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MESENCHYMAL STROMAL CELLS AS RESCUE THERAPY FOR REFRACTORY CHRONIC LUNG ALLOGRAFT DYSFUNCTION: EXPERIENCE OF AN ITALIAN MONOCENTRIC COHORT

Coordinatore:

Chiar.mo Prof. Stefano Perlini

Relatore:

Chiar.mo Prof. Lorenzo Cavagna

Tesi sperimentale di dottorato

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Sara Lettieri

Matricola n. 527161

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ABBREVIATION LIST

ACR= Acute cellular rejection

AMR= Antibody Mediated Rejection

ARAD= azithromycin-responsive allograft dysfunction

ARDS= Acute Respiratory Distress Syndrome

ATG= antithymocyte globulin

BMI= body mass index

BAL= bronchoalveolar lavage

BOS= bronchiolitis obliterans syndrome

BSI= Bronchiectasis Severity Index

CF= Cystic fibrosis

CLAD= Chronic lung allograft dysfunction

CMV= Cytomegalovirus

CNI= calcineurin inhibitor

COPD= chronic obstructive pulmonary disease

CPD= Cumulative population doubling

CsA= cyclosporine A

CT= computed tomography

CTMP= Cell Therapy Medicinal Products

DCD= Donation after Cardiac Death

dd-cfDNA = Donor-derived cell-free DNA

DLCO= diffusion lung capacity for CO

DMEM= Dulbecco's Modified Eagle Medium

DMSO= Dimethyl sulfoxide (DMSO)

DSAs= donor-specific antibodies

EBV= Epstein Barr Virus

ECMO= extracorporeal membrane oxygenation

ECP= extracorporeal photopheresis

EVLP= Ex Vivo Lung Perfusion

FEV1= forced expiratory volume in 1 second

FVC= forced vital capacity

GMP= Good Manufacturing Practice

GvHD= graft-versus-host disease

HLA= human leukocyte antigen

ILD= interstitial lung disease

INR= International Normalized Ratio C

IPF= idiopathic pulmonary fibrosis

IQR= interquartile range

IRR= Incidence Rate Ratio

ISHLT= International Society for Heart and Lung Transplantation

ITSA= Interrupted Time Series Analysis

LAM= lymphangioleiomyomatosis

LAS= Lung Allocation Score

LDH= lactate dehydrogenase

MHC= Major Histocompatibility Complex

MMF= Mycophenolate mofetil

MPS= mycophenolate sodium

MSCs= Mesenchymal stromal cells

mTOR= Mammalian target of rapamycin

NTM= non-tuberculous mycobacteria

PAH= pulmonary arterial hypertension

PFT= pulmonary function tests

PGD= Primary graft Dysfunction

PH= pulmonary hypertension

PJP= Pneumocystis jirovecii

P *n* =Passage *n*. (i.e P0= Passage 0)

PRES= Posterior reversible encephalopathy syndrome

PT= protrombin time

PTLD= post-transplant lymphoproliferative disorder

RCP= reactive C protein

R&D= research and development

QC= quality controls

RAS= restrictive allograft syndrome

TLC= total lung capacity

TLI= total lymphoid irradiation

UIP= Usual Interstitial Pneumonia

1. OVERVIEW ON LUNG TRANSPLANT

1.1 History

Transplantation has fascinated humankind since ancient times, with early references found in mythology and historical texts. Accounts from Hindu and Chinese traditions describe symbolic forms of tissue and organ replacement, reflecting early attempts to conceptualize restoration of bodily integrity. Similarly, the well-known “Miracle of the Black Leg” illustrates early cultural representations of transplantation.

The first documented scientific approaches to transplantation emerged in the 16th century. In 1599, Gaspare Tagliacozzi described autologous tissue reconstruction and recognized individual specificity as a barrier to transplantation, anticipating the concept of graft rejection. Subsequent attempts at tissue and bone transplantation in the 17th and 18th centuries, including xenotransplantation and non-vascularized grafts, were largely unsuccessful.

During the 19th century, progress was achieved primarily in skin transplantation. Autologous skin grafting became an established therapeutic approach through the work of Baronio, Cooper, Reverdin, Ollier, Wolfe, Krause, and Thiersch. Early corneal transplantation experiments further supported the feasibility of tissue replacement.

Systematic experimental work on solid organ transplantation began in the late 19th and early 20th centuries. Kocher demonstrated functional thyroid autotransplantation in 1883. In 1902, Alexis Carrel developed vascular anastomosis techniques, enabling experimental organ transplantation in animals, although graft rejection remained the principal obstacle. In the same period, Ullmann performed the first experimental kidney transplant, and Schöne later proposed the immunological basis of graft rejection.

Advances in experimental transplantation continued through the mid-20th century. Demikhov performed heterotopic heart transplantation and the first orthotopic lung transplant in animal models, demonstrating technical feasibility. The first human kidney transplant was performed by Voronoy in 1933, although survival was limited. During World War II, Medawar’s studies on skin grafting established histocompatibility as genetically determined, laying the foundations of modern transplant

immunology. The subsequent identification of the major histocompatibility complex by Dausset represented a pivotal milestone.

Clinical transplantation became feasible with the advent of effective immunosuppression. In 1954, Joseph Murray performed the first successful kidney transplant between identical twins. The introduction of pharmacological immunosuppression in the 1960s enabled transplantation between unrelated individuals. In 1963, James D. Hardy performed the first human lung transplant; although the recipient (Figure 1) survived only 18 days, this procedure marked the beginning of clinical lung transplantation [1]. Early attempts were limited by bronchial anastomotic complications and acute rejection, resulting in poor long-term outcomes.

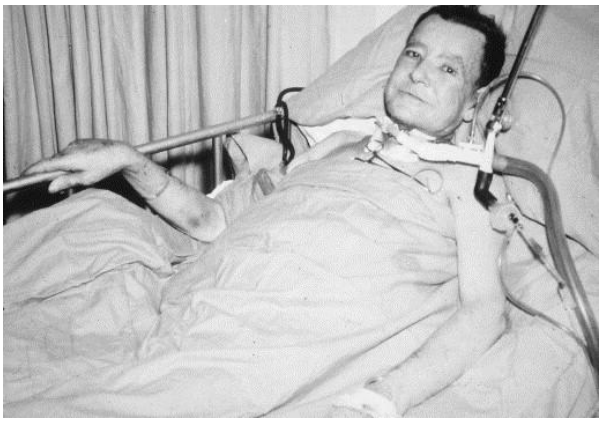


Figure 1. JR, the first man to receive a lung transplant,
photographed after the procedure (Hardy, 1999).

On December 3, 1967, in Cape Town, Christian Barnard performed the first human-to-human heart transplant; although the procedure was technically successful and gained worldwide attention, the recipient survived only 18 days due to fatal pneumonia related to intensive immunosuppression [2].

Beetwen 1967 and 1978, multiple attempts at lung transplantation were made; however, complications at the bronchial anastomosis and acute rejection resulted in short survival, rarely exceeding one year.

Major progress occurred following the discovery of cyclosporine A in 1971, which dramatically reduced acute rejection and improved post-transplant survival. These improvements, combined with advances in surgical techniques, organ preservation, and immunosuppressive protocols, transformed transplantation into a viable clinical therapy.

The first lung transplants in Italy were performed in Rome at the Umberto I Polyclinic in 1991 by Costante Ricci and in Pavia in 1992, following delayed institutional authorization, with a technically successful procedure carried out on a patient with end-stage idiopathic pulmonary fibrosis by Mario Viganò, Gino Volpato, and collaborators. [4].

Lung transplantation initially emerged as combined heart-lung transplantation. Subsequently, due to the scarcity of suitable organs, the growing need for transplants driven by rising incidence of end-stage lung disease, and improvements in surgical techniques, the heart-lung procedure was progressively abandoned in favor of single-lung transplantation or double-lung transplantation, depending on the underlying disease.

To address the shortage of available organs, living-donor lung transplantation was also developed in some centers, especially for pediatric recipients, typically involving transplantation of a single lung lobe. This procedure is rarely performed due to its technical complexity, the risks to the donor, and ethical implications.

In recent years, lung transplants from non–heart-beating donors (Donation after Cardiac Death, DCD) have been successfully performed. Tom Egan and his group first revisited the preclinical foundations for DCD in 1991. They demonstrated at least 8 hours of life sustenance after transplanting a non-ventilated canine lung, which was procured 1 hour after cardiac arrest, into a recipient with a ligated contralateral pulmonary artery [5]. In 1995, D'Alessandro *et al.* reported the first successful case of lung transplantation using a controlled DCD donor [6]. More recently, several series have described the growing experience with DCD [7]. Today, controlled DCD is used in most countries. In this type of organ procurement, life-sustaining therapies are withdrawn, triggering cardiac arrest. At this time, the recovery team procures and cold-preserves the organs in a usual fashion. Although the number of DCD donors in the United States has risen from 0 in 2000 to 32 in 2012, DCD accounts for just 1.9% of all deceased donor lung [8]. Patients who receive organs from DCD and Donation after brain death have similar outcomes, as demonstrated by the results of the first meta-analysis [9] and an International Society for Heart and Lung Transplantation (ISHLT) DCD registry review comparing these groups [10].

In these and other cases involving compromised organs, lungs can be assessed and reconditioned, allowing the use of organs that would previously have been discarded [11]. The first transplant after Ex Vivo Lung Perfusion (EVLP) was performed in Sweden in 2001 [12]. Since then, hundreds of thousands such transplants have been carried out worldwide, and the number is rapidly increasing.

With this technique, dysfunctional lungs—most often affected by interstitial fluid accumulation impairing gas exchange—are perfused with a hyperoncotic acellular solution (STEEN Solution™) that draws fluid from the extracellular compartment, thereby reducing edema (figure 2). The solution also contains dextran, which protects the endothelium from leukocyte-mediated injury and prevents intravascular thrombosis. Its electrolyte composition (low K+) reduces free-radical generation and prevents vasospasm under normothermic conditions, thus improving gas exchange and enabling the use of lungs initially deemed unsuitable [13]. Moreover, this technique allows true ex vivo graft treatment: antibiotics can be infused to treat bacterial infection, or cytokines such as IL-10 can be administered via a vector, reducing pro-inflammatory cytokine production, promoting recovery of alveolar epithelial junctions, and decreasing vascular resistance. The HELP Trial reported the successful transplantation of 20 high-risk donor lungs that were physiologically stabilized using EVLP; the results of those transplants were comparable to the results of transplants carried out with conventionally selected lungs [11]. The 2013 FDA-mandated NOVEL trial [14] and its 2014 update [15] confirmed the satisfactory outcomes of lungs transplanted from marginal donors, comparing them to contemporary controls. Today, EVLP is a safe technique that has increased the number of DCD lung transplantations, but further studies are needed to better define its role in this context [16].

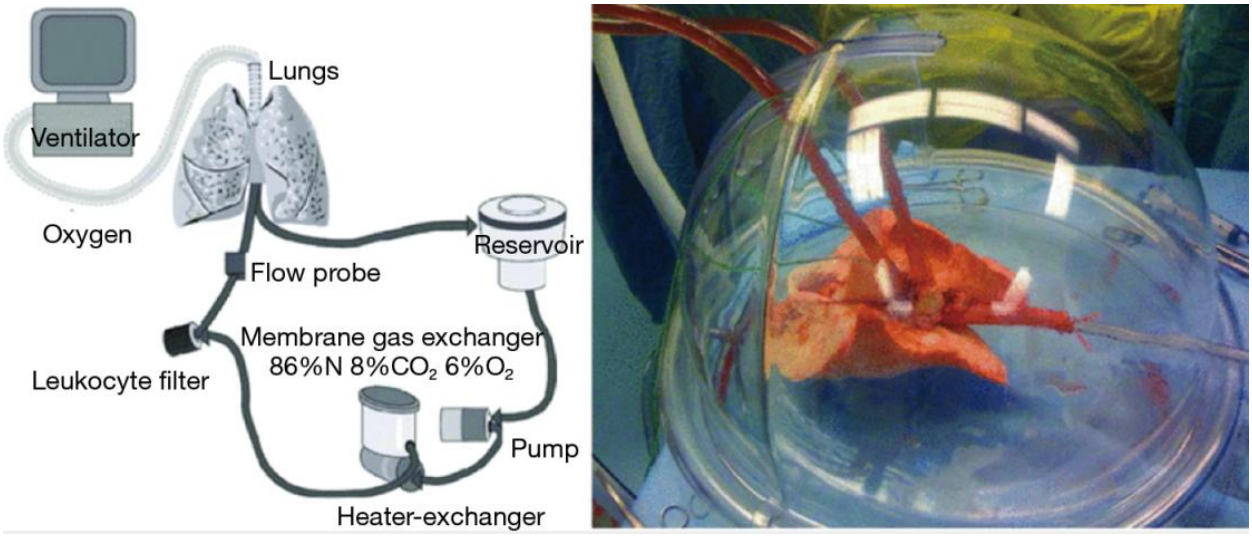


Figure 2. The *ex vivo* lung perfusion circuit. Reprinted with permission from Cypel [11]

In recent years, “bridge-to-transplant” therapies have been developed, enabling patients with severe respiratory failure to survive until transplantation thanks to extracorporeal membrane oxygenation (ECMO). ECMO is an extracorporeal device that prolongs patient survival before transplantation and can also be used as support during the surgical procedure [17]. The most modern devices allow patients to ambulate while connected, helping maintain muscle tone and physical condition.

Thanks to these techniques and the increase in donors, the number of transplants has risen in recent years. According to ISHLT registry data, 69786 lung transplants and 2207 heart-lung transplants have been performed worldwide in the period 1992-2024 [18]. Lung transplantation has come a long way from its beginning (figure 3), evolving from experimental procedures with high mortality into a well-established, life-saving therapy. Advances in surgical techniques, organ preservation, immunosuppression, and post-operative care have dramatically improved outcomes, offering hope and extended survival to patients with end-stage lung disease.

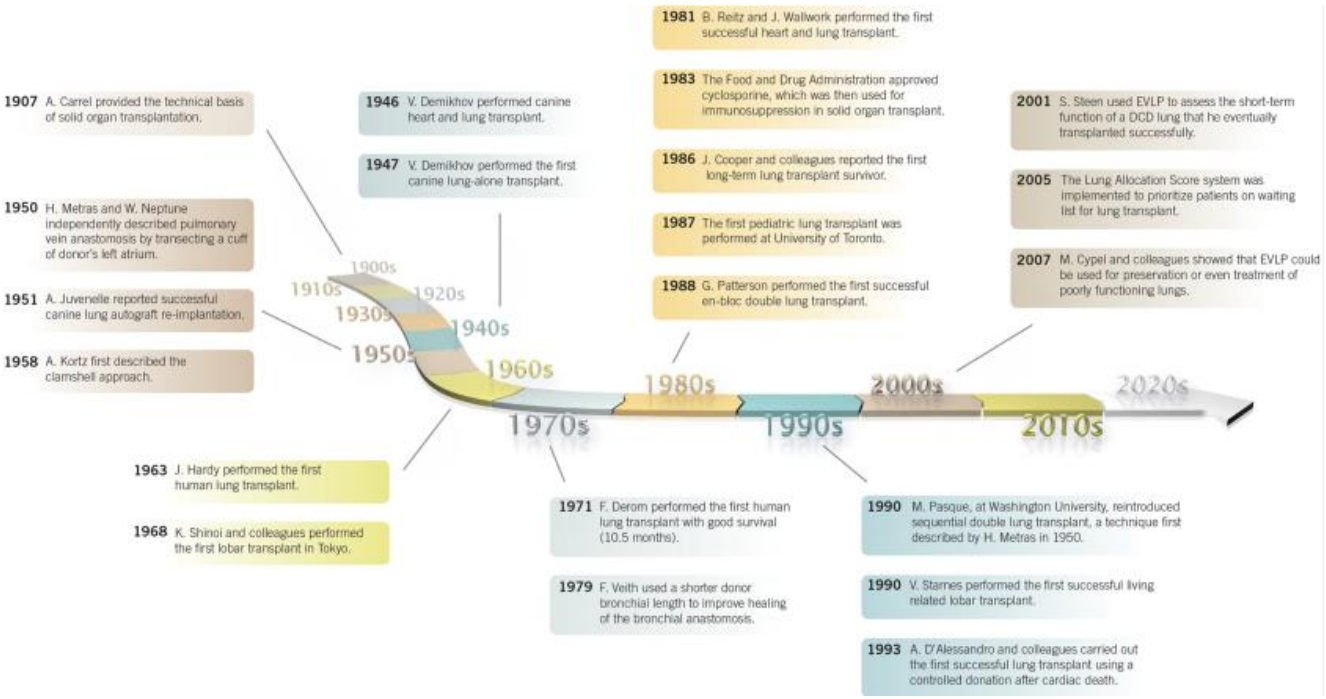


Figure 3. Major milestones in the field of lung transplantation. From [19]

1.2 Indications for lung transplant

Lung transplantation currently represents the only therapeutic option for patients with end-stage chronic lung disease who are no longer responsive to medical or surgical treatment and have an estimated life expectancy of less than two years.

In general, indications for transplantation derive from obstructive, restrictive, or vascular lung diseases associated with a markedly increased risk of death (2-year survival <50%), together with a high probability (>80%) of 5-year survival after the procedure. Until the early 2000s, the most

common indication for lung transplantation was chronic obstructive pulmonary disease (COPD), accounting for approximately 40% of cases. More recently, interstitial lung diseases (ILDs), particularly idiopathic pulmonary fibrosis (IPF), which carries the poorest prognosis, have become the leading indication for lung transplantation (27%), followed by COPD (24%), cystic fibrosis (10%), and pulmonary vascular diseases such as primary pulmonary hypertension (PH) and pulmonary hypertension secondary to congenital heart disease, including Eisenmenger syndrome. Less common indications include pulmonary Langerhans cell histiocytosis and lymphangioleiomyomatosis (LAM). Systemic diseases with pulmonary involvement, such as sarcoidosis or connective tissue disorders, may also be treated with transplantation; however, these cases require particular caution, as extrapulmonary involvement may adversely affect post-transplant outcomes [20, 21].

The ISHLT has developed four editions of recipient selection guidelines, published in 1998, 2006, 2015, and 2021 [21-23]. These guidelines represent the most comprehensive evidence-based recommendations for the appropriate selection of lung transplant candidates.

The process involves referral of the patient to a transplant center and possible listing if selection criteria are met. Importantly, the decision to refer a patient should not be based on a single factor, as no individual variable is sufficiently predictive of early mortality. Instead, multiple clinical factors (such as infection rate, frequency of intensive care unit admissions, oxygen requirements, and weight loss), laboratory parameters (PaO_2 and $PaCO_2$), and functional indicators (pulmonary function tests, echocardiography, and exercise capacity tests) should be considered. Experts also agree on the importance of early referral once other therapeutic options have failed [21].

Listing a patient for lung transplantation requires a complex assessment that includes clinical status, psychosocial factors, and program- or region-specific considerations (such as donation policies and blood group distribution within the population). In addition, absolute and relative contraindications must be carefully evaluated, as lung transplantation is associated with significant morbidity and mortality that may negatively impact outcomes.

Timing is crucial for lung transplant referral and listing. Early referral allows sufficient time for evaluation and for modifiable barriers to transplantation—such as frailty, deconditioning, abnormal body weight, and medical comorbidities—to be addressed. Patients referred “too early” can be monitored closely at 3- to 6-month intervals. Conversely, patients referred “too late” may present barriers that are difficult or impossible to overcome.

