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Microbiome and virome dynamics after lung transplantation and its impact on recipients' long-term prognosis

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DOCTORAL THESIS

Microbiome and virome dynamics after lung transplantation and its impact on recipients' long-term prognosis

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We do not need magic to change the world, we carry all the power we need inside ourselves already; **we have the power to imagine better**.

J.K Rowling

LIST OF ABBREVIATIONS

A1ATD Alpha-1 Antitrypsin Deficiency

ACR Acute Cellular Rejection

ANCOM-BC: Analysis of Microbiome Composition with Bias Correction

AMR Antibody Mediated Rejection

ASV Amplicon Sequence Variant

AZA Azathioprine

BAL Bronchoalveolar Lavage

BOS Bronchiolitis Obliterans Syndrome

CARV Community-Acquired Respiratory Viruses

CF Cystic Fibrosis

CLAD Chronic Lung Allograft Dysfunction

CMV Cytomegalovirus

CNI Calcineurin Inhibitor

COPD Chronic Obstructive Pulmonary Disease

DNA Deoxyribonucleic Acid

DSA Donor Specific Antibodies

EDTA Ethylenediaminetetraacetic acid

FC Fold Change

FDR False Discovery Rate

FEV_1 Forced expiratory volume in 1 second

FVC Forced Vital Capacity

HC: Healthy Controls

HCoV Human Coronavirus

HLA Human Leukocyte Antigen

hMPV Human metapneumovirus

ICTV: International Committee on Taxonomy of Viruses

ILD Interstitial Lung Disease

IPF Idiopathic Pulmonary Fibrosis

IQR Interquartile Range

ISHLT International Society of Heart and Lung Transplantation

ITS Internal Transcribed Spacer

L-AmB Liposomal Amphotericin B

LRT Lower Respiratory Tract

LT Lung Transplantation

MDS Multidimensional Scaling

MMF Mycophenolate Mofetil

mTOR Mammalian Target Of Rapamycin

NFAT Nuclear Factor of Activated T cells

NP Nasopharyngeal

ONT Organización Nacional de Trasplantes

OTU Operational Taxonomic Unit

PAH Pulmonary Arterial Hypertension

PBS Phosphate Buffered Saline

PCA Principal Component Analysis

PCoA Principal Coordinate Analysis

PCR Polymerase Chain Reaction

PERMANOVA Permutational Multivariate Analysis of Variance

PGD Primary Graft Dysfunction

PMP Per Million Population

PSB Protected Specimen Brushing

RAS Restrictive Allograft Syndrome

RNA Ribonucleic Acid

rRNA Ribosomal RNA

RSV Respiratory Syncytial Virus

SD Standard Deviation

SISPA Sequence-Independent Single-Primer Amplification

SMS Shotgun metagenomics sequencing

TTV Torque teno virus

URT Upper Respiratory Tract

VLP Viral-Like Particle

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ABSTRACT

Lung transplantation (LT) represents the last therapeutic option for patients suffering from various end-stage respiratory diseases. Despite all the advances made in recent years, the life expectancy of LT remains the lowest of all solid organ transplants. This is conditioned by the occurrence of infections, which account for 37% of deaths in the first year after LT and chronic lung allograft dysfunction (CLAD), which is the main limitation for long-term survival, with a mortality of 25-30% between 3-5 years after LT. Besides, both complications are related, as infections in LT recipients are a well-known risk factor for CLAD development.

In recent years, metagenomic methods have made it possible to characterise the bacterial microbiome of LT recipients, although its role in the pathogenesis of CLAD is unclear. In the case of the viral component, there is even less information, as the entire virome, including DNA and RNA viruses, has not been studied in depth and, moreover, neither the long-term dynamics of the virome after LT nor its relationship to the prognosis of LT recipients has been clearly assessed.

Therefore, the main objective of the present thesis was to characterise in detail the bacterial microbiome (part 1) and the complete virome composition (part 2) in LT recipients using metagenomic approaches to assess the temporal dynamics, its eventual restoration and the potential impact on long-term prognosis and CLAD development.

The first study (part 1) contends that the alpha diversity of the nasopharyngeal microbiome decreased in the early post-LT stages and that, one year after LT, CLAD-free recipients presented a bacterial composition more closely to that of healthy patients, a finding that could be related to the better outcome observed in this group. Further, it is suggested that upper respiratory tract sampling could be an effective and less invasive alternative to study the respiratory bacterial microbiome.

The second study (part 2) states that alpha diversity normalises two years after LT and that virome composition differs between LT recipients and healthy patients. However, no differences in global virome composition between CLAD and CLAD-free recipients were observed. Further, it is suggested that immunosuppression may alter virome composition

Overall, the studies included in this thesis contribute to a better understanding of the dynamics of respiratory bacterial microbiome and plasma virome after LT and their implication on long-term outcomes, and open up new perspectives for future research.

RESUMEN

El trasplante de pulmón (TP) representa la última opción terapéutica para aquellos pacientes que padecen de alguna enfermedad respiratoria en fase terminal. Aunque en los últimos años se han hecho muchos avances, la esperanza de vida en el TP es la más baja de entre todos los trasplantes de órgano sólido. Esto es debido a la incidencia de infecciones, que representan que suponen el 37% de las muertes en el primer año tras el TP, y la disfunción crónica del injerto pulmonar (DCI), que es la principal limitación para la supervivencia a largo plazo, con una mortalidad del 25-30% entre 3-5 años tras el TP. Además, ambas complicaciones están relacionadas, ya que las infecciones en los receptores de TP son un factor de riesgo bien conocido para el desarrollo de DCI.

En los últimos años, los métodos metagenómicos han permitido caracterizar el microbioma bacteriano de los receptores de TP, aunque su papel en la patogénesis de la DCI no está claro. En el caso del componente vírico, la información es aún menor, ya que no se ha estudiado en profundidad todo el viroma, incluidos los virus de ADN y ARN y, además, no se ha evaluado claramente ni la dinámica a largo plazo del viroma tras el TP ni su relación con el pronóstico de los trasplantados.

Por lo tanto, el objetivo principal de la presente tesis fue caracterizar en detalle el microbioma bacteriano (parte 1) y la composición completa del viroma (parte 2) en receptores de TP utilizando enfoques metagenómicos para evaluar la dinámica temporal, su eventual restauración y el impacto potencial en el pronóstico a largo plazo y el desarrollo de DCI.

