

Gastrointestinal Reflux Related Aspiration (GRASP) and Airway Injury after Lung Transplantation: Insights into Pathophysiology and Role of Airway Biomarkers for Risk Stratification

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Introduction

Lung transplantation remains the only long term treatment for end-stage pulmonary disease. The main limitation to allograft survival beyond the first year post transplant is development of chronic lung allograft dysfunction (CLAD). Among survivors, CLAD will develop in virtually all recipients and often will show signs of airway injury before any clinical manifestations appear. There is currently no specific treatment available and management depends largely on modification of risk factors. Recent efforts by our group and others have focused on understanding the underlying pathophysiology and development of clinical biomarkers to diagnose CLAD early and allow risk stratification before irreversible allograft injury.

Definition, Mechanism and Risk Factors for CLAD

Chronic lung allograft dysfunction is defined by the International Society for Heart and Lung Transplantation as a persistent decline >20% in forced expiratory volume at 1 second (FEV1) after other causes have been ruled out [1]. Historically, this was defined solely based on FEV1 parameters with a predominantly obstructive pattern, denominated as bronchiolitis obliterans syndrome (BOS). While this is the most common form of CLAD, a restrictive or mixed restrictive pattern has been described denominated restrictive allograft syndrome (RAS) [1,2]. In these cases, there is an associated decline in >10% of total lung capacity with or without an

obstructive pattern on pulmonary function tests.

The pathophysiology of CLAD is poorly understood and is likely multifactorial. Risk factors described include infections, acute cell or antibody mediated rejection, gastrointestinal related reflux aspiration (GRASP), among others. This results in airway inflammation, development of subepithelial tissue and eventual obliteration of distal airways.

Treatment is based on control of risk factors to prevent further injury to the airways. This includes corticosteroids for acute rejection, antibiotics for documented or suspected infection, correction of reflux, among others. Regardless of the cause, allograft dysfunction will eventually progress and the only available long-term treatment is re-transplantation.

Gastro-intestinal Reflux Related Aspiration (GRASP) and Lung Transplant Outcomes

Lung transplantation is associated with gastroparesis and changes in esophageal sphincter and diaphragm function [3-6].

The prevalence of gastroesophageal reflux disease (GERD) has been reported to be as high as 75% of recipients [7,8]. Gastroparesis and esophageal dysmotility characterized by depressed LES and esophageal function are prevalent in lung transplant candidates and recipients [5,6,9,10]. pH testing before and after transplant are not unidirectional

with evidence of normalization of findings after transplant in some recipients while new onset of acid reflux in others [11]. This is likely related in some to the normalization of the lower esophageal high pressure zone thanks to the normalized apposition of the extrinsic (the crural diaphragm), and the intrinsic lower esophageal sphincter while in others to the new onset of gastroparesis and esophageal dysmotility [5,6,12].

GRASP is associated with lung injury and worsened pulmonary function as well as other outcomes, such as acute rejection and mortality [13]. This has been of particular importance for lung recipients given the frequent correlation reported between reflux and worsened survival and/or rates of CLAD. The first reports looking at this interaction and the role of anti-reflux surgery came from Davis *et al.*, who reported decreased survival after lung transplantation among patients with GERD diagnosed by pH probe [14]. Those that underwent a fundoplication after transplant were associated with improved survival, improved pulmonary function test parameters and BOS-free survival [15,16]. More specifically, anti-reflux procedures tend to decrease the rate of allograft function decline (defined by the decrease in FEV1 from baseline). This has been supported by findings from several studies and a metanalysis [17-20].

Given the proposed benefits of a fundoplication to decrease allograft deterioration, efforts have been done to identify the population as well as the timing of the procedure that best improves outcomes. We reviewed patients with first time lung transplantation at our institution and stratified them by those that underwent an anti-reflux procedure to those that did not using propensity matching to address selection bias [21]. Interestingly, we noted that diagnosis of acid gastroesophageal reflux was not related to adverse outcomes, while fundoplication before a diagnosis of CLAD was associated with an improved overall survival after matching. In multivariable analysis, patients with an age <65 years, those with a restrictive disorder and both double and single lung transplants benefited from anti-reflux treatment. Furthermore, the effect was present both among patients before and after CLAD diagnosis, supporting a decrease in the rate of allograft deterioration. The best timing for fundoplication is currently unknown, however published literature suggest that those with earlier procedure have better outcomes [16].