To qualify for lung transplantation, a patient must have disease severity sufficient to justify the procedure while still being medically fit to tolerate it. Since 2005, the United States has adopted the Lung Allocation Score (LAS) system to prioritize candidates based on estimated waitlist mortality and projected post-transplant survival benefit [22]. Europe introduced this system in 2016. The LAS is calculated using 12 demographic and physiological variables known to influence mortality in patients with advanced lung disease. It is derived by subtracting the predicted 1-year survival without transplantation from the predicted 1-year survival with transplantation and normalizing the result to a scale ranging from 0 to 100 [24]. Organs are allocated preferentially to candidates with higher LAS values. Studies have demonstrated that the LAS outperforms clinical judgment in predicting waitlist mortality, with a hazard ratio of 1.06 per unit increase in LAS [25]. This allocation system prioritizes medical urgency over geographic proximity, resulting in higher transplantation rates and reduced waitlist mortality for patients with severe diseases such as IPF, who typically have elevated LAS values [26].

The ISHLT has established disease-specific criteria for referral and listing to guide clinicians in appropriate patient management.

COPD

Although COPD remains a common indication for lung transplantation, accounting for approximately 40% of procedures worldwide, determining the optimal timing of listing is challenging because disease progression and prognosis are highly heterogeneous.

Celli et al. evaluated 207 individuals with COPD and identified four readily measurable factors associated with increased mortality risk: body mass index (BMI), airflow obstruction severity (O), dyspnea severity assessed by the Medical Research Council (MRC) dyspnea scale (D), and exercise capacity measured by the 6-minute walk distance (6MWD). These variables were combined to create the BODE index, ranging from 0 (lowest risk) to 10 (highest risk) [27]. The BODE index has been externally validated in multiple cohorts and is cited by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as the preferred prognostic model [23]. However, studies focusing on lung transplant candidates have shown that the BODE index tends to overestimate mortality, including in patients with alpha-1 antitrypsin deficiency. Despite these limitations, it remains the most widely used prognostic indicator.

<u>Timing of referral</u>

- Patients with a BODE index of 5–6 and at least one additional risk:
  - Frequent acute exacerbations

- Increase in BODE index >1 over past 2 years
- Pulmonary artery/aorta ratio >1 on CT scan
- FEV1 20%–25% predicted

- Clinical deterioration despite maximal treatment including medication, pulmonary rehabilitation, oxygen therapy, and, as appropriate, nocturnal non-invasive positive pressure ventilation.
- Poor quality of life unacceptable to the patient

For a patient who is a candidate for bronchoscopic or surgical lung volume reduction (LVR), simultaneous referral for both lung transplant and LVR evaluation is appropriate.

Timing of listing

Patients with a BODE index of 7–10 and at least one of the following:
- FEV1 <20% predicted
- Moderate to severe pulmonary hypertension
- Severe exacerbations
- Chronic hypercapnia

ILDs

Interstitial lung diseases are associated with poor prognosis, particularly idiopathic pulmonary fibrosis, which has a median survival of approximately 3–5 years in the absence of treatment. This supports early consideration of lung transplantation at the time of diagnosis. Patients with fibrotic ILD are also at risk of acute exacerbations, which are associated with very high mortality [27]. The introduction of antifibrotic therapies, including nintedanib and pirfenidone, has significantly altered the natural history of IPF by slowing functional decline, reducing the incidence of acute exacerbations, and improving overall survival [29]. Similar benefits have been demonstrated in progressive fibrosing non-IPF ILDs [30].

Timing of referral

- Referral should be made at time of diagnosis, even if a patient is being initiated on therapy, for histopathological Usual Interstitial Pneumonia (UIP) or radiographic evidence of a probable or definite UIP pattern.
- Any form of pulmonary fibrosis with Forced Vital Capacity (FVC) of < 80% predicted or Diffusion lung capacity for CO (DLCO) < 40% predicted.
- Any form of pulmonary fibrosis with one of the following in the past 2 years:

  - Relative decline in FVC 10%
  - Relative decline in DLCO 15%
  - Relative decline in FVC 5% in combination with worsening of respiratory symptoms or radiographic progression
- Supplemental oxygen requirement either at rest or on exertion.
- For inflammatory ILDs, progression of disease (either on imaging or pulmonary function) despite treatment.
- For patients with connective tissue disease or familial pulmonary fibrosis, early referral is recommended as extrapulmonary manifestations may require special consideration.

Timing of listing

- Any form of pulmonary fibrosis with one of the following in the past 6 months despite appropriate treatment:
  - Absolute decline in FVC > 10%
  - Absolute decline in DLCO > 10%
  - Absolute decline in FVC > 5% with radiographic progression.
- Desaturation to < 88% on 6 minute walk test or > 50 m decline in 6 minute walk test distance in the past 6 months
- Pulmonary hypertension on right heart catheterization or 2-dimensional echocardiography (in the absence of diastolic dysfunction)
- Hospitalization because of respiratory decline, pneumothorax, or acute exacerbation.

Cystic fibrosis (CF)

Until recently, CF was a common indication for transplant. The advent of CF modulator therapies has dramatically affected overall disease course for patients who are candidates for those therapies, with the resultant decline in the need for lung transplants for CF. However, in patients who are ineligible for CFTR modulator therapy, the natural course of the disease, together with its infectious and non-infectious complications, underscores the importance of careful timing and optimization of referral for lung transplantation.

Timing of referral

Referral for lung transplantation should occur for an individual with CF meeting any of the following criteria despite optimal medical management including a trial of elexacaftor / tezacaftor / ivacaftor if eligible:

- FEV1 < 30% predicted in adults (or < 40% predicted in children)
- FEV1 < 40% predicted in adults (or < 50% predicted in children) and any of the following:
  - Six-minute walk distance < 400 meters
  - PaCO2 > 50 mmHg
  - Hypoxemia at rest or with exertion
  - Pulmonary hypertension (PA systolic pressure > 50 mmHg on echocardiogram or evidence of right ventricular dysfunction)
  - Worsening nutritional status despite supplementation
  - 2 exacerbations per year requiring intravenous antibiotics
  - Massive hemoptysis (>240 mL) requiring bronchial artery embolization
  - Pneumothorax
- FEV1 < 50% predicted and rapidly declining based on pulmonary function testing or progressive symptoms
- Any exacerbation requiring positive pressure ventilation

Timing of listing

Listing for lung transplantation should occur for an individual with CF meeting any of the above referral criteria in combination with any of the following:

- FEV1 < 25% predicted
- Rapid decline in lung function or progressive symptoms (>30% relative decline in FEV1 over 12 months)
- Frequent hospitalization, particularly if > 28 days hospitalized in the preceding year
- Any exacerbation requiring mechanical ventilation
- Chronic respiratory failure with hypoxemia or hypercapnia, particularly for those with increasing oxygen requirements or needing long-term non-invasive ventilation therapy
- Pulmonary hypertension (Pulmonary arterial systolic pressure > 50 mmHg on echocardiogram or evidence of right ventricular dysfunction)
- Worsening nutritional status particularly with BMI < 18 kg/m2 despite nutritional interventions
- Recurrent massive hemoptysis despite bronchial artery embolization
- World Health Organization functional class IV

Non CF bronchiectasis

Non-CF bronchiectasis accounts for approximately 2.7% of all lung transplants performed between 1995 and 2018 [31]. Indications for lung transplantation are guided by disease severity as assessed by validated scoring systems, including the FACED score and the Bronchiectasis Severity Index, (BSI) together with FEV_1.

The FACED score incorporates FEV_1 (<50% predicted), age (<70 years), chronic colonization (presence of *Pseudomonas aeruginosa*), disease extension (number of lobes affected), and dyspnea severity assessed using the modified Medical Research Council scale [32]. The BSI includes age, body mass index, FEV_1, prior hospital admissions, exacerbation frequency, dyspnea (modified Medical Research Council score), *Pseudomonas* colonization, other bacterial colonization, and radiologic extent of disease. A small observational cohort study found no significant difference between the FACED score and the BSI in predicting survival [33].

Lung transplant evaluation and prognostication in patients with non-CF bronchiectasis remain areas of ongoing investigation. Compared with cystic fibrosis–matched cohorts, patients transplanted for non-CF bronchiectasis appear to have a lower risk of death, with an estimated 5-year mortality of approximately 25%.

Timing of referral and listing

For individuals with non-CF bronchiectasis, similar criteria as with CF for referral and listing for lung transplantation is reasonable, though providers should recognize that prognosis is highly variable with many patients experiencing a more stable course.

Pulmonary arterial hypertension (PAH)

Lung transplantation in PAH remains particularly challenging, as the LAS does not fully capture either waitlist mortality or the potential for sudden clinical deterioration. Current guidelines therefore recommend serial risk assessment using validated tools such as REVEAL 2.0 and/or the 2015 ESC/ERS risk stratification models to guide clinical management [34].

Failure to achieve a low-risk profile after three to six months of optimized medical therapy should prompt escalation of PAH treatment. Patients who continue to demonstrate a high-risk profile despite maximal therapy should be referred for lung transplant evaluation. In addition to clinical risk scores, cardiopulmonary exercise testing and assessment of right ventricular function using transthoracic echocardiography and/or cardiac magnetic resonance imaging provide important prognostic information [35]. Determining the optimal timing for listing remains complex; however, the recently

proposed four-stratum COMPERA 2.0 risk model may assist in identifying patients most likely to benefit from lung transplantation by allowing more granular risk stratification [36].

Timing of referral

- ESC/ERS intermediate or high risk or REVEAL risk score 8 despite appropriate PAH therapy
- Significant RV dysfunction despite appropriate PAH therapy
- Need for IV or SC prostacyclin therapy
- Progressive disease despite appropriate therapy or recent hospitalization for worsening of PAH
- Known or suspected high-risk variants such as PVOD/PCH, scleroderma, large and progressive pulmonary artery aneurysms
- Signs of secondary liver or kidney dysfunction due to PAH
- Potentially life-threatening complications such as recurrent hemoptysis

Timing of listing

- ESC/ERS high risk or REVEAL risk score >10 on appropriate PAH therapy, including IV or SC prostacyclin analogues
- Progressive hypoxemia, especially in patients with PVOD or PCH
- Progressive, but not end-stage, liver or kidney dysfunction due to PAH
- Life-threatening hemoptysis

LAM

LAM is a rare cystic lung disease that is an uncommon indication for lung transplantation. mTOR inhibitor therapy can prevent progression of disease and prevent the need for lung transplantation.

Timing of referral

Presence of any of the following despite mTOR inhibitor therapy:

- Severely abnormal lung function (e.g. FEV1 < 30% predicted)
- Exertional dyspnea (NYHA class III or IV)
- Hypoxemia at rest
- Pulmonary hypertension
- Refractory pneumothorax

Timing of listing

Listing for lung transplantation should occur for an individual with LAM who meets the above referral criteria and has evidence of disease progression despite mTOR inhibitor therapy.

Cessation of mTOR inhibitor therapy should occur at the time of transplant but cessation should not be required for placement on the waiting list. It may be preferable to use everolimus and target trough levels in the lower therapeutic range for patients on the waiting list.

Adenocarcinoma In Situ and Minimally Invasive Adenocarcinoma

Thoracic malignancy is a rare and declining indication for lung transplantation, accounting for less than 0.1% of all transplants performed between 1995 and 2018 [31]. The principal concerns associated with lung transplantation for malignancy include increased recurrence rates and reduced survival, with the exception of incidentally detected malignancies identified in explanted lungs [37]. These cases require the implementation of rigorous lung cancer surveillance protocols within transplant programs [38].

Timing of referral and listing

Lung transplant should be limited to very select cases of lung-limited adenocarcinoma in situ, minimally invasive adenocarcinoma, or lepidic predominant adenocarcinoma for patients in whom (1) surgical resection is not feasible either because of multifocal disease or significant underlying pulmonary disease; (2) multifocal disease has resulted in significant lung restriction and respiratory compromise; (3) medical oncology therapies have failed or are contraindicated; and (4) lung transplant is expected to be curative.

Acute Respiratory Distress Syndrome (ARDS)

The rapid onset and severity of ARDS complicate pre-transplant evaluation and candidate selection. However, given the increasing incidence of ARDS, several criteria may assist in identifying patients who could benefit from lung transplantation. Prognostic factors associated with favorable post-transplant outcomes in ARDS include younger age, absence of significant medical comorbidities or extrapulmonary organ dysfunction, a primary pulmonary etiology of ARDS, and the use of extracorporeal life support as a bridge to transplantation [39]. In contrast, intensive care unit–acquired muscle weakness has been associated with increased early post-transplant mortality [40].

Patients may be considered for lung transplantation if supported with ECMO for more than three weeks without evidence of clinical improvement, in the presence of persistent parenchymal infiltrates and severely reduced lung compliance, as reflected by elevated driving pressures [41].

Timing of referral

Persistent requirement for mechanical ventilatory support and /or ECMO without expectation of clinical recovery and with evidence of irreversible lung destruction.

1.3 Controindications to lung transplant

Lung transplantation represents a complex therapeutic approach associated with significant perioperative risks and potential mortality. Therefore, a thorough evaluation of contraindications and relevant comorbidities is essential. There are some risk factors that are considered absolute contraindications to lung transplantation, as outlined by ISHLT. Several additional factors are associated with varying degrees of increased risk in lung transplantation [23]. Although none of these relative contraindications alone necessarily precludes lung transplantation, their associated risks may be additive or even synergistic. Therefore, an individualized risk–benefit assessment should be performed, taking into account the experience and expertise of the transplant center.

1.3.1 Absolute Contraindications

- Malignant tumors within the past five years, with the exception of cutaneous squamous cell carcinoma and basal cell carcinoma, for which a disease-free interval of two years is recommended;
- Advanced, untreatable organ failure (heart, liver, kidney, brain), unless combined organ transplantation is feasible. Untreatable coronary artery disease not amenable to percutaneous or bypass revascularization and associated with significant left ventricular dysfunction constitutes an absolute contraindication to lung transplantation, although in selected cases combined heart–lung transplantation may be considered;
- Chronic infection with highly virulent and/or resistant pathogens that cannot be adequately controlled prior to transplantation;
- Active infection with *Mycobacterium tuberculosis*;
- HIV infection with detectable viral load;
- Septic shock;
- Non-adherence, or a history of prolonged or repeated episodes of non-adherence to medical therapy, which may increase the risk of post-transplant non-compliance;
- Psychiatric or psychological conditions associated with an inability to cooperate with the medical team and/or adhere to medical therapy;

- Progressive cognitve impairment;
- Lack of an adequate social support system;
- Severely limited functional status with poor rehabilitative potential;
- Substance abuse or dependence, including alcohol, cigarette smoking, marijuana, or other drugs in the previous 6 months. Prior to transplant candidacy, patients must actively and/or long-term participate in substance abuse treatment programs, and abstinence should be verified by repeated blood and urine toxicology testing.

1.3.2 Risk factors with high risk

- Age > 70 years;
- BMI >35 or < 16 Kg/m^2;
- Impaired functional status with poor rehabilitative potential;
- Relevant organ failure (e.g. coronary artery disease requiring coronary artery bypass grafting at the transplant, chronic heart failure with left ventricular ejection fraction < 40%, severe esophageal dismotility, significant cerebrovascular disease, uncorrectable bleeding diathesis);
- Psychiatric, psycological or cognitive impairment with potential to interfere with medical adherence;
- Infections with virulent/highly resistant pathogens (*Mycobacterium abscessus, Lomentospora prolificans, Burkholderia cenocepacia* or *gladioli*, Hepatitis B or C wirth detectable viral load and liver fibrosis);
- ECMO;
- Retransplant < 1 year after lung transplant;
- Retransplant for restrictive Chronic lung allograft dysfunction (CLAD) or for Antibody mediated rejection (AMR) as cause of CLAD

1.3.3 Other risk factors

- Age among 65 and 70 years
- Clinical conditions with potential unfavorable implications after transplant (e.g. mild to moderate coronary artery disease, severe CAD treatable with percutaneous revascularization before transplantation, peripheral vascular disease, connective tissue disease, severe gastroesophageal reflux, osteoporosis, anemia, leukopenia or

thrombocytopenia with high probability of persistence after transplant, mellitus diabetes poorly controlled)

- BMI among 16-17 kg/m^2 or among 30-34.9 kg/m^2
- Frailty
- Hypoalbuminemia
- HIV infection with undetectable viral load or *Scedosporium apiospermum* infection
- Mechanical ventilation. These patients require careful evaluation: if they have no additional acute or chronic organ dysfunction, can actively participate in a rehabilitation program, and are expected to achieve rapid postoperative recovery, they may undergo successful transplantation;
- Prior thoracic surgery or pleurodesis
- Retransplant > 1 year for obstructive CLAD.

1.4 Notes on surgical technique

Lung transplantation is performed under general anesthesia using a double-lumen endotracheal tube to allow independent lung ventilation. Central venous access is obtained via a triple-lumen catheter for medication administration, fluid management, and central venous pressure monitoring. Most centers also establish femoral vascular access to allow rapid percutaneous cannulation for ECMO if required; the venous catheter is preferentially placed on the left side to accommodate potential postoperative ECMO needs. A Swan–Ganz catheter is inserted to monitor pulmonary artery pressures, providing continuous assessment of hemodynamic status. Transesophageal echocardiography is routinely used, given the close interaction between cardiac and pulmonary function during transplantation, and allows real-time monitoring throughout the procedure.

The patient is positioned supine and prepped from the neck to the knees. Single-lung transplantation is typically performed through an anterolateral thoracotomy, whereas bilateral lung transplantation is traditionally carried out via a clamshell incision, consisting of a bilateral thoracotomy in the fourth intercostal space connected by a transverse sternotomy. This approach offers optimal exposure of both hili and the heart, particularly when central cannulation for extracorporeal support is required.

The choice of which lung to transplant first depends on both donor and recipient factors. Ventilation–perfusion scanning is useful in guiding this decision; in general, the functionally worse lung is transplanted first to reduce the need for extracorporeal support. If donor lungs differ in quality, the

better lung is typically implanted first. Pneumonectomy is performed in a standard fashion, with stapling of the pulmonary artery and veins as peripherally as possible. The bronchus is prepared centrally and divided with a scalpel, and the specimen is removed. Two 4-0 polydioxanone stay sutures are placed at the junction between the membranous and cartilaginous portions of the main bronchus

To improve exposure, a traction suture is placed on the diaphragmatic dome, and the pericardium is opened to facilitate hilar dissection. Adhesions are carefully released using electrocautery to avoid injury to surrounding structures. The inferior pulmonary ligament is divided, and systemic heparinization is administered. The pulmonary artery and veins are then divided using an endostapler, leaving adequate stumps for anastomosis. Circumferential pericardial opening allows preparation of the left atrium, while excessive denudation of the recipient bronchus beyond the anastomotic site must be avoided to prevent ischemic complications.

Implantation begins with the bronchial anastomosis, performed using a running 3-0 polypropylene suture, often in a telescoping fashion to ensure airtightness. The pulmonary artery is then anastomosed end-to-end with a running 4-0 polypropylene suture, followed by completion of the pulmonary vein anastomosis. Careful de-airing of the graft is performed before final tightening of the sutures to prevent air embolism.

Before graft reperfusion, an initial dose of methylprednisolone (500–1000 mg, according to recipient body weight) is administered. The remaining immunosuppressive regimen is initiated postoperatively in the intensive care unit. Accurate hemostasis is essential prior to implantation, as certain areas—particularly the posterior mediastinum—are difficult to access once the lung is in place. In patients with septic lung disease, such as cystic fibrosis, the pleural cavity is irrigated with antiseptic solution. Throughout the procedure, particular care is taken to avoid injury to the phrenic nerve and, on the left side, the recurrent laryngeal nerve.

After implantation, ventilation is initiated with a low fraction of inspired oxygen to reduce the risk of reperfusion injury, and ventilatory parameters are adjusted gradually. When necessary, cardiopulmonary bypass or ECMO is used to provide intraoperative hemodynamic and respiratory support. In bilateral transplantation, the same procedure is subsequently repeated on the contralateral side.

At the end of the operation, 24-French chest drains are placed in the costodiaphragmatic sinus and toward the apex on each side. Some centers additionally place a small posterior Jackson–Pratt drain

to allow earlier removal of standard chest tubes while maintaining adequate drainage. The thoracotomy is closed in layers, including pectoral fascia, subcutaneous tissue, and skin.

