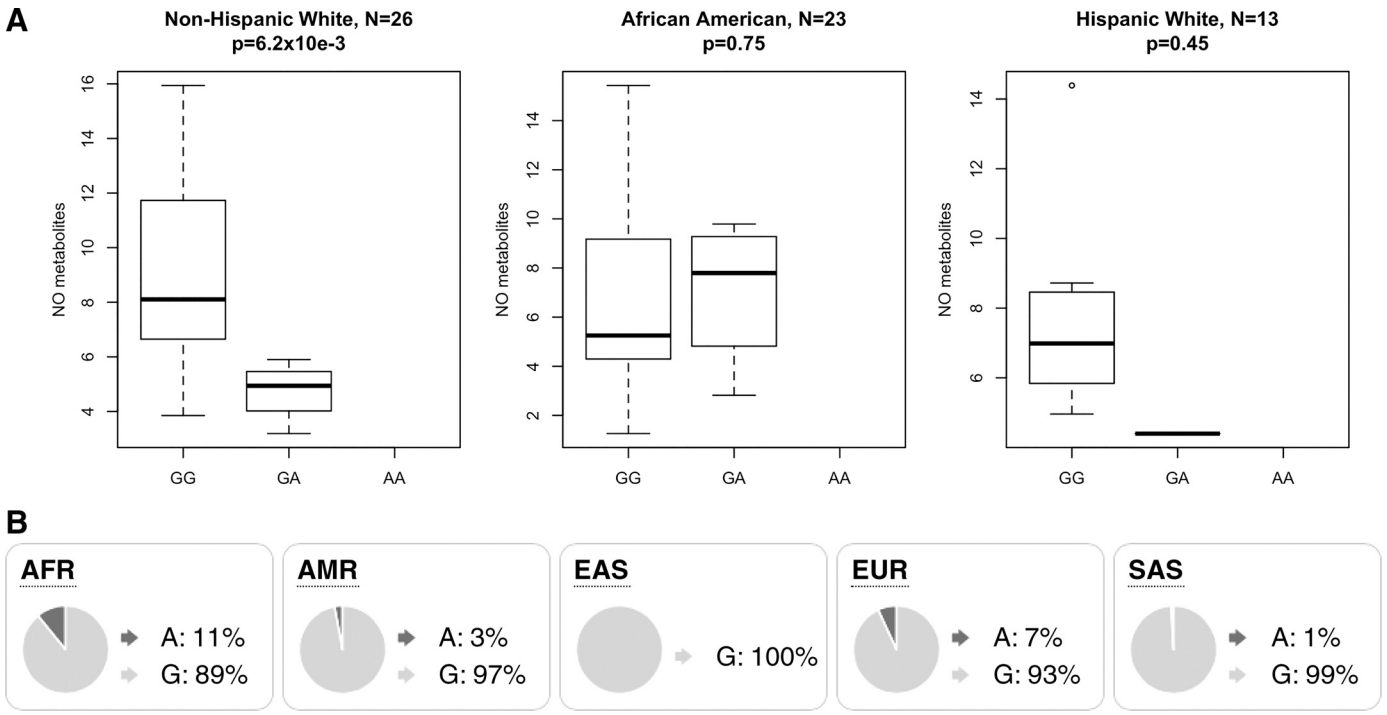

Fig. 5. *A*: box plot showing levels of urinary NO metabolites by genotype at rs372271081 in preterm infants following treatment with inhaled nitric oxide at 5 ppm. *B*: frequency of rs372271081 in populations from the Phase 3 1000 Genomes Project. A and G, alleles; AFR, African; AMR, Admixed American; EAS, East Asian; EUR, European; SAS, South Asian.

Table 4. *Top genes associated with survival without BPD in a meta-analysis across racial/ethnic groups including 246 cases and 106 controls*

Chr	Gene	No. of SNPs	*P* Value
5	*RICTOR*	17	4.5×10^{-5}
4	*MED28*	6	1.8×10^{-4}
12	*IL23A*	8	2.2×10^{-4}
19	*ZNF492*	14	2.5×10^{-4}

Gene-based statistics were calculated using versatile gene-based association study (VEGAS). None of the genes was statistically significant following Bonferroni correction for the total number of genes examined ($n = 17{,}671$). BPD, bronchopulmonary dysplasia; Chr, chromosome; SNPs, single-nucleotide polymorphisms.

different variants of African ancestry in genes of key NO-related pathways. Alternatively, there may be differential gene-environment interactions between racial/ethnic groups. For example, the protective effect of African ancestry may only be relevant under specific environmental exposures that vary by racial/ethnic group.