En el primer estudio (parte 1) se sostiene que la diversidad alfa del microbioma nasofaríngeo disminuyó en las primeras fases post-TP y que, un año después, los receptores sin DCI presentaban una composición bacteriana más parecida a la de los pacientes sanos, un hallazgo que podría estar relacionado con el mejor pronóstico observado en este grupo. Además, se sugiere que el muestreo del tracto respiratorio

superior podría ser una alternativa eficaz y menos invasiva para estudiar el microbioma bacteriano respiratorio.

En el segundo estudio (parte 2) se afirma que la diversidad alfa se normaliza dos años después de la LT y que la composición del viroma difiere entre los receptores de TP y los pacientes sanos. Sin embargo, no se observaron diferencias en la composición global del viroma entre los trasplantados con y sin DCI. Además, se sugiere que la inmunosupresión puede alterar la composición del viroma.

En general, los estudios incluidos en esta tesis contribuyen a una mejor comprensión de la dinámica del microbioma bacteriano respiratorio y el viroma plasmático tras el TP y su implicación en los resultados a largo plazo, y abren nuevas perspectivas para futuras investigaciones

1. INTRODUCTION

1.1 BRIEF HISTORY OF LUNG TRANSPLANTATION

Lung transplantation (LT) is a type of surgery that replaces a diseased or failed lung with a healthy donor lung. LT was initiated in 1946 with animal experimentation, although without success (1). A few years later, in 1963, LT was performed for the first time to a human patient in the Mississippi Medical Center. Unfortunately, the patient died 18 days later due to a renal failure (2). From that moment on, different studies and attempts were developed in order to improve the patient survival, especially focused on immunosuppression treatments, but also on infections and surgical techniques for proper bronchial healing (3–5). However, it was not until 1983 when Dr. Cooper and his team achieved for the first time that a patient survived long-term after LT, specifically for 7 years (6). After many years of effort, LT was finally recognized in the late 1980s as a therapeutic option (7). Since 1992, according to data from the International Society of Heart and Lung Transplantation (ISHLT), the number of LT procedures has been progressively increasing and more than 70.000 procedures have been performed worldwide (8). At present, it represents an established therapeutic option for patients suffering from various end-stage respiratory diseases, once all alternative treatments have been exhausted.

1.2 LUNG TRANSPLANTATION DATA

1.2.1 Lung transplantation activity

In Spain, the first LT program was initiated in 1990 in Hospital Universitari Vall d'Hebron in Barcelona (9). Since then six more centers in Spain have started their LT activity and the Organización Nacional de Trasplantes (ONT) has been recording those procedures since 2001. From then to 2020, 4612 procedures have been performed in the country. The evolution of the number of LT shows a continuous rise although in 2020 there was a considerable decline due to the SARS-CoV-2 pandemic. Lately, double-LT has been the most frequent option, representing a 76% from all LT recorded in 2020, while the single-LT accounts for only 24% from the total (figure 1) (10).

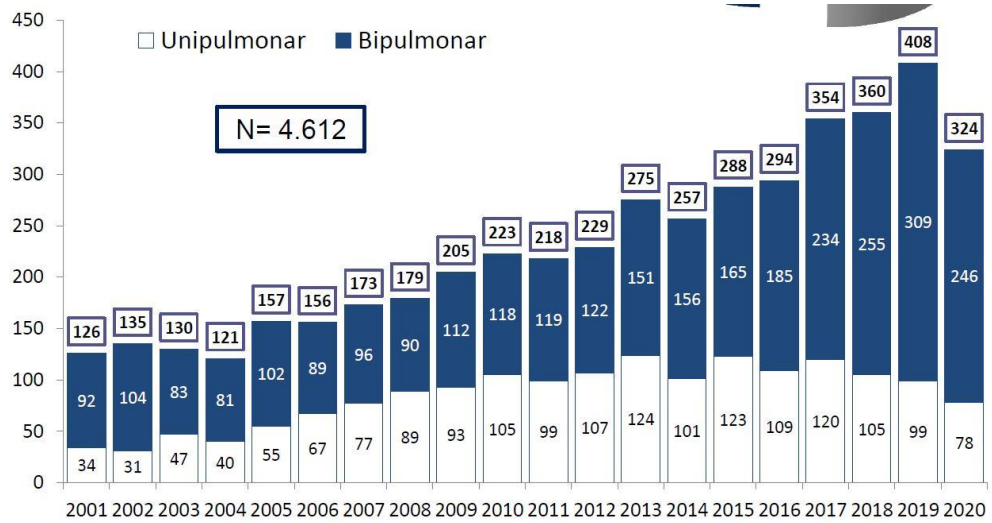

Figure 1. Evolution of the number of lung transplants in Spain reported to the ONT by year (2001-2020 period) (10).

At the international level, the ISHLT recorded LT data for the first time in 1988. However, the last update was done in 2017. During this period, the number of surgeries performed increased every year and more than 69200 LT have been recorded worldwide. As is the case in Spain, the most frequent procedure is double-LT, which in 2017 accounted for 81% of all procedures (11).

According to the European Directorate for the Quality of Medicines and Healthcare, Spain is the third most lung transplant-performing country in Europe, with an annual rate of 7.8 per million of population (pmp) in 2021. Second and first place went to Belgium (8.1 pmp) and Austria (13.7 pmp), respectively (12).

1.2.2 Characteristics of lung-transplanted recipients

Of the 4612 patients who underwent LT between 2001 and 2020 in Spain, 64% were men and 36% women. Regarding the age at which patients underwent surgery, in this period, the median was of 52.3 years old (10).

Furthermore, the main indications for LT in Spain are grouped into different categories. The main ones are: interstitial lung disease (ILD): 1767 patients (38.3%); chronic obstructive

pulmonary disease (COPD)/emphysema/alpha-1 antitrypsin deficiency (A1ATD): 1687 patients (36.6%); bronchiectasis/cystic fibrosis (CF): 626 patients (13.6%), pulmonary arterial hypertension (PAH): 148 patients (3.2%) and retransplant: 122 patients (2.6%). The minority indications are bronchiolitis obliterans (BO), occupational lung disease, among others (10). The Spanish data are extrapolable, since the percentages of LT indications between 1995 and 2018 were very similar worldwide (11).