Postoperatively, the double-lumen tube is exchanged for a single-lumen endotracheal tube. Bronchoscopy is performed to assess the bronchial anastomoses and clear secretions or clots. A nasogastric tube is placed to ensure adequate nutritional support during the early postoperative period.

Total operative time typically ranges from 6 to 10 hours. Patients are transferred to the intensive care unit for postoperative management, where mechanical ventilation is continued until adequate graft function and clinical stability are achieved. Peripheral venovenous ECMO may be employed to support primary graft dysfunction when indicated. Pain control, fluid balance, hemodynamic monitoring, and immunosuppressive therapy are carefully managed to optimize outcomes.

Although minor technical variations exist among centers, lung transplantation is currently performed in a largely standardized manner worldwide [42].

1.5 Immunosoppressive treatment

Immunosuppressive therapy is fundamental to the success of lung transplantation and is required lifelong to prevent immune-mediated injury to the allograft. Compared with other solid organ transplants, lung transplantation is associated with a higher risk of rejection, reflecting the lung's continuous exposure to environmental antigens, its extensive lymphoid tissue, and the presence of donor-derived immune cells. Consequently, lung transplant recipients require intensive and carefully balanced immunosuppressive regimens aimed at preventing acute and chronic rejection while minimizing treatment-related complications such as infection, malignancy, nephrotoxicity, and metabolic disorders.

Immunosuppressive strategies after lung transplantation are conventionally divided into induction therapy, administered in the perioperative period, and maintenance therapy, continued lifelong. Additional therapeutic escalation is required for the treatment of acute cellular rejection and CLAD.

1.5.1 Induction Immunosuppression

Induction immunosuppression consists of potent, short-term therapy administered at the time of transplantation to attenuate the early, robust T-cell–mediated immune response to the lung allograft. Although induction therapy was initially adopted by only a subset of transplant centers, its use has increased substantially over time. According to contemporary registry data from ISHLT,

approximately 70–80% of lung transplant recipients now receive some form of induction therapy [31].

The most frequently used induction agents are interleukin-2 receptor antagonists, particularly basiliximab, followed by antithymocyte globulin (ATG) and, less commonly, alemtuzumab. Basiliximab and daclizumab are chimeric monoclonal antibody directed against the CD25 subunit of the IL-2 receptor expressed on activated T lymphocytes, thereby inhibiting IL-2–mediated T-cell proliferation without causing lymphocyte depletion. Daclizumab was removed from the US market in 2009 due to the development of several cases of severe, often fatal, inflammatory brain diseases, so basiliximab remains the only IL-2RA available for clinical use at this time. Basiliximab is administered intraoperatively or within two hours after transplantation, with a second dose typically given on post-operative day four. It is generally well tolerated, with no increase in adverse advents compared to placebo in clinical trials [43]. Its favorable safety profile has made it the preferred first-line induction agent at many centers. ATG, a polyclonal antibody preparation derived from equine or rabbit sources, induces profound T-cell depletion and exerts additional immunomodulatory effects, including impacts on B cells, dendritic cells, and regulatory T-cell populations. ATG is administered using weight-based dosing starting on day one, often intraoperatively. Two to four additional doses are typically given every 24 hours, though single dose regimens have been described. Premedication with glucocorticoids, antihistamines and antipyretics is frequently used to prevent or reduce infusion related symptoms. Common adverse effects of ATG include chills, anxiety, abdominal pain, nausea, hyperkalemia, leukopenia, and thrombocytopenia. More serious adverse reactions, including infusion reactions, immune complex mediated glomerulonephritis, serum sickness, and cytokine release syndrome have also been reported [44]. Alemtuzumab, a monoclonal antibody targeting CD52, causes antibody-dependent cell lysis, and can cause sustained T-cell depletion for up to 3 years, and B-cell depletion for up to several months [45]. Alemtuzumab also results in the depletion of monocytes, macrophages, and some subsets of dendritic cells, all of which express CD52, and impacts additional immune cell maturation resulting in a tolerogenic immune environment [4]. Alemtuzumab is given as a single intravenous or subcutaneous injection at the time of organ reperfusion or immediately following transplantation. It is used selectively because of concerns regarding prolonged immunosuppression and infectious or malignant complications (figure 4).

Agent	Mechanism of action	Dosing	Adverse effects	Notes on use
Basiliximab	Monoclonal antibody that binds to the IL-2 receptor, preventing T-cell proliferation and differentiation	Intravenous. 20 mg at the time of implantation and on post-operative day 4	Rare hypersensitivity reactions	Most commonly used induction agent. 1st line at many centers due to side effect profile
ATG	Polyclonal immunoglobulin preparation containing antibodies to human T-cells that act through Fc receptors to deplete cytotoxic T-cells	Intravenous. Weight based dosing on day one, with 2–4 additional doses every 24 hours. Single dose regiments also described	Chills, anxiety, abdominal pain, nausea, hyperkalemia, pancytopenia, infusion reactions, immune complex mediated glomerulonephritis, serum sickness, and cytokine release syndrome	Not commonly used as 1st line therapy in lung transplantation
Alemtuzumab	Monoclonal antibody to the CD52 antigen present on all B- and T-lymphocytes. Binding causes antibody-dependent cell lysis resulting in B- and T-cell depletion	Intravenous or subcutaneous. One 30 mg dose at the time of allograft reperfusion or immediately following transplantation	Pancytopenia, insomnia, anxiety, infusion reaction, cytokine release syndrome, secondary autoimmunity	Used as 1st line therapy at some centers. Use limited by concern for prolonged immunosuppression (B-cell depletion for months, T-cell depletion for up to 3 years)

ATG, anti-thymocyte globulin; IL-2, interleukin-2.

Figure 4. Agents for induction immunosuppression. From [44]

High-dose corticosteroids are routinely administered intraoperatively, typically immediately before reperfusion of the lung allograft, to reduce ischemia–reperfusion injury. While corticosteroids are not universally classified as formal induction agents, they likely contribute significantly to early immunosuppression.

Despite its widespread use, the clinical benefit of induction immunosuppression in lung transplantation remains controversial. Large retrospective analyses of national and international registries suggest associations with lower rates of acute rejection and improved graft and patient survival [47,48], and selected cohorts have demonstrated reduced incidence of bronchiolitis obliterans syndrome (BOS) or CLAD [49]. In contrast, randomized controlled trials and meta-analyses have yielded inconsistent results, with several studies failing to demonstrate significant reductions in mortality, graft loss, or chronic rejection compared with no induction [50,51]. Concerns regarding increased risks of infection and malignancy—particularly with lymphocyte-depleting agents—persist, although these have not been consistently confirmed in prospective studies. Consequently, induction therapy remains individualized, guided by recipient risk profile, donor characteristics, and center-specific experience.

1.5.2 Maintenance Immunosuppression

Maintenance immunosuppression is lifelong and aims to prevent both acute and chronic rejection while minimizing cumulative drug toxicity. The standard approach in lung transplantation is triple-drug therapy consisting of a calcineurin inhibitor (CNI), an antiproliferative agent, and corticosteroids (Figure 5). According to 2019 ISHLT registry data, the most commonly used regimen is tacrolimus, mycophenolate mofetil/mycophenolic acid (MMF/MPA), and prednisone [31]. Mammalian target of rapamycin (mTOR) inhibitors have emerged as adjunctive agents in selected patients.

CNIs represent the cornerstone of maintenance immunosuppression. Cyclosporine binds cytoplasmic cyclophilins, whereas tacrolimus binds FK-binding proteins; in both cases, the resulting complex inhibits calcineurin, a phosphatase required for activation of transcription factors regulating cytokines such as IL-2 and TNF-α. Inhibition of calcineurin suppresses T-lymphocyte activation and proliferation.

Cyclosporine is available in intravenous form and as non-modified (Sandimmune®) and modified microemulsion (Neoral®) oral formulations. The non-modified formulation exhibits variable absorption due to bile dependence, whereas the microemulsion provides more consistent bioavailability; the two formulations are not interchangeable. Dosing is guided by trough or 2-hour post-dose levels, typically targeting trough concentrations of 100–450 ng/mL or 2-hour levels of 800–1,400 ng/mL, with higher targets often used during the first year after transplantation. Major adverse effects include neurotoxicity, hypertension, dyslipidemia, nephrotoxicity, electrolyte disturbances, diabetes, and hirsutism; rare but severe complications include posterior reversible encephalopathy syndrome and thrombotic microangiopathy.

Tacrolimus is 10–100 times more potent than cyclosporine and is available in intravenous and oral formulations. Although no oral suspension exists, sublingual administration can be used at reduced doses. Extended-release formulations are approved in kidney transplantation and have shown promising safety data in lung transplant recipients, with ongoing studies evaluating efficacy. Target trough levels typically range from 5–15 ng/mL, with higher targets early after transplantation. Adverse effects overlap with those of cyclosporine, with some evidence suggesting higher rates of neurotoxicity and post-transplant diabetes.

Tacrolimus has largely replaced cyclosporine in contemporary practice, as multiple studies demonstrate lower rates of acute rejection and chronic rejection phenotypes, including BOS, although overall survival benefits are modest.

Antiproliferative agents inhibit lymphocyte expansion and antibody production. Mycophenolate mofetil (MMF) and mycophenolate sodium (MPS) reversibly inhibit inosine monophosphate dehydrogenase, blocking de novo purine synthesis in B and T lymphocytes. Azathioprine, an older agent, is metabolized to 6-mercaptopurine, which interferes with purine nucleotide synthesis, thereby reducing lymphocyte proliferation and immunoglobulin production.

MMF is available in intravenous and oral formulations (target dose 1–1.5 g twice daily), while MPS is available orally (720 mg twice daily); doses are not interchangeable. Common adverse effects include cytopenias and gastrointestinal intolerance. Azathioprine is administered orally or intravenously (typically 2 mg/kg/day) and is associated with hematologic toxicity, hepatotoxicity, and pancreatitis. Its use requires caution with xanthine oxidase inhibitors and in patients with thiopurine-S-methyltransferase deficiency. Due to variable tolerability and toxicity, azathioprine is now used less frequently, with mycophenolate generally favored.

Concerns regarding CNI-related nephrotoxicity have increased interest in mTOR inhibitors, primarily sirolimus and everolimus. These agents inhibit mTOR signaling by binding FK-binding protein, resulting in cell cycle arrest at the G1–S phase and suppression of lymphocyte proliferation. Both are administered orally and are associated with cytopenias, dyslipidemia, hyperglycemia, impaired wound healing, pneumonitis, and thromboembolic events.

Clinical experience with mTOR inhibitors in lung transplantation is limited. Available data suggest that mTOR-based regimens may allow CNI dose reduction and preservation of renal function. In randomized trials, everolimus-based low-CNI strategies improved renal function without increasing acute rejection, CLAD, or mortality. However, despite early interest in their antifibrotic properties, larger studies have failed to demonstrate consistent or durable protection against chronic lung allograft dysfunction, and discontinuation rates due to adverse events remain high[52-55].

Early post-transplant use of mTOR inhibitors is contraindicated because of a strong association with impaired wound healing and catastrophic airway complications, including bronchial anastomotic dehiscence. Consequently, these agents are generally introduced no earlier than three months after transplantation [56,57].

Agent	Mechanism of action	Dosing	Adverse effects	Notes on use
Calcineurin inhibitors				
Tacrolimus	Macrolide antibiotic that binds to intracellular FK-binding proteins. Drug-receptor complex inhibits calcineurin, which decreases cytokine production, and subsequent activation and proliferation of T-lymphocytes	Oral, sublingual, or intravenous. Dosed by drug level, with goal trough concentrations of 5–15 ng/mL	Tremor, headache, neuropathy, seizures, hypertension, hyperlipidemia, nephrotoxicity, hyperkalemia, hypomagnesemia, diabetes, PRES, TMA	1st line therapy at most centers. 10–100 times more potent than cyclosporine. Higher rates of neurotoxicity and diabetes compared to cyclosporine
Cyclosporine	Lipophilic peptide that binds to intracellular FK-binding proteins. Drug-receptor complex inhibits calcineurin, which decreases cytokine production, and subsequent activation and proliferation of T-lymphocytes	Oral (modified and non-modified), intravenous. Dosed by drug level: goal trough 100–450 ng/mL, goal 2-hour post-dose 800–1,400 ng/mL	Tremor, headache, neuropathy, seizures, hypertension, hyperlipidemia, nephrotoxicity, hyperkalemia, hypomagnesemia, diabetes, PRES, TMA	2nd line therapy at most centers. Used for patients unable to tolerate tacrolimus
Antiproliferatives				
Mycophenolate	Reversible inhibitor of inosine monophosphate dehydrogenase, decreasing purine synthesis and their B- and T-lymphocyte proliferation	MMF: Oral, intravenous. 1,000–1,500 mg twice daily. MPS: Oral. 720–1,080 mg twice daily (oral formulation only)	Pancytopenia, nausea, abdominal pain, diarrhea	1st line therapy at most centers
Azathioprine	Metabolized to 6-MP, which produces compounds that interfere with purine synthesis resulting in a decrease in production of B- and T-lymphocytes	Oral, intravenous. 2 mg/kg daily (50–150 mg/day)	Pancytopenia, hepatotoxicity, pancreatitis	2nd line therapy. Excess toxicity can occur when used with xanthine oxidase inhibitors (i.e., Allopurinol), or in patients with low or absent TPMT activity
mTOR inhibitors				
Sirolimus and everolimus	Bind to FK binding protein, inhibiting mTOR, causing arrest of the cell cycle in the G1-S phase and preventing cell cycle progression and lymphocyte proliferation	Sirolimus: Oral. Dosed by drug level, goal trough 5–13 ng/mL. Everolimus: Oral. Dosed by drug level.	Pancytopenia, hyperlipidemia, hyperglycemia, impaired wound healing, pneumonitis, venous thromboembolism.	Can be used as adjunct to conventional immunosuppression to limit toxicity of those agents. Due to complications of airway dehiscence, initiation must be delayed until 3 months after transplantation

PRES, posterior reversible encephalopathy syndrome; TMA, thrombotic microangiopathy; MMF, mycophenolate mofetil; MPS, mycophenolate sodium; mTOR, mammalian target of rapamycin; TPMT, thiopurine-S-methyltransferase.

Figure 5. Agents for manteinance immunosuppression. From [44]

Corticosteroids remain a key component of maintenance immunosuppression. Following high-dose perioperative administration, steroids are gradually tapered to low-dose maintenance therapy, typically 2.5–5 mg of prednisone daily, although protocols vary by center. Their immunosuppressive effects involve inhibition of lymphocyte proliferation, suppression of pro-inflammatory cytokines, sequestration of lymphocytes within the reticulo-endothelial system, and modulation of immune cell trafficking [58]. Despite long-term adverse effects, including metabolic complications, osteoporosis, and increased infection risk, complete steroid withdrawal is rarely attempted outside carefully selected, stable recipients.

Belatacept, a costimulatory pathway inhibitor that blocks CD28-mediated T-cell activation, has shown renal benefits in kidney transplantation in terms of improved renal function, reduced formation of de novo donor-specific antibodies, and favorable graft survival compared with CNI–based regimens[58-60]. However, experience in lung transplantation is limited. Early retrospective studies suggested possible benefit in CNI-intolerant patients [61,62], but a recent randomized controlled trial of de novo belatacept-based immunosuppression was terminated early due to excess mortality. A total of five deaths occurred among patients receiving belatacept, whereas no deaths were observed in the control group [63]. Additional reports describe severe acute cellular rejection following conversion from CNIs [64,65]. Based on current evidence, belatacept is not recommended for maintenance immunosuppression in lung transplantation.

1.6 Non immunological complications after lung transplant

The lung allograft is uniquely susceptible to injury because of continuous exposure to the external environment, high baseline immunologic activity, ischemia–reperfusion injury, and the need for intensive immunosuppression. Consequently, post-transplant complications are heterogeneous and include surgical, infectious, immunological, and systemic events. Early outcomes are often dominated by primary graft dysfunction and technical complications, whereas late morbidity is increasingly driven by chronic rejection and the long-term consequences of immunosuppression. Among these, CLAD remains the principal limitation to long-term graft survival.

Complications can be categorized according to time after transplantation as: immediate (≤72 hours), early (≤3 months), intermediate (4–12 months), and late (>1 year). From a mechanistic perspective, complications are commonly grouped into surgical, medical, infectious, and immunological events, acknowledging that these categories frequently overlap.

1.6.1 Surgical complications after lung transplant

1.6.1.1 Airways complications

Airway complications are a distinctive source of morbidity after lung transplantation compared with other solid-organ transplants. Reported incidence ranges from 2% to 33%, reflecting heterogeneity in definitions, surveillance strategies, and the lack of a universally adopted classification system. Most contemporary series report an overall incidence of approximately 5–15%, with higher rates in

recipients transplanted for infectious lung diseases such as cystic fibrosis and bronchiectasis [66,67]. In these populations, chronic colonization with aggressive bacteria and fungi—including *Pseudomonas aeruginosa*, *Aspergillus* spp., *Scedosporium*, and *Penicillium*—is associated with airway inflammation, impaired mucosal healing, and increased risk of severe anastomotic complications [68].

Airway complications contribute to prolonged hospitalization, increased healthcare utilization, impaired lung function, reduced quality of life, and increased mortality. They can occur early or late after transplantation and are commonly classified as anastomotic or non-anastomotic. The most frequent entities include anastomotic dehiscence, airway stenosis, granulation tissue formation, bronchial fistulas, anastomotic infections, and post-transplant bronchomalacia [68].

The pathogenesis is multifactorial, with airway ischemia playing a central role. During transplantation, the bronchial circulation is interrupted and airway perfusion initially depends on retrograde flow from the pulmonary circulation until collateralization develops, typically over 2–4 weeks. During this vulnerable period, ischemia may trigger inflammation, impaired epithelial repair, necrosis, and abnormal remodeling of the airway wall.

Additional risk factors include prolonged ischemic time, primary graft dysfunction, perioperative hemodynamic instability, prolonged mechanical ventilation, acute cellular rejection, and early exposure to mTOR inhibitors (e.g., sirolimus). Infectious factors—especially fungal colonization or infection at the anastomotic site—further impair healing. Surgical factors also contribute: excessive donor bronchial length, donor–recipient size mismatch, and certain anastomotic techniques may increase susceptibility to ischemia-related injury. Bronchial artery revascularization has been explored but is not routinely performed because of technical complexity and potential prolongation of ischemic time [69].

Anastomotic dehiscence is an early, potentially life-threatening complication, usually occurring within the first five weeks after transplantation. Reported incidence ranges from 1% to 10%, although clinically significant cases occur in a smaller subset. Dehiscence is primarily driven by airway necrosis from severe ischemia and may be exacerbated by infection, high-dose corticosteroids, and rejection. Clinical manifestations include persistent air leak, pneumothorax, subcutaneous emphysema, and respiratory compromise. Bronchoscopy is the diagnostic gold standard, while computed tomography provides complementary assessment of extraluminal air and associated complications [68].

Management depends on severity. Small, contained defects without ongoing air leak may be managed conservatively with antimicrobial therapy and close bronchoscopic surveillance [68]. Clinically significant dehiscence often requires endobronchial stenting. Uncovered metallic stents may promote granulation tissue and epithelialization, whereas covered metallic stents can be used for larger defects. Platelet-rich plasma has been reported as an adjunct in selected small dehiscences to promote healing [70]. When conservative and endoscopic approaches fail, surgical options include reanastomosis and flap bronchoplasty; retransplantation may be considered when other strategies are not feasible [69].

Anastomotic stenosis is the most common airway complication and has been reported in up to 40% of patients. It typically develops within 2–9 months after transplantation [68,69] and is associated with ischemia, severe reperfusion injury, and early rejection. Stenosis may remain localized to the anastomosis or extend distally and, in severe cases, progress toward vanishing airway syndrome, which carries a poor prognosis [68].