Airway Biomarkers for GRASP and Post-transplant Outcomes

Gastro esophageal reflux has been traditionally diagnosed by distal esophageal pH probes. This method is not effective at measuring non-acid reflux as well as reflux of deleterious molecules and pathogens that may cause airway injury if aspirated. Further, it fails to document proximal esophageal reflux which has ultimately been associated with post-transplant outcomes [22]. In fact, the data on GER diagnosed

by standard pH monitoring and its association with adverse outcomes has been inconsistent [23,24]. Similarly, the data using impedance monitoring, which can detect reflux episodes independent of pH change have not consistently shown an association with adverse outcomes [21,25]. A reason for this is that the association with CLAD and/or mortality is not related to distal esophageal acid exposure, but rather by other molecules or pathogens within the refluxate that may be aspirated.

This has led researchers to study alternative methods of assessing GRASP directly within the bronchial lumen by means of airway biomarkers (Table 1). These allow to be collected during post-transplant surveillance bronchoscopy without the need of an additional procedure, which can be a limitation for systematic screening for reflux. The best studied airway biomarkers for GRASP among transplant recipients are bile acids (BA) and pepsin.

Bile acids

Bile acids are water soluble amphipathic steroid salts that are mainly produced in the liver as adjuncts for fat digestion [26]. These can be primary or secondary based on whether they are produced endogenously by humans vs bacterial flora, respectively. Furthermore, these molecules can be conjugated by amino-acids such as taurine or glycine which change some of the chemical properties. Given that these compounds are mostly found within the enteric lumen, several groups have studied them as markers of airway injury due to aspiration. Studies have shown detectable levels of BA in airways of lung transplant recipients (**Table 1**). Bronchioalveolar lavage (BAL) was the initial method used for measurement of BA in airways. Early studies have shown correlation between BA level and airway neutrophils, pro-inflammatory cytokines, lower surfactant proteins A and D, and earlier development of CLAD [13,27]. While they correlate with the presence of proximal GER by pH studies, they are not reduced by treatment with proton pump inhibitors or prokinetic agents such as azithromycin [25,28]. Conversely, anti-reflux surgery has been shown to decrease the concentration of specific BA as well as inflammatory proteins [29]. In addition, they correlate with gastroparesis and visits to the emergency department and hospitalizations [30]. More recently, an alternative method of collection of samples by large airway bronchial wash (LABW) has been explored. As opposed to BAL, which collects fluid from the distal airways after wedging the bronchoscope, LABW involves the instillation of 20 ml of saline solution at the proximal airway of the allograft. This allows monitoring of proximal changes as well as a more concentrated sample with better yield of examined molecules [31,32]. As early as 3 months after transplant, samples with a high level of BA (defined by the highest tertile in the cohort) had a decreased overall survival and freedom from CLAD [32]. This remained a predictor for both outcomes after adjusting for

