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Oxidant stress regulatory genetic variation in recipients and donors contributes to risk of primary graft dysfunction after lung transplantation

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Abstract

Objective—Oxidant stress pathway activation during ischemia reperfusion injury may contribute to the development of primary graft dysfunction (PGD) after lung transplantation. We hypothesized oxidant stress gene variation in recipients and donors is associated with PGD.

Methods—Donors and recipients from the Lung Transplant Outcomes Group (LTOG) cohort were genotyped using the Illumina IBC chip filtered for oxidant stress pathway genes. Single nucleotide polymorphisms (SNPs) grouped into SNP-sets based on haplotype blocks within 49 oxidant stress genes selected from gene ontology pathways and literature review were tested for

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Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

EC and RJS participated in study design and coordination and drafted the manuscript. WL and ZJD performed the statistical analysis. JMD, YS, JHE, CFB, GAA and NJM helped to draft the manuscript. JDC, RA, and RF conceived of the study, and participated in its design and coordination and helped to draft the manuscript. MR performed laboratory analyses and aided in study design. All authors read and approved the final manuscript.

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PGD association using a sequencing kernel association test. Analyses were adjusted for clinical confounding variables and population stratification.

Results—392 donors and 1038 recipients met genetic quality control standards. 30% of subjects developed grade 3 PGD within 72 hours. Donor NADPH Oxidase 3 (*NOX3*) was associated with PGD (p=0.01) with 5 individual significant loci (p-values between 0.006 and 0.03). In recipients, variation in glutathione peroxidase (*GPX1*) and NRF-2 (*NFE2L2*) was significantly associated with PGD (p=0.01 for both). The *GPX1* association included 3 individual loci (p-values between 0.006 and 0.049) and the *NFE2L2* association included 2 loci (p=0.03 and 0.05). Significant epistatic effects influencing PGD susceptibility were evident between three different donor blocks of *NOX3* and recipient *NFE2L2* (p=0.026, p=0.017 and p=0.031).

Conclusions—Our study prioritizes *GPX1*, *NOX3*, and *NFE2L2* genes for future research in PGD pathogenesis, and highlights a donor-recipient interaction of *NOX3* and *NFE2L2* that increases PGD risk.

Keywords

Primary Graft Dysfunction; Lung Transplantation; Oxidant Stress; Genes

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Primary graft dysfunction (PGD) is a form of acute lung injury that develops within 72 hours of lung transplantation. It is defined by the presence of hypoxemia and radiographic infiltrates in the allograft, [1] and is the major cause of death in the early post-transplant period. [2] PGD affects 10–30% of all subjects receiving lung transplantation, [3] and is associated with an increased risk of bronchiolitis obliterans syndrome, prolonged hospitalization, and increased short and long-term mortality. [1, 3, 4, 5]

Although the pathophysiology of PGD is incompletely understood, ischemia reperfusion injury leading to activation of the oxidant stress pathway is thought to be a major contributor to its development. [6] In support of this notion, Williams, et al identified antioxidant system dysfunction prior to lung transplantation that persisted for up to a year after transplantation. [7] Furthermore, the antioxidant N-acetylcysteine was protective against graft dysfunction in a small randomized trial. [8] Additionally, targeted delivery of catalase, an anti-oxidant enzyme, reduced ischemia reperfusion injury in an experimental model of lung transplantation. [9] These and other studies provide evidence that oxidative stress is an important mediator of lung allograft dysfunction after transplantation.

The oxidant stress pathway is rich in genetic variation, thought due to evolutionary pressures from infectious and environmental stimuli. Prior studies have implicated genetic variation in oxidant stress genes altering acute lung injury (ALI) risk. [10–12] In this study, we sought to identify donor and recipient variation in oxidative stress genes associated with PGD using a large multicenter cohort study and a candidate gene platform. This study has previously been registered as clinical trials.gov # NCT00457847.