First-line management is balloon bronchoplasty, often combined with mechanical debulking or cold techniques to remove fibrotic tissue. Refractory or recurrent stenosis frequently requires endobronchial stenting, which may improve airway patency and outcomes compared with dilation alone. Silicone and metallic stents are widely used; biodegradable stents are promising but long-term data are limited [71]. Additional bronchoscopic therapies include cryoablation, laser photoresection, electrocautery, and airway brachytherapy. Cold techniques (e.g., cryotherapy, cryospray) are often preferred over thermal modalities because they reduce mucosal injury and granuloma formation [72]. Topical mitomycin-C [73] and submucosal steroid injection have been used to delay restenosis, although controlled trials are lacking. When endoscopic management fails, surgical approaches such as wedge bronchoplasty, sleeve resection of the bronchus intermedius, lobectomy, or retransplantation may be considered [69].

Granulation tissue formation develops in up to 20% of recipients and is often associated with fungal infection, particularly *Aspergillus* spp. It may cause fixed airway obstruction, functional decline, and recurrent infections. Treatment is bronchoscopic and severity-guided; cryotherapy is commonly preferred to limit mucosal injury and recurrent inflammation [69].

Bronchial fistulas (bronchopleural, bronchomediastinal, bronchovascular) arise from prolonged ischemia and necrosis. Bronchopleural fistulas may present with dyspnea, hypotension, subcutaneous emphysema, and pneumothorax, and require tube thoracostomy when pneumothorax is present, together with broad-spectrum antimicrobials and antifungal therapy. Management may be surgical (open drainage, direct closure with flap, thoracoplasty, transsternal bronchial closure) [73] or

bronchoscopic (fibrin glue for small defects; metallic stents for larger proximal fistulas). Occlusion devices (e.g., Amplatzer) may be used in selected cases [69] .

Bronchomediastinal fistulas typically present with mediastinal infection. Aerosolized antibiotics are standard, but severe cases may require mediastinal debridement. Bronchovascular fistulas are associated with very high morbidity and risk of massive hemorrhage. Emergent bilobectomy or pneumonectomy may succeed in selected cases, but outcomes are generally poor [75]. Retransplantation may be considered when other strategies fail.

Post-transplant bronchomalacia is characterized by dynamic expiratory airway collapse, localized or diffuse. Patients may present with dyspnea, chronic cough, recurrent infections, and progressive airflow limitation. Diagnosis requires dynamic bronchoscopy. Management is symptom-driven and ranges from airway clearance and noninvasive ventilation to temporary or permanent stenting in severe cases. Surgical options (resection, reconstruction, tracheoplasty) and retransplantation are rarely pursued [69].

Anastomotic infections, particularly fungal infections, are clinically significant due to impaired mucociliary clearance, local immune dysfunction, and intense immunosuppression. Distinguishing colonization from invasive infection may be challenging. Diagnosis relies on bronchoscopic sampling, and management includes prophylaxis when indicated, targeted antimicrobial therapy, and close surveillance to prevent progression to necrosis or dehiscence [69].

1.6.1.2 Vascular complications

Vascular complications are relatively uncommon (≈1–3%) but carry high morbidity and mortality. They primarily involve pulmonary veins or arteries and typically present early after transplantation.

Pulmonary venous complications (stenosis, thrombosis) are more common than arterial events and often involve lower pulmonary veins, particularly the left lower vein, likely due to anatomical factors. Increased incidence has been described in women with pulmonary fibrosis, possibly related to smaller thoracic dimensions. Venous obstruction usually occurs within hours of transplantation and may present with severe hypoxemia, pulmonary edema, and lobar infiltrates [76]. Diagnosis relies on echocardiography or, more commonly, CT angiography, which provides detailed assessment of venous patency and anastomotic integrity. Partial obstruction may be managed with anticoagulation and close monitoring, whereas complete obstruction or clinical deterioration may require urgent

surgical intervention, including lobectomy or retransplantation. In selected stable patients, delayed endovascular approaches (angioplasty, stenting) may be considered.

Pulmonary arterial complications are less frequent and may present later with persistent hypoxemia and pulmonary hypertension. CT angiography or arteriography is essential for diagnosis [76]. Mild narrowing is often clinically insignificant; however, significant stenosis due to kinking or thrombosis may require balloon dilation, stenting, and anticoagulation [77].

1.6.1.3 Pleural and thoracic complications

Pleural and thoracic complications include hemothorax, pneumothorax, prolonged air leak, and chylothorax. Hemothorax is the most frequent surgical complication and the leading cause of early reintervention. It is rarely due to vascular anastomotic bleeding and more commonly results from bleeding at divided pleural adhesions. Risk factors include extensive adhesions, prior thoracic surgery (especially pleurodesis), cardiopulmonary bypass, and pretransplant antiplatelet/anticoagulant therapy. Management typically requires prompt surgical re-exploration to achieve hemostasis and correct coagulopathy.

Prolonged postoperative air leak is uncommon but may follow parenchymal injury during explantation or graft handling, particularly with dense adhesions. Bronchial anastomotic complications should be excluded by bronchoscopy. Most cases resolve with conservative management and continued drainage.

Pneumothorax is relatively uncommon and may develop after chest tube removal when donor–recipient size mismatch prevents full graft expansion, particularly in recipients with severe emphysema and pretransplant hyperinflation. In single-lung transplantation, pneumothorax may also involve the native lung.

In single-lung transplantation for emphysema, persistent hyperinflation of the native lung may cause mediastinal shift and compromise the allograft; early extubation is often recommended to mitigate this risk.

Chylothorax is rare and usually results from thoracic duct injury during mediastinal dissection. Most cases are managed conservatively with dietary modification and prolonged drainage [76].

1.6.1.4 Wound and nerve complications

Wound complications are relatively uncommon. In single-lung transplantation, partial thoracotomy dehiscence may occur, particularly in obese patients or those receiving high-dose preoperative corticosteroids. Minor dehiscences are generally managed conservatively, whereas larger defects affecting respiratory mechanics may require early surgical revision. In bilateral transplantation, transverse sternotomy dehiscence can occur, especially in emphysema with an expanded thorax; reinforced sternal wiring reduces risk. Wound hematomas usually arise from bleeding along muscle planes and are more common with prior antiplatelet/anticoagulant therapy; most are managed with compression, with drainage reserved for larger collections. Surgical site infections are uncommon despite immunosuppression and typically require debridement only if deep tissues are involved.

Injury to the phrenic, recurrent laryngeal, or vagus nerves may occur during mediastinal dissection. Phrenic nerve injury is clinically most relevant, impairing diaphragmatic function and cough and potentially delaying extubation; it is more likely in patients with dense adhesions (e.g., bronchiectasis, silicosis). In bilateral transplantation, dysfunction may also result from traction and is often transient. Recurrent laryngeal nerve injury is more common on the left, particularly during dissection in the aortopulmonary window, and may cause vocal cord paralysis, dysphonia, and aspiration risk, with partial compensation often occurring over weeks. Vagus nerve injury is less common but may occur with extensive posterior mediastinal adhesions; bilateral injury can markedly impair gastrointestinal motility and cause delayed gastric emptying [76].

1.6.2 Primary graft Dysfunction (PGD)

PGD is a frequent and severe early complication after lung transplantation, typically occurring within the first 72 hours. It represents ischemia–reperfusion injury and clinically resembles ARDS. PGD is a leading cause of early post-transplant mortality and is strongly associated with adverse long-term outcomes, particularly the development of CLAD [78].

PGD is defined by hypoxemia and radiographic pulmonary edema in the absence of alternative explanations (e.g. infection, rejection, or mechanical complications). To standardize diagnosis and facilitate research, the ISHLT published consensus diagnostic criteria in 2005 and updated them in 2016 [79] (Figure 6). PGD severity is graded according to chest imaging findings and the PaO_2/FiO_2 ratio assessed at defined time points during the first 72 hours post-transplant. The updated definition accounts for contemporary supportive strategies, including high-flow oxygen therapy, pulmonary vasodilators, and ECMO; patients requiring ECMO are classified as PGD grade 3.

Grade at T_0, T_{24}, T_{48}, T_{72}	Radiographic infiltrates consistent with diffuse pulmonary edema	PaO_2:FiO_2* (P:F Ratio)
0	Absent	Any
1	Present	>300
2	Present	200-300
3	Present	<200

*measured on FiO2 = 1.0 and PEEP 5 cm H_2O

Figure 6. ISHLT diagnostic criteria for PGD

Clinically, PGD most often emerges on postoperative day 1, peaks around day 3, and gradually resolves over the subsequent week, although the clinical course varies according to recipient and procedural factors.

The pathogenesis of PGD involves a complex inflammatory cascade initiated at reperfusion, with activation of donor- and recipient-derived immune cells, neutrophil recruitment, oxidative stress, and endothelial injury, ultimately leading to increased alveolar-capillary permeability and pulmonary edema [80,81]. Beyond acute injury, this inflammatory milieu may enhance allograft immunogenicity and contribute to late dysfunction. An immunological link has been suggested by the observation of increased class II donor-specific HLA antibodies at 5 years in patients with PGD [82]. PGD severity is also associated with increased risk of BOS [83]. More recently, PGD has been linked to baseline lung allograft dysfunction (BLAD), reflecting failure of lung function to normalize after transplantation [84].

Risk factors for PGD include donor factors (e.g., advanced age, smoking history, infection), recipient characteristics (e.g., pulmonary hypertension, idiopathic pulmonary fibrosis, obesity), and procedural variables such as prolonged ischemic time, use of cardiopulmonary bypass, and single-lung transplantation. Preventive strategies include optimized donor management and lung-protective ventilation tailored to donor lung size [85].

Management of PGD is primarily supportive and largely parallels ARDS care, emphasizing lung-protective ventilation, conservative fluid management, and early ECMO in severe cases. However, PGD management typically includes less frequent use of prone positioning and neuromuscular

blockade than standard ARDS protocols, and pharmacologic strategies used in ARDS (e.g., inhaled β_2-agonists, renin–angiotensin–aldosterone system inhibitors, antioxidants) have not shown consistent benefit [86]. Importantly, donor-size–based tidal volume calculation (rather than recipient-size–based) reduces the risk of severe PGD at 48–72 hours and has been associated with lower 1-year mortality [87]. Ex vivo lung perfusion has expanded donor availability and may reduce PGD severity, although its long-term impact on outcomes remains under investigation.

1.6.3 Infectious complications after lung transplant

The lung allograft is uniquely exposed to environmental pathogens and is characterized by impaired mucociliary clearance, disrupted lymphatic drainage, denervation with reduced cough reflex, and frequent airway ischemia. Combined with potent lifelong immunosuppression, these factors place lung transplant recipients at particularly high risk for both common and opportunistic infections. Infectious complications contribute substantially to early postoperative mortality and adversely affect long-term outcomes through their association with acute rejection and CLAD [88].

The epidemiology of post-transplant infections follows a characteristic timeline that reflects the net state of immunosuppression and the source of pathogens. In the early postoperative period (first month), infections are predominantly bacterial and are commonly related to donor-derived organisms, recipient colonization, nosocomial exposure, or surgical complications (e.g., airway ischemia and anastomotic dehiscence). During the intermediate period (1–6 months), opportunistic infections become more prominent, including cytomegalovirus (CMV), invasive fungal infections, and *Pneumocystis jirovecii* (PJP). In the late post-transplant period (>6 months), community-acquired respiratory viruses and infections associated with CLAD and chronic immunosuppression predominate. Awareness of this timeline is essential to guide diagnostic evaluation, prophylaxis, and empiric therapy [89].

Bacterial infections are the most frequent infectious complications after lung transplantation and remain a leading cause of hospitalization. The lower respiratory tract is the predominant site, reflecting direct exposure of the allograft to inhaled pathogens. Gram-negative organisms—particularly *Pseudomonas aeruginosa*—are common, especially in recipients with CF or bronchiectasis who frequently have longstanding pre-transplant colonization. Gram-positive pathogens, including *Staphylococcus aureus*, also contribute substantially, particularly early after transplantation.

Beyond pneumonia, bacterial infections may involve the pleural space, bloodstream, surgical sites, and intravascular catheters. Persistent colonization and recurrent infections are frequent long-term and have been associated with progressive decline in graft function. Mycobacterial infections, including *Mycobacterium tuberculosis* and non-tuberculous mycobacteria (NTM), are clinically challenging because of delayed diagnosis, prolonged multidrug therapy, and interactions with immunosuppressive regimens [90].

CMV is the most clinically important viral infection after lung transplantation and occurs more frequently than in other solid organ recipients. Manifestations range from asymptomatic viremia to CMV syndrome (fever, malaise, myalgias/arthralgias, leukopenia and thrombocytopenia) and tissue-invasive disease, including pneumonitis, encephalitis, retinitis, hepatitis, and colitis. CMV has also been linked to acute rejection, CLAD, and reduced long-term survival, and may exert broader immunomodulatory effects that predispose to other opportunistic infections [88].

Two preventive strategies are used:

- **Universal prophylaxis** for high- and intermediate-risk recipients (typically all except D−/R−), most commonly with oral valganciclovir.
- **Pre-emptive therapy**, in which valganciclovir is initiated once viral replication exceeds a defined threshold on surveillance testing, aiming to prevent progression to clinical disease.

Current international consensus guidelines generally favor prophylaxis because of its established efficacy and safety [91]. The AST Infectious Diseases Community of Practice recommends 12 months of prophylaxis for CMV D+/R− lung transplant recipients and 6–12 months for D+/R+ and D−/R+ recipients, individualized according to risk of reactivation, drug toxicity, and viral load monitoring. Prophylaxis is typically started with intravenous ganciclovir (with renal dose adjustment) and transitioned to oral valganciclovir once oral absorption is reliable. After treatment with anti-lymphocyte antibodies or pulse steroids, CMV prophylaxis should be extended for at least 1–3 months following completion of the anti-rejection regimen. CMV immune globulin may be used as an adjunct in selected high-risk patients but should not be used [92]. A pre-emptive approach is often useful after prophylaxis ends, based on close viral load monitoring.

Active CMV infection is treated with ganciclovir or valganciclovir, with dose adjustment for renal function. Both agents can cause significant myelotoxicity (leukopenia, neutropenia, thrombocytopenia). Oral valganciclovir is non-inferior to intravenous ganciclovir for non–life-threatening CMV disease and is preferred in these [93], whereas intravenous ganciclovir remains standard for severe or tissue-invasive disease. Therapy is continued until clinical resolution and

virologic clearance, commonly defined as two consecutive negative viral load measurements at least one week apart, for a minimum of two weeks, followed by secondary prophylaxis with oral valganciclovir for 1–3 months to reduce relapse risk. In confirmed ganciclovir-resistant CMV, foscarnet or cidofovir may be considered, although nephrotoxicity and limited experience constrain their use.

Influenza, respiratory syncytial virus, parainfluenza, adenovirus, and rhinovirus are increasingly recognized with modern molecular diagnostics. These infections may precipitate acute graft dysfunction and have been implicated in the development and progression of CLAD.

Donor-to-recipient transmission is a major route of Epstein Barr Virus (EBV) acquisition, particularly in EBV-seronegative recipients receiving an organ from a seropositive donor; approximately 10% of lung transplant recipients have this high-risk mismatch. In the setting of impaired T-cell surveillance, EBV-driven B-cell proliferation can progress to post-transplant lymphoproliferative disorder (PTLD), with reported incidence of 5–15% in lung transplant recipients. Routine EBV viral load surveillance by quantitative PCR is increasingly used for early detection. Antiviral prophylaxis with acyclovir or ganciclovir has been used in some centers for high-risk mismatches, but evidence that it reduces PTLD incidence is limited [94].

Fungal infections are among the most severe complications after lung transplantation and are associated with high mortality. *Aspergillus* species are the most common pathogens (typically *A. fumigatus*). Infection is acquired via inhalation and ranges from colonization to tracheobronchitis and invasive pulmonary disease. Risk factors include graft dysfunction, intense immunosuppression, airway colonization, CMV infection, and single-lung transplantation. Tracheobronchial aspergillosis is particularly characteristic of lung transplant recipients and often involves the bronchial anastomosis, where it can contribute to stenosis, dehiscence, or bleeding.

Even when non-invasive, *Aspergillus* colonization is clinically relevant because it increases the risk of BOS/CLAD and progression to invasive disease [95]. Surveillance with chest CT and bronchoscopy supports early detection. Voriconazole is generally first-line therapy for both tracheobronchial and invasive aspergillosis, often combined with inhaled amphotericin B for airway disease; severe cases may require combination therapy with an echinocandin. Drug–drug interactions with immunosuppressive agents require careful monitoring.

Other important fungal pathogens include *Candida*, *Cryptococcus*, mucormycetes, *Scedosporium*, and endemic fungi, which often require prolonged treatment and multidisciplinary management [90].

Effective prophylaxis has markedly reduced *PJP* pneumonia, although breakthrough cases still occur and can be severe. Other opportunistic infections (e.g., *Toxoplasma gondii* and rare parasitic or fungal pathogens) are uncommon but may cause disseminated disease. Vaccination-preventable infections remain an important consideration, underscoring the need for optimized immunization before and after transplantation.

Diagnosing infection in lung transplant recipients is challenging because inflammatory responses may be blunted and clinical features overlap with rejection. Evaluation typically requires a combination of imaging, bronchoscopy with bronchoalveolar lavage, microbiologic cultures, and molecular diagnostics. Preventive strategies—including antimicrobial prophylaxis, structured surveillance, vaccination, and careful adjustment of immunosuppression—are central to reducing infectious risk while preserving graft function.

Pathogen	Timing	Prevention	Treatment
Bacterial Infections	**Early Post-transplant (0-1 month)**	- Antimicrobial prophylaxis based on donor/recipient screening. - Strict infection control practices.	- Empirical broad-spectrum antibiotics (e.g., Piperacillin/tazobactam, Meropenem).
Pseudomonas aeruginosa		- Prophylaxis for high-risk patients (e.g., CF, bronchiectasis). - Early antibiotic initiation.	- Beta-lactam/beta-lactamase inhibitor combination (e.g., piperacillin-tazobactam), or cephalosporins (e.g., cefepime), or carbapenems (e.g., meropenem).
Staphylococcus aureus		- Empirical broad-spectrum antibiotics.	- MRSA: Vancomycin or Linezolid. - MSSA: Nafcillin or Cefazolin.
Mycobacterium tuberculosis	**Post-Transplant (3-12 months)**	- Pre-transplant screening for latent TB. - Prophylaxis if indicated.	- Standard TB regimen (e.g., Rifampin, Isoniazid, Pyrazinamide, Ethambutol) for active infection.