1.2.3 Survival after lung transplantation

Globally, in Spain the median estimated survival during the period 2001-2020 was of 6.4 years after LT. Interestingly, the median survival of double-LT exceeds that of single-LT, being 8.1 years after LT and 4.6 years after LT, respectively (10). Internationally, a similar effect is observed as between 1992-2017, double-LT had a median survival of 7.8 years, while among single-LT recipients it was only 4.8 years (figure 2) (11).

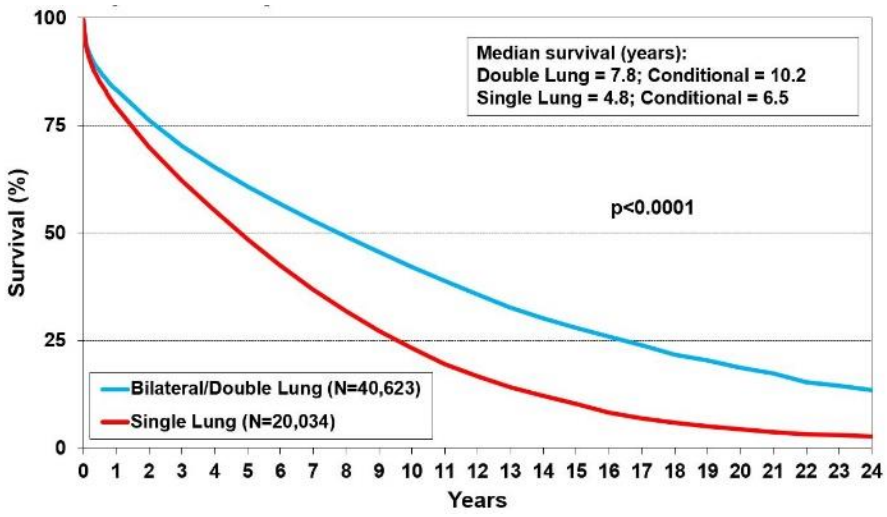

Figure 2. Kaplan-Meier survival analysis for adult lung-transplanted recipients reported to the ISHLT. Registry by type of LT (1992-2017 period) (11).

When survival rates in Spain were studied in separate time periods (2001-2005, 2006-2010, 2011-2015 and 2016-2020) significant differences were observed. Better results are shown between 2011 and 2020 despite the widening of donor acceptability in recent years and the inclusion of older patients on the waiting lists. The most updated data (corresponding to the

2011-2020 period) showed survival rates of 81.5% one year after LT and 59.3% at year 5 (10).

Internationally, patients undergoing primary LT in the most recent era (2010-2017), the median survival was 6.7 years. Although survival after LT is significantly longer in the most recent era (2010-2017), the survival curves for this and the earlier period, (2002-2009) have not diverged as impressively as in previous eras (figure 3) (11).

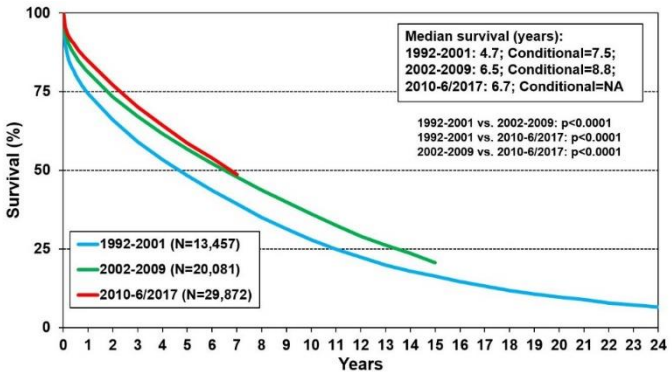

Figure 3. Kaplan-Meier survival analysis for adult lung-transplanted recipients reported to the ISHLT. Registry by eras (blue: 1992-2001, green: 2002-2009 and red: 2010-2017 period)

Moreover, in Spain, LT indications showed differences in survival rates. Five years after LT, survival rates according to LT indications were 70.6% (bronchiectasis/CF), 55.3% (COPD/emphysema/A1ATD), 54.9% (PAH), 50.5% (ILD) and 32.4% (retransplantation). These differences could be related to two facts: First, 57% of LTs in patients with ILD were single-LT and, as mentioned previously in this section, single-LTs have lower survival rates than double-LTs. Secondly, patients with CF tend to be transplanted at younger ages compared to the other indications (10).

According to the ISHLT, for patients who survived one year after primary LT, there were also significant differences depending on the LT indication. Between 1992-2017, the median survival was 12.4 years for bronchiectasis/CF, 12 for PAH, 8.4 for COPD/emphysema/A1ATD and 7.9 for ILD. All pairwise comparisons were significant at $p < 0.05$ except COPD/emphysema/A1ATD vs. ILD and bronchiectasis/CF vs. PAH (11).

1.2.4 Mortality after lung transplantation

In Spain, during the period 2001-2020, the most frequent cause of mortality up to the first year after LT was infections, with 41.7%. However, in the long-term, chronic lung allograft dysfunction (CLAD) becomes the first cause of mortality after LT with 34.2% during the third and fifth year (10).

Worldwide, similar rates were observed, as between 1995-2018, infections had the highest incidence of mortality during the first year after LT (35%) but CLAD represented the major cause of mortality five years after LT (29.3%) (11).

1.3 COMPLICATIONS AFTER LUNG TRANSPLANTATION

1.3.1 Primary graft dysfunction

Primary graft dysfunction (PGD) is the clinical syndrome of acute lung injury that occurs in the first 72h after LT (13,14). PGD is characterized by pulmonary edema with decreased distensibility, impaired gas exchange and increased pulmonary vascular resistance (13). Unfortunately, PGD development is linked to the multiple injuries suffered by pulmonary allografts that are inherent to the surgery, such as pulmonary ischemia or preservation, which makes it difficult to distinguish between these complications. PGD has an incidence of 10-30% in LT recipients and represents an important cause of short-term mortality after LT (14) and a risk factor for CLAD development (15–17).

1.3.2 Acute rejection

Acute rejection is considered one of the most frequent complications during the first year after LT, with an incidence of approximately 27% (11). Importantly, it has been identified as a potential risk factor for CLAD development (18,19). This complication includes two different clinical phenotypes: acute cellular rejection (ACR), and antibody-mediated rejection (AMR).