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METHODS

Study Population and Data Collected

Subjects were selected from the Lung Transplant Outcomes Group (LTOG), which is a multi-center, prospective cohort study of lung transplant recipients that has been previously described. [13–17] University of Pennsylvania institutional review board (Federal-wide Assurance # 00004028) approval and informed written consent was obtained prior to recruitment of subjects. Consecutive subjects transplanted between January 1, 2002 and December 31, 2009 from 10 LTOG centers were enrolled. Clinical data were collected prospectively as described elsewhere. [18, 19] PGD grade was determined using the consensus definition of the International Society of Heart and Lung Transplantation using two blinded readers as previously described. [20, 21] We used any Grade 3 PGD occurring within the first 72 hours following lung transplantation as our primary PGD definition (noted as "PGD" henceforth).[21] Potential confounding variables including both recipients' and donors' characteristics were tested for their association with PGD. Recipient age, predisposing diagnosis, and use of cardiopulmonary bypass were significant at the 0.05 level and thus were included in the genetic models.[18]

Collection and Processing of Biological Samples

Whole blood collected prior to organ procurement (donor) or organ implantation (recipient) was centrifuged, and buffy coat fractions were aliquoted and stored at −80°C until DNA extraction using the Qiagen Qiamp 96 blood kit (Qiagen, Valencia, CA). Negative controls were included with all DNA extraction runs. Extracted DNA from PGD and non-PGD subjects were plated together on each 96-well microplate, and lab personnel were unaware of the PGD status of each sample at the time of laboratory analysis.

Genotyping and Quality Control

We used the HumanCVD BeadChip (IBC chip), a custom 50K single nucleotide polymorphism (SNP) genotyping array designed to assay SNPs in candidate genes and pathways affecting cardiovascular, pulmonary, inflammatory, and metabolic phenotypes (llumina, Inc.®, San Diego, CA). [22] The array was designed to evaluate all non-synonymous coding SNPs with minor allele frequencies (MAF) > 0.01, as well as provide coverage for a number of loci with MAF > 0.02 of potential importance to cardiac, pulmonary, and metabolic phenotypes. [22] Quality control thresholds for each SNP to be included in the analysis included genotyping call 95%; chi-square testing of Hardy- Weinberg equilibrium (HWE) on the whole population yielding a p value 10^{-6}; and MAF 0.01 overall. We sought to apply a computational methodology (SNPset analysis) which incorporated oxidant stress as a biologically plausible mediator of PGD. To accomplish this, we filtered the IBC BeadChip for 49 oxidant stress genes identified through PubMed search conducted May 2011 using the search terms "oxidant stress", "polymorphism", "genetic" limited to human and manually curated search results. Identified genes were further expanded using gene ontology pathways (See online supplement for list of genes, eTable 1).

Following genotyping, outliers were detected and removed using a genome-wide similarity metric. [23] Population stratification was determined using multidimensional scaling (MDS)

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analysis using all markers [23] and the resulting components were used in adjustment for population stratification.

SNPset Analysis

SNPs were grouped into sets based on haplotype blocks within 49 genes identified to be in the oxidant stress pathway. The haplotype blocks were initially determined using the confidence interval method [24] implemented in Haploview and small blocks were modified to include at least three common SNPs.

We used a logistic kernel machine method, sequencing kernel association test (SKAT), to test the joint effect of SNP-sets on PGD. The method has previously been shown to have improved power to detect association by reducing the total number of tests being performed, jointly testing the multiple SNPs surrounding causal variants, and incorporating nonlinear and epistatic effects. [25] Detailed methods of SNPset analyses are presented in the supplemental material. We first tested the associations between PGD and each recipient's SNPset and then between PGD and each donor's SNPset, adjusting for recipient age, recipient diagnosis, use of cardiopulmonary bypass, and population stratification.[26]

After donor and recipient genes with significant association were identified, we used the quadratic terms to test the interactions between significant matched donor and recipient SNPsets using SKAT. [27] For each interaction test of donor SNP set 1 and recipient SNP set 2, all pair-wise cross-products of SNPs within set 1 and SNPs within set 2 were included in the function h of the model (Equation 1 in Supplement). We fitted a multivariate logistic model with coded SNPs as an approximation to the nonparametric function and the top SNPs that were most associated with PGD were chosen as covariates to be adjusted in the model for testing nonlinear effect of gene-set interactions. Due to the multiple comparison adjustment inherent in SNPset analysis, a p-value of 0.05 was considered significant for all SNPset comparisons.