Viral Infections	**Early Post-transplant (0-3 months)**	- CMV prophylaxis (valganciclovir/ganciclovir). - Risk-based strategies based on donor-recipient serology.	- CMV: Valganciclovir or Ganciclovir. - EBV: Rituximab (in cases of PTLD).
Cytomegalovirus (CMV)		- Preemptive therapy based on viral load monitoring.	- Ganciclovir or Valganciclovir.
Epstein-Barr Virus (EBV)	**Post-Transplant (3-12 months)**	- EBV viral load monitoring to adjust immunosuppression. - Prophylaxis for high-risk patients.	- Rituximab (for PTLD or EBV reactivation).
Respiratory Viruses	**Late Post-transplant (6 months+)**	- Annual influenza vaccination. - Early identification and management of respiratory infections.	- Supportive care (e.g., antivirals for influenza: Oseltamivir). - Steroids for severe inflammation (e.g., RSV).
Influenza, Respiratory Syncytial Virus, Parainfluenza		- Avoidance of sick contacts.	- Antiviral therapy for Influenza (Oseltamivir). - RSV: Ribavirin (in severe cases).
Fungal Infections	**Post-Transplant (1-6 months)**	- Systemic antifungal prophylaxis (e.g., voriconazole, itraconazole). - Inhaled amphotericin B for high-risk patients.	- Aspergillus: Voriconazole, or Amphotericin B. - Candida: Fluconazole or Echinocandins (e.g., Caspofungin).
Aspergillus spp.		- Routine fungal screening (e.g., galactomannan assay).	- Voriconazole, Amphotericin B (for invasive cases).
Candida spp.		- Early initiation of antifungal therapy for invasive infections.	- Fluconazole or Echinocandins (Caspofungin).
Opportunistic Infections	**Post-Transplant (0-3 months)**	- Pneumocystis jirovecii pneumonia (PJP) prophylaxis (trimethoprim-sulfa ↓ oxazole). - Early screening and treatment of *Nocardia* and *Clostridioides difficile*.	- PJP: Trimethoprim-sulfamethoxazole (TMP-SMX) or Dapsone. - Nocardia: Trimethoprim-sulfamethoxazole. - C. difficile: Metronidazole or Vancomycin.
Pneumocystis jirovecii		- Prophylactic antibiotics for *Nocardia*. - Stool monitoring for *C. difficile* infections.	- TMP-SMX for PJP. - Metronidazole or oral Vancomycin for C. difficile.
Mycobacterial Infections	**Post-Transplant (3-12 months)**	- Screening for *Nontuberculous Mycobacteria* (NTM) pre-transplant. - Antimycobacterial therapy for active infections.	- Antimycobacterial therapy (e.g., Clarithromycin, Rifampin, Ethambutol).
Nontuberculous Mycobacteria		- Early identification and tailored therapy.	- Mycobacterium avium complex (MAC): Clarithromycin, Ethambutol, Rifampin.
Other Pathogens	**Late Post-transplant (6 months+)**	- Prophylaxis and surveillance based on p ↓ t risk factors.	- Aggressive antifungal treatment (for invasive Cryptococcus, Aspergillus).

Cryptococcus spp.	- Fungal culture and antigen testing in symptomatic patients.	- Fluconazole or Amphotericin B for invasive Cryptococcus.
Aspergillus fumigatus	- Aggressive antifungal treatment for invasive infections.	- Voriconazole, Amphotericin B (for invasive Aspergillus).

Table 1 summarizes the most relevant infections by timing of onset and outlines common prevention and treatment strategies.

1.6.4 Medical complications after lung transplant

Medical complications after lung transplantation are common and are largely driven by lifelong immunosuppression, perioperative stress, and pre-existing comorbidities, although overall cardiovascular mortality remains relatively low (<5%). Frequent metabolic and cardiovascular complications include post-transplant diabetes mellitus—often developing within the first year and promoted by CNIs and corticosteroids—hypertension, which tends to increase over time and is influenced by volume overload, calcineurin inhibitor exposure, and acute kidney injury, and dyslipidemia, which frequently requires dietary intervention and pharmacologic treatment with statins or fibrates. When lipid-lowering therapy is used, careful attention to drug–drug interactions and the risk of rhabdomyolysis, particularly in combination with calcineurin inhibitors, is required. Rare but severe metabolic complications, such as hyperammonemia—sometimes related to donor-derived infections (e.g., *Ureaplasma* or *Mycoplasma*)—may be rapidly fatal and require early recognition and targeted antimicrobial therapy. Atrial arrhythmias are particularly common in the early postoperative period and are associated with prolonged mechanical ventilation and adverse outcomes [96].

Renal dysfunction represents a major medical complication after lung transplantation. Acute kidney injury occurs in a substantial proportion of recipients early after transplantation and is associated with hemodynamic instability, blood transfusions, extracorporeal membrane oxygenation, prolonged mechanical ventilation, nephrotoxic antimicrobial exposure, and CNI use. Acute kidney injury increases length of hospitalization, short-term mortality, and the risk of progression to chronic kidney disease, which becomes increasingly prevalent with longer post-transplant survival. Preventive strategies include meticulous fluid and renal perfusion management, close monitoring of serum creatinine and urine output, and careful therapeutic drug monitoring of CNIs. In selected high-risk patients, induction strategies that allow delayed initiation or minimization of CNIs may be beneficial [96].

Gastrointestinal complications range from early, potentially life-threatening events—such as ileus or bowel perforation—to chronic disorders including gastroesophageal reflux disease and gastroparesis. These chronic conditions are of particular clinical importance, as reflux and impaired gastrointestinal motility have been associated with worse allograft outcomes. Management typically includes acid suppression and prokinetic agents, with early anti-reflux surgery considered in selected patients. Gastrointestinal bleeding requires prompt evaluation, as etiologies may include peptic ulcer disease, ischemic or CMV colitis, post-transplant lymphoproliferative disorder, and other transplant-related conditions [97].

Hematologic complications are frequently medication-related and include cytopenias and anemia, which require exclusion of infection and nutritional deficiencies[96]. CNIs may rarely cause thrombotic microangiopathy, which is managed primarily by withdrawal or substitution of the offending agent; supportive therapies such as plasmapheresis are sometimes employed, although their benefit remains uncertain [98]. Venous thromboembolism is common in the early post-transplant period and may be clinically significant, including pulmonary embolism; standardized thromboprophylaxis and anticoagulation protocols are therefore essential [99].

Neurologic complications—including delirium, seizures, encephalopathy, and stroke—may occur throughout the post-transplant course and are influenced by cardiopulmonary bypass or ECMO exposure, acute kidney injury, severe primary graft dysfunction, hypertension, and immunosuppressant-related neurotoxicity. Posterior reversible encephalopathy syndrome (PRES) is a rare but important complication associated with calcineurin inhibitors, typically occurring in the early postoperative period. PRES manifests with altered mental status, seizures, visual disturbances, or cortical blindness, and brain MRI reveals characteristic cortical and subcortical hyperintensities in the occipito-parietal regions consistent with vasogenic edema. Management includes temporary cessation of calcineurin inhibitors, substitution with alternative immunosuppressive agents (e.g., basiliximab), blood pressure control, and seizure management. Prognosis is generally favorable, and recurrence is uncommon when precipitating factors are adequately addressed [100].

Bone and musculoskeletal complications are prominent following lung transplantation. Many candidates have pre-existing low bone mineral density, and post-transplant corticosteroid exposure and immobility further accelerate osteoporosis and fracture risk. Preventive and therapeutic strategies include calcium and vitamin D supplementation, weight-bearing physical activity, and bisphosphonate therapy when indicated, along with minimization of steroid exposure when feasible. Deconditioning and muscle weakness—including steroid myopathy and critical illness–associated

weakness—are common and can substantially impair quality of life, making structured rehabilitation a cornerstone of post-transplant care [101].

Psychiatric complications are frequent after lung transplantation. Mood and anxiety disorders affect approximately one-third of recipients within the first post-transplant year and often persist long term. Pre-existing psychiatric disease is the strongest risk factor, while reduced functional status, limited social support, prolonged waiting time, female sex, and early post-transplant complications further increase vulnerability. These disorders are associated with poorer clinical outcomes and higher complication rates. Post-traumatic stress disorder may also develop, particularly following traumatic intensive care experiences, with younger age, prior psychiatric illness, and ECMO as a bridge to transplantation identified as risk factors. Post-transplant delirium is common early after surgery and is associated with longer hospitalization and worse neurologic outcomes, although not with increased mortality; management is primarily supportive and focused on early identification and correction of contributing factors [96].

Finally, lung transplantation is associated with a markedly increased risk of malignancy due to long-term immunosuppression and impaired immune surveillance. Cancer represents the second leading cause of death five to ten years after transplantation, accounting for approximately 17% of late mortality [102]. Cutaneous squamous cell carcinoma is the most common post-transplant malignancy, and routine dermatologic surveillance is recommended for all recipients. Single-lung transplant recipients are at increased risk of lung cancer in the native lung—particularly those with emphysema or idiopathic pulmonary fibrosis—while recipients with cystic fibrosis remain predisposed to gastrointestinal malignancies [103].

Virus-associated malignancies are especially prevalent, including PTLD, Kaposi sarcoma, and anogenital cancers. PTLD comprises a spectrum of lymphoid proliferations most commonly driven by EBV infection. Its incidence in lung transplant recipients ranges from 2% to 9%, but is substantially higher in EBV-seronegative recipients receiving organs from EBV-seropositive donors. PTLD may occur at any time after transplantation and is associated with poor long-term outcomes. Management centers on careful reduction of immunosuppression, with adjunctive therapies including rituximab, chemotherapy, and localized interventions as indicated. Preventive strategies such as EBV viral load monitoring remain key components of post-transplant surveillance [96].

1.7 Immunological complications after lung transplant

Immunological complications remain a major determinant of both short- and long-term outcomes after lung transplantation. Owing to the lung's continuous exposure to environmental antigens and its rich population of resident immune cells, the lung allograft is particularly susceptible to immune-mediated injury. Despite major advances in immunosuppressive therapy, alloimmune responses directed against the donor organ continue to pose significant clinical challenges. These complications encompass a broad spectrum, ranging from hyperacute and acute rejection to AMR and CLAD, each characterized by distinct pathophysiological mechanisms and clinical implications.

1.7.1 Hyperacute rejection

Hyperacute rejection is a form of antibody-mediated rejection driven by pre-existing circulating antibodies in the recipient that recognize antigens expressed on the donor lung endothelium. These antibodies are most commonly directed against human leukocyte antigen (HLA) molecules or ABO blood group antigens [104, 105]. Sensitization may result from prior blood transfusions, previous organ transplantation, or pregnancy. Upon reperfusion of the transplanted lung, recipient antibodies bind to their corresponding antigens on the vascular endothelium of the allograft, triggering immediate activation of the complement cascade. Complement activation leads to endothelial injury, increased vascular permeability, platelet aggregation, and widespread intravascular thrombosis. The resulting microvascular occlusion produces severe ischemia and acute alveolar injury, rapidly rendering the graft nonfunctional. Histopathologically, hyperacute rejection is characterized by diffuse capillary thrombosis, endothelial necrosis, hemorrhage, and extensive pulmonary edema. Once established, this cascade results in irreversible graft damage and necrosis [106].

Clinically, hyperacute rejection manifests dramatically at the time of transplantation or shortly thereafter. The transplanted lung may become acutely edematous and congested during surgery, with visible swelling and impaired ventilation shortly after reperfusion. Severe hypoxemia, pulmonary hypertension, and hemodynamic instability rapidly ensue. Radiographic imaging typically demonstrates diffuse, homogeneous infiltrates involving the entire allograft, consistent with widespread pulmonary edema.

The clinical course is often fulminant, progressing quickly to graft failure despite maximal supportive care. If the affected lung remains implanted, systemic inflammatory responses may develop, including fever, malaise, and multi-organ dysfunction [107].

The diagnosis of hyperacute rejection is primarily clinical, based on the timing of graft failure in relation to transplantation and the exclusion of other causes such as primary graft dysfunction, technical complications, or infection. The presence of pre-formed donor-specific antibodies (DSAs), positive crossmatch testing, or known ABO incompatibility strongly supports the diagnosis. Histologic confirmation is rarely feasible in the acute setting but may demonstrate extensive vascular thrombosis and complement-mediated endothelial injury.

Therapeutic options for hyperacute rejection are limited and generally ineffective once irreversible injury has occurred. Interventions such as plasma exchange, aggressive immunosuppression, and complement inhibition have been attempted with limited success. Emergency retransplantation may be considered in select cases but is rarely feasible due to the rapid deterioration of the patient and limited organ availability. Consequently, the prognosis of hyperacute rejection remains extremely poor, with mortality approaching 100% in reported series.

The incidence of hyperacute rejection has dramatically declined due to improvements in pre-transplant immunologic evaluation, including sensitive crossmatch techniques and detailed screening for anti-HLA and anti-ABO antibodies. As a result, this form of rejection is now exceedingly rare in contemporary lung transplantation practice.

ABO-incompatible transplantation is generally avoided in adult recipients but may be performed in selected pediatric patients, particularly infants, whose immune systems are not fully mature and lack isohemagglutinins. Ongoing organ shortages and prolonged waitlist mortality have renewed interest in expanding ABO-incompatible transplantation strategies in carefully selected populations; however, this approach requires specialized protocols and remains investigational in lung transplantation [108].

1.7.2 Acute cellular rejection (ACR)

ACR is one of the most frequent immunological complications following lung transplantation and a major contributor to early graft dysfunction and long-term allograft failure. Despite advances in surgical techniques and immunosuppressive strategies, ACR remains common and is a well-established risk factor for the development of CLAD, particularly BOS [109]. Understanding its incidence, pathogenesis, diagnosis, and management is therefore central to post-transplant care.

Reported ACR incidence varies widely according to patient population, surveillance intensity, biopsy protocols, and treatment thresholds. According to the ISHLT registry, approximately 28% of lung transplant recipients experience at least one treated episode of acute rejection within the first post-

transplant year [31]. In contrast, data from the Organ Procurement and Transplantation Network and Scientific Registry of Transplant Recipients report a lower incidence of approximately 17% [110].

Higher rates of ACR have consistently been reported in randomized controlled trials evaluating immunosuppressive regimens. Studies comparing MMF with everolimus in combination with cyclosporine report first-year rejection rates exceeding 35–45%. Similarly, trials comparing tacrolimus- and cyclosporine-based regimens show that nearly half of recipients experience at least one histologic rejection episode[48]. More recent studies using systematic surveillance biopsies have identified histologic ACR in up to 60% of recipients, underscoring the high prevalence of subclinical rejection [111]. These differences largely reflect variability in surveillance strategies, biopsy timing, histopathologic interpretation, and treatment criteria.

ACR is predominantly a T-cell–mediated immune process driven by recipient recognition of donor MHC or HLA molecules via both direct and indirect allorecognition pathways. Early after transplantation, donor antigen-presenting cells directly activate recipient T cells, whereas over time recipient antigen-presenting cells process donor antigens and stimulate indirect responses. Ischemia–reperfusion injury and innate immune activation further amplify these adaptive responses, leading to endothelial and epithelial injury through cytokine release and cytotoxic mechanisms [112].

Clinically, ACR spans a broad spectrum. Many episodes are asymptomatic and detected only by surveillance biopsies. When present, symptoms are nonspecific and include dyspnea, cough, sputum production, low-grade fever, hypoxemia, or a decline in pulmonary function, most commonly reduced FEV_1. Symptomatic disease is more often associated with higher-grade rejection (≥A2). In severe cases, ACR may present with diffuse alveolar damage and a clinical picture resembling acute respiratory distress syndrome. Radiographic findings are variable and nonspecific, requiring exclusion of infection, airway complications, or primary graft dysfunction [112].

Diagnosis relies on histopathologic assessment of transbronchial lung biopsies. ISHLT guidelines recommend sampling at least five pieces of alveolated lung parenchyma to improve diagnostic yield. Histologically, ACR is characterized by perivascular and interstitial mononuclear infiltrates and is graded from A0 (no rejection) to A4 (severe rejection) based on the extent and intensity of inflammation. Although higher grades generally correlate with greater clinical severity, sampling error and interobserver variability remain important limitations [113].

Lymphocytic bronchiolitis, defined as small-airway inflammation without identifiable infection, is graded separately from vascular rejection (B1R–B2R) (figure 7). It has been independently associated with increased risk of BOS and mortality, even in the absence of concurrent vascular rejection, although optimal management remains uncertain [111].

	Grade	Meaning	Appearance
A-grade: Perivascular inflammation	0	None	Normal lung parenchyma.
	1	Minimal	Scattered, infrequent small mononuclear perivascular infiltrates. No eosinophils.
	2	Mild	More frequent perivascular infiltrates identifiable at low magnification. Eosinophils may be present.
	3	Moderate	Dense perivascular infiltrates, eosinophils and neutrophils common. Pathognomonic feature is extension into alveolar septae and airspaces.
	4	Severe	Diffuse perivascular, interstitial and air-space infiltrates with pneumocyte damage and features of acute lung injury.
B-grade: Airway-associated inflammation	0	None	No evidence of bronchiolar inflammation.
	1R	Low grade	Single-layer mononuclear cells in bronchiolar submucosa.
	2R	High grade	Larger infiltrates of larger and activated lymphocytes in bronchiolar submucosa, with potential involvement of eosinophils and plasmacytoid cells.
	X	Ungradable	No bronchiolar tissue available.

R = revised

Figure 7. Histopathologic grading of acute cellular rejection according to ISHLT criteria [113]

Because ACR is frequently asymptomatic, many centers perform routine surveillance bronchoscopy during the first post-transplant year; approximately two-thirds of programs report this practice [112]. Evidence supporting routine surveillance is mixed, with some studies showing no difference in rejection rates or BOS-free survival compared with clinically indicated bronchoscopy, while others demonstrate detection of clinically significant rejection and concurrent infections [114,115]. Consequently, surveillance strategies are largely center-specific.

There is broad consensus that moderate to severe ACR (grade ≥A2) warrants treatment. First-line therapy consists of high-dose intravenous methylprednisolone for three days, followed by an oral prednisone taper, although optimal dosing and duration have not been established in randomized trials. Management of minimal or asymptomatic ACR (grade A1) remains controversial; although associated with increased BOS risk, routine treatment has not consistently demonstrated a survival benefit. Follow-up bronchoscopy is often used to assess resolution or progression [116].

Refractory or recurrent ACR remains a major therapeutic challenge. Management options include repeated corticosteroid pulses, optimization of baseline immunosuppression, and antibody-based lymphocyte-depleting therapies such as antithymocyte globulin or alemtuzumab. Additional strategies, including extracorporeal photopheresis (ECP), total lymphoid irradiation (TLI), or

introduction of mTOR inhibitors, have been reported, although supporting evidence is largely observational [116].

1.7.3 Antibody mediated rejection (AMR)

AMR is a distinct form of acute rejection in lung transplantation driven predominantly by humoral immune mechanisms (table 2). In its classical form, AMR results from recipient B-cell and plasma-cell production of DSAs directed against donor HLA molecules expressed on the pulmonary microvascular endothelium. In addition to HLA-directed responses, antibodies against non-HLA self-antigens—including collagen V, K-α1 tubulin, angiotensin II type 1 receptor, and endothelin type A receptor—have been implicated in antibody-mediated graft injury and are associated with an increased risk of CLAD [117,118]

The immunobiology of pulmonary AMR is centered on two interconnected processes: generation and persistence of circulating antibodies—either preformed in sensitized recipients or arising de novo after transplantation—and antibody-mediated endothelial injury [118]. Endothelial damage may occur through complement-dependent pathways, reflected by activation products such as C4d, or via complement-independent mechanisms involving Fc-receptor–mediated recruitment and activation of neutrophils, macrophages, and natural killer cells, with downstream cytokine signaling and microvascular inflammation [119]. Importantly, the presence of circulating DSA does not uniformly translate into clinical AMR, as some patients remain stable despite detectable antibodies, whereas others develop progressive graft dysfunction, underscoring the challenge of distinguishing benign alloimmunization from subclinical or evolving injury.

The epidemiology of pulmonary AMR remains incompletely defined, with reported incidence ranging from approximately 4% to over 50%, reflecting heterogeneity in diagnostic criteria, surveillance intensity, and study populations [120]. In one cohort, the median time to diagnosis was approximately eight months, although AMR may occur at virtually any time after transplantation. Recognized risk factors include the presence of preformed or de novo DSA, recurrent or severe infections—particularly *Pseudomonas aeruginosa*—lymphocytic bronchiolitis, and, in some series, CF [121]. The absence of standardized diagnostic criteria prior to 2016 substantially limited accurate estimation of incidence and outcomes across studies.

Clinically, AMR presents across a broad spectrum, ranging from subtle and progressive declines in lung function to acute hypoxemic respiratory failure with radiographic features of diffuse lung injury [118]. AMR frequently overlaps with other post-transplant complications, including bacterial or viral

infections and high-grade acute cellular rejection, complicating recognition and delaying diagnosis [117]. Capillaritis-associated diffuse alveolar hemorrhage is an important diagnostic clue. A rare hyperacute form of AMR, driven by high-titer preformed antibodies and occurring within minutes of reperfusion, has become uncommon with modern crossmatching and ABO compatibility testing [117].

