and CLAD, especially the impact of antibiotic therapies for pathogenic taxa and antirejection therapies that might promote dysbiosis. CLAD phenotypes were not provided to allow consideration of airway versus parenchymal pathology, nor were the implications of single versus bilateral LTx explored exhaustively. Whether protective bacterial genera exist to assist in homeostasis and prevent dysbiosis is not elaborated but is a potential area of great interest in septic lung disease states and, possibly, in GERD (15). Notably, the analysis was developed using an "extreme phenotype" that excluded two-thirds of LTx recipients, so caution is warranted in interpretation. Future studies in the intermediate GERD group, to test the relationships identified here, might prove informative.

Notwithstanding these criticisms, the results provide powerful new information regarding the impact of GERD on the bacterial component of the pulmonary microbiome and demonstrate how new-generation technologies can advance our understanding of the many factors affecting the health of the lung allograft. On the basis of the aforementioned findings, perhaps the time has come to reconsider the paradigm of GERD management and focus on the pathogenic components of the pulmonary microbiome for which compelling evidence is accumulating regarding their role in inflammation, injury, and CLAD. ■

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Lung Allograft Dysfunction: Does Aspiration Hold the Key?

Despite advances in management, the long-term survival of lung transplant recipients remains poor compared with that of other solid organ transplant recipients. Chronic lung allograft dysfunction (CLAD), defined as a substantial decline in FEV_1 that persists over at least 3 months after transplantation, is the leading cause of death in the late posttransplant period (1). Although CLAD is a heterogeneous disorder, multiple studies have linked CLAD to various clinical factors, including the lung microbiome. CLAD has been associated with gastroesophageal reflux disease (GERD) (2, 3), lung inflammation (4), increased lung bacterial biomass (5), and changes in lung microbiome composition (5–8). Although mouse studies have shown that microaspiration of oral commensals provokes a normal protective lung immune response (9), it is unknown if this beneficial

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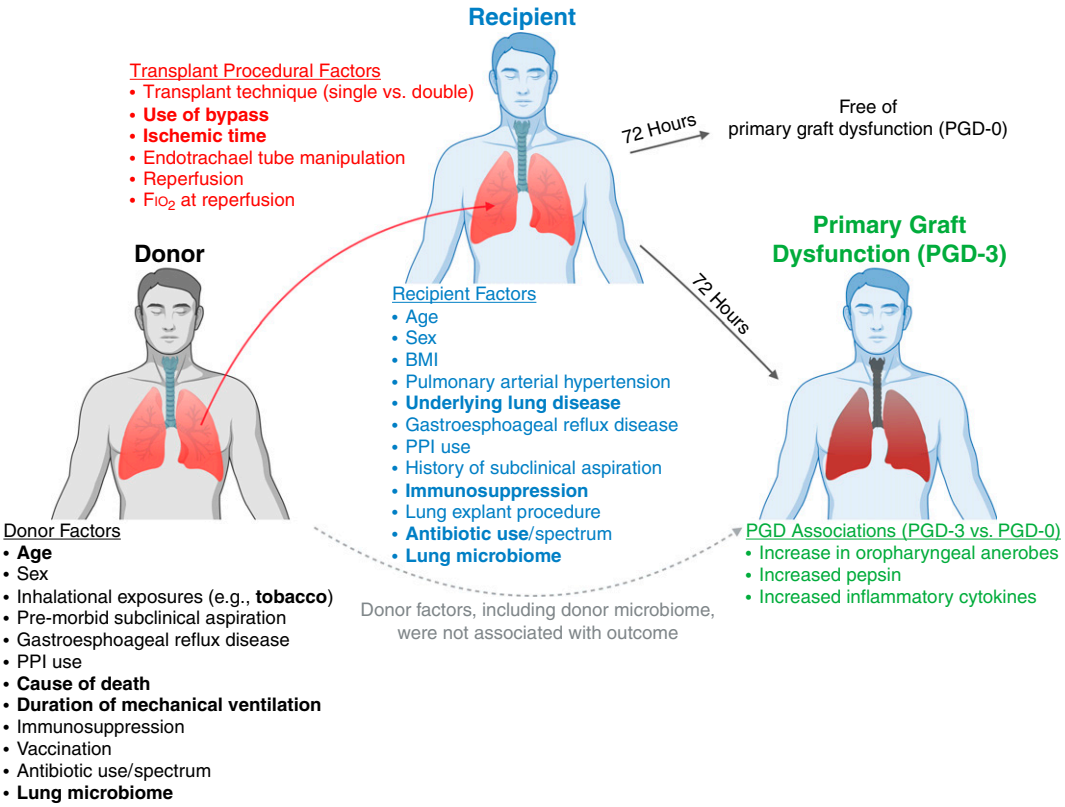

Figure 1. Primary graft dysfunction (PGD) may be associated with donor, recipient, and perioperative factors. The study by McGinniss and colleagues is represented schematically. Donor lung samples were obtained by bronchoscopy in the operating room immediately before organ procurement. Recipient allograft samples were obtained from the same organ by bronchoscopy immediately after implantation and reperfusion. Subjects were followed for 72 hours after transplantation, and PGD was assessed. Severe PGD (PGD-3) versus no PGD (PGD-0) was associated with recipient allograft microbiome composition changes (an increase in oropharyngeal anaerobes), increased pepsin (a marker of gastric aspiration), and an increase in inflammatory cytokines. PGD-3 was not associated with donor factors, including the donor lung microbiome. Clinical factors (donor, recipient, or perioperative) known (10) or hypothesized to mediate the relationship between the lung allograft microbiome and PGD are included here. Bolded clinical factors were assessed in the present study (11). Illustration by Shane W. Hodgson. BMI = body mass index; PPI = proton pump inhibitor.

response exists in the posttransplant setting of immunosuppression and antibiotic therapy.