1.3.2.1 Acute cellular rejection

The pathway of ACR involves recruitment and activation of recipient lymphocytes (predominantly effector T cells) into the lung allograft, resulting lung allograft injury and loss of function (20). ACR is mainly diagnosed by bronchoscopic transbronchial biopsy (21) and is based on the histological observation of lymphocytic infiltrates in a perivascular (acute perivascular inflammation) or peribronchiolar (lymphocytic bronchiolitis) distribution (22).

1.3.2.2 Antibody-mediated rejection

In AMR, allospecific B-lymphocytes and plasma cells produce donor-specific anti-human leukocyte antigen (HLA) antibodies (DSA). The antigen-antibody complex results in an amplified immune response, via complement-dependent and independent pathways, leading to abnormal lung tissue pathology and graft dysfunction to varying degrees (23). Both pre-LT DSA and de novo DSA after LT are associated with graft dysfunction (24,25).

1.3.3 Chronic Lung Allograft Dysfunction

In 2019, an international consensus (22) was established to facilitate the definition and phenotypes of CLAD, research into its pathogenesis, and the development of appropriate prevention and treatment for each phenotype among transplant centers. Currently, CLAD is defined as a substantial and persistent decline in FEV_1 of ≥20% from baseline FEV_1 (defined as the mean of the two best postoperative FEV_1 measurements taken at least three weeks apart) (26). It the decline in FEV_1 is confirmed on a second pulmonary function test at least 3 weeks after from the first decline, and after alternative causes of the decline have been addressed, this indicates probable CLAD (26,27). The diagnosis of definite CLAD is made when the decrease persists after 3 months and other causes have been discarded. After the diagnosis of CLAD, the stage can be assigned according to the severity of FEV_1 decline (from CLAD 0 to 4) and can be classified into 4 different phenotypes: BOS (Bronchiolitis Obliterans Syndrome), RAS (Restrictive Allograft Syndrome), mixed and undefined or unclassified.

1.3.3.1 Bronchiolitis obliterans syndrome

BOS is defined as CLAD with obstructive ventilatory defect (FEV_1/FVC <0.7) and absence of persistent radiologic lung opacities or pleural thickening (26). BOS is the most frequent phenotype (70% of CLAD patients) (28), with different series describing a prevalence of 50% at 5 years post-LT (23), and a median survival after onset of about 3.9 years (28).

1.3.3.2 Restrictive allograft syndrome

The consensus for RAS diagnosis included three conditions: First, a diagnosis of CLAD must be present. In addition, a further decline ≥ 10% in TLC (compared with baseline, defined as the average of the two measurements obtained at the same time as or very close to the two best post-operative FEV_1 measurements) and the presence of persistent opacities (29) for more than 3 months (frequently ground glass or consolidations). The prevalence of RAS ranges from 25-35 % and has worse prognosis than the other phenotypes, with a median survival after onset around 1.5 years (28).

1.3.3.3 Mixed phenotype

The mixed phenotype includes all cases that share histopathologic findings of both BOS and RAS phenotypes, an obstructive ventilatory defect, decline ≥ 10% of TLC and the presence of opacities (26).

1.3.3.4 Undefined or unclassified phenotype

Undefined means definite CLAD, but with two possible combinations of variables, making it difficult to classify into BOS, RAS or mixed phenotypes (26).

To date, no effective treatments for CLAD are available. Overall, CLAD is the leading cause of death, accounting for more than 40% of mortality after the first year post-LT (18).

1.3.4 Infections

Infection is one of the leading causes of death after LT, causing 33% of deaths between 30 days and one year post-LT, although it remains a major complication at all time points after

LT. Unlike other solid organ transplant patients, LT recipients are especially vulnerable to infections due to their immunosuppressed state, continuous exposure of the lungs to the external environment, blunted cough reflex and impaired mucociliary cleareance (30,31). Further, infections are known to be a risk factor for CLAD development (32–39).

1.3.4.1 Bacterial infections

The most frequent aetiology for infections after LT is bacterial pathogens; in fact, some studies describe that up to 85% of patients present respiratory complications due to bacterial infections after transplantation (40), with an incidence ranging from 35% to 66%. However, these episodes have lower mortality rates than viral or fungal infections.

Most bacterial infections occur during the first 3 months after LT (41). In the first month, infections with hospital-acquired multidrug-resistant pathogens predominate. For instance, *Enterococcus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* are the most frequent ones (42,43).

P. aeruginosa is the most frequently isolated microorganism in pneumonia, followed by *S.aureus* and *Acinetobacter* (44). Although bacterial pneumonia is more frequent during the first month after LT (44), the risk persists in the long term after surgery and community-acquired pathogens such as *Streptococcus pneumoniae* become more frequent (45).

The importance of *P. aeruginosa* infection derives not only from its high incidence (46) but also from its possible association to CLAD development (36,37). Some studies suggest that infection with *P. aeruginosa* triggers cytokines expression, leading to airway inflammation and destruction (41).

1.3.4.2 Viral infections

Viral infections account for up to 30% of all infectious complications in LT recipients, and remain a major cause of morbidity and even mortality, mainly due to Herpesvirus and respiratory viruses.

Human cytomegalovirus (CMV), which belongs to the *Herpesviridae* family, remains latent after infection, making immunocompromised individuals more vulnerable to reactivation. When this occurs, CMV can cause pneumonitis, but also increase susceptibility to other infections and to CLAD development (38).

The risk of CMV disease after LT is related to both donor and recipient status, with the seropositive-donor and seronegative-recipient combination being the most dangerous (47). CMV is responsible for approximately 1% mortality in LT recipients (11).

Other viral respiratory pathogens with clinical relevance include influenza A and B viruses, respiratory syncytial virus (RSV), parainfluenza virus (PIV), human metapneumovirus (hMPV), human coronaviruses (HCoV), picornaviruses and adenovirus. In particular, acute mortality rates of 6-20% have been reported in PIV, RSV and hMPV (48–50). Respiratory viral infections have also been associated to CLAD development (39).

1.3.4.3 Fungal infections

Among all solid organ transplant patients, LT recipients have the highest risk of developing an invasive fungal infection (IFI). According to the Transplant Associated Infection Surveillance Network (TRANSNET), 8.6% of LT recipients will develop IFIs in the first 3 to 12 months after LT (51). The most common IFI among LT recipients is caused by *Aspergillus* spp (51,52) which can lead to tracheobronchitis or invasive pulmonary aspergillosis (IPA), the most dangerous phenotype. *Aspergillus* infections have incidence rates around 14%; however, invasive disease ranges from 1.5% to 12.2%. Similarly, mortality rates vary depending on the clinical symptoms: 3% in non-invasive forms versus approximately 40% in invasive aspergillosis disease (53).