Genetic ancestry does not form a direct causal relationship but rather indicates differences in the underlying patterns of genetic variation in infants with/without BPD that differ by continental origin. If only small proportions of African ancestry are required for a protective effect, this would suggest a highly polygenic contribution to BPD distributed throughout the genome. Admixture mapping in black/AA infants identified two suggestive, but not statistically significant, peaks, at 10q22 and 18q21, whereby African ancestry was associated with a decreased and increased risk of death/BPD, respectively. Therefore, admixture mapping further supports the hypothesis that the effect of ancestry is not limited to a single locus of large effect. It is possible the relationship between ancestry and BPD is restricted to infants receiving iNO; this is supported by prior studies that identified racial differences in endogenous NO levels or metabolism in infants (8) and adults (32, 34, 35, 44).

In our agnostic scan including ~9 million genotyped and imputed variants, no individual variant was genome-wide significantly associated with survival without BPD. This was not unexpected, given our small sample size, and is consistent with prior GWAS that similarly failed to identify individual variants with large effects at genome-wide significance levels (4, 29, 43, 61). However, numerous biologically plausible genes have been implicated with top GWAS variants identified at suggestive levels of significance, including *ADARB2* (4), *CRP* (43), *SPOCK2* (29), and an intergenic region on chromosome 18 (61). We were unable to replicate any of the top findings from prior GWAS, but note that our current study has many unique characteristics as outlined above.

The biggest limitation of our study is the sample size, given that any one genetic variant is likely to have a small effect. Small/modest sample size is a common limitation to genetic studies of preterm infants, and thus there is a need to integrate additional biological measurements to increase power of discovery. For example, after implicating variants around *CRP* at suggestive levels of statistical significance, Mahlman et al. (43) identified CRP protein as being significantly elevated in infants that went on to develop BPD at 36 wk. Ambalavanan et al. (4) queried gene expression in BPD lungs and validated suggestive associations identified through GWAS at variants in *CD44* and the miR-219 pathway. By taking a similar integrative approach, we found our top BPD-associated variant, rs372271081, was significantly associated with differences in NOx in white infants, whereby the protective allele for BPD was associated with decreased levels of NOx in the urine following treatment of iNO (but not before treatment). This finding lends supports for a true genetic association with BPD at rs372271081 and indicates the association is likely specific to iNO treatment and an effect through differential drug metabolism. However, we found no significant association between genotype and NO metabolites in black/AA or Hispanic white infants, which may reflect the limited statistical power given the smaller sample sizes, the lower frequency of the allele, varying patterns of linkage disequilibrium, and/or the presence of genetic and environmental interactions.

rs372271081 Lies within an intron of neuroblastoma 1, DAN family BMP antagonist (*NBL1*), which is a highly plausible candidate gene for contributing to BPD susceptibility via differential response to iNO. Numerous studies in mice indicate that the BMP pathway is important for lung development, including branching morphogenesis in early gestation and distal lung epithelial cell differentiation, alveolization, and vasculogenesis in late gestation (16, 23, 62). The transforming growth factor-β-BMP signaling pathway is disrupted by hyperoxia (1), which is known to play a role in the development of BPD (1, 2). In humans, disrupted BMP signaling has been implicated in the pathogenesis of heritable pulmonary arterial hypertension and hereditary hemorrhagic telangiectasis (27, 48). Finally, in addition to ligand inhibition of BMP, DAN family members are known to modulate Wnt and VEGF signaling pathways that have a role in lung development and injury/repair (48). Overall, there is strong biological plausibility for a role of genetic variation in *NBL1* and respiratory

Table 5. *List of NO-related candidate genes/variants previously associated with disease*