In contrast to CLAD, we understand less about lung allograft dysfunction occurring early in the posttransplant period. Primary graft dysfunction (PGD), an acute lung injury occurring in the first few days after lung transplantation, is the leading cause of death during the early period after a lung transplant. In addition, it is associated with an increased risk of CLAD at later time points. PGD has been linked to donor, recipient, and perioperative clinical risk factors (10), but no previous studies on PGD and the lung microbiome have been reported.

In this issue of the *Journal*, McGinniss and colleagues (pp. 1508–1521) provide the first report on the lung microbiome in primary graft dysfunction (11). Their single-center prospective cohort study of the lung microbiome assesses donor lungs both immediately before procurement ("donor lung") and immediately after implantation and reperfusion ("recipient allograft"). At both time points, BAL specimens were collected for lung microbiome analyses. Their study enrolled 139 transplant recipients, obtaining donor lung samples from 109 subjects and recipient allograft samples from 136 subjects. After quality control filtering, paired donor lung and recipient allograft samples were available from 67 of the 139 subjects. Paired samples were assessed to discover lung microbiome changes associated with the transplant surgery. An "extreme phenotype analysis" was also employed to compare the 15 subjects who consistently exhibited severe PGD over the immediate 72-hour period after transplant with the 40 subjects who were consistently free of PGD over the same period.

The donor lung microbiome differed from the healthy lung microbiome, exhibiting decreased α diversity (defined as heterogeneity within each sample) and altered microbiome composition. Recipient allograft samples had higher α diversity, greater microbial biomass, and altered microbiome composition (enrichment with oropharyngeal taxa) compared with the donor lung microbiome. Despite the significant changes observed in recipient allograft samples compared with donor lung samples as a group, there was a strong correlation between paired donor lung and recipient allograft samples.

The extreme phenotype analysis of severe PGD versus no PGD did not detect any associations between the donor lung

microbiome (biomass, diversity, or composition) and PGD outcome. In contrast, recipient allograft samples from subjects who subsequently experienced severe PGD demonstrated altered microbiome composition when compared with the composition of subjects who subsequently experienced no PGD. The relationship between the recipient allograft microbiome and PGD was examined to determine if any relevant clinical factors mediated the PGD outcome. These included recipient factors such as primary pulmonary diagnosis, antibiotic exposure, and immunosuppression before transplantation; intraoperative factors such as ischemic time; and donor factors such as cause of death and smoking history. None reached significance, indicating that they did not mediate the relationship between recipient allograft microbiome and PGD outcome (Figure 1).

In addition, the authors assessed pepsin (a gastric enzyme) and 41 inflammatory markers in the available recipient allograft BAL samples. Pepsin concentrations were significantly higher in recipient allograft samples from subjects who developed severe PGD, suggesting that aspiration of gastric contents is associated with severe PGD. Inflammatory markers were broadly and consistently elevated in recipient allograft samples and were significantly different in those who experienced severe PGD versus those who did not develop PGD.

There is much to commend in this *Journal* article. Study data and samples were prospectively obtained from a large number of subjects over 3 years, a significant achievement at a time when other studies of the lung microbiome must rely on smaller sample sizes, retrospective analyses of biobank samples, and prolonged enrollment periods. The latter can be especially problematic as management after lung transplant has changed significantly over time and may confound the analyses. The inclusion of paired pre- and postsurgery samples also provides an important opportunity to assess the role of perioperative clinical factors in lung transplant PGD outcomes. This is a remarkable achievement for the research team because paired samples could only be acquired after the consent of both the donor surrogate and the transplant recipient. In addition, their findings implicating perioperative aspiration of gastric or oropharyngeal contents in PGD broadly support other findings implicating GERD in CLAD after transplant (2, 3).

Despite these strengths, several limitations remain. It is puzzling that the donor lung microbiome was closely associated with the recipient microbiome, and the recipient microbiome was associated with PGD outcome, but the donor lung microbiome was not associated with PGD outcome (Figure 1). It is possible that the study was simply underpowered to detect associations between donor factors and PGD outcomes. Likewise, only approximately half of the subjects had paired microbiome samples available after quality control filtering. This limits the generalizability of the findings, as these missing samples may not be missing at random.