Histopathologic findings in AMR are heterogeneous and non-specific, typically reflecting patterns of acute lung injury rather than a single diagnostic lesion. Common findings include neutrophilic capillaritis, intracapillary neutrophil accumulation, diffuse alveolar damage, organizing pneumonia, and interstitial pneumonitis [122]. Although immunohistochemical staining for C4d is a well-established marker of antibody-mediated injury in kidney and heart transplantation, its diagnostic utility in lung transplantation is limited by non-specific staining, focal positivity, and inconsistent correlation with circulating DSA. Consequently, C4d-negative AMR is increasingly recognized, and absence of C4d does not exclude the diagnosis [118,123].

To address these challenges, the ISHLT published a consensus definition in 2016 establishing standardized diagnostic criteria for pulmonary AMR [119]. This framework distinguishes between clinical AMR, characterized by graft dysfunction, and subclinical AMR, in which graft function is preserved. Diagnostic certainty is graded as definite, probable, or possible based on the fulfillment of four elements: exclusion of alternative causes of graft dysfunction, compatible histopathology, detection of circulating DSA, and supportive evidence such as C4d staining. This multidisciplinary approach emphasizes integration of clinical, immunologic, and pathologic data rather than reliance on any single test.

Management of pulmonary AMR remains largely empirical, as no randomized controlled trials or standardized treatment algorithms exist. Therapeutic strategies are extrapolated from experience in other solid organ transplants and aim to reduce circulating antibodies, suppress B-cell and plasma-cell activity, and limit ongoing immune-mediated injury. Common components include plasmapheresis or immunoadsorption for antibody removal, intravenous immunoglobulin for immunomodulation, B-cell–directed therapy with rituximab, and, in selected or refractory cases, proteasome inhibitors such as bortezomib or carfilzomib [117,118,121,124]. Complement inhibition has been reported in limited contexts, though evidence in lung transplantation remains sparse. In patients with moderate to severe graft dysfunction, combination regimens—often repeated cycles of plasmapheresis alternating with IVIG—are frequently applied, with escalation guided by clinical response and DSA kinetics. Persistent DSA is associated with an increased risk of CLAD and reduced survival [125].

Because histopathologic diagnosis relies on invasive transbronchial biopsy and is limited by sampling error and interobserver variability, substantial effort has focused on developing noninvasive biomarkers for acute rejection. Donor-derived cell-free DNA (dd-cfDNA) reflects donor cell injury and apoptosis and has emerged as a promising tool. Elevated dd-cfDNA levels correlate with acute lung allograft dysfunction and may precede histologic confirmation of AMR [126]. Longitudinal changes appear more informative than absolute thresholds, although elevations may also occur during symptomatic respiratory viral infections, limiting specificity [127].

In parallel, transcriptomic profiling of lung tissue and bronchoalveolar lavage fluid has demonstrated distinct molecular signatures during acute rejection, including increased expression of genes associated with cytotoxic T-cell activity [128]. Donor-derived extracellular vesicles, particularly exosomes, represent another emerging biomarker platform, with altered RNA profiles identified in bronchoalveolar lavage samples during acute rejection [129].

	Acute cellular rejection	Antibody-mediated rejection
Pathophysiology	T-cell mediated	B-cell/plasma-cell mediated
Incidence	17–55% in 1st year	4–50%
Risk factors	• HLA Mismatch • Younger Age • Immunosuppression choice	• Pseudomonal infection • Lymphocytic bronchiolitis • Preformed DSA • De novo DSA posttransplant
Diagnosis	Transbronchial biopsy	Allograft dysfunction, other causes excluded, lung histology, C4d staining, DSA
Histopathology	Mononuclear cell infiltration of arterioles and bronchioles	Capillary neutrophilic inflammation, capillaritis, acute lung injury, diffuse alveolar damage
Treatment	• High dose glucocorticoids • Lymphodepletion therapy in refractory cases	• Plasmapheresis • IVIG • Bortezomib • Rituximab

Table 2. Acute rejection subtypes summary. From [116]

1.7.4 Chronic rejection or CLAD

CLAD represents the principal limitation to long-term survival following lung transplantation and remains the leading cause of late graft failure and mortality. Despite substantial advances in surgical techniques, immunosuppressive regimens, and perioperative management, CLAD continues to affect a significant proportion of lung transplant recipients, resulting in progressive respiratory impairment,

reduced health-related quality of life, and increased healthcare utilisation. Overall, CLAD develops in up to 50% of patients within five years after lung transplantation [43] , although reported incidence varies considerably among transplant centres, with some institutions describing lower rates ranging from approximately 18% to 33% [130].

CLAD is defined as a persistent and otherwise unexplained decline in lung function after lung transplantation. According to ISHLT consensus documents, CLAD is diagnosed when a sustained reduction in FEV_1 of at least 20% from baseline is observed. Baseline FEV_1 is defined as the mean of the two highest post-transplant FEV_1 values obtained at least three weeks apart [131].

The diagnostic process for CLAD follows a stepwise approach (Figure 8). An initial decline in FEV_1 of ≥20% identifies possible CLAD. If lung function fails to recover after appropriate treatment of reversible causes and remains impaired for at least three weeks, probable CLAD is diagnosed. A definitive diagnosis is established when the decline persists for three months or longer despite adequate evaluation and management of alternative etiologies, including infection, acute rejection, airway complications, or pleural disease. Once CLAD is confirmed, disease severity is staged according to the degree of FEV_1 reduction, ranging from stage 1 (>65–80% of baseline) to stage 4 (≤35%)[131].

CLAD stage	FEV_1 (% of baseline)
1	>65–80%
2	>50–65%
3	>35–50%
4	≤35%

Figure 8. Staging of CLAD as defined in the 2019 ISHLT consensus document [131].

CLAD is not a single disease entity but rather a heterogeneous syndrome encompassing multiple phenotypes with distinct functional, radiological, pathological, and prognostic characteristics. The two principal phenotypes are BOS and restrictive allograft syndrome (RAS). In addition, mixed, undefined, and unclassified phenotypes are increasingly recognised.

A subset of lung transplant recipients develops an obstructive form of graft dysfunction that may be partially or completely reversible following azithromycin therapy, termed azithromycin-responsive allograft dysfunction (ARAD). This condition is characterised by a predominantly inflammatory

milieu within the allograft, typically reflected by marked neutrophilic inflammation in bronchoalveolar lavage (BAL) fluid, with neutrophils exceeding 15% in the absence of active infection. In contrast to CLAD, which generally follows a progressive and irreversible course, ARAD is distinguished by measurable improvement in lung function after initiation of low-dose azithromycin. Approximately 40% of patients initially suspected of having BOS demonstrate a clinically relevant response to azithromycin, commonly defined as an increase in FEV_1 of at least 10%. Notably, BAL neutrophilia is not specific to ARAD and may also be observed in BOS, particularly in the presence of superimposed infection [132,133].

1.7.4.1 Bronchiolitis Obliterans Syndrome (BOS)

BOS is the most frequent clinical phenotype of CLAD, accounting for approximately 60–75% of cases across multiple cohorts [134,135]. It is defined by a persistent obstructive ventilatory defect characterised by a reduced FEV_1/FVC ratio (<0.7), in the absence of sustained parenchymal opacities or pleural thickening on thoracic imaging. Lung volume measurements may demonstrate hyperinflation, reflecting small airway obstruction and air trapping, best visualised on expiratory computed tomography (CT).

CT imaging commonly reveals indirect signs of small airway disease, including bronchial wall thickening, cylindrical or varicoid bronchiectasis, mosaic attenuation, and distal airway obstruction. These features support the functional diagnosis but are not independently diagnostic [136].

BOS is regarded as the clinical surrogate of the pathological entity bronchiolitis obliterans (BO), also referred to as obliterative bronchiolitis [131]. Histologically, BO is characterised by progressive fibroproliferative narrowing and eventual obliteration of small airway lumina, typically without significant involvement of the alveolar parenchyma. Due to the patchy distribution of lesions and limited sensitivity of transbronchial biopsies, histological confirmation is rarely achievable in vivo, necessitating reliance on clinical and functional criteria [131].

Pathogenetically, BOS is believed to result from repeated epithelial microinjury caused by environmental exposures, gastro-oesophageal reflux, infections, and immune-mediated insults such as acute rejection. These injuries promote chronic inflammation, aberrant repair with epithelial-to-mesenchymal transition, recruitment of (myo)fibroblasts and circulating fibrocytes, and ultimately irreversible luminal obliteration of small airways, while adjacent alveolar tissue remains largely preserved [137].

1.7.4.2 Restrictive Allograft Syndrome (RAS)

RAS is a restrictive phenotype of CLAD defined by a sustained ≥20% decline in FEV_1 from baseline together with an additional ≥10% reduction in total lung capacity (TLC), accompanied by persistent (>3 months) parenchymal abnormalities on imaging after exclusion of alternative causes [138].

High-resolution CT typically demonstrates ground-glass opacities, consolidation, architectural distortion, and traction bronchiectasis, often with upper-lobe predominance. Using current criteria, RAS accounts for approximately 9–35% of CLAD cases, with lower prevalence reported in single-lung transplant recipients, likely reflecting challenges in interpreting TLC measurement [132, 139].

Histopathologically, RAS is frequently associated with pleuroparenchymal fibroelastosis, characterised by subpleural elastotic fibrosis with abrupt transition to relatively preserved parenchyma, distinguishing it from BOS, in which fibrosis is largely confined to the small airways [140].

Clinically, RAS follows a particularly aggressive course, with more rapid functional decline and significantly reduced survival compared with BOS. Reported median survival ranges from 8 to 12 months after diagnosis in some cohorts, compared with approximately 36 months in obstructive CLAD [139,141,142].

1.7.4.3 Mixed phenotype of CLAD

The mixed phenotype describes patients with combined obstructive and restrictive ventilatory defects together with persistent parenchymal opacities. This phenotype may be present at initial CLAD diagnosis or emerge during disease evolution, most commonly through progression from BOS. Reported prevalence ranges between 3% and 9%, with approximately 11% of BOS patients evolving to a mixed phenotype [137, 138, 143].

1.7.4.4 Undefined and unclassified phenotypes of CLAD

Undefined and unclassified phenotypes include CLAD patients who do not clearly meet criteria for established phenotypes. These cases require close longitudinal follow-up and reassessment, with heightened suspicion for alternative diagnoses mimicking CLAD [144]. Up to 28% of single-lung transplant recipients remain unclassified, compared with approximately 15% of bilateral recipients, largely due to confounding effects of the native lung on physiological measurements [134, 140].

1.7.4.5 Pathophysiology of CLAD

Obliterative bronchiolitis is a common pathological feature across CLAD phenotypes and represents the primary driver of airflow limitation [140]. It arises from repeated epithelial injury due to environmental, infectious, and immune-mediated insults, leading to chronic inflammation, dysregulated repair, epithelial-to-mesenchymal transition, fibroblast recruitment, and irreversible airway fibrosis.

In RAS, airway pathology is compounded by extensive alveolar and pleural involvement, with pleuroparenchymal fibroelastosis reflecting unresolved alveolar injury and persistent fibroinflammatory responses. Mixed phenotypes exhibit features of both airway obliteration and parenchymal fibrosis.

1.7.4.6 Risk factors for CLAD

CLAD results from a complex interplay of recipient-related, alloimmune, and environmental factors. Acute cellular rejection, particularly recurrent or moderate-to-severe episodes (≥A2), is strongly associated with BOS development. Lymphocytic bronchiolitis, primary graft dysfunction, respiratory infections, microbial colonisation, gastro-oesophageal reflux, air pollution exposure, antibody-mediated rejection—particularly for RAS—and nonadherence to immunosuppression all significantly increase CLAD risk [145,146].

1.7.4.7 Treatment strategies

Therapeutic options for established chronic lung allograft dysfunction remain limited and are largely aimed at slowing functional decline rather than reversing disease. Consequently, preventive strategies are of paramount importance. Macrolide therapy—particularly azithromycin—has emerged as a cornerstone immunomodulatory intervention, with evidence supporting its role in delaying CLAD onset and improving lung function in a subset of patients, especially those with neutrophil-predominant airway inflammation. Azithromycin is therefore frequently introduced early after transplantation or at the first sign of lung function decline [147]. Once CLAD is established, there are limited treatment options, which mostly result in a temporary stabilisation of the FEV1 decline (figure 9). There is no consensus on the best treatment algorithm for CLAD. High-dose systemic corticosteroids are discouraged due to toxicity and lack of sustained benefit. Optimisation of maintenance immunosuppression, including conversion from cyclosporine to tacrolimus, is

commonly employed, and aggressive management of gastroesophageal reflux—potentially including anti-reflux surgery—constitutes an essential component of care. Additional immunomodulatory approaches, such as ECP, leukotriene receptor antagonists (e.g. Montelukast), lymphocyte-depleting agents, TLI, and antithymocyte globulin, may stabilise lung function in selected patients; however, supporting evidence is derived predominantly from observational studies. ECP involves incubating isolated recipient leukocytes with 8-methoxypsoralen and exposing them to ultraviolet A light, leading to lymphocyte apoptosis. ECP at regular intervals may lead to immunomodulation and is well tolerated, but like the other treatment options, there is a lack of randomised clinical trials and it is not available or reimbursed in all countries [148]. TLI is a potent immunosuppressive therapy that targets lymphoid tissues, selectively and durably reducing radiosensitive T- and B-cell populations while sparing relatively radioresistant regulatory and natural killer T cells, thereby shifting immune balance toward regulation; this effect is further enhanced by TLI-induced TGF-β–mediated regulatory T-cell activation, and the therapy is typically delivered as ten fractions of 0.8 Gy administered twice weekly using mantle, paraaortic, and inverted-Y fields. Although data are relatively scarce, TLI seems to be more effective in CLAD patients with a rapid decline in lung function at the time of treatment initiation [149].

Treatment options for RAS are even more limited, and no intervention has reliably altered its natural history. Immunomodulatory therapies, including ECP and lymphocyte-depleting agents such as alemtuzumab (which targets T-, B- and natural killer lymphocytes thorugh blocking of CD52), have demonstrated variable and generally modest benefit in selected cases, particularly in the presence of active inflammatory features. Antifibrotic therapy has generated interest in this context: pirfenidone has been associated with attenuation of lung function decline in small studies, although its impact on clinically meaningful outcomes remains uncertain, and concerns regarding safety and infectious risk persist.

Re-transplantation remains the only definitive therapeutic option for advanced CLAD but is reserved for carefully selected patients. Outcomes after re-transplantation are inferior to those of primary transplantation, with particularly poor survival observed in patients retransplanted for RAS, mandating stringent patient selection and careful consideration of competing risks [150].

Lymphocyte depletion/modulation
Total lymphoid irradiation
Extracorporeal photopheresis
Antithymocyte globulin
Alemtuzumab
Immunomodulatory treatments
Azithromycin
Montelukast
Antifibrotic treatment (RAS phenotype)
Nintedanib
Pirfenidone
Palliative/supportive care
Re-transplantation (very selected cases)

Figure 9. Therapeutic options for treatment of CLAD. From [137]

1.8 Mesenchymal stromal cells (MSCs): definition, biology, and functional properties

Mesenchymal stromal cells (MSCs) are multipotent, non-hematopoietic cells originally identified in the bone marrow for their capacity to support hematopoiesis and differentiate into mesodermal lineages. First described by Friedenstein and colleagues in the late 1960s, MSCs were initially characterized as fibroblast-like, plastic-adherent cells capable of osteogenic differentiation. Over subsequent decades, MSCs have been identified in a wide range of adult and perinatal tissues, including adipose tissue, umbilical cord, placenta, lung, and other organs, indicating that they represent a ubiquitous stromal cell population rather than a tissue-restricted progenitor pool [151-153].

As understanding of MSC biology has evolved, it has become evident that the term *mesenchymal stem cell* inadequately reflects their primary biological role. Although MSCs retain multilineage differentiation potential in vitro, their therapeutic effects in vivo are now recognized to be largely independent of durable engraftment or differentiation. Consequently, the term *mesenchymal stromal cell* is preferred, emphasizing their role as dynamic regulators of tissue microenvironments rather than classical stem cells [154].

MSCs are operationally defined by a combination of physical, phenotypic, and functional criteria. They are plastic-adherent under standard culture conditions and express a characteristic surface

antigen profile, typically including CD73, CD90, and CD105, while lacking expression of hematopoietic and endothelial markers such as CD45, CD34, CD14, CD19, and HLA-DR. While these criteria facilitate standardization, they do not fully capture the biological and functional heterogeneity of MSC populations derived from different tissue sources [156].

Importantly, MSCs are not rare cells. They reside predominantly as perivascular stromal cells in most tissues, where they contribute to structural support, extracellular matrix homeostasis, and regulation of local immune responses. Their widespread distribution supports the concept that MSCs function as tissue-resident sentinel cells that respond to stress, inflammation, and injury.

A defining feature of MSC biology is their context-dependent functional plasticity. MSCs do not exert fixed or constitutive effects; rather, their behavior is shaped by signals from the surrounding microenvironment. In the presence of inflammatory stimuli—such as activated immune cells or pro-inflammatory cytokines—MSCs adopt an immunomodulatory phenotype characterized by the release of anti-inflammatory and tolerogenic mediators. Under homeostatic conditions, by contrast, MSCs primarily provide trophic and structural support. This responsiveness allows MSCs to act as biological sensors that integrate environmental cues and dynamically adjust their functional output [156].

One of the most extensively studied properties of MSCs is their capacity to modulate both innate and adaptive immune responses. MSCs interact with multiple immune cell populations predominantly through paracrine signaling, although direct cell–cell contact also contributes. MSCs suppress proliferation and effector functions of activated T cells, promote expansion of regulatory T cells, and inhibit dendritic cell differentiation and maturation. They also influence B-cell function by reducing antibody production and promoting regulatory B-cell phenotypes under specific conditions. Within the innate immune compartment, MSCs modulate macrophage polarization toward an anti-inflammatory, tissue-repair phenotype and regulate natural killer cell activation [157].

These immunomodulatory effects are mediated by a broad array of soluble factors, including prostaglandin E_2, indoleamine 2,3-dioxygenase, transforming growth factor-β, interleukin-10, nitric oxide, and other immunoregulatory mediators. Importantly, MSC-mediated immune modulation does not induce global immunosuppression but rather selectively attenuates excessive or pathological immune activation (figure 10) [158].

Accumulating evidence indicates that the therapeutic activity of MSCs is mediated predominantly through their secretome rather than through long-term engraftment. The MSC secretome comprises

cytokines, chemokines, growth factors, lipid mediators, extracellular matrix components, and extracellular vesicles such as exosomes and microvesicles [158].

Extracellular vesicles derived from MSCs carry bioactive proteins, lipids, and nucleic acids, including microRNAs, capable of modulating gene expression in recipient cells. These vesicles reproduce many of the immunomodulatory and tissue-protective effects of whole MSCs in experimental models, supporting the concept that MSCs function primarily as paracrine regulators of tissue homeostasis [159] .

Contrary to early assumptions, intravenously administered MSCs do not engraft extensively or persist long term in recipient tissues. Instead, they undergo rapid clearance from the circulation, predominantly within the pulmonary vasculature, through mechanisms involving complement activation, coagulation pathways, and immune-mediated cytotoxicity. This results in MSC apoptosis and subsequent phagocytosis by monocytes and macrophages.

Rather than representing a limitation, this transient presence appears central to MSC function. Phagocytosis of apoptotic MSCs by innate immune cells induces a tolerogenic immune response, promoting anti-inflammatory signaling and systemic immune regulation. This mechanism explains why MSC efficacy does not depend on donor–recipient HLA matching and why biological effects are observed despite minimal tissue persistence [160].