Table 1. Airway biomarkers after lung transplant and relationship with outcomes.					
Study	Biomarker	Source	Population	Sampling time*	Outcomes
Bile acids and subtypes					
Zhang, 2022	TCA, GCA, CA	LABW BAL	LTR (n=61)	3 mo	- Higher concentrations of BA and inflammatory proteins in LABW than in BAL - BA levels correlated with inflammatory proteins
Urso, 2021 [32]	Total BA Conjugated BA	LABW	LTR (n=50)	3 mo	- Highest tertile (>16.57 nM) independent predictor of mortality and CLAD on MV analysis - Both the BA concentration as well as the percentage of conjugated BA independent predictors of CLAD, mortality and percent positive bacterial cultures - Primary conjugated BA levels associated with increased proinflammatory cytokines and lipidome dysregulation
Rosen, 2021 [30]	Total BA BA subtypes	BAL Gastric	Pediatric Non-Tx (n=48) LTR (n=22)	8.1 ± 17.4 mo	- Higher level of BAL BA among patients with gastroparesis - No correlation between gastric and BAL BA concentrations - Higher total BA and conjugated BA level among patients with CLAD - Higher BA level a/w hospitalizations and ED visits
Zhang, 2020 [29]	TCA, GCA, CA	BAL	LTR (n=76)	3 mo	- Higher levels of BAL TCA, IL-1beta, IL-12p70 and CCL5 among those with GERD compared to no GERD - Elevated TCA and GCA were associated with ALAD and inflammatory proteins - Reduced levels of TCA and inflammatory after fundoplication
Neujahr, 2014 [42]	Total BA	BAL	LTR (n=51)	N/A	BA associated with microbial metabolism pathways and biomarkers of lung injury
Reder, 2014 [43]	Total BA	BAL	LTR (n=85)	N/A	Sensitivity of 67% and specificity 80% for GERD with pH monitoring
Mertens, 2011 [28]	Total BA	BAL	LTR (n=37)	N/A	- BA a/w progression of BOS1 or higher and mortality - Azithromycin did not decrease BA levels and did not protect against mortality
Blondeau, 2008 [25]	Total BA	BAL	LTR (n=45)	N/A	- BA was detectable in 50% of patients - Higher rate of BA present among patients with BOS - BA levels not reduced by PPI
D'Ovidio, 2006 [27]	Total BA	BAL	LTR (n=43)	3 mo	72% of patients with abnormal pH study had detectable BA - Higher BA associated with BOS, lower SP-A and SP-D, DPPC and higher SM levels
D'Ovidio, 2005 [13]	Total BA	BAL	LTR (n=120)	N/A	- BA associated with airway neutrophilia and IL-8 - Higher BA associated with earlier BOS

Other biomarkers					
Ramendra, 2024 [39]	PGA4	LABW	LTR (n=200)	2-4 mo	- PGA4 is expressed in human stomach but not lung <i>in vitro</i> and <i>ex vivo</i> - Detectable airway PGA4 was associated with aspiration based on BA and pepsin levels and increased risk of CLAD as well as clinical airway infection by bronchoscopy - Anti-reflux surgery was associated with decreased PGA4 levels
McGinniss, 2022 [44]	Pepsin Lung microbiome	BAL	LTR (n=139)	Within 1 hr of implant	- Recipients with PGD associated with oropharyngeal anaerobic pathogens - Post implant Prevotella/Streptococcus ratio, pepsin and cytokines correlated with development of severe PGD during the 72h post implant period
Reder, 2014 [43]	Pepsin	BAL	LTR (n=85)	N/A	- Atypical GERD symptoms (aspiration and bronchitis) correlated better with BAL pepsin - Sensitivity of 60% and specificity of 45% for GERD by pH monitoring
Fisichella, 2011 [37]	Pepsin	BAL	LTR (n=60)	21 [1-168] mo	- Higher levels of pepsin in BAL among those that did not have anti-reflux surgery - Pepsin associated with BOS progression and acute rejection
Blondeau, 2008 [25]	Pepsin	BAL	LTR (n=45)	N/A	- Pepsin was present in all patients - No correlation with BOS - Pepsin was not reduced by PPI
Stovold, 2007 [36]	Pepsin	BAL	LTR (n=36)	N/A	Higher levels of pepsin among transplants with ACR grade A2 and above
Ward, 2005 [35]	Pepsin	BAL	LTR (n=13)	N/A	Pepsin measurable in all recipients, no correlation with neutrophils
LABW: Large Airway Bronchial Wash; BA: Bile Acid; LTR: Lung Transplant Recipients; TCA: Taurocholic Acid; GCA: Glycocholic Acid; CA: Cholic Acid; BAL: Bronchioalveolar Lavage; EBC: Exhaled Breath Condensate; GERD: Gastroesophageal Reflux Disease; ALAD: Acute Lung Allograft Dysfunction; PPI: Proton Pump Inhibitors; SP-A: Surfactant Protein A; SP-D: Surfactant Protein D; DPPC: Dipalmitoylphosphatidylcholine; SM: Sphingomyelin. *Post transplant					

other underlying risk factors. Furthermore, not only the total concentration of BA was a predictor but also the percentage of conjugated BA from the total. In addition, conjugated BA correlated with higher rates of positive bacterial cultures within the first year after transplant as well as increased levels of proinflammatory cytokines and a dysregulation of the airway lipidome. Given that BA are conjugated in the liver prior to excretion to the duodenum, these findings likely show that enteric BA are more specifically correlated with adverse outcomes. Whether BA are direct effectors, markers of airway injury, or both is topic of active investigation [33].