Other potentially informative data were not available for analysis. Pepsin and inflammatory marker measurements were not available from the donor lung samples, limiting analyses of donor premorbid microaspiration, GERD, or lung inflammation in PGD. Furthermore, the authors were unable to include all potentially relevant clinical factors in their assessment of PGD associations (as described in Reference 10, the lung microbiome literature, and Figure 1). Antibiotic therapy, with or without anaerobic activity, given to either the donor or recipient, may have shaped the lung microbiome and/or PGD outcome (12). Recent work on the lung microbiome in humans and mice has shown that hyperoxia is associated with shifts in lung community composition as well as lung injury (13). In these experiments, lung injury could be modulated by microbiome composition and antibiotic administration. It is possible that hyperoxia and antibiotic administration, or other confounders, are contributing to PGD as well. Lastly, we must resist the temptation to equate correlation with causation in observational studies, even when the events of interest occur in close and apparent chronological order.

With this publication, GERD and/or microaspiration have now been implicated in acute postoperative lung allograft dysfunction as well as CLAD. Aspiration prevention may indeed hold the key to improved outcomes after a lung transplant. However, it would be premature to focus solely on aspiration at the expense of other considerations. ■

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Understanding Genotype–Phenotype Correlations in Patients with *TBX4* Mutations: New Views Inside and Outside the Box

Gene mutations associated with pulmonary arterial hypertension (PAH) have greatly advanced our understanding of the underlying etiologies of pulmonary vascular disease in adults and children (1, 2). In contemporary series, nearly 20% of sporadic and 70% of familial cases of PAH are found to have mutations in one of several identified PAH-associated genes (3). Mutations of *TBX4* (T-Box transcription factor 4 gene) on chromosome17q23.2 are associated with idiopathic and heritable PAH (4, 5) and small patellar syndrome (SPS) (6), underscoring the important role of developmental pathways in the pathogenesis of pulmonary vascular and lung parenchymal diseases. TBX4 is a DNA-binding protein that, with transcription factor TBX5, is critical for lung growth and branching during embryogenesis (7). Phenotypes associated with *TBX4* mutations include not only infantile, pediatric, and adult pulmonary hypertension but also acinar dysplasia and lethal neonatal lung disorders, as well as bronchial and parenchymal lung abnormalities (8–11).

In this issue of the *Journal*, Prapa and colleagues (pp. 1522–1533) provide important genotype–phenotype correlations for patients with *TBX4* mutations (12). Notably, their study combines detailed phenotypic clinical investigation with *in vitro* functional analysis of genetic variants to understand how loss or gain of protein function might affect the clinical manifestations of *TBX4* mutations. They identified 137 subjects with TBX4 variants from 22 published studies and analyzed phenotypic and demographic data. Twenty-one subjects had a primary diagnosis of SPS, and 116 subjects had lung disease, with 45% presenting in adulthood, 36% in childhood, and 19% in the perinatal period, a complete range of ages that adds value to this analysis.

Mutations were localized to protein domains, including the highly conserved T-Box domain containing the first nuclear localization segment (NLS1) as well as the predicted second nuclear localization segment (NLS2) at the C-terminus and the transactivating region. Remarkably, 108 distinct *TBX4* variants were identified among the 116 subjects, including 43 missense, 54 truncation (39 frameshift and 15 nonsense), 3 splice site mutations, 6 indels, and 1 *TBX4* promotor variant. A key feature of this study was the determination of functionality for many of the TBX4 mutations, using site-directed mutagenesis to create defined variants of TBX4, and gauging protein function by measuring binding to T-Box–binding motifs with a readout on the basis of a downstream luciferase reporter assay. All indels, and 23 of 42 missense variants, caused TBX4 loss of function (LoF), whereas 11 missense variants were found to be benign. Intriguingly, eight missense variants resulted in TBX4 protein gain of function (GoF). Of three splice site variants, two resulted in exon skipping expected to alter protein structure.

Analysis of mutation localization and functional assessment identified novel genotype–phenotype correlations. Mutations within the T-Box and nuclear localization domains were associated with younger age at diagnosis and a higher incidence of interstitial and developmental lung disease. GoF mutations were associated with later-age presentation compared with LoF. Secondary skeletal manifestations of small patellar syndrome were found with higher frequency in variants outside the T-Box and NLS2 domains and in those with protein-truncating versus missense variants. Among 89 subjects with follow-up, variants localized to the T-Box domain were associated with shorter event-free survival, though younger age at diagnosis also remained a significant adverse factor. The authors compared patients with TBX4-related PAH against those harboring mutations in another common genetic contributor to PAH, *BMPR2,* and those without known variants in PAH-associated genes. Compared with PAH patients with *BMPR2* or no identified variants, patients with *TBX4* variants had a younger age at presentation, better performance on the 6-minute-walk test, worse pulmonary function testing, higher frequency of airway abnormalities, and longer event-free survival.

The data show that not all *TBX4* mutations are the same. Mutations in specific domains are associated with different ages at presentation, the presence of interstitial lung disease, and survival. In particular, mutations in the T-Box domain are associated with earlier presentation, greater interstitial lung disease, and shorter event-free survival. Although most genetic mutations are linked to loss of function, the investigators found eight variants with GoF

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