In addition to immune regulation, MSCs exert cytoprotective and reparative effects on injured tissues. They enhance epithelial and endothelial barrier integrity, reduce apoptosis, promote angiogenesis, and limit oxidative stress. MSCs have also been shown to transfer mitochondria to injured cells via extracellular vesicles or tunneling nanotubes, restoring cellular bioenergetics and improving cell survival [161].

Collectively, these functions position MSCs as facilitators of tissue repair rather than drivers of tissue regeneration, reinforcing their role as modulators of injury responses rather than stem cells in the classical sense.

Across a wide range of clinical applications, MSC therapy has demonstrated a favorable safety profile. Large meta-analyses and long-term follow-up studies have not identified consistent signals of increased malignancy, ectopic tissue formation, or severe infusion-related toxicity. Transient adverse events such as fever or mild inflammatory reactions have been reported, but serious complications remain rare [162].

Nevertheless, MSCs are biologically active cellular products, and their effects are highly dependent on tissue source, manufacturing conditions, and host immune context. These variables underscore the importance of rigorous characterization, standardization, and controlled clinical application.

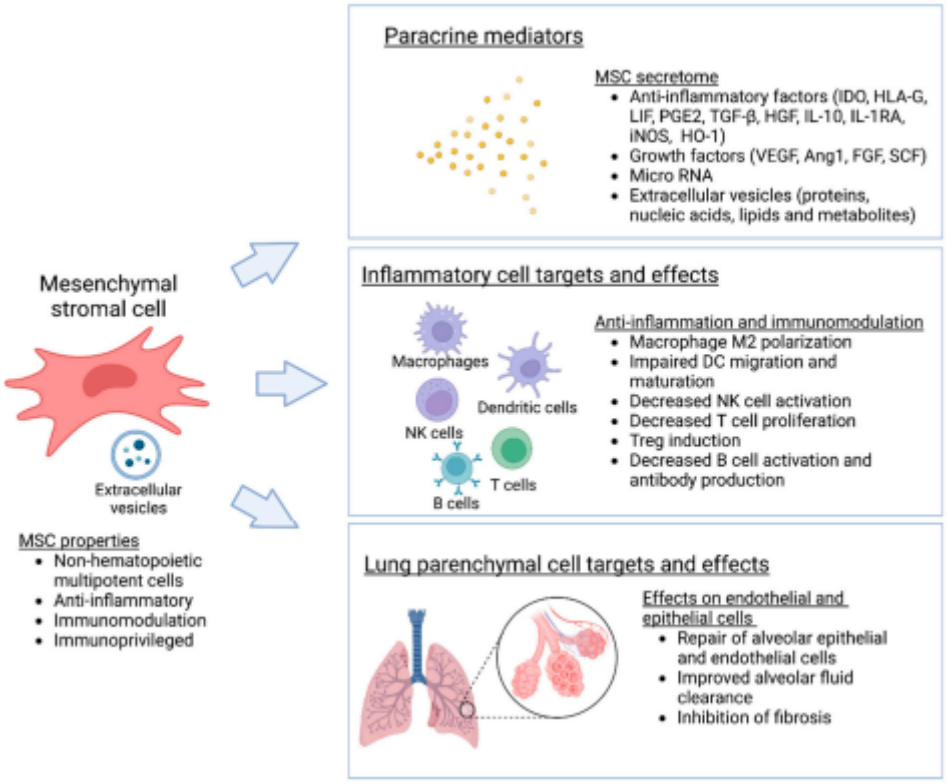


Figure 10. Biological properties and effects of MSCs. From [158].

1.8.1 Application of MSCs in lung diseases and graft-versus-host disease (GvHD)

The therapeutic properties of MSCs have been extensively investigated in both preclinical and clinical settings across a broad range of disorders, including cardiovascular, neurodegenerative, immune-mediated, pulmonary, hepatic, renal, and orthopedic diseases, with variable success [163]. Among these indications, MSC-based therapies have been most actively explored in lung diseases—such as ARDS and ILDs—as well as in GvHD.

The lung represents a particularly attractive target for MSC-based interventions because of its susceptibility to inflammatory injury and its central role in immune regulation. In addition, following systemic administration, MSCs are preferentially sequestered within the pulmonary

microvasculature. Initially regarded as a limitation, this phenomenon is now recognized as a potential biological advantage, as it concentrates therapeutic cells at sites of lung injury and inflammation.

In IPF, MSCs were hypothesized to counteract epithelial injury and aberrant repair processes driving progressive fibrosis. Phase I clinical studies using bone marrow–, adipose tissue–, or placenta-derived MSCs administered intravenously or endobronchially have consistently demonstrated a favorable short-term safety profile, with no treatment-related serious adverse events. Although some studies reported relative stabilization of lung function during follow-up, no consistent evidence of disease modification has been observed [164].

Similarly, MSC therapy in ARDS has demonstrated a robust safety profile across preclinical models and early-phase clinical trials, with no significant infusion-related toxicity, even at higher doses or with repeated administrations. Biologically, MSC treatment has been associated with reductions in pro-inflammatory mediators, attenuation of oxidative stress, enhancement of alveolar–capillary barrier integrity, and promotion of epithelial and endothelial repair, partly mediated through paracrine signaling and mitochondrial transfer. Clinically, although large randomized trials have not consistently shown reductions in mortality or ventilator-free days, several studies have reported improvements in oxygenation, decreases in lung injury biomarkers, and trends toward better outcomes in selected subgroups, particularly younger patients and those receiving higher doses of viable cells [165].

The rationale for MSC therapy in CLAD is strongly supported by mechanistic and clinical insights derived from GvHD, the condition in which MSCs have been most extensively studied. Although CLAD and GvHD differ in anatomical targets and clinical manifestations, both represent prototypical syndromes of alloimmune-driven tissue injury characterized by chronic inflammation and dysregulated repair. Experience in GvHD has challenged early assumptions regarding MSC engraftment and persistence, instead highlighting their role as context-dependent immunomodulators whose efficacy depends on host immune interactions rather than durable cell survival.

In GvHD, MSC therapy has demonstrated an excellent safety profile with clinically meaningful responses, particularly in steroid-refractory acute disease. Higher response rates have been reported in pediatric patients and in those with skin and gastrointestinal involvement. These results culminated in the approval by the U.S. Food and Drug Administration on 18 December 2024 of Ryoncil (remestemcel-L-rknd), an allogeneic bone marrow–derived MSC therapy for the treatment of steroid-refractory acute GvHD in pediatric patients aged two months and older [166].

1.8.2 Application of MSCs in CLAD

Building on robust preclinical evidence demonstrating the immunomodulatory and anti-fibrotic properties of MSCs in chronic lung allograft injury, early-phase clinical studies have evaluated the feasibility, safety, and preliminary efficacy of MSC therapy in lung transplant recipients with CLAD, predominantly BOS.

The first clinical experience was reported by Chambers et al., who conducted an open-label phase I study administering intravenous third-party allogeneic bone marrow–derived MSCs to lung transplant recipients with BOS grade ≥1 or early BOS with risk factors for progression. Patients received a single dose of 2×10^6 cells/kg. The intervention was well tolerated, with no infusion-related serious adverse events. Although the study was not powered to assess efficacy, post-treatment lung function analysis suggested a reduction in the rate of FEV_1 decline compared with the pre-treatment period. These findings supported the hypothesis that MSCs may attenuate ongoing alloimmune-mediated airway injury rather than reverse established structural damage [167].

Subsequently, Erasmus et al. extended these observations in a cohort of patients with moderate-to-severe BOS, evaluating lower MSC doses (0.5 or 1×10^6 cells/kg) administered intravenously. Consistent with the Chambers study, MSC infusion was feasible and safe, with no treatment-limiting toxicity observed. Importantly, longitudinal lung function assessment demonstrated a significant decline in FEV_1 prior to MSC therapy, followed by stabilization after infusion, suggesting a potential disease-modifying effect. These results reinforced the concept that MSC therapy may be most effective when administered at a stage in which inflammatory and immune-mediated mechanisms remain active drivers of CLAD progression [168].

Building upon these phase I studies, the ASSIST-CLAD trial represented the first multicentre, phase II, double-blind, randomized controlled evaluation of MSC therapy in CLAD. Lung transplant recipients with new-onset CLAD were randomized to receive either allogeneic bone marrow–derived MSCs or placebo. MSCs were administered intravenously at a dose of 2×10^6 cells/kg twice weekly for two weeks, reflecting a strategic shift toward repeated dosing to enhance and sustain immunomodulatory effects. The primary endpoint was progression-free survival at 12 months, defined as freedom from both all-cause mortality and CLAD progression, the latter measured as a ≥10% decline in FEV_1 from baseline. Secondary endpoints included all-cause and CLAD-related mortality, as well as the slopes of FEV_1 and FVC decline over 12 months. By incorporating placebo control, standardized GMP-grade MSC manufacturing, and clinically meaningful composite endpoints, the ASSIST-CLAD trial addressed key limitations of earlier studies. Although final

efficacy results are pending, this trial represents a critical step toward determining whether MSC therapy can alter the natural history of CLAD rather than solely establishing safety [169].

Collectively, these studies indicate that intravenous administration of third-party allogeneic bone marrow–derived MSCs is safe and feasible in lung transplant recipients with CLAD, with consistent signals suggesting stabilization of lung function, particularly in BOS. However, heterogeneity in dosing regimens, disease severity at treatment initiation, and study design underscores the need for adequately powered randomized trials and the development of biomarker-guided strategies to optimize patient selection.

2 MSCs AS RESCUE TREATMENT FOR CLAD: ANALYSIS OF IMMUNOREGULATORY EFFECTS IN VITRO AND PRELIMINARY SAFETY DATA IN VIVO

2.1 Rationale and aim of the study

Lung transplantation is the only treatment available for several end-stage respiratory diseases, such as COPD, ILDs, pulmonary hypertension and cystic fibrosis. Although its life-saving potential, the median survival after lung transplant is about 7,8 years [170]. CLAD is the main factor limiting long term survival [131]. It is caused by repeated immunological and non-immunological insults, leading to damage of the epithelial barrier, associated with decreased epithelial regeneration and reduced secretion of protective proteins [171] which finally trigger exaggerated fibrotic repair associated to a progressive and irreversible graft function deterioration. CLAD is currently being regarded as an umbrella definition encompassing several presentations, with two main phenotypes recognized, named BOS and RAS. Other well defined but less common phenotypes are the mixed, with coexisting features of BOS and RAS, and the undefined one [131,172]. The therapeutic options for CLAD include azithromycin [147,163], montelukast [174], lymphodepletion with ATG or alemtuzumab [175,176] or immune-regulation by means of TLI or ECP [177-182]. Most of these approaches have been reported , in retrospective studies, to stabilize graft function or slower the rate of decline in up to 50-60% of treated cases. Although these interventions may stabilize lung function in some cases, in others progressive decline is relentless [180]. Thus re-transplantation remains the last curative option for progressive CLAD but is associated to worse prognosis than the primary lung transplant and is generally reserved to highly selected , younger CLAD patients, mainly with the obstructive phenotype [183]. In this context, the evaluation of new possible therapeutic approaches in CLAD is crucial.

MSCs are multipotent, non-hematopoietic progenitor cells with properties of self-renewal and differentiation into several cell types. Recently, they have gained significant attention in the field of regenerative medicine due to their potential to promote tissue repair, modulate immune responses, and reduce inflammation. These effects are exerted through a wide secretoma composed by cytokines, growth factors, microRNAs, and extracellular vescicles, acting in paracrine way [158]. MSCs resident in the lungs have a protective effect by promoting epithelial [184] and endothelial repair [185,186] and polarizing macrophages towards an immunosuppressive M2-like phenotype for the restoration of tissue homeostasis [187]. If administered systemically for therapeutic purposes,

they can home and accumulate to the sites of damage, with a high rate of pulmonary accumulation as first passage organ, thus providing an advantage for the potential treatment of chronic inflammatory pulmonary diseases [164].

Several clinical studies have shown intravenous MSCs safety and potential efficacy in the treatment of both Covid and non- Covid ARDS [164,165,188].

Some experimental evidences in vitro and ex vivo suggest a role of MSC in the regulation of allo-immune response in the context of solid organ and bone marrow transplantation [189]. As for lung transplantation MSCs have been administered to the donor lungs to prevent the ischemia-reperfusion injury (IRI) and the consequent PGD [189]. Bone marrow–derived MSCs have also been studied as a novel therapeutic strategy for BOS post lung transplantation. Preclinical models, including heterotopic tracheal transplantation in rats, demonstrated that MSCs delivered via endotracheal or intravenous routes reduced fibrotic obliteration in bronchioles, decreased airway fibrosis and edema, and promoted reparative processes[191]. In an early phase I "first-in-man" trial (NCT01175655), allogeneic BM-MSCs were administered to patients with moderate-to-severe BOS. The treatment was safe and well tolerated; most recipients experienced stabilization of lung function (FEV_1/FVC), with no serious infusion-related adverse events [167]. A subsequent phase II randomized trial, ASSIST-CLAD, confirmed safety and feasibility of IV MSC infusions in new-onset CLAD cases, paving the way for efficacy endpoints [169]. More recently, a low-dose MSC infusion study in lung transplant recipients with established obstructive CLAD showed that intravenous BM-MSCs were well tolerated and appeared to slow the decline of FEV_1 over one year compared to pre-treatment trajectories (no significant post-infusion deterioration). Preliminary clinical studies on BOS, however, have employed short treatment schedules and heterogeneous dosing regimens (number of infused cells per kg of body weight). Consequently, uncertainties remain regarding the safety, tolerability, and potential clinical effects of prolonged administration of low-dose MSCs, in analogy to what has been proposed in the context of GvHD [192,193] .

Thus the primary aim of this study is, therefore, to evaluate feasibility and safety of repeated intravenous administration of allogeneic, bone marrow–derived, HLA–unmatched MSCs in patients with advanced CLAD who have not responded to multiple lines of therapy. We have measured survival, incidence of lung infections and other serious adverse effects. The secondary objectives were to observe the changes of functional parameters, expressed as FEV_1 and FVC, as well as trends in laboratory findings before and after MSC administration.

2.2 Materials and Methods

Between November 2020 and May 2023 we enrolled six lung-transplanted recipients diagnosed with CLAD grade 3-4, who had clinical or functional deterioration (decline in FEV_1 >20% over the previous 12 months, progression in CLAD grade, or new-onset pulmonary opacities) despite optimization of immunosuppressive treatment, ECP, azithromycin, and montelukast.

On a compassionate use basis, patients received monthly infusions of allogeneic BM-MSCs at a dose of 1×10^6 cells/kg body weight. Patients with a history of thromboembolic events, active infections, or pulmonary colonization by difficult to treat pathogens (i.e extended drug resistant bacteria, fungi) were excluded. The study was approved by the local Ethics Committee and conducted in accordance with the Declaration of Helsinki.

After thawing MSCs were diluted in saline solution + 5% human albumin under a class A laminar hood and intravenously infused within one hour at the dose of $1x10^6$/Kg body weight. Infusions were delivered slowly over 10 minutes via a large peripheral vein. Vital signs, including heart rate, blood pressure, and peripheral oxygen saturation, were continuously monitored for one hour post-infusion. Physical examination, laboratory tests, and pulmonary function tests (PFTs) were performed prior to each MSC administration.

Initially, patients were approved to receive MSC administration for 6–9 months. Given the favourable outcomes, authorization to continue treatment was subsequently granted, and patients remained on therapy.

2.2.1 MSCs preparation

MSC were expanded from bone marrow of screened third-party Hematopoietic Stem Cell Transplantation donors in compliance with European GMP at the GMP Facility "Cell Factory" of the Fondazione IRCCS Policlinico San Matteo, Pavia and cryopreserved until used.

Materials and reagents for Cell Therapy Medicinal Products (CTMP)/MSC production included

- Commercial platelet lysate
- Cell separation medium (Ficoll)
- Dulbecco's Modified Eagle Medium + 1 g/L D-glucose (DMEM + GlutaMAX)
- Albumin

- Physiological saline
- Recombinant trypsin
- Dimethyl sulfoxide (DMSO)

Isolation and expansion of MSCs involve the following steps:

- **Establishment of MSC cultures:**
  Bone marrow mononuclear cells (derived from approximately 50 mL of bone marrow aspirate supplemented with anticoagulant) used to establish the cultures are obtained from healthy hematopoietic stem cell donors. Mononuclear cells, isolated by density gradient separation, are seeded in T175 flasks at a density of 160,000 cells/cm²; this step is defined as passage 0 (P0).
- **Culture medium replacement:**
  Complete medium replacement is performed twice weekly until cultures reach a confluence of ≥80%.
- **Expansion:**
  Once a confluence of ≥80% is achieved, adherent cells are trypsinized, counted, and reseeded in T175 flasks at a density of 4,000 cells/cm² (corresponding to 7×10^5 MSCs per T175 flask). With each trypsinization, the passage number increases, up to detached passage P3, conventionally defined as P4 (final product).

At the trypsinization of passages P0, P1, and P2 (intermediate passages, which when cryopreserved are conventionally referred to as P1, P2, and P3, respectively), based on cell recovery, after reseeding for expansion, any remaining MSCs are cryopreserved for subsequent expansion. Microbiological quality controls (QC) are performed at each cryopreservation of intermediate passages. At passage P1, one aliquot of cells (~2×10^6) is cryopreserved for flow cytometric characterization and two backup aliquots (~1×10^6 each) are stored. Upon cryopreservation of the final product (P4), microbiological QC is performed and one aliquot (~1×10^6 cells) is cryopreserved as backup, two aliquots (~1×10^6 cells each) for regulatory control, one aliquot (~1×10^6 cells) for research and development (R&D), and one aliquot (~2×10^6 cells) for flow cytometric characterization.

The table below (table 3) summarizes the quality controls performed at intermediate passages and the aliquots cryopreserved

Passage	Quality controls	Cryopreserved aliquots
P1 (intermediate product)	Sterility and flow cytometry	P1, QC, backup
P2 (intermediate product)	Sterility	P2
P3 (intermediate product)	Sterility	P3
P4 (final product)	Viability, morphology, cumulative population doubling (cPD), sterility, flow cytometry, genotypic identity	P4, QC, backup, R&D, counter-sample

Table 3

- **Expansion from intermediate products:**
  Expansion of the PMTC to the final product may be performed by thawing aliquots of intermediate products (P1, P2, or P3) and following the production protocol steps described above.
- **Cryopreservation of PMTC/MSC (intermediate and final products):** At each trypsinization (for intermediate products P1, P2, and P3) and at the end of expansion of passage P3 (defined by convention as the final product, P4), cells are cryopreserved in physiological saline containing 5% human albumin and 10% DMSO, with a final volume of 1 mL. For intermediate products, aliquots containing up to 16×10^6 MSCs are frozen in 1.8 mL cryovials. For final products, aliquots containing up to 40×10^6 MSCs are cryopreserved. The final product is transferred to a storage dewar (nitrogen vapor container) and stored under quarantine conditions pending the results of the quality control tests required for product release.
- **Validation of PMTC/MSC:**
  Batch specification analyses of PMTC/MSC are performed by the Quality Control laboratories of the Cell Factory. In particular, purity, potency, and identity analyses are carried out by the Biological QC Laboratory, while sterility testing is performed by the Microbiological QC Laboratory. Morphological assessment is performed by Quality Control personnel within the production laboratory prior to trypsinization of the final PMTC product. At trypsinization of both intermediate and final products, a portion of the MSCs is sent to the QC laboratories for testing.

PMTC/MSC batches are characterized on the basis of phenotypic and functional specifications performed on PMTC/MSC after cryopreservation. These specifications were selected based on preclinical validation studies.

Purity

- Morphology: Assessed by optical microscopy on cells in culture, adherent to plastic.
- Cell viability at freezing: Evaluated by viability counting using Trypan Blue exclusion.
- Cell phenotype: Assessed by flow cytometry through monoclonal antibody staining and analysis of the expression of CD105, CD90, and CD73, as well as the absence/low expression of CD45, CD34, CD19, and HLA-DR.