1.4 TREATMENTS IN LUNG TRANSPLANTATION

1.4.1 Immunosuppressive therapy

The purpose of lifelong immunosuppression is to prevent acute and chronic rejection. Finding a balance is crucial because of the adverse side effects and increased risk of

opportunistic infections and malignancies caused by this therapy. The conventional maintenance strategy usually consists of triple therapy including a calcineurin inhibitor (CNI) (tacrolimus or cyclosporine), a cell cycle inhibitor, (mycophenolate (MMF) or azathioprine (AZA)) and corticosteroids. According to the 2018 ISHLT registry report, the most commonly used combination one year after LT is tacrolimus, MMF and corticosteroids (54). The use of tacrolimus is more frequent than cyclosporine in LT because it has been associated to decreased risk of BOS development, as well as better control of persistent rejection (55). MMF and AZA are antimetabolites, which inhibit nucleotide synthesis in lymphocytes but using different mechanisms. Ultimately, proliferation of de novo T and B-lymphocytes is suppressed (56). Although acute and chronic rejection and survival appear to be similar between patients receiving MMF and AZA, MMF may slow the progression of chronic rejection (57), and more patients discontinued AZA treatment (58). Corticosteroids have been used in solid organ transplant since the beginning, as induction and maintenance immunosuppression. The most commonly used are prednisone and methylprednisolone. Corticosteroids are known for their antiinflamatory properties and exert their effect in different pathways. For instance, they inhibit cytokine production (59) and the translocation of the nuclear factor κB (NF-κB) to the nucleus (60).

There is another group of drugs called mammalian target of rapamycin (mTOR) inhibitors (sirolimus and everolimus), which are used as alternative cell cycle inhibitors for those patients who cannot tolerate CNI or in specific circumstances (CMV replication, malignancies, etc).

1.4.2 Antimicrobial therapy

LT recipients undergo an antimicrobial therapy, which includes antibacterial, antiviral and antifungal drugs.

1.4.2.1 Antibacterial prophylaxis

Cotrimoxazole is used to prevent *Pnemocystis jirovecci* infection but it also acts against *S. pneumoniae*, *Listeria monocytogenes*, *Staphylococcus*, *Nocardia* and Enterobacteriaceae (61). The combination of trimethoprim and sulfamethoxazole block different enzymes that bacteria need for the synthesis of tetrahydrofolic acid, a cofactor necessary for the synthesis of nucleotide bases (62).

Azithromycin is a macrolide with immunomodulatory effects. As an antibiotic, it acts by binding to 23S rRNA of the bacterial 50S ribosomal subunit. Thus, 50S subunit cannot be assembled and bacterial protein synthesis is stopped (63). Interestingly, randomized clinical trials have shown that prophylactic treatment with azithromycin significantly reduces the incidence of BOS development at early stages (64) but also at advanced post-LT period (65). Further, once BOS is diagnosed it reduces the risk of mortality in patients with this chronic dysfunction (66).

1.4.2.2 Antiviral prophylaxis

According to the Transplantion Society guidelines from 2017, valganciclovir is currently the most commonly used drug for CMV prophylaxis (67). Further, a duration of prophylaxis of 6 and 12 months is recommended for positive donor/negative LT recipients as they are considered high risk (67). Although seropositive recipients often require shorter courses of prophylaxis, longer protocols have been associated with a significantly lower incidence of late CMV infections (68,69).

1.4.2.3 Antifungal prophylaxis

Different antifungal prophylactic strategies have been reported but currently, inhaled amphotericin B (L-AmB) is the most used drug. L-AmB is a polyene that binds to the ergosterol placed at the membrane of the fungal cells. L-AmB acts by pore-forming or sequestering the ergosterol and this results in membrane disruption causing cell death (70,71). L-Amb has been shown to be good in terms of tolerability (53,72), allowing it to be

maintained for life. It is also specific for the respiratory tract, as systemic absorption does not occur (72,73). L-AmB is used on its own or in combination with azoles; however, L-AmB has shown to have a lower incidence of systemic side effects and does not interact with immunosuppressive drugs (73).

1.5 THE HUMAN MICROBIOME

The term microbiome was coined by Joshua Ledelberg in 2001 and was literally defined as the ecological community of commensal, symbiotic, and pathogenic microorganisms that hare our body space and have been virtually ignored as determinants of health and disease (74). Initially, the term microbiome was of concern to researchers, as it was usually confused with microbiota (75). Today, we know the microbiota as the microbial community associated with a defined habitat and the microbiome as the genetic information of the gene products of the microbiota. Globally, the human microbiome is defined as the microbiome collectively found in internal and external habitats of the human body, including bacteria, viruses and fungi (75–77). The human microbiome comprises more than 100 trillion microbes distributed in different compartments (see table 1). Although the densest community is found in the gut, other locations such as the skin, the vagina or the respiratory tract also contain their own ecosystems (78,79).

Table 1. Key words from the microbiome field
Microbiota: Microbial community in a habitat
Microbiome: Genetic information of the microbiota in a community
Virome: Genetic information of the virus present in a community
Mycobiome: Genetic information of the fungi present in a community
Dysbiosis: Deviation from the normal, optimal, or healthy microbiome in a community
Taxon: A grouping of microbes at any level such as phylum, family, genus or species
Metagenomics: Genomic analysis of microorganisms directly in their natural environments, bypassing the need of isolation or culture (80)
16S ribosomal RNA gene: Conserved bacterial gene which allows their identification
18S ribosomal RNA gene: Conserved fungal gene which allows their identification
Internal Transcribed Spacer Sequence (ITS): Region in the rRNA locus used for targeted fungi sequence
Shotgun metagenomics sequencing (SMS): Sequencing technique that previously requires breaking the genome into small fragments and then reassembling using overlapping sequences
Sequenced-Independent Single-Primer Amplification (SISPA): Random priming method that allows enrichment of the viral genome in only a few steps
Richness: Number of different species present in a community
Eveness: Evenness of distribution among species in a community
Alpha diversity: Microbiome diversity within a sample or community
Beta diversity: Microbiome diversity among samples or communities
OTUs: Operational Taxonomic Units. Clusters with similar sequencing reads
ASVs: Amplicon sequence variants. Sequences obtained from a denoising bioinformatics model to classify bacterial reads

1.5.1 The human bacterial microbiome

Research on the human microbiome using culture-independent approaches began with the bacterial part in the 1970s (81,82). For years, the focus was mainly on bacteria, so many studies referred to the microbiome only as the bacterial microbiome. In addition, most reports speak of the gut microbiome, as it contains the highest bacterial load (76,83). In fact, the first study that characterised the lung microbiome by culture-independent techniques dates back to 2010 (84). After years of study, it is now accepted that the number

of bacterial cells associated with the human body is approximately ~10^{13}, the same number of human cells (78). Further, it has been estimated that between 500-1000 bacterial species coexist in the human body (76).