Gene	Variant	Disease (Measurement) (Ref.)
NOS2	rs944722	Radiation lung injury (lung function) (58)
		Infant RSV-related respiratory morbidity (22)
	rs2274894, rs7215373	Tuberculosis susceptibility (59)
	rs3794767	Malaria susceptibility (blood *Plasmodium*/NO) (57)
NOS3	rs1799983, rs2070744	Coronary artery disease (54, 67)
	G894T	Essential hypertension (40)
		Hypoxic ischemic encephalopathy (65)
	−922 G>A, −786 T>C	Ischemic stroke susceptibility (30)
GUCY1A3	A680T	Pulmonary hypertension (63)
LYRM9	rs3751972	Asthma (FeNO*) (58)
GSDMB	rs8069176	Asthma (FeNO*) (58)
GSR	rs2253409	Lupus (NO production) (55)
KALRN	rs9289231	Coronary artery disease (15)
TSNAX-DISC1	rs821722	Nicotine dependence (25)
PON1	Q192R	Coronary artery disease (49)
IFNGR1	rs1327474	Tuberculosis susceptibility (59)
PDE5	G1142T	Congestive heart failure response to inhaled NO (20)

A, C, G, and T, alleles; RSV, respiratory syncytial virus. *FeNO, fractional concentration of nitric oxide in exhaled air.

Table 6. *PANTHER and MSigDB pathways showing a significant enrichment of genes associated with survival without BPD at $P < 0.05$*

Pathway/Gene Set	*P* Value	FDR	Fold Enrichment	No. of Genes
PANTHER				
Toll receptor signaling pathway	2.3×10^{-4}	0.035	3.1	13
MSigDB				
Genes within amplicon 1q21 identified in a copy number alterations study of 191 breast tumor samples	1.5×10^{-15}	5.1×10^{-12}	8.6	21
Genes with low-CpG-density promoters bearing H3K4me3 marks in embryonic fibroblasts	4.1×10^{-7}	6.8×10^{-4}	2.5	35
Nearest neighbors of TAL1, based on the close agreement of their expression profiles with that of TAL1 in pediatric T cell acute lymphoblastic leukemia	6.7×10^{-6}	0.0076	5.0	11
Genes upregulated in lung tissue on LPS aspiration with mechanical ventilation	2.4×10^{-5}	0.020	2.2	32

Foreground set of 1,024 genes with association *P* value <0.05 was compared with a background set of 17,640 genes using Genomic Regions Enrichment of Annotations Tool (GREAT). Gene-based association *P* values were calculated using versatile gene-based association study (VEGAS). BPD, bronchopulmonary dysplasia; FDR, false discovery rate; MSigDB, Molecular Signature Database; PANTHER, Protein Analysis THrough Evolutionary Relationships.

outcome in iNO-treated infants based on *1*) the critical role of BMP signaling in lung development and disease, *2*) the mediation of BMP action via NO, *3*) the expression of *NBL1* and BMPs in human fetal lung (35), and *4*) the racial differences in BPD and NO metabolism (12).

Although *NBL1* has not been specifically implicated in prior GWAS/exome sequencing studies, genes involved in lung development are strong candidates for a role in BPD, which only occurs in immature lungs. For example, a common variant in *SPOCK2*, an extracellular matrix protein, was implicated in BPD through GWAS and found to be upregulated during lung alveolar development and after exposure to hyperoxia in rats (29). Furthermore, pathway analyses have implicated other genes involved in pulmonary structure and functions (39). Replication and both laboratory and functional validation are necessary to confirm a causal relationship of variants in *NBL1* and BPD in infants treated with iNO. Currently, there are no other cohorts of premature infants treated with iNO with DNA samples available for validation studies.

We further performed hypothesis-driven tests of association with BPD using a set of 21 genes that are dysregulated in BPD lungs (14) and 11 genes in the nitric oxide pathway that are reported to have variants associated with disease (Table 5). First, we hypothesized that genes showing differential expression in BPD-dysregulated vs. control lungs may contain variants that contribute to survival without BPD. We found a significant association with genetic variation in a single gene, *CCL18*, a cytokine involved in the immune response that promotes collagen production in lung fibroblasts (42) and is associated with pulmonary fibrosis and interstitial lung diseases in adults (51, 66). Inflammation is known to be important in the pathogenesis of BPD, and anti-inflammatory therapy (dexamethasone) suppresses a variety of inflammatory mediators, including CCL18, and reduces BPD (12, 26). Second, because all infants in the study received iNO, we hypothesized that variation in genes in the NO pathway may contribute to differential response to iNO treatment as indicated by survival without BPD. However, no individual variant or candidate gene (based on known association with human disease) was significantly associated with survival without BPD following correction for multiple tests.