Identity

- Identity concordance between starting biological material and PMTC: Assessed by analysis of short tandem repeat (STR) polymorphisms.

Potency

- Cumulative population doubling (cPD): Defined using the formula: log(number of harvested cells / number of seeded cells) / log2.

Safety

- Sterility testing:
  a) Cultures for aerobic and anaerobic bacteria and fungi
  b) Bacterial endotoxin detection (Limulus Amebocyte Lysate, LAL test)
  c) PCR testing for mycoplasma

A batch is considered validated if it complies with the requirements deemed critical for the release of PMTC (table 4).

Parameter	Release criterion
Morphology	Spindle-shaped
Viability	≥ 70%
Cumulative population doubling (cPD)	≥ 2 and ≤ 20
Molecular identity (STR)	Concordance between starting biological material and PMTC
Phenotypic analysis (flow cytometry – surface markers)	
CD73	≥ 95%
CD90	≥ 95%
CD105	≥ 95%
Sum of HLA-DR, CD34, CD45, CD19	≤ 5%
Sterility	
Aerobic and anaerobic bacterial and fungal cultures	Negative
Bacterial endotoxin testing	Negative
Mycoplasma PCR	Negative

Table 4. Criteria for quality evaluation of MSCs

2.2.2 Statistical analysis

Interrupted Time Series Analysis (ITSA) was used to assess the effect of an intervention over time, focusing on changes and change in trends before and after the index date (start of treatment). To model this, we applied splines to introduce a knot at the index date and fitted a mixed linear model

with random intercepts (patients) and random slopes for time-related variables. This approach allowed us to estimate the pre-intervention trend, the immediate change in outcome after the intervention, and the difference in slopes before and after the index date.

2.3 Results

2.3.1 Study population

The study included six bilateral lung transplant recipients (4 males and 2 females). The underlying diseases were COPD (n =1), ILDs (n=3), among which 2 were IPF and 1 was fibrotic hypersensitivity pneumonitis (fHP), obliterative bronchiolitis (OB) in rheumatoid arthritis (n= 1), re-transplant for BOS (n=1). All patients received a triple maintenance immunosuppressive treatment with a calcineurin inhibitor, steroid and a cell cycle inhibitor, except for one patient, who received only Tacrolimus and steroids for severe side effects with the third agent. CLAD was diagnosed, according to ISHLT guidelines [131], as persistent and significant decline (> 20%) of the measured FEV1 compared with the mean of the two best post-operative FEV1 measurements, taken at least 3 weeks apart, and exclusion of other causes of FEV1 decline.

Three patients had a BOS, three had a mixed phenotype.

DSAs were tested and resulted negative, except for patient n°5 who was persistently positive for de novo anti class II DSA (HLA DR3) Mean Fluorescence Intensity 1000-3000 by Luminex single-antigen bead, and had received, 6 months before CLAD diagnosis, a treatment for a possible antibody-mediated rejection according to ISHLT guidelines [119], treated with a high dose steroid course, plasma exchange , and high dose intravenous Immunoglobulines.

In all patients , at CLAD onset, a treatment course with Azytromicin and Montelukast together with an optimization of immunosuppression (when possible a shift from mycophenolate to everolimus) was initiated and at progression to grade 2 CLAD, long term offline ECP was proposed. At failure of ECP recipients were enrolled to MSC protocol.

The medium age at the start of MSC treatment was 57.6 years (SD +- 8.42) and median follow up post transplantation was 10.5 years (+- IQR 13.75). Further clinical and demographic features of the patients are summarized in the table 5.

Patient	Sex	Indication for lung tx	Year of lung tx	Time from tx to CLAD (years)	Stage and CLAD phenotype	Immunosuppressive treatment at the beginning of MSCs therapy	Age at MSCs treatment	Time from tx to MSCs (years)	Month and year of MSCs initiation	Frequent bronchial exacerbations (>= 3/Year)	History of AMR
Patient 1	M	Fibrotic hypersensitivity pneumonitis	2016	3	IV, BOS	Tacrolimus, everolimus, prednison	47	4	November 2020	yes	no
Patient 2	M	Obliterative bronchiolitis in rheumatoid arthritis	2005	10	IV, BOS	Tacrolimus, micophenolate, prednison	71	16	February 2021	yes	no
Patient 3	F	Re-transplant for BOS	2000	12	IV, BOS	Tacrolimus, prednison	48	22	July 2022	yes	no
Patient 4	M	IPF	2016	4,5	III, mixed	Tacrolimus, everolimus, prednison	62	6	August 2022	no	no
Patient 5	F	COPD	2019	3,8	III, mixed	Cyclosporine, everolimus, prednison	62	3	October 2022	no	yes
Patient 6	M	IPF	2007	11	III, mixed	Tacrolimus, everolimus, prednison	56	15	May 2023	yes	no

Table 5. Clinical features of enrolled patients

2.3.2 Safety

Overall a total of 251 monthly infusions were performed in the 6 patients enrolled (median treatment 37.5 months (IQR 22.5) with a minimum follow up of 28 months and a maximum follow up of 58 months.

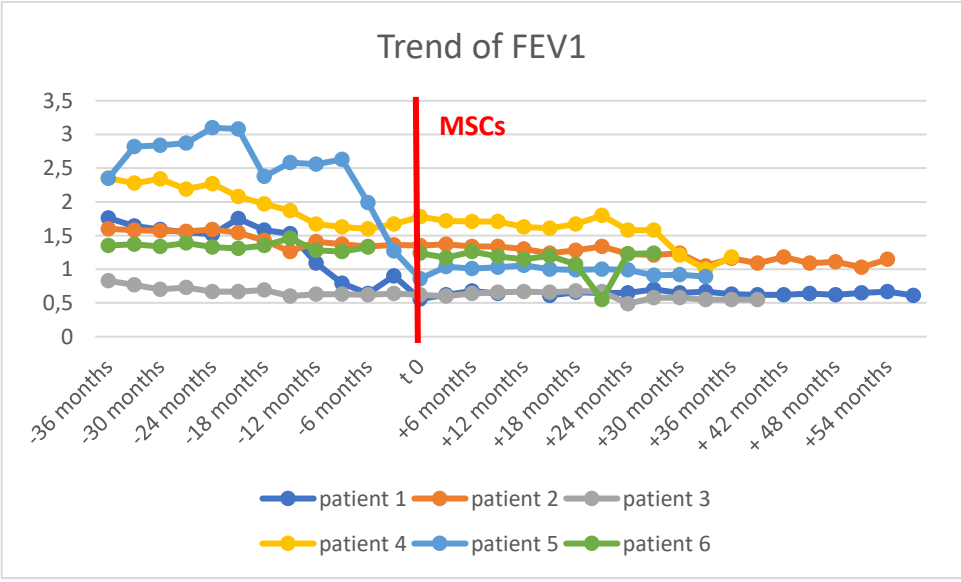
Even taking into account heterogeneity in the length of MSC treatment and follow up, all patients completed a minimum of 24 months of follow up. Survival after 24 months of monthly infusion with MSCs was 100%. One patient was listed for lung re-transplantation after 34 months from the initiation of MSC treatment for progression of CLAD and died 37 months later due to a severe pulmonary infection. The other five patients (83.3%) are still alive and still under treatment (median treatment 37 months (IQR 25) with a minimum follow up of 28 months and a maximum follow up of 58 months at the time of present analysis. The incidence of lung infections did not significantly increase post-treatment (IRR = 1.33, IC 95%: 0.67–2.61; p = 0.41), suggesting that immunomodulation by MSCs did not increase overall immunosuppression rate and infection susceptibility. Four patients (67%) had respiratory infections (table 6). Globally, treatment with MSCs was well tolerated. No alterations of vital signs after infusion and no serious adverse events attributable to MSCs were recorded, one patient (17%) reported dizziness and one patient (17%) had episodes of metrorrhagia requiring treatment with Gonadotropin-Releasing Hormone Agonists. No fever, anaphylactic reactions or thromboembolic events were reported.

Patient	Number infections pre-MSC	Number infections post-MSC	Pathogens pre- MSC	Pathogens post-MSC
Patient 1	7	6	Pseudonomas aueruginosa, Aspergillus, Moraxella catharralis	Pseudonomas aueruginosa, Aspergillus, RSV
Patient 2	0	3		Pseudomonas aeruginosa, Haemophilus influenzae, Aspergillus
Patient 3	4	8	Pseudomonas aeruginosa, Staphilococcus aureus	Aspergillus, Klebsiella pneumoniae, Serratia marcescens, Staphilococcus aureus, Enterobacter cloacae
Patient 4	1	0	Aspergillus	
Patient 5	1	3	Sars Cov 2	Staphilococcus epidermidis
Patient 6	2	2	Mycobacterium chimerae	

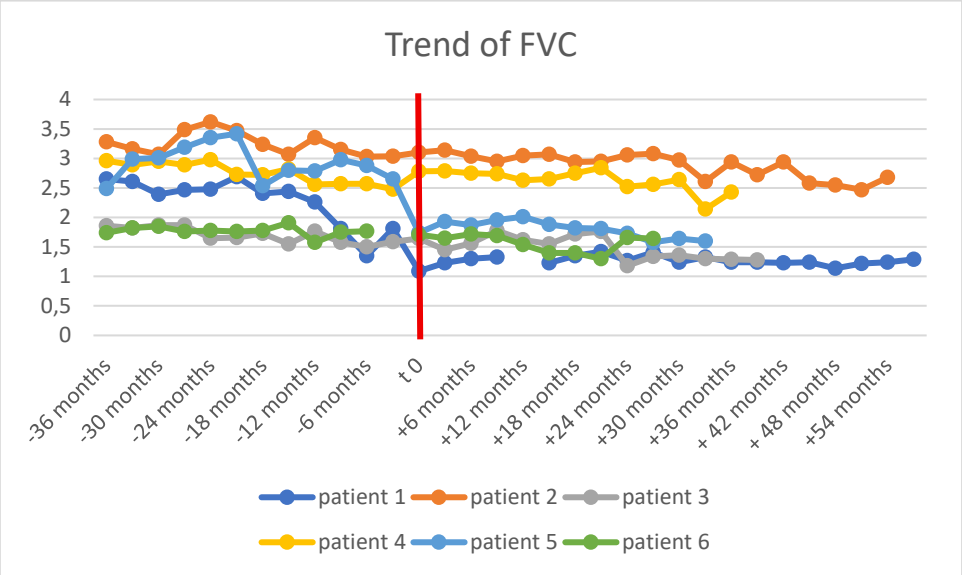
Table 6. Spectrum of infections before and after initiation of MSCs.

2.3.3 Lung function

From a functional perspective, there were no statistically significant changes of FEV1 (coefficient -0.031, IC95%: -0.15; 0.09, p = 0.6) and FVC (coefficient -0.038, IC95%: -0.16; 0.09, p = 0.55) before and after treatment. However, interestingly, the slope of decline significantly decreased over time (FEV_1 $\beta = -0.0189$, $p = 0.003$; FVC $\beta = -0.0139$, $p = 0.008$). These results could suggest that MSC therapy may attenuate the progressive loss of pulmonary function typical of advanced CLAD. The trend of FEV1 and FVC over time is reported in the figure 11.



a



b

Figure 10 a,b. Trend of FEV1 and FVC on time

2.3.4 Blood parameters

No significant changes over the whole treatment were detected in the following variable values hemoglobin, leucocyte and platelet counts, protrombin time (PT), International Normalized Ratio (INR), creatinine, potassium, liver enzymes, C reactive C protein (RCP), and lactate dehydrogenase (LDH) confirming the absence of a systemic toxicity of this therapeutic approach (figure 2). A near-significant rise in Calcium levels was detected post-therapy (IRR = 3.04, IC 95%: 0.9- 9.5, p = 0.057). Although it could theoretically indicate an effect of MSCs infusion, it is more likely to reflect biological variability given the wide confidence interval and lack of correlated clinical deterioration.

Blood parameter	Median pre-infusion (IQR)	Median post-infusion (IQR)	Incidence rate ratio (IRR)	Standard deviation (SD)	P-value
Hemoglobin (gr/dl)	14 (11,9-14,7)	14,3 (12,7-15,3)	1,0	0,2	0,9
White blood cells (cells/mmc)	7,12 (6,3-8,91)	8,25 (7,04-9,42)	3,8	3	0,09
Platelets (cells/mmc)	198 (178-223)	231 (208-246)	0,65	0,37	0,47
Creatinine	0,99 (0,91-1,31)	1,01 (0,9-1,31)	1,19	0,38	0,59
Potassium	3,96 (3,72-4,33)	3,9 (3,8-4,2)	1,0	0,1	0,99
Calcium	9,2 (8,9-9,6)	9,3 (8,7-9,6)	3,05	1,78	0,05
LDH	189 (169-216)	208 (180-228)	1,16	0,39	0,43
ALT	16 (13-19)	16,5 (14-20)	1,08	1,08	0,93
GGT	13 (13-23,75)	19,5 (15-30)	1,05	1,05	0,9
RCP	0,21 (0,06-0,54)	0,195 (0,08-0,6)	1,09	0,35	0,78
PT	101 (90-109)	104 (90,75-118)	1,0	0,3	0,9
INR	0,98 (0,92-1,01)	0,97 (0,92-1,01)	0,7	0,47	0,6

Figure 2. Median of blood parameters measured monthly 36 months before and after MSCs administration

2.4 Discussion

CLAD remains the major barrier to long-term survival after lung transplantation but , despite great advances in the management of lung transplant recipients, we still lack effective disease-modifying treatments. Its pathogenesis involves recurrent immunologic and non-immunologic injury, impaired epithelial repair, persistent inflammation, and progressive fibro-proliferation, ultimately resulting in irreversible decline in graft function [194]. Current therapeutic strategies, including azithromycin, leukotriene receptor antagonists, intensified immunosuppression, ECP, and TLI, may offer transient stabilization in selected patients, but none have consistently demonstrated the ability to halt disease progression. In this context, the use of a regenerative, immunomodulatory treatment can be an attractive approach.

MSCs are able to modulate immune cell proliferation, activation, and effector function, inhibit the expression of pro-inflammatory cytokines, promoting alveolar epithelial and endothelial repair and restoration of tissue homeostasis via paracrine mechanisms. Interestingly, when they are infused intravenously, they remain partially entrapped, producing the “pulmonary first-pass effect” [195].This phenomenon is especially advantageous for lung-directed therapies and increases the

likelihood that MSCs reach areas of active injury, mitigating inflammation and facilitating endogenous repair processes [196].

Preclinical models and early human studies in acute lung injury, ischemia–reperfusion damage, idiopathic pulmonary fibrosis, Graft Versus Host Disease after hematopoietic stem cell transplantation and chronic rejection [165,188,197-204] have demonstrated favourable safety and biological activity, and two phase I studies in BOS further supported the feasibility of MSC-based therapies in lung transplant recipients [167,168].

Notably, *Chambers et al* reported a marked decline in the rate of lung function deterioration, with FEV_1 decline slowing from 120 mL/month pre-treatment to 30 mL/month post-MSC therapy in 10 CLAD patients who received twice-weekly infusions of bone marrow derived, allogenic MSCs at a dose of 2×10^6 cells per kilogram of bodyweight for each infusion, over a short treatment period of two weeks [167]. Similarly, *Erasmus et al* documented stabilization of lung function over a 12-month period in 13 patients receiving MSCs in two administrations [168]. Treatment schedules and dosage of cells /infusion significantly differed from what we report in our experience. This is, to our knowledge, the first reported experience of long-term treatment with allogeneic bone marrow–derived MSCs in CLAD. Our study shows that this therapeutic approach, even in advanced stages of CLAD refractory to previous treatment strategies—including ECP—is well tolerated and does not result in increased susceptibility to infections or other significant adverse events. Moreover, the use of HLA-unmatched MSCs did not induce new donor-specific antibodies, further supporting the immunological safety of this approach.

During the study period we observed 100% survival (6/6 treated CLAD grade 3 or 4 patients) at 2 years and only 1 over 6 patients experienced a progression of CLAD and died of respiratory failure after 37 months of MSC treatment. These outcomes are more favourable than those recently reported in the literature by two large European lung transplant centres, which documented a 2-year survival rate of 55% for patients enrolled in follow-up at CLAD stage 3[183].

Interestingly, even if the study was not targeted for the evaluation of MSC therapeutic efficacy, our data indicate that MSC infusion was associated to a significant decrease of the rate of graft function decline both FEV_1 and FVC conferring a long patient survival. In such a condition typically characterized by relentless deterioration, a slowdown in functional loss represents a clinically meaningful outcome and prolongs survival.

Our protocol of mesenchymal cell administration — involving long-term, low-dose monthly infusions — may be associated with a sustained immunoregulatory activity at the graft level.

Our study has several limitations. First of all, the small sample size and the lack of a control group preclude definitive conclusions regarding therapeutic efficacy. Secondly, the clinical and demographic features of the patients were heterogeneous, potentially introducing confounding elements and reducing internal validity of data, although patients were all affected by advanced CLAD with BOS or mixed phenotype. Nevertheless, such variability is justified by the compassionate-use context and the absence of alternative therapeutic options for these patients. Thirdly, while the use of interrupted time-series analysis strengthens the inference of temporal association, residual confounding cannot be excluded. Finally, we didn't assess biological markers of MSC activity, such as circulating extracellular vescicles, miRNAs or cytokine levels, and we should integrate them into future studies. Future research should focus on controlled trials to define optimal dosing, timing, and patient selection, and to evaluate MSC-derived products, such as extracellular vesicles, as potential cell-free therapies.

2.5 Conclusion

In conclusion, this study demonstrates that long-term, repeated monthly administration of allogeneic bone marrow–derived MSCs in patients with advanced CLAD is feasible and well tolerated, even in a population with severe disease refractory to multiple established therapeutic strategies. Importantly, prolonged MSC therapy was not associated with an increased incidence of serious adverse events, systemic toxicity, or infectious complications, supporting the long-term safety of this immunomodulatory approach in lung transplant recipients. The absence of de novo donor-specific antibody development further reinforces the immunological safety of administering HLA-unmatched MSCs in this setting.

Beyond safety, longitudinal functional analyses revealed a significant attenuation in the rate of decline of pulmonary function, as reflected by both FEV_1 and FVC trajectories. Although this study was not designed or powered to establish definitive efficacy, the observed reduction in the slope of functional deterioration is clinically meaningful in a condition typically characterized by relentless progression and limited therapeutic options. In this context, stabilization or slowing of lung function decline may translate into prolonged survival, delayed need for retransplantation, and preservation of quality of life.

These findings support the hypothesis that sustained, low-dose MSC administration may exert ongoing immunoregulatory and tissue-protective effects at the allograft level, counteracting chronic immune-mediated injury and dysregulated repair rather than reversing established structural damage.

This paradigm is consistent with emerging evidence that MSCs function primarily as dynamic modulators of inflammation and tissue homeostasis through paracrine mechanisms, rather than as engrafting or regenerative cells.

While acknowledging the limitations inherent to a small, non-randomized, compassionate-use study, this work provides novel clinical evidence supporting the potential role of MSC-based therapies as a rescue strategy in advanced CLAD. Importantly, it extends prior short-term investigations by demonstrating the feasibility and safety of long-term treatment schedules, thereby addressing a critical knowledge gap in the field.

Taken together, these results justify further investigation of MSC therapy in CLAD through prospective, controlled clinical trials aimed at defining optimal dosing regimens, treatment duration, timing of intervention, and patient selection. Future studies should also incorporate mechanistic biomarkers—including immune profiling and MSC-derived extracellular vesicles—to better elucidate biological responses and identify predictors of therapeutic benefit. Ultimately, MSC-based approaches may represent a valuable addition to the currently limited therapeutic armamentarium for CLAD, with the potential to meaningfully improve long-term outcomes after lung transplantation

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