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# Anti-Oxidant Response Genes sequence variants and BPD susceptibility in VLBW infants

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## Abstract

**Background—**Lung injury resulting from oxidative stress contributes to bronchopulmonary dysplasia (BPD) pathogenesis. Nuclear factor erythroid-2 related factor-2 (*NFE2L2*) regulates cytoprotective responses to oxidative stress by inducing enzymes containing anti-oxidant response elements (ARE). We hypothesized that ARE genetic variants will modulate susceptibility or severity of BPD in very low birth weight (VLBW) infants.

**Methods—**Blood samples obtained from VLBW infants were used for genotyping variants in the *SOD2*, *NFE2L2*, *GCLC*, *GSTP1*, *HMOX1* and *NQO1* genes. SNPs were genotyped utilizing TaqMan probes (Applied Biosystems (ABI), Grand Island, NY), and data was analyzed using the ABI HT7900. Genetic dominance and recessive models were tested to determine associations between SNPs and BPD.

**Results—**In our cohort (n=659), 284 infants had BPD; 135 of whom developed severe BPD. Presence of the hypomorphic *NQO1* SNP (rs1800566) in a homozygous state was associated with increased BPD while presence of the *NFE2L2* SNP (rs6721961) was associated with decreased severe BPD in the entire cohort and in Caucasian infants. In regression models that adjusted for epidemiological confounders, the *NQO1* and the *NFE2L2* SNPs were associated with BPD and severe BPD, respectively.

**Conclusions—**Genetic variants in NFE2L2-ARE axis may contribute to the variance in liability to BPD observed in preterm infants. These results require confirmation in independent cohorts.

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## INTRODUCTION

Bronchopulmonary dysplasia (BPD), a chronic lung disease that develops in 16–20% of very low birth weight infants (VLBW, birth weight<1500g) remains the major cause of pulmonary morbidity and mortality during infancy (1,2). In contrast with fetal lung development in a relatively hypoxic intrauterine environment, postnatal lung development in preterm infants is encumbered by increased oxidative stress that portends the development of BPD in some VLBW infants (3,4). Exposure to hyperoxia, mechanical ventilation and bacterial infections increases production of reactive oxygen species in the lung which trigger inflammation and mucosal injury contributing to the development of BPD (3,5). Markers of cellular oxidative damage such as oxidized surfactant phospholipids, 8-Oxo-2′-deoxyguanosine, uric acid and F2-isoprostanes, are elevated in tracheal lavage fluid, urine and/or serum of infants who develop BPD (3,6). Further, multiple clinical trials have attempted to decrease the use of supplemental oxygen therapy to reduce the incidence of BPD (7). Although a number of studies have shown that genetic factors can contribute to the risk of developing BPD, whether inherited differences in the host antioxidant response enzymes modulate susceptibility or severity of the disease in premature infants remains unknown (8–10).

Animal data and human studies demonstrate that both constitutive and stress-dependent pulmonary antioxidant defenses are developmentally programmed and mature late in gestation (3,11). In this setting of increased oxidative stress and sub-optimal antioxidant defenses, functional genetic variation in antioxidant enzyme genes may contribute to increase oxidative damage and lung injury in preterm infants and predispose to BPD. The NF-E2-related factor-2 (Nrf2)-dependent antioxidant response elements (ARE) pathway genes are master regulators of host responses to oxidative stress and cellular injury (12). Nrf2, encoded by the gene *NFEL2* is a basic leucine zipper transcription factor which is kept inhibited in the cytoplasm by being bound to Kelch like-ECH-associated protein 1 (12,13). Oxidative stress and other stress signals activate Nrf2 which then binds to the ARE promoter sequences ensuring coordinated up-regulation of antioxidant enzymes like superoxide dismutase 2 (*SOD2*), NAD(P)H: quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO1), glutamate-cysteine ligase catalytic subunit (GCLC), and detoxification enzymes like glutathione S-transferases isoforms (GST) and cytochrome P450 oxidases(12,14). Functional loss of ARE genes have been shown to modulate lung injury in response to environmental toxicants, hyperoxia and smoking in animal models (14,15). In humans, sequence variants in anti-oxidant genes have been implicated in modulating susceptibility to chronic obstructive pulmonary disease (COPD), acute lung injury and environmental pollutants (16–18). However, whether function- or expression-altering genetic variants in the Nrf2-ARE axis alter susceptibility or severity of BPD in VLBW infants remains unknown. In this study, we investigated the relationships between six single nucleotide polymorphisms (SNPs) in the ARE pathway genes and BPD outcomes in VLBW infants.

# RESULTS

## BPD outcomes in our study

In our cohort (n=659) of VLBW infants, 284 infants had BPD; 135 of whom developed severe BPD. The distribution of epidemiological/clinical variables among infants with no BPD, BPD and severe BPD is shown in table 1. When compared to infants without BPD, infants with BPD had lower birth weights ($p<0.001$), were more premature ($p<0.001$), were more likely male ($p<0.02$), and were more likely to have patent ductus arteriosus (PDA; $p<0.001$). Similar results were obtained when comparing infants with severe BPD to infants without severe BPD. Compared to infants without BPD, infants with BPD ($p<0.006$) were more likely to be Caucasian (CAU). Rates of clinical chorioamnionitis and prenatal steroid treatment were similar among the three groups.

## Association between BPD outcomes and ARE variants

Hardy-Weinberg equilibrium was confirmed at all loci. The distribution of ARE genotypes among infants with no BPD, BPD and severe BPD is shown in table 2. The *SOD2*, *HMOX1*, *GSTP1*, and *GCLC* SNPs were not associated with BPD or severe BPD. Infants homozygous for the *NQO1* (rs1800566) SNP had higher rates of BPD when compared to infants who were not homozygous for the SNP (21/35 (60%) *vs*. 261/621 (42%); p=0.037). Presence of the homozygous state for the *NQO1* SNP was not associated with severe BPD (10/35 (28.6%) *vs*. 124/621 (20%); p=0.20). Infants who had the *NFE2L2* SNP (rs6721961) had decreased severe BPD (18/140 (12.9%) *vs*. 116/515 (22.5%); p=0.015) when compared to infants without the variant. There was no association between the *NFE2L2* SNP and BPD. The associations between the *NQO1* SNP and BPD, and the *NFE2L2* SNP and severe BPD did not meet the Bonferroni significance level of $p<0.008$.

To control for potential confounders, associations between the ARE SNPs and BPD or severe BPD were evaluated with time-sequence logistic regression models. Gestational age (GA) ≤ 26wk ($p<0.001$), birth weight ≤ 800g (p=0.001), male gender (p=0.03), and PDA ($p<0.001$) were associated with increased BPD risk (Table 3a). African American (AA) race ($p<0.001$), and presence of the *NFE2L2* SNP (p=0.023) were associated with decreased BPD risk. Infants who were homozygous for the *NQO1* SNP were at increased risk of BPD (p=0.007). In models for severe BPD, we again noted that GA ≤ 26wk ($p<0.001$), birth weight ≤ 800g (p=0.002), PDA ($p<0.001$), and male gender (p=0.003) were associated with increased risk of severe BPD (Table 3). The *NFE2L2* SNP ($p<0.001$) and AA race (p=0.02) were associated with decreased risk of severe BPD (Table 3). There was a marginal association between presence of the *NQO1* SNP in a homozygous state and severe BPD.

## Relationship between *NFE2L2* and *NQO1* SNPs and demographic variables

We next examined whether the *NQO1* and *NFE2L2* SNPs were associated with demographic variables. There was no significant difference in birth weight, GA, gender, prenatal steroids use or chorioamnionitis among infants with or without the *NQO1* and *NFE2L2* SNPs. Although the *NQO1* SNP was not associated with race, infants who had the *NFE2L2* SNP were more likely to be CAU (CAU vs. AA; 114/481 vs. 22/142, p=0.038). The allele frequency of this SNP in our cohort is similar to that reported by other

investigators (16,19). To minimize the effect of population stratification on the relationships between *NQO1* and *NFE2L2* SNPs and BPD outcomes, and because race was an effect modifier on both the outcome (BPD or severe BPD) and variant examined (*NFE2L2*) we examined CAU infants separately.

### ARE SNPs and BPD outcomes among Caucasian infants

Among 475 CAU infants, 220 infants had BPD; 101 of whom developed severe BPD. Similar to our results in the entire cohort we did not find significant associations between the *SOD2*, *HMOX1*, *GSTP1*, and *GCLC* SNPs and BPD or severe BPD. Genotype frequencies of the *NQO1* and *NFE2L2* SNPs in CAU infants categorized by BPD outcomes are shown in table 4. Infants homozygous for the *NQO1* SNP had higher rates of BPD (17/24 (71%) *vs.* 204/452 (45%); p=0.014, OR = 2.95, 95% CI; 1.13 – 8.1) when compared to infants who were not homozygous for the *NQO1* SNP. However, this association did not meet the Bonferroni adjusted significance level of p<0.008. Infants homozygous for the *NQO1* SNP did not have significantly higher rates of severe BPD (8/24 (33%) *vs*. 93/452 (20.7%); p=0.14). Infants who had the *NFE2L2* SNP had decreased rates of severe BPD (13/101 (12.9%) *vs*. 100/374 (26.7%); p=0.004; OR − 0.40, 95% CI − 0.21 – 0.78) but not BPD when compared to infants without the SNP. The association between the *NFE2L2* SNP and severe BPD met the Bonferroni significance level (p<0.008).

In time-sequence regression models for BPD in CAU infants, GA ≤ 26wk (p<0.001), birth weight ≤ 800g (p=0.002), male gender (p=009), and PDA (p<0.001) were associated with increased BPD (Table 5). Presence of the *NFE2L2* SNP (p=0.005) was associated with decreased BPD. Infants homozygous for the *NQO1* SNP were at increased risk of BPD (p=0.006). In similar models for severe BPD, we noted that GA ≤ 26wk (p<0.001), birth weight ≤ 800g (p=0.01), PDA (p<0.001), and male gender (p=0.02) were associated with increased risk of severe BPD (Table 5). Presence of the *NFE2L2* SNP was associated with decreased risk of developing severe BPD (p<0.001).