Nonetheless, because exposure to iNO appears to influence the differential rates of BPD between racial/ethnic groups (6, 60), we hypothesized that genetic variants that contribute to BPD may act through differential response to iNO. In support of this, the protective allele for BPD at rs372271081 is significantly associated with decreased NOx and is more common in populations with African ancestry. Several studies indicate reduced bioavailability of NO in African Americans vs. Caucasians, likely, in part, due to increased oxidation of NO. In laboratory studies, release of NO from umbilical venous endothelial cells was substantially lower in African-American vs. Caucasian infants (34, 44). Levels of urinary NOx are lower in African-American and Hispanic premature infants vs. Caucasian infants regardless of iNO treatment, reflecting baseline differences in NO metabolism and thus bioavailability (8). In adults, African Americans are known to have increased frequency of hypertension and cardiovascular disease, and a NO-targeted medication (isosorbide dinitrates and hydralazine) is indicated therapy for heart failure specifically in African Americans (i.e., a racially directed therapy; Refs. 32, 35). However, further studies are needed to evaluate the contribution of rs372271081 to racial/ethnic differences in NO bioavailability and differential response to iNO.

Pathway analyses identified pathways and sets of genes that were significantly enriched for genes with association *P* values <0.05. Across IPA, PANTHER, and MSigDB data sets, a common theme that emerged was genes involved in immune function, including granulocyte and agranulocyte adhesion and diapedesis from IPA canonical pathways, Toll receptor signal-

Table 7. *Canonical pathways from Ingenuity Pathway Analysis with a significant enrichment of genes with association $P < 0.01$ for survival without BPD*

Canonical Pathway	No. of Genes (%)	Genes in Pathway with $P < 0.01$	*P* Value
Agranulocyte adhesion and diapedesis	9/181 (4.8)	CCL3, CCL4, CCL17, CCL18, CCL22, CLDN17, CX3CL1, MYL9, RDX	3.06×10^{-5}
Granulocyte adhesion and diapedesis	8/181 (4.4)	CCL3, CCL4, CCL17, CCL18, CCL22, CLDN17, CX3CL1, RDX	1.22×10^{-4}

Statistical significance was determined using a Bonferroni adjustment for 209 canonical pathways tested ($\alpha = 2.39 \times 10^{-4}$). BPD, bronchopulmonary dysplasia.

ing pathway from PANTHER, and genes upregulated in response to LPS exposure and mechanical ventilation from MSigDB. These results suggest that variation in immune response, including recruitment of leukocytes and lymphocytes, contributes to survival without BPD.

Overall, our results for this cohort of iNO-treated, high-risk infants suggest that genomic African ancestry is protective for BPD and that an intronic variant in *NBL1* may contribute to BPD via differential activity of the transforming growth factor-β-BMP pathway and production/metabolism of NO. Furthermore, we implicated variation in genes involved in the immune response, including *CCL18*, as contributing to differences in respiratory outcomes of preterm infants.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

D.G.T., P.L.B., R.L.K., E.G.B., and R.A.B. conceived and designed research; D.G.T., P.L.B., R.L.K., C.E., E.G.B., and R.A.B. performed experiments; D.G.T., P.L.B., R.L.K., S.S.O., S.H., D.H., C.E., and R.A.B. analyzed data; D.G.T., P.L.B., R.L.K., and R.A.B. interpreted results of experiments; D.G.T. prepared figures; D.G.T. and P.L.B. drafted manuscript; D.G.T., P.L.B., R.L.K., E.G.B., and R.A.B. edited and revised manuscript; D.G.T., P.L.B., R.L.K., S.S.O., S.H., D.H., C.E., E.G.B., and R.A.B. approved final version of manuscript.

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