RESULTS

There were 1038 lung transplant recipients and 392 lung donors who met genetic quality control standards (Figure 1) with 338 representing a donor recipient pair. 30% (95% confidence interval: 27.5, 33.0) of subjects (314 out of 1038) developed grade 3 PGD within 72 hours. Baseline demographics for study subjects are summarized in Table 1. Recipient race, pre-transplant diagnosis and cardiopulmonary bypass use differed by PGD. These differences have previously been described as associated with PGD.[3]

Table 2 presents the results of the main effect SNPset associations. Four recipient genes were associated with elevated PGD risk including, glutathione peroxidase (*GPX1*), an enzyme important in detoxification of hydrogen peroxide, (p=0.01); and nuclear factor (erythroid-derived 2)-like 2 (*NFE2L2)*, encoding the NRF-2 transcription factor that up-regulates expression of several phase I and phase II reactive oxygen species (ROS) detoxifying proteins (block 1 p=0.024 and block 2 p=0.005). There was also borderline significance for nitric oxide synthase 3 (*NOS3*), responsible for production of nitric oxide (p=0.028); and glutathione S-transferase mu 2 enzyme (*GSTM2*) which regulates glutathione

production (p=0.048). Individual recipient SNPs that differed most between PGD and non PGD included 3 SNPs within *GPX1* and 2 within *NFE2L2*. Within *GPX1* PGD associations included two SNPs in tight linkage disequilibrium (LD), rs3811699 (p=0.006) and rs1800668 (p=0.012), located in the 5′ promoter region and first exon, which have previously been described to have functional consequences in transcription, [28] and rs9818758 (p=0.046) located in the intergenic region near *GPX1*, has been associated with inflammatory bowel disease. [29] Within *NFE2L2*, there were 2 SNPs located in the first intron that were significantly associated with PGD; rs6726395 (p=0.029) and rs1806649 (p=0.049). These SNPs were in moderate LD (R^2=0.35; D′=0.93) with SNPs previously reported to have function and ALI association by our group (eFigure 1). [10]

In donors, *NOX3* (p=0.01), nitric oxide synthase 1 adaptor protein (*NOS1AP,* p=0.048) and paraoxonase-1 (*PON1*, p=0.03) were associated with the development of PGD. Within *NOX3*, rs3749930, a coding non-synonymous SNP had the strongest association with PGD (p=0.006). The SNP marks a G/T nucleotide conversion resulting in a threonine to lysine substitution at position 171 in a transmembrane portion of the NOX-3 protein. The variant T allele resulting in a lysine was associated with a decreased risk of PGD compared with the ancestral G allele. Additionally, several intronic SNPs within *NOX3* associated with increased PGD risk.

Sensitivity analyses of individual donor and recipient SNPs with respect to cardiopulmonary bypass and IPF demonstrated only small effects on point estimates. Only one of the 10 variants (rs231948) demonstrated a change in the directionality of the risk (eTable 3). Taken together, these results suggest the effects of the individual SNPs are important and not the result of known unbalanced clinical risk factors.

Individual SNP interactions between the 338 matched donor/recipient pairs are presented in Figure 2. Within recipient *NFE2L2*, blocks 1 and 2 (5 kb and 34 kb in length) are separated by 10 kb with one SNP in the intervening region not in LD with either block. Donor *NOX3,* was divided into 5 blocks. Block 1 was the largest of the blocks (21 kb) containing 14 SNPs with a 4 kb intervening region with 7 SNPs upstream of block 2. Table 2 displays the significant interactions that were detected between donor and recipient genes. As displayed in Figure 2, donor *NOX3* block 3 and block 5 were demonstrated to have significant epistatic effects with recipient *NFE2L2* blocks 1 and 1 and 2 in their association with development of PGD (p=0.026, p=0.017 and p=0.031, respectively).