## DISCUSSION

BPD is a complex disease influenced by interactions between genetic factors, fetal environment and postnatal risk-factors that contribute to lung injury (2,20). While pulmonary oxidative stress is implicated in neonatal lung injury the relationships between anti-oxidant stress response sequence variants and BPD remain understudied (3,4). In this study we followed a pathway approach to investigate the impact of functional ARE SNPs on susceptibility or severity of BPD in VLBW infants. We report an association between a missense hypomorphic *NQO1* (p.P187S) SNP and increased BPD as well as a promoter *NFE2L2* (−617C>A) variant and decreased severe BPD. We also demonstrate that common SNPs in the *SOD2*, *HMOX1*, *GSTP1* and *GCLC* are not associated with BPD or severe BPD. Although we report new data, lack of replication in an independent cohort limits the generalizability of our results. Widening our genotyping approach to examine rare ARE genetic variants and inclusion of a replication cohort are directions for future research.

NQO1 is a flavoprotein enzyme that catalyzes two electron reduction of a variety of substrates including quinones, and is transcriptionally activated by Nrf2 (12). The *NQO1*

SNP (rs1800566;P187S) investigated in this study abolishes cellular NQO1 activity in the homozygous state (21). Among VLBW infants there was a 50% increase in BPD rates among individuals who were homozygous for the *NQO1* SNP. This association persisted after adjusting for potential confounders in the entire cohort and in Caucasian infants. However, this association did not meet Bonferroni significance after correcting for 6 SNPs, possibly due to an inadequate sample size. While the homozygous variant *NQO1* genotype was more prevalent in infants with severe BPD when compared to infants without severe BPD this association was not statistically significant. This suggests that the *NQO1* SNP may not modulate disease severity in BPD. Alternatively, it may suggest that our sample size was not adequate to demonstrate an independent effect on severe BPD. Multiple reports have demonstrated associations between this SNP (rs1800566) and breast cancer, bladder cancer and tardive dyskinesia (22–24). The mechanism is suggested to be a loss in NQO1-dependent, p53-mediated pro-apoptotic signaling leading to cancer survival (22). Relationships between the *NQO1* SNP and lung disease phenotypes have shown inconsistent results with respect to lung cancer, atopy and asthma (25,26). In VLBW infants, we speculate that loss of NQO1 activity resulting from the T/T genotype at the *NQO1* locus diminishes lung protective responses against hyperoxia, bacteria or oxidant-mediated pulmonary inflammation and remodeling (27). The relationship between *NQO1* genotype, markers of oxidative injury and BPD outcomes need to be examined in other VLBW cohorts to determine the importance of *NQO1* in BPD.

*NFE2L2* encodes Nrf2, the master transcriptional activator of cellular anti-oxidant enzymes that protect against hyperoxia, sepsis and electrophile chemicals (12). We found that the promoter *NFE2L2* SNP (rs6721961; −617C>A) was not associated with increased BPD. Marzec et al. (16) reported an increased risk of acute lung injury after major trauma in adults with this variant. Both in-vitro and in-vivo studies have shown that this SNP decreases basal Nrf2 mRNA expression (16,28). Whereas BPD is a phenotype for chronic lung injury in the immature lung, pulmonary injury after trauma is representative of an acute phenotype. Further, the relevance of the heterozygous state on Nrf2 expression levels in the preterm lung, and our inability to test a genetic recessive model due to limited number of infants homozygous for this SNP may have contributed to our results. Paradoxically, we found that the *NFE2L2* SNP was associated with decreased risk of severe BPD even after adjusting for confounding variables such as GA, gender and race. It is unclear how presence of the carrier state (most infants with the *NFE2L2* SNP in our study were heterozygous) protects against severe BPD. Studies that examined associations between this SNP and chronic lung phenotypes such as childhood-onset asthma or adult COPD have yielded negative results (29,30). However, heterozygous carriers of this SNP had better survival after lung cancer supporting a protective effect (19). A larger cohort could have given us more statistical power to investigate the relationships between the different genotypes on BPD outcomes. In summary, the above studies suggest limited penetrance of this variant in complex diseases. Future studies are needed to examine the effect of different *NFE2L2* SNP (rs6721961) genotypes on BPD, severe BPD, lung function and Nrf2 expression in premature infants.

The *SOD2* SNP (rs4880) queried in this study encodes a missense (*Ala*47*Val*) variant that results in decreased manganese superoxide dismutase (MnSOD) activity (31). We did not

find an association between this variant and BPD using genetic dominance or recessive models. Giusti et al. (32) did not report significantly increased BPD rates with this variant among infants with GA<30wk. A potential explanation is the decreased amount of MnSOD protein in the fetal lung and the presence of other dismutases that can compensate for MnSOD function (11). *GSTP1* encodes glutathione S-transferase pi, an enzyme which detoxifies electrophile compounds using reduced glutathione. The *GSTP1* variant is a missense variant (Ile104Val) that alters enzymatic binding to specific substrates (33). In adult studies, the heterozygous variant genotype was associated with a protective effect against COPD in contrast with the homozygous variant genotype which showed a trend towards increased COPD(17,18). In our cohort we did not find any associations between this variant and BPD outcomes.