Pepsin and pepsinogen

Pepsin is the active metabolite of pepsinogen after

activation by gastric acid and is produced within the mucosa of this organ [34]. Given the secretion of this enzyme in gastric fluid and potential effector as proteolytic molecule, it has been studied as a marker of reflux and airway injury. Pepsin measured in BAL has been shown to be detectable among recipients and correlates with severe acute cellular rejection as well as CLAD progression in some studies [25,35-37]. This effect on outcomes has been less consistent compared to those results of BA levels. A potential cause is that some of the pepsinogens such as pepsinogen C can be expressed in type II pneumocytes which confound the relationship between airway pepsin and aspiration [38]. For this reason, Ramendra *et al.* studied the presence of pepsinogen A4 (PGA4) in LABW and outcomes after lung transplant [39]. This subtype is only expressed in gastric cells. Detectable PGA4 in LABW was

associated with increased concentration of conjugated BA and decreased freedom from CLAD. In addition, there was a decreased level of PGA4 after anti-reflux surgery showing promise as an adjunct to detect the presence or absence of bronchial gastroenteric aspiration. Similar to BA in airway, the role of pepsin or pepsinogen as a marker of aspiration vs effector molecule remains to be investigated.

Strengths and Limitations of Airway Biomarkers

These biomarkers present tools for early risk stratification of recipients at risk for CLAD. At this time, BA levels hold the most promise given the most consistent association with adverse outcomes as well as an earlier ability to detect risk for mortality and CLAD, which can be as early as 3 months after transplant. In addition, they show not only a quantitative association but also a qualitative correlation based on the percentage of conjugated BA. In contrast, pepsin and pepsinogen has shown less of an association with outcomes, however it is possible that gastric-specific subtypes such as PGA4 may improve risk stratification.

There are several limitations to the available biomarkers. First, they all require an invasive procedure which limits the timing of collection to when other interventions are performed. Second, the exact concentration of BA or conjugated BA is still being investigated and require validation through a multicenter effort [40,41]. Third, it is unknown whether the presence and/or concentration of BA is a surrogate for other of the known risk factors for CLAD [33]. For this reason, the specific treatment plan after detection of high LABW levels remains to be elucidated. It is likely that this will involve a combination of antimicrobial treatment, anti-reflux procedure, optimization of immunosuppression, among others.

Future Directions

While the role of gastrointestinal reflux related aspiration and allograft injury is being elucidated, there is a clear relationship between the presence of GRASP and worsened outcomes after lung transplantation. This has allowed better understanding into the pathophysiology of CLAD development and whether GRASP is one of the multiple injuries to the allograft that cause it. We now have early objective evidence of lung injury and presence of GRASP using biomarkers such as BA which will allow to better risk stratify patients at a higher risk of worsened outcomes. This will allow to take early action by correcting risk factors for reflux and/or treating GRASP to prevent clinical pulmonary deterioration. The best way to stratify patients in need of anti-reflux surgery on the basis of LABW BA level remains to be studied prospectively. A multicentric prospective study with standardized analysis of BA or subtype concentrations will allow refinement of cutoff levels for risk stratification, which is currently underway [41]. This would also require to be followed by a treatment plan in terms of anti-reflux interventions with long term follow up.

However, it is likely that an early intervention to treat not only gastroesophageal reflux but GRASP in particular is warranted to slow down progression into CLAD.

Conclusions

Chronic lung allograft dysfunction continues to limit survival after lung transplantation. Given the lack of available lasting treatments, efforts have been made to diagnose recipients early to risk stratify and correct risk factors prior to clinical development of spiro metric changes. The presence of GRASP by airway bile acid concentration as well as the percentage of conjugated bile acids correlate with earlier development of CLAD as well as inflammatory cytokines, lipidomic dysregulation, airway hyperreactivity and presence of bacterial pathogens. Anti-reflux surgery is associated with improved overall survival, whether performed prior to or after the onset of CLAD. The specific role of bile acids as markers of injury vs direct effector molecules on lung injury is being determined. This however provides insight into early airway changes prior to clinical development of CLAD and allows for early intervention for patients at high risk for allograft dysfunction to allow longer survival.

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