CONCLUSIONS

PGD is a complex trait that has been associated with both inherent donor and recipient clinical factors.[3] In this study, we have defined both the main effects and the interactions in key oxidant stress genes from lung transplant donors and recipients that increase post-transplant PGD risk, using SNPset analysis. Like most complex traits, PGD most likely involves many SNPs with modest effects; therefore this technique is particularly well suited. [30] This study is the first to employ these techniques in a lung transplant cohort, and our results identify that *GPX1, NFE2L2, NOS3, GSTM2, NOX3, NOS1AP* and *PON1* should be prioritized for future research efforts. Most importantly, the demonstration of donor-

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recipient interaction between putatively functional SNPs in genes encoding NOX3 and NRF2 (*NFE2L2*) should focus future research efforts on these mediators.

NFE2L2 encodes NRF-2, a zinc finger transcription factor responsible for the up-regulation of several key reactive oxygen detoxification enzymes. [31] A hypofunctioning promoter variant in NF2EL2 (rs6721961) has previously been show by our group to have an association with increased risk for acute lung injury following major trauma. [10] This variant and others previously described by our group are not contained within the IBC chip used in our current study. However, the individual SNP's within *NFE2L2* described in our current study (rs6726395 and rs1806649) are significantly associated with PGD in lung transplant recipients and are in moderate LD with the previously described promoter SNP not in the IBC chip (rs6721961, D′ =1; R^2= 0.118 and D′ =1; R^2= 0.052, respectively; eFigure 2b). Variation in this gene in lung transplant recipients may increase susceptibility to PGD by reducing transcription of ROS detoxifying enzymes, and a resultant dampened oxidant stress response.

Interactions between recipient *NFE2L2* and donor *NOX3* were also associated with PGD risk. However, the functional consequences of the Thr171Lys substitution (rs3749930) in *NOX3* is unknown and the function of the *NOX3* intronic variants (rs231956, rs13207865, rs23948, and rs1546894) have thus far not been described. Because any hypothesized mechanism behind the interaction of recipient *NFE2L2* and donor *NOX3* on PGD susceptibility is speculative at this time, fine mapping of variants and/or cellular functional studies aimed at clarifying this finding are logical next research steps. Our study is useful to prioritize these two genes for further research into their function of increased PGD risk.

GPX1 is an intracellular anti-oxidant enzyme that reduces harmful hydrogen peroxide to water. [32] Genetic polymorphisms in *GPX1* have been demonstrated to affect transcription and alter response to vascular oxidant stress and risk of cardiovascular disease in humans.[28] In our study, three *GPX1* SNPs (rs1800668, rs9818758, rs3811699) were significantly associated with PGD. Two of the three SNPs (rs1800668, rs3811699) are in tight LD (D′=1, R^2=0.88, eFigure 2) and have previously been shown to enhance oxidant injury in cell culture through decreased expression of *GPX1* in the setting of oxidant stress. [28] Our results demonstrate protective effects associated with these three SNP (rs1800668, rs9818758, rs3811699) minor alleles, indicating that either lower GPX1 expression is protective, or that the putative functional inference from prior studies does not apply to pulmonary ischemia reperfusion injury. Therefore, future study is warranted in suitable models that apply to PGD.

There are several limitations of this study. First, although several of our implicated variants have suggested function in regulating transcription from prior studies, it is not possible to establish functional causation with our study design. However, our results serve to prioritize these specific oxidant stress genes for future study. Second, gaps in our knowledge in oxidant stress gene inducers, regulators and modulators may have led us to exclude genes with important function in regulating oxidative stress. [30] We designed a strategy that was as inclusive as possible, relying both on existing gene ontology pathways as well a comprehensive literature search. Additionally, SNPset methods as we have employed them

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assume that SNPs within known genes associate with a complex trait, an approach that ignores potential trait-associated SNPs which are not located within genes.[30] Third, although we did not perform a simple adjustment for multiple comparisons in our post-analysis p-values (such as a Bonferroni adjustment), SNPset methods do not require post-analysis multiple comparison adjustment, as the method accounts for the number of comparisons. [25] Additionally, our methods did not employ multiple adjustments for donor-specific factors given the limited information available. Finally, our findings have not been replicated in an independent dataset. Unfortunately, there is no existing cohort to validate our multicenter findings available at this time. Work is underway by our group and others to standardize prospective sample collection in order to develop cohorts from which validation of identified genetic variants and pathways important in PGD can be tested. Given the many possible confounding factors inherent in lung transplantation and the modest effects of potential factors, only large multi-institutional cohort studies will possess enough statistical power to clarify effects of these identified factors).

In summary, we have demonstrated an association between genes regulating oxidative stress and PGD. The effects of these associations suggest importance of oxidant stress detoxifiers in lung recipients and ROS generators in lung donors. Effects of their interaction suggests a complex mechanism of injury of which oxidant stress is a component and therefore should stimulate further inquiry of *GPX1*, *NFE2L2*, *NOX3*, *PON1* and *GSTM2* participation in PGD pathogenesis. Additionally, if validated, these gene variants may allow improved risk stratification of transplant recipients for therapies targeted at oxidant balance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PGD	primary graft dysfunction
ALI	acute lung injury
LTOG	Lung Transplant Outcomes Group
SNP	single nucleotide polymorphism
MAF	minor allele frequencies
HWE	Hardy- Weinberg equilibrium
MDS	multidimensional scaling
SKAT	sequencing kernel association test

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IPF	idiopathic pulmonary fibrosis
COPD	chronic obstructive pulmonary disease
NOX3	NADPH oxidase-3 gene
GPX1	glutathione peroxidase-1 gene
NFE2L2	nuclear factor (erythroid-derived 2)-like 2 gene
GSTM2	glutathione S-transferase mu 2 gene
ROS	reactive oxygen species
NOS3	nitric oxide synthase 3 gene
LD	linkage disequilibrium
NOS1AP	nitric oxide synthase 1 adaptor protein gene
PON1	paraoxonase-1 gene

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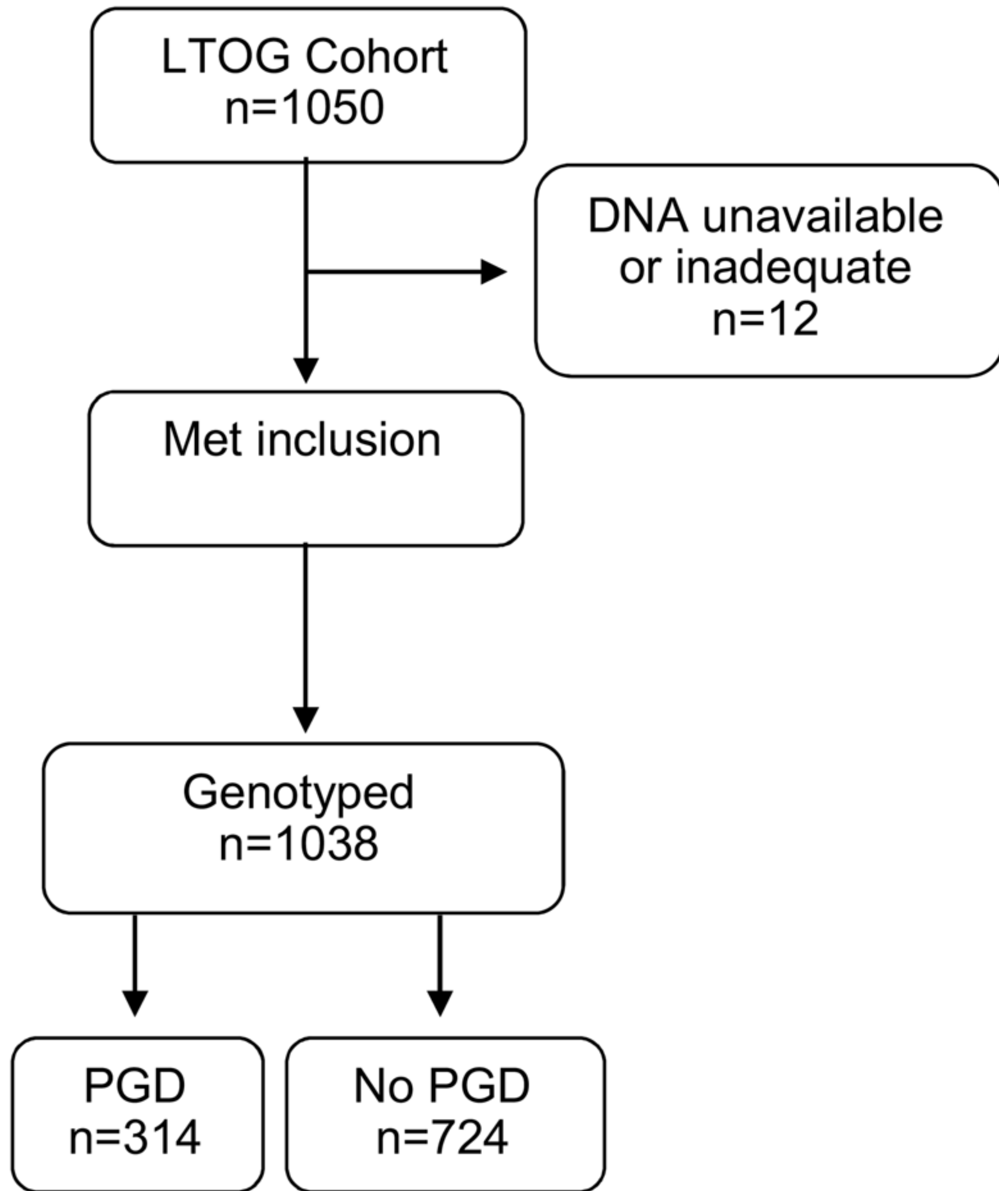

FIGURE 1.
Enrollment Design

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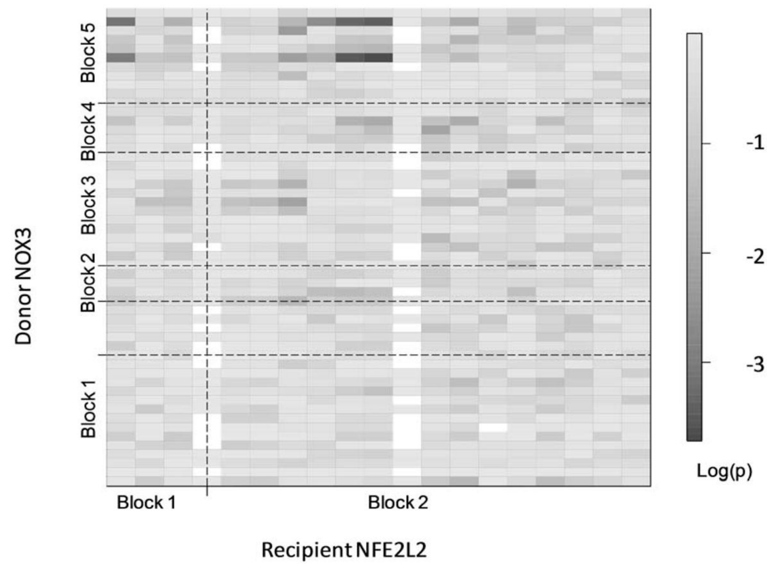

FIGURE 2.

SNPset Block interaction

Individual SNPs are represented by a rectangle. Blocks are delineated by dotted lines. The highest intensities are evident in donor *NOX3* block 3 with recipient *NFE2L2* block 1 and in donor *NOX3* block 3 with recipient *NFE2L2* blocks 1 and 2. SNP, single nucleotide polymorphism.

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TABLE 1

Donor and Recipient Characteristics[a]

	No PGD (n=724)	PGD (n=314)	p-value
Donor Age	34.48±14.31	34.64±13.97	0.872
Female Donor	277 (38%)	136 (43%)	0.128
Donor Race			0.965
Caucasian	457 (63%)	205 (65%)	
African American	145 (20%)	59 (19%)	
Hispanic	85 (12%)	34 (11%)	
Other	30 (4%)	13 (4%)	
Recipient Age	54.02±12.91	53.09±12.57	0.277
Female Recipient	322 (44%)	143 (46%)	0.786
Recipient Race			0.001
Caucasian	632 (87%)	245(78%)	
African American	49 (7%)	46 (15%)	
Hispanic	24 (3%)	14 (4%)	
Other	18 (2%)	9 (3%)	
Diagnosis			<0.001
COPD	109 (15%)	26 (8%)	
IPF	282 (39%)	177 (56%)	
Other	333 (46%)	111 (35%)	
Bilateral Tx	73 (10.1%)	29 (9.2%)	0.758
CPB use	221 (31%)	174 (55%)	<0.001

[a]Comparison of individual cohorts with and without donor/recipient pairing is available in the supplemental materials. COPD, chronic obstructive pulmonary disease; CPB, cardiopulmonary bypass; IPF, idiopathic pulmonary fibrosis; PGD, primary graft dysfunction.

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TABLE 2

Genes associated with PGD[a]

Cohort	Gene	P-value	No. SNPs
Recipients (n=1038)	*GPX1* block 1	0.014	8
	NFE2L2 block 1	0.024	3
	NFE2L2 block 2	0.005	13
	NOS3 block 1	0.028	8
	GSTM2 block 1	0.048	6
Donors (n=392)	*NOX3* block 2	0.01	3
	NOX3 block 3	0.05	12
	NOS1AP block 4	0.048	11
	PON1 block 4	0.035	13
Interaction (n=338)	Donor *NOX3* block 3 with recipient		
	NFE2L2 block 1	0.026	
	Donor *NOX3* block 5 with recipient		
	NFE2L2 block 1	0.017	
	Donor *NOX3* block 5 with recipient		
	NFE2L2 block 2	0.031	

[a] statistical tests of significance generated using the quadratic kernel function.

PGD, primary graft dysfunction; SNP, single nucleotide polymorphism.

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TABLE 3

Individual SNPs associated with PGD

Cohort	SNP	MA	MAF_A	OR	95% CI	P-value	Gene	Location
Recipient	rs9818758	A	0.13	0.74	0.54, 0.99	0.048	*GPX1*	Intergenic
	rs1800668	T	0.25	0.74	0.58, 0.94	0.012	*GPX1*	UTR
	rs3811699	G	0.26	0.72	0.57, 0.91	0.006	*GPX1*	5′ upstream
	rs6726395	T	0.43	0.79	0.64, 0.98	0.029	*NFE2L2*	Intron
	rs1806649	A	0.18	0.76	0.58, 0.99	0.049	*NFE2L2*	Intron
Donor	rs3749930	T	0.06	0.38	0.20, 0.76	0.006	*NOX3*	Coding non-synonymous
	rs231956	C	0.53	1.49	1.05, 2.12	0.024	*NOX3*	Intron
	rs13207865	T	0.29	1.57	1.06, 2.33	0.024	*NOX3*	Intron
	rs231948	T	0.54	0.57	0.3, 1.0	0.037	*NOX3*	Intron
	rs1546894	A	0.02	0.31	0.11, 0.89	0.029	*NOX3*	Intron

A, adenine; C, cytosine; CI, confidence interval; G, guanine; MA, minor allele; MAF_A, minor allele frequency of those affected; OR, odds ratio; PGD, primary graft dysfunction; SNP, single nucleotide polymorphism; T, thymine; UTR, untranslated region.