1.5.2 The human virome

As mentioned above, viruses are considered part of the microbiome, although there is a term to describe the collection of genomic information of viruses present in a habitat, which is called virome (79). It consists of bacteriophages that infect bacteria, viruses that infect other microorganisms such as archaea and viruses that infect human cells (85–87). Further, their genomes can be categorized in DNA or RNA, and both of them can be double-stranded or single-stranded (88). Because of their high mutation rates, viruses are more difficult to evaluate than bacteria (79). In fact, the first study of a viral population was in a marine environment in 2002 (89), in which ~10^{7} viral-like particles (VLPs) per millilitre of seawater were reported. Therefore, it was not surprising the large proportion of viral "dark matter" (unclassified viral sequences awaiting characterization) that they also found (89). Since then, different studies about human virome have been published; and it has been observed that the number of viruses found in humans is of a similar order than bacterial and human cells (~10^{13}) (90). The vast majority of the human virome is composed of bacteriophages (79,88). Their distribution, as well as that for eukaryotic viruses, varies depending on the body site (79).

1.5.3 The human mycobiome

As with viruses, the fungal genomic part of the microbiome is recognized by a specific term: the mycobiome. The term mycobiome was first coined in 2010 when metagenomics was used to characterize the oral mycobiome in healthy controls finding greater diversity when compared to culture techniques (91). It consists in microeukaryotes that can be found on different mucosal surfaces such as the lungs, oral cavity, gut, vaginal and urinary tracts and skin (92) although its abundances varies substantially depending on the body site (92).

1.5.4 Methods for microbiome analysis

Table 2. Advantages and disadvantages of different metagenomics techniques

16S/18S/ITS sequencing	Shotgun metagenomic sequencing
Only catches targeted genes	Many reads may be from host
Amplification bias	No amplification bias
Viruses cannot be captured	Detects bacteria, fungi, archaea and viruses
Lower taxonomic resolution	Higher taxonomic resolution
Fast and cost-effective	Slow and expensive (requires high reads count)
Easier bioinformatics analysis	More complex bioinformatics analysis

In recent years, the reduction in cost and increase in accuracy and speed of metagenomics techniques, together with advances in bioinformatic approaches, have made it possible to identify microbial taxa that were difficult or even impossible to culture (75,76). Two different metagenomics methods are available to evaluate the microbiome: targeted sequencing and shotgun metagenomics sequencing (see table 2).

1.5.4.1 Targeted sequencing

Targeted sequencing focuses on the sequencing of specific target regions (amplicons). Currently, 16S ribosomal RNA (rRNA) gene sequencing is the most extended method in bacterial microbiome analysis (93). The 16S rRNA gene is highly conserved among different species of bacteria and archaea. Besides, it contains nine hypervariable regions (from V1 to V9), allowing bacterial identification and the study of bacterial composition, abundance and diversity (94) (figure 4). Isolated bacterial sequences are identified by homology analysis with previous existing sequences in databases. The closest match obtained becomes the species assigned to the new sequence. However, there is a limitation: in many cases, the difference between the closest match and the next closest match is not large enough to allow species identification with confidence. Thus, inaccuracy in species level classification is the main limitation of this technique (93). Further, as previously mentioned, 16S rRNA gene sequencing is limited to the bacterial microbiome. Other targeted

sequencing methods include 18S rRNA gene (95) or nuclear ribosomal internal transcribed spacer (ITS) region, both specific for mycobiome (96).

Figure 4. Representation of the 16S rRNA gene. It contains ~1500 bp and it is composed of nine hypervariable regions (V1-V9) flanked by the conserved regions (grey color). Hypervariable regions allow bacterial identification (93,94,97).

1.5.4.2. Shotgun metagenomic sequencing

Shotgun metagenomic sequencing (SMS) is an alternative approach, which allows for parallel sequencing of DNA from all organisms within the community, with high coverage for species-level detection. SMS is based on the production of random DNA fragments that are assembled using bioinformatics tools to find overlapping ends between DNA fragments (98). Thus, SMS covers the two limitations of 16S rRNA gene sequencing, as it is more accurate in the species-level classification of bacteria and allows the classification of other genomes, such as the viral microbiome. However, SMS is more expensive and requires more extensive bioinformatics analysis.

However, this technique is frequently hampered by the relatively lower abundance of virus genomes within a huge sequence background from other cellular sources. To overcome this problem, several approaches for enriching the viral nucleic acid content in the specimen have been developed (99). Based on random priming and nonspecific amplification, the Sequence-Independent, Single-Primer-Amplification (SISPA) is a frequently employed method for this purpose (100–105). The method uses a barcoded amplification primer with random nucleotides at the 3′ end that bind to template DNA and a second round of single primer PCR amplification (106). The amplified products can be used directly in next generation sequencing library construction. However, some genomics regions may be underrepresented due to variations in priming efficiency (100).

1.5.5. Assessment of microbiome composition

As mentioned above, bacteria, viruses and fungi compose the microbiome and specific strategies are needed to study their diversity, i.e., to characterise the number of different species of microorganisms present and their distribution. For all three of them, the most extended ways to study diversity are alpha and beta diversity measurements.

1.5.5.1 Alpha diversity

Alpha diversity is the distribution of species abundance in a given sample that depends on species richness and evenness (107). Richness refers to the total number of species in a community (or sample). The simplest richness index is the number of species observed (observed richness). However, assuming limited sampling of the community, this may underestimate the true species richness. Further, evenness focuses on species abundance, so it may complement the number of species (107). An alpha diversity estimator is the Shannon index, which is defined as the sum of the proportion of each species relative to the total number of species in the community analysed and thus takes into account both richness and evenness (108).

1.5.5.2 Beta diversity

Beta diversity quantifies (dis-)similarities between communities (samples) (107). Some of the most popular beta diversity measures in microbiome research are the Bray-Curtis dissimilarity (for compositional data) (109) and the Aitchison distance (Euclidean distance for abundances transformed to log-ratio centered (clr), with the aim of avoiding compositional bias).

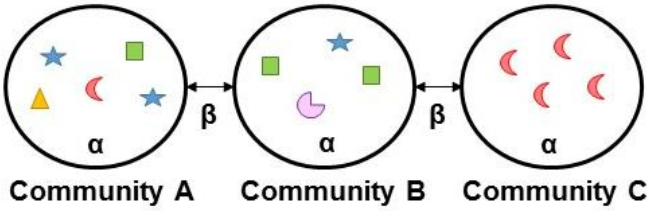

Figure 5. Schematic definition of alpha (α) and beta (β) diversities. Each shape represents one specie. Community A has four different shapes, community B has three different shapes and community C has one. This means that community A has the highest alpha diversity. If we compare between communities A and B, there are three different shapes that they do not share, as is also the case when comparing communities A and C. While communities B and C contain four shapes that they do not share, so the composition of these two communities is the most different.

In addition, the technique called Ordination can represent distances in lower dimensions for better visualization. Some of the most common ordination methods are Principal Component Analysis (PCA), Principal Coordinate Analysis (PCoA) or metric multidimensional scaling (MDS) (107).

1.6 THE RESPIRATORY MICROBIOME

For years, lungs were considered sterile organs, as traditional cultures provided negative results (110). However, metagenomics techniques have established that lungs are not only not sterile organs, but also have their own microbiome (77,111).

Even so, the respiratory microbiome has received less attention due to different factors. For example, respiratory samples are considered low biomass, meaning that the bacterial load in the respiratory tract is considerably low compared to other compartments such as the gut (77,111). In addition, the connection of the upper respiratory tract (URT) to the lower respiratory tract (LRT) may introduce experimental bias and, finally, all the sampling difficulties of the LRT.

1.6.1 Sampling the respiratory tract

The respiratory tract is divided into two main areas: the URT, which includes the nasal cavity, paranasal sinuses, pharynx and supraglottic portion of the larynx, and the LRT,

which includes the trachea, bronchi and lungs. Both tracts offer alternatives for sampling. For instance, for the URT, preferred samples include nasal, nasopharyngeal (NP) or oropharyngeal swabs as well as oral wash or nasal lavage fluid, because access to these areas is easier (112,113). As for LRT, bronchoalveolar lavage (BAL) represents the gold standard for analysing the microbiome, as it shows what is happening in the lungs (77,111). However, it requires an invasive procedure to obtain it. Similar is the case for lung or bronchial biopsies or protected specimen brushings (PSB), which also represent the LRT but can only be obtained with invasive techniques. Another representative sample of LRT is sputum. Sputum is usually limited to patients with infection or CF because the healthy population does not expectorate spontaneously (77). (77). However, sputum can be induced by a non-invasive procedure involving inhalation of a nebulised hypertonic saline solution, which liquefies airway secretions, promotes coughing, and allows expectoration of respiratory secretions (114). It is accepted that the best sampling procedure depends on the research topic (77). For example, to study diseases with a bronchial component, sputum would be a more accurate sample; however, to obtain information on the peripheral bronchial tree or alveolar surfaces, BAL, PSB, bronchial or lung biopsies are required (77).

Although different studies have shown that the influence of contamination of the pharyngeal microbiome that can be carried by the bronchoscope to BAL samples is minimal (115,116), parallel sampling of both URT and LRT may be useful to discriminate both microbiomes, especially in disease.

The low-biomass nature of all these samples makes them easy to contaminate at any stage of processing, including nucleic acid extraction and amplification (77,117,118). To avoid external or environmental contamination, it is necessary to decontaminate all laboratory equipment and to use sterile reagents and materials. In addition, negative controls need to be introduced during extraction and amplification, and all protocols should be as efficient as possible to recover as much of the scarce genetic material present in the samples without adding contamination (77,117,118).

1.6.2 The respiratory microbiome in health

The respiratory microbiome in health is characterised by the balance between immigration and elimination mechanisms, such as cough, mucociliary escalation or inflammatory cells activation and concentration, but also with the reproduction rates of microorganisms (118).

Analysis of the respiratory microbiome in the healthy population is challenging, specifically for LRT. Although sputum can be induced, not all individuals can do so successfully and bronchoscopy is needed to obtain both BAL and lung biopsy.

1.6.2.1 The respiratory bacterial microbiome in health

Even with these difficulties, the bacterial microbiome in healthy lungs has been described. Since the first study characterising the lung bacterial microbiome by metagenomics in healthy subjects, it has been observed that healthy lungs contain a diverse bacterial microbiome (84,119). In spite of methodological differences, it is now accepted that healthy lungs are especially colonised by the following phyla: Firmicutes (especially *Streptococcus*, *Veillonella* and *Staphylococcus*), Bacteroidetes (mainly *Prevotella*), Proteobacteria (*Pseudomonas*) (116,120) and Actinobacteria (mainly *Corynebacterium* and *Propionibacterium*), although this phylum is more abundant in the URT (119,121). In addition, an overlap in bacterial composition has also been observed between the LRT and URT (116,119,120), although the URT contains a higher bacterial burden than the lungs (116,119).

1.6.2.2 The respiratory virome in health

Different viral communities populate the healthy human respiratory tract. Bacteriophages appear to be the most abundant viruses found and are mainly derived from the abundant bacterial populations of the URT (79). However, a high proportion of unidentified sequences are also found (79). The most common phages are *Caudovirales* (122–124). Regarding eukaryotic viruses, among the DNA one, *Anelloviridae* is the most common family (122–124) followed by *Redondoviridae* (125). Other frequent DNA families include *Adenoviridae*,

Herpesviridae and *Papillomaviridae* (122–124). As in the respiratory tract, the *Anelloviridae* are the most frequent viruses found in blood, accounting for 70% (126).

1.6.3 The respiratory microbiome in disease

In contrast to what happens in health, in disease there is a dysregulation of microbiome composition, called dysbiosis, which occurs in both URT and LRT. However, it remains unclear whether the microbial dysbiosis itself is the cause of the disease or a consequence of the pathogenic process (127). For instance, in diseases with impaired mucociliary clearance mechanisms, microbial dysbiosis might be a consequence of inaccurate microbial elimination (111) and immigration has been found to be accelerated by gastro-oesophageal reflux, a co-morbidity with high prevalence among patients with chronic diseases (128). In contrast, Dickson *et al.* proposed that an inflammatory cascade is activated by an inflammatory trigger with the release of cytokines that promote selective growth of some bacterial species (118). In turn, macrophages are recruited and activated to kill bacteria. Consequently, a negative selective pressure gradient is created between species. Further, there is an increase of mucus production that facilitates persistent colonisation of disease-associated microbes (118), making it possible for potential pathogens to persist for longer periods of time (77,111). There is also evidence to suggest that a persistent exposure to certain microbes not only perpetuates inflammation, but also leads to immune exhaustion. For example, Segal *et al.* demonstrated that if the microbiome of the LRT was dominated by oral commensals, there was a weakening of the Toll-like receptor 4 response of alveolar macrophages(129). Later, the same group observed that microbial products such as short-chain fatty acids had important immunomodulatory properties and weakened IFN-γ and IL-17 responses to pathogen-associated molecular patterns (130).

1.6.3.1 The respiratory bacterial microbiome in disease

The first study in the respiratory field using metagenomics to analyse the microbiome, focused not only on healthy subjects, but also in patients with asthma, observed that their

airways contained different microorganisms compared to healthy subjects, mainly enriched with Proteobacteria (84). Since then, different reports have demonstrated the existence of an altered bacterial microbiome in different respiratory diseases. For example, *Prevotella*, *Streptococcus* and *Moraxella* genera in bronchial brush samples were observed to discriminate between patients with mild-to-moderate COPD and healthy controls (131). Other studies with stable COPD reported an association between some pathogens (*Haemophilus, S. pneumonia, Moraxella catarrhalis, S. aureus* and *P. aeruginosa*) with neutrophilic inflammation and increased expression of cytokines, such as IL-8 and TNF in the LRT (132–135). The contribution of the microbiome to COPD remains poorly understood. However, a study in macaques showed that an enrichment of the LRT with oral commensals contributed to COPD development, suggesting a causal role of the microbiome (136). For severe COPD, many studies have shown a shift from the phylum Bacteroidetes to potentially pathogenic genera of the phylum Proteobacteria, such as *Pseudomonas* or *Haemophilus* in sputum, BAL and lung tissue (137–139). Apparently, with disease progression, chronic inflammation alters the innate immune system of the lung and, consequently, an increase of pathogens occurs. For instance, one study showed association between *Haemophilus* in sputum with increased IL-1β and TNF levels (140). In contrast, COPD patients enriched in *Veillonella* and *Prevotella* had a more dynamic microbiome over time and increased levels of IL-17A (140).

Although traditionally idiopathic pulmonary fibrosis (IPF) was not associated with bacterial pathogenesis, different studies showed associations between microbiome and IPF progression. For instance, *Staphylococcus* and *Streptococcus* genera seem to correlate positively with IPF progression (141) and bacterial burden in BAL has been associated with exacerbations (142) or even increased mortality rates (143). More recently, a decrease in bacterial diversity has been observed in patients with lower FVC and early mortality in IPF, as well as an increase in Firmicutes and a decrease in Proteobacteria related to IPF progression (144). In another study, it was observed that, in IPF, lower alpha diversity was

associated with higher concentrations of alveolar inflammatory and fibrotic cytokines such as IL-1Ra, IL-1β, CXCL8, MIP1α, G-CSF and EGF (145). Further, alveolar IL-6 concentration correlated positively with the relative abundance of Firmicutes while IL-12p70 correlated negatively with relative abundance of pulmonary Proteobacteria (145). In addition, other studies pointed to an association between dysbiosis of the lung microbiome with transcriptomic expression of peripheral blood mononuclear cell of immune pathways and with survival (146,147). However, human studies have the limitation of being unable to prove the directionality of the observed associations, although murine studies suggested that certain microbial exposures could act as persistent stimuli for alveolar injury contributing to pulmonary fibrosis (145).

1.6.3.2 The respiratory virome in disease

A limited number of studies have been published on the respiratory virome in disease compared to the bacterial microbiome, although some have demonstrated the influence of the virome on respiratory diseases. The first disease in which the virome was characterized by metagenomics was CF. Sputum of these patients showed a poorly diverse virome compared to that of healthy individuals. Further, the eukaryotic viral communities in CF were dominated mainly by Herpesviruses, but also Retroviruses, which appeared to be present in the genome as DNA intermediates (148). However, only DNA viruses were evaluated. In another study in which DNA and RNA viruses were sequenced, more than 39 viral species were observed in NP aspirates from adults with severe LRT infection, with *Paramyxoviridae*, *Orthomyxoviridae* and *Picornaviridae* (all with RNA genome) being the most frequents families (149). For moderate and severe COPD, a recent study using sputum samples demonstrated that DNA viruses of the *Anelloviridae* and *Herpesviridae* families, RNA virus from the *Retroviridae* family, and phages from the *Shiphoviridae* family shape the main respiratory virome in this disease; however, in healthy subjects, Herpesvirus were partially substituted by members of the *Anelloviridae* family (150). On the other hand, one study showed that patients with Hepatitis C virus infection had a higher incidence of IPF compared

to patients with Hepatitis B virus infections, although these findings could be altered by other confounding factors (151). Recently, a new family of DNA called *Redondoviridae* has been identified in oropharyngeal specimens from patients with periodontal disease (125). In addition, *Redondoviridae* was detected at higher levels in endotracheal aspirates from critically ill patients than in healthy controls. Interestingly, this viral family could not be detected in the blood of these patients, suggesting that it may be limited to the respiratory tract (152).

Different studies have suggested that bacteriophages stimulate the host immune response. For instance, phagocytosis of *P. aeruginosa* phages by dendritic cells appears to trigger the production of Toll-like receptor 3 and type I interferon, promoting the antiviral response (153). Further, it has also been observed that phages can induce an inflammatory response by activating macrophages and inducing the release of IL-1β and TNF (154). However, the role of bacteriophages and mainly eukaryotic viruses in the dysbiosis of the microbiome and in the interaction with the immune system is not well understood.

1.6.4 Challenges of studying the human respiratory microbiome

Although research on the human microbiome has grown in recent years, standardized protocols