The *GCLC* (−129C/T) variant investigated in this study decreases expression of the catalytic sub-unit of glutamate cysteine ligase; an enzyme that catalyzes the rate limiting step of glutamate synthesis (34). Glutamate is a major intracellular antioxidant highly expressed in the lung (11). In adults, this variant is associated with a rapid decline in lung function as well as increased COPD risk (35,36). In VLBW infants we did not find an association between this variant and BPD. The lack of enough infants who were homozygous for this variant may have confounded our results. *HMOX1* encodes the inducible form of heme oxygenase, which aside from it's role in heme catabolism is important for the cellular anti-oxidant response (37). The variant (rs2071747) examined in this study encodes a missense change (Asp7His) that is in linkage with a functional $(GT)_n$ promoter variant known to be associated with emphysema (38,39). We did not find any association between this variant and BPD outcomes in our study cohort. While this variant has not been investigated with relation to lung phenotypes in children, in adults Tanaka et al (39) reported the lack of association with lung function decline in adults.

A recent GWAS study did not identify any SNPs that were associated with BPD in premature infants at a genome-wide significance level of $5 \times 10^{-8}$ (9). Our results with regard to the *GSTP1*, *HMOX1*, *SOD2* and *GCLC* SNPs are consistent with data reported by Wang et al.(9). The association between the *NQO1* SNP and BPD in our cohort was found using a genetic recessive model whereas in their study dominant and additive models were used (personal communication Dr. Hugh O'Brodovich). This suggests that this hypomorphic variant may be penetrant only in the recessive state. In contrast with Wang et al. (9) who did not examine relationships between SNPs and severe BPD, the *NFE2L2* SNP was only associated with severe BPD. The use of different genetic models and analysis of severe BPD as a separate outcome may have contributed to disparate results between Wang et al. and our study. Although our data need to replicated in an independent cohort, it is possible that for a complex, multi-factorial disease such as BPD certain genetic variants will modify disease severity in the presence of clinical risk-factors.

In summary, we examined the impact of functional ARE variants on BPD outcomes in VLBW infants. Our data suggest that a hypomorphic *NQO1* variant is associated with increased BPD while the *NFE2L2* variant is associated with decreased severe BPD in our cohort. Although several studies suggest that inherited factors influence liability to BPD, identification of genetic biomarkers that can predict disease remains elusive. Future studies

have to consider approaches using recessive models, characterizing sub-phenotypes or extreme phenotypes, and incorporate testing for rare variants to characterize genetic risk-factors for BPD.

## METHODS

### Recruitment of study subjects

VLBW infants were recruited prospectively from neonatal intensive care units at Children's Hospital of Wisconsin (Milwaukee, WI), St. Joseph's Hospital (Milwaukee, WI), Kosair's Children's Hospital (Louisville, KY), Children's Hospitals and Clinics of Minnesota (Minneapolis, MN), and University of Iowa Children's Hospital (Iowa City, IA) after institutional review board approval. After informed consent, 0.5mL of blood was collected in coded sample containers, and shipped to Children's Hospital of Wisconsin where DNA extraction and genotyping was done. For study subjects from Iowa, de-identified DNA samples were sent to Children's Hospital of Wisconsin. De-identified clinical and epidemiological data were assigned a study code and entered into a password-protected database.

### Eligibility criteria

Premature infants born with a birth-weight ≤ 1500 grams (VLBW) admitted to the participating centers were eligible. Infants with major congenital anomalies of the heart, gastro-intestinal tract, renal or respiratory tract were excluded.

### Definition of case

BPD was defined as the need for supplemental oxygen at a postmenstrual age (PMA) of 36 weeks. Because genetic factors may contribute to the disease susceptibility or severity we also examined severe BPD. We defined severe BPD among infants with BPD if they required ≥ 30% oxygen or positive pressure airway support at 36 weeks PMA (2). For infants on nasal cannula effective $FiO_2$ was calculated as per criteria published previously (40).

### Selection of SNPs

ARE pathway genes were targeted based on; a) whether they are transcriptionally activated by *NFE2L2*, and ii) functional relevance to pulmonary anti-oxidant responses (12,17,18). SNPs in candidate genes were identified by searching public databases (pubmed and dBSNP) and selected based on whether: i) variants were reported to be associated with lung injury phenotypes, ii) variants had a functional effect, and iii) mean allele frequency (MAF) > 2% in the Caucasian population.

### Laboratory procedure

Genomic DNA was extracted from blood samples using the FlexiGene DNA kit (Qiagen, Inc., Valencia, CA) and stored at 4_ C. To genotype the *NFE2L2* (rs6721961), *SOD2* (rs4880), *GSTP1* (rs1695), *NQO1* (rs1800566), *GCLC* (rs17883901) and *HMOX1* (rs2071747) SNPs we performed a 5′ nuclease Taqman assay (Applied Biosystems, Foster

City, CA) as per the manufacturer's instructions using custom/predesigned TaqMan® SNP Genotyping Assay probes (ABI, Foster City, CA). The principle of the assay involves amplification of the genomic region of interest followed with ligation with allele-specific probes that emit a distinct fluorescent signal specific to the variant or reference allele. Samples were analyzed on ABI HT7900 with SDS 2.3 software package (probes available on request). Genotyping was done by personnel blinded to clinical outcomes. ***Quality control:*** 5% of the samples were re-genotyped by an independent technician blinded to prior results. There was >99% concordance for all samples.

### Statistical analysis

Chi-square analyses were used for comparisons between dichotomous demographic or clinical variables among infants with and without BPD or severe BPD. Gestational age and birth-weight were compared between the groups using the Wilcoxon-Mann-Whitney rank sum test. We used genetic dominance (a single copy of the SNP confers disease risk) and recessive models (two copies of the SNP are required to confer disease risk) to examine BPD outcomes as studies in adults suggests that ARE SNPs exert dominant or recessive effects (17,18). Variant allele frequencies were compared among groups using the Pearson's Chi-square test or Fisher's exact test. In pre-specified a priori analysis, relationships between SNPs and BPD outcomes would also be examined in Caucasian infants (largest racial group). **Power:** A genetic dominance model was used for calculations. Using a case-control design (1 case: 1.3 controls), we estimated that a sample size of 650 infants would give us 80% power with a p=0.008 (Bonferroni correction) to detect an 8 – 14% difference in the prevalence of the variant allele between infants with and without BPD. Assuming ~50% of the infants with BPD will develop severe BPD, we will have 80% power (p=0.008) to detect a 10–16% difference in the prevalence of the variant allele between infants with or without severe BPD.

To control for potential confounders, we analyzed data using time-sequence logistic regression with backward elimination where the probability of removal was set at P≥0.05. In this model, birth variables (gestational age, birth-weight, clinical chorioamnionitis, antenatal steroid exposure, race, sex) along with ARE SNPs were examined for association with BPD. Variables were removed from the model in a stepwise fashion till only those associated with BPD (p<0.05) remained. Patent ductus arteriosus (clinical or echocardiograph diagnosis) was then added to the model and backward elimination done until only variables associated with outcomes remained. Risk factors for severe BPD were modelled in a similar fashion. SPSS 18.0 (SPSS Inc., Chicago, IL) and SAS 9.2 (SAS Inc., NC) were used for data analysis.

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## References


1. Bancalari E, Claure N, Sosenko IR. Bronchopulmonary dysplasia: changes in pathogenesis, epidemiology and definition. Semin Neonatol. 2003; 8:63–71. [PubMed: 12667831]
2. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. American journal of respiratory and critical care medicine. 2001; 163:1723–9. [PubMed: 11401896]
3. Saugstad OD. Oxygen and oxidative stress in bronchopulmonary dysplasia. J Perinat Med. 2010; 38:571–7. [PubMed: 20807008]
4. Asikainen TM, Raivio KO, Saksela M, Kinnula VL. Expression and developmental profile of antioxidant enzymes in human lung and liver. American journal of respiratory cell and molecular biology. 1998; 19:942–9. [PubMed: 9843929]
5. Vento M, Moro M, Escrig R, et al. Preterm resuscitation with low oxygen causes less oxidative stress, inflammation, and chronic lung disease. Pediatrics. 2009; 124:e439–49. [PubMed: 19661049]
6. Joung KE, Kim H-S, Lee J, et al. Correlation of urinary inflammatory and oxidative stress markers in very low birth weight infants with subsequent development of bronchopulmonary dysplasia. Free Radic Res. 2011; 45:1024–32. [PubMed: 21651454]
7. Saugstad OD, Aune D. Optimal oxygenation of extremely low birth weight infants: a meta-analysis and systematic review of the oxygen saturation target studies. Neonatology. 2014; 105:55–63. [PubMed: 24247112]
8. Bhandari V, Bizzarro MJ, Shetty A, et al. Familial and genetic susceptibility to major neonatal morbidities in preterm twins. Pediatrics. 2006; 117:1901–6. [PubMed: 16740829]
9. Wang H, St Julien KR, Stevenson DK, et al. A Genome-Wide Association Study (GWAS) for Bronchopulmonary Dysplasia. Pediatrics. 2013; 132:290–7. [PubMed: 23897914]
10. Lavoie PM, Pham C, Jang KL. Heritability of bronchopulmonary dysplasia, defined according to the consensus statement of the national institutes of health. Pediatrics. 2008; 122:479–85. [PubMed: 18762515]
11. Asikainen TM, White CW. Pulmonary antioxidant defenses in the preterm newborn with respiratory distress and bronchopulmonary dysplasia in evolution: implications for antioxidant therapy. Antioxidants & redox signaling. 2004; 6:155–67. [PubMed: 14713347]
12. Cho HY, Reddy SP, Kleeberger SR. Nrf2 defends the lung from oxidative stress. Antioxidants & redox signaling. 2006; 8:76–87. [PubMed: 16487040]
13. Tian H, Zhang B, Di J, et al. Keap1: one stone kills three birds Nrf2, IKKβ and Bcl-2/Bcl-xL. Cancer Lett. 2012; 325:26–34. [PubMed: 22743616]
14. Cho HY, Kleeberger SR. Genetic mechanisms of susceptibility to oxidative lung injury in mice. Free radical biology & medicine. 2007; 42:433–45. [PubMed: 17275675]
15. Asikainen TM, Huang T-T, Taskinen E, et al. Increased sensitivity of homozygous Sod2 mutant mice to oxygen toxicity. Free Radic Biol Med. 2002; 32:175–86. [PubMed: 11796207]
16. Marzec JM, Christie JD, Reddy SP, et al. Functional polymorphisms in the transcription factor NRF2 in humans increase the risk of acute lung injury. Faseb J. 2007; 21:2237–46. [PubMed: 17384144]
17. Forsberg L, de Faire U, Morgenstern R. Oxidative stress, human genetic variation, and disease. Arch Biochem Biophys. 2001; 389:84–93. [PubMed: 11370676]
18. Bentley AR, Emrani P, Cassano PA. Genetic variation and gene expression in antioxidant related enzymes and risk of COPD: a systematic review. Thorax. 2008; 63:956–61. [PubMed: 18566111]
19. Okano Y, Nezu U, Enokida Y, et al. SNP (−617C>A) in ARE-like loci of the NRF2 gene: a new biomarker for prognosis of lung adenocarcinoma in Japanese non-smoking women. PLoS ONE. 2013; 8:e73794. [PubMed: 24040073]
20. Pietrzyk JJ, Kwinta P, Wollen EJ, et al. Gene expression profiling in preterm infants: new aspects of bronchopulmonary dysplasia development. PLoS ONE. 2013; 8:e78585. [PubMed: 24194948]
21. Siegel D, Anwar A, Winski SL, Kepa JK, Zolman KL, Ross D. Rapid polyubiquitination and proteasomal degradation of a mutant form of NAD(P)H:quinone oxidoreductase 1. Mol Pharmacol. 2001; 59:263–8. [PubMed: 11160862]

22. Fagerholm R, Hofstetter B, Tommiska J, et al. NAD(P)H:quinone oxidoreductase 1 NQO1*2 genotype (P187S) is a strong prognostic and predictive factor in breast cancer. Nat Genet. 2008; 40:844–53. [PubMed: 18511948]
23. Zai CC, Tiwari AK, Basile V, et al. Oxidative stress in tardive dyskinesia: Genetic association study and meta-analysis of NADPH quinine oxidoreductase 1 (NQO1) and Superoxide dismutase 2 (SOD2, MnSOD) genes. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 2010; 34:50–6. [PubMed: 19778569]
24. Lajin B, Alachkar A. The NQO1 polymorphism C609T (Pro187Ser) and cancer susceptibility: a comprehensive meta-analysis. Br J Cancer. 2013; 109:1325–37. [PubMed: 23860519]
25. Kiyohara C, Yoshimasu K, Takayama K, Nakanishi Y. NQO1, MPO, and the risk of lung cancer: a HuGE review. Genet Med. 2005; 7:463–78. [PubMed: 16170238]
26. Reddy P, Naidoo RN, Robins TG, et al. GSTM1, GSTP1, and NQO1 polymorphisms and susceptibility to atopy and airway hyperresponsiveness among South African schoolchildren. Lung. 2010; 188:409–14. [PubMed: 20526719]
27. Potts-Kant EN, Li Z, Tighe RM, et al. NAD(P)H:quinone oxidoreductase 1 protects lungs from oxidant-induced emphysema in mice. Free Radic Biol Med. 2012; 52:705–15. [PubMed: 22198263]
28. Suzuki T, Shibata T, Takaya K, et al. Regulatory nexus of synthesis and degradation deciphers cellular Nrf2 expression levels. Mol Cell Biol. 2013; 33:2402–12. [PubMed: 23572560]
29. Córdova EJ, Jiménez-Morales S, Centeno F, et al. NFE2L2 gene variants and susceptibility to childhood-onset asthma. Rev Invest Clin. 2011; 63:407–11. [PubMed: 22364041]
30. Sandford AJ, Malhotra D, Boezen HM, et al. NFE2L2 pathway polymorphisms and lung function decline in chronic obstructive pulmonary disease. Physiol Genomics. 2012; 44:754–63. [PubMed: 22693272]
31. Sutton A, Imbert A, Igoudjil A, et al. The manganese superoxide dismutase Ala16Val dimorphism modulates both mitochondrial import and mRNA stability. Pharmacogenet Genomics. 2005; 15:311–9. [PubMed: 15864132]
32. Giusti B, Vestrini A, Poggi C, et al. Genetic polymorphisms of antioxidant enzymes as risk factors for oxidative stress-associated complications in preterm infants. Free Radic Res. 2012; 46:1130–9. [PubMed: 22574884]
33. Zimniak P, Nanduri B, Pikuła S, et al. Naturally occurring human glutathione S-transferase GSTP1-1 isoforms with isoleucine and valine in position 104 differ in enzymic properties. Eur J Biochem. 1994; 224:893–9. [PubMed: 7925413]
34. Koide S, Kugiyama K, Sugiyama S, et al. Association of polymorphism in glutamate-cysteine ligase catalytic subunit gene with coronary vasomotor dysfunction and myocardial infarction. J Am Coll Cardiol. 2003; 41:539–45. [PubMed: 12598062]
35. Tang W, Bentley AR, Kritchevsky SB, et al. Genetic variation in antioxidant enzymes, cigarette smoking, and longitudinal change in lung function. Free Radic Biol Med. 2013; 63:304–12. [PubMed: 23688726]
36. Siedlinski M, Postma DS, van Diemen CC, Blokstra A, Smit HA, Boezen HM. Lung function loss, smoking, vitamin C intake, and polymorphisms of the glutamate-cysteine ligase genes. Am J Respir Crit Care Med. 2008; 178:13–9. [PubMed: 18420959]
37. Choi AM, Alam J. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. Am J Respir Cell Mol Biol. 1996; 15:9–19. [PubMed: 8679227]
38. Yamada N, Yamaya M, Okinaga S, et al. Microsatellite polymorphism in the heme oxygenase-1 gene promoter is associated with susceptibility to emphysema. Am J Hum Genet. 2000; 66:187–95. [PubMed: 10631150]
39. Tanaka G, Aminuddin F, Akhabir L, et al. Effect of heme oxygenase-1 polymorphisms on lung function and gene expression. BMC Med Genet. 2011; 12:117. [PubMed: 21902835]
40. Walsh M, Engle W, Laptook A, et al. Oxygen delivery through nasal cannulae to preterm infants: can practice be improved? Pediatrics. 2005; 116:857–61. [PubMed: 16199694]

## Table 1

Distribution of clinical and epidemiological risk-factors for BPD in our cohort:

| Variable | Infants without BPD (n=375) | Infants with BPD (n=284) | Infants with severe BPD (n=135) |
|---|---|---|---|
| Gestational age (wk) | 29 (28; 30) | 26 (25; 28)* | 26 (24; 27)* |
| Birth-weight (grams) | 1190 (678; 1352) | 876 (675; 1107)§ | 760 (635; 991)§ |
| Race - Caucasians | 68% | 77.8%† | 75.5% |
| African American | 21.3% | 10.2% | 11.9% |
| Others | 10.7% | 12.0% | 12.6% |
| Prenatal steroid use | 85% | 91.1% | 91% |
| Male sex | 48.8% | 57.8%§§ | 63.0%§§ |
| Clinical chorioamnionitis | 7.5% | 11.7% | 10.3% |
| Patent ductus arteriosus | 22.6% | 58.3%** | 65.9%** |

Data is represented as median ± interquartile range or as raw numbers with percentages.

*P<0.001 (no BPD *vs*. BPD; severe BPD *vs*. others),

§P=0.001 (no BPD *vs*. BPD; severe BPD *vs*. others),

†P=0.006 (% CAU infants, no BPD *vs*. BPD),

§§P<0.02 (no BPD *vs*. BPD; severe BPD *vs*. others),

**P<0.001 (no BPD *vs*. BPD; severe BPD *vs*. others).

Clinical chorioamnionitis was diagnosed in the presence of maternal fever >38°C plus one additional criteria (uterine tenderness, malodorous vaginal discharge, maternal leukocytes >15,000 cells/$mm^3$ or fetal heart-rate of >160/min)

## Table 2

Distribution of ARE genetic variants by BPD outcomes in our cohort:

| Variant rs number | No BPD (n=375) genotype frequency (%) | BPD (n=284) genotype frequency (%) | Severe BPD (n=135) genotype frequency (%) |
|---|---|---|---|
| GSTP1 rs1695 | AA - 148 (39.6)<br>AG - 171 (45.7)<br>GG - 55 (14.7) | AA - 118 (41.8)<br>AG - 125 (44.3)<br>GG - 39 (13.9) | AA - 53 (39.3)<br>AG - 64 (47.4)<br>GG - 18 (13.3) |
| SOD2 rs4880 | TT - 95 (25.5)<br>CT - 183 (49)<br>CC - 95 (25.5) | TT - 67 (23.8)<br>CT - 149 (52.8)<br>CC - 66 (23.4) | TT - 31 (23.3)<br>CT - 73 (54.9)<br>CC - 29 (21.8) |
| NQO1 rs1800566 | CC - 222 (59.4)<br>CT - 138 (36.9)<br>TT - 14 (3.7) | CC - 173 (61.3)<br>CT - 88 (31.2)<br>TT - 21 (7.5)* | CC - 83 (61.9)<br>CT - 41 (30.6)<br>TT - 10 (7.5) |
| NFE2L2 rs6721961 | CC - 289 (77.3)<br>CA - 84 (22.5)<br>AA - 1 (0.2) | CC - 226 (80.4)<br>CA - 51 (18.2)<br>AA - 4 (1.4) | CC - 116 (86.6) †<br>CA - 16 (11.9)<br>AA - 2 (1.5) |
| GCLC rs17883901 | CC - 315 (84.2)<br>CT - 57 (15.2)<br>TT - 2 (0.6) | CC - 240 (85.1)<br>CT - 39 (13.8)<br>TT - 3 (1.1) | CC - 114 (85.7)<br>CT - 19 (14.3)<br>TT - 0 |
| HMOX1 rs2071747 | GG - 344 (92.5)<br>GC - 27 (7.3)<br>CC - 1 (0.2) | GG - 254 (90)<br>GC - 26 (9.2)<br>CC - 2 (0.8) | GG - 120 (89.6)<br>GC - 13 (9.7)<br>CC - 1(0.7) |

Genotype frequencies of study subjects stratified by BPD outcome are presented. rs number; reference SNP accession ID number.

*P=0.05 (BPD *vs.* infants without BPD; recessive model);

†P=0.01 (severe BPD *vs*. infants without severe BPD; dominant model).

Genetic recessive model; two copies of the SNP is required to confer disease risk, genetic dominance model; a single copy of the SNP can confer disease risk.

## Table 3

Logistic regression models for BPD (3a) and severe BPD (3b) in our cohort:

| Variable | Odds ratio | 95% CI | P - value |
|---|---|---|---|
| **BPD** | | | |
| GA ≤ 26wk | 3.4 | 2.1 – 5.7 | <.0001 |
| Birth weight ≤ 800g | 2.5 | 1.4 – 4.2 | 0.001 |
| Male | 1.5 | 1.04 – 2.2 | 0.03 |
| *NFE2L2* CA or AA vs. CC | 0.6 | 0.4 – 0.9 | 0.023 |
| AA vs. CAU | 0.3 | 0.2 – 0.5 | <.0001 |
| *NQO1* TT vs. CC or CT | 3.0 | 1.4 – 6.8 | 0.007 |
| PDA | 3.1 | 2.1 – 4.5 | <.0001 |
| **Severe BPD** | | | |
| GA ≤ 26wk | 3.8 | 2.1 – 6.7 | <.0001 |
| Birth weight ≤ 800g | 2.5 | 1.4 – 4.5 | 0.002 |
| Male | 2.0 | 1.3 – 3.1 | 0.003 |
| *NFE2L2* CA or AA vs. CC | 0.3 | 0.2 – 0.6 | 0.0004 |
| AA vs. CAU | 0.5 | 0.3 – 0.9 | 0.02 |
| *NQO1* TT vs. CC or CT | 2.5 | 1.01 – 6.0 | 0.049 |
| PDA | 2.3 | 1.5 – 3.7 | 0.0003 |

Epidemiological variables available at birth, ARE variants, and postnatal variable (PDA) were investigated with logistic regression to model BPD and severe BPD risk (*see full description in methods section*). The final model representing significant factors ($p<0.05$) associated with BPD and severe BPD are depicted.

**Table 4**

Distribution of *NFE2L2* (rs6721961) and *NQO1* (rs1800566) SNPs categorized by BPD outcomes in Caucasians:

| Variant rs number | No BPD (n=255) genotype frequency (%) | All BPD (n=220) genotype frequency (%) | Severe BPD (n=101) genotype frequency (%) |
|---|---|---|---|
| NFE2L2 rs6721961 | CC - 186 (74.9)<br>CA - 69 (25.1)<br>AA - 0 | CC - 176 (80.0)†<br>CA - 40 (18.2)<br>AA - 4 (1.8) | CC - 88 (87.1) ‡<br>CA - 11 (10.9)<br>AA - 2 (2) |
| NQO1 rs1800566 | CC - 154 (60.4)<br>CT - 94 (36.9)<br>TT - 7 (2.7) | CC - 131 (59.3)<br>CT - 73 (33.0)<br>TT - 17 (7.7) * | CC - 63 (62.4)<br>CT - 30 (30.6)<br>TT - 8 (7.5) |

† P=0.07 (BPD *vs*. infants without BPD; dominant model);

‡ P=0.004 (severe BPD *vs*. infants without severe BPD; dominant model);

* P=0.014 (BPD *vs*. infants without BPD; recessive model).

## Table 5

Logistic regression models for BPD (5a) and severe BPD (5b) among Caucasian infants:

| Variable | Odds ratio | 95% CI | P - value |
|---|---|---|---|
| **BPD** | | | |
| GA ≤ 26wk | 3.2 | 1.8 – 5.7 | <.0001 |
| Birth weight ≤ 800g | 2.7 | 1.4 – 5.2 | 0.002 |
| Male gender | 1.8 | 1.2 – 2.7 | 0.009 |
| *NQO1* TT vs. CC or CT | 4.2 | 1.5 – 11.1 | 0.006 |
| *NFE2L2* CA or AA vs. CC | 0.48 | 0.3 – 0.8 | 0.005 |
| PDA | 2.9 | 1.9 – 4.6 | <.0001 |
| **Severe BPD** | | | |
| Gestational age ≤ 26wk | 3.6 | 1.9 – 6.7 | <.0001 |
| Birth weight ≤ 800g | 2.3 | 1.2 – 4.5 | 0.01 |
| Male gender | 1.9 | 1.1 – 3.2 | 0.02 |
| *NFE2L2* CA or AA vs. CC | 0.3 | 0.1 – 0.6 | 0.0003 |
| PDA | 2.6 | 1.5 – 4.4 | 0.0004 |

Epidemiological variables available at birth, ARE variants and PDA were investigated with logistic regression to model BPD and severe BPD risk. Risk-factors that remained ($p<0.05$) associated with BPD and severe BPD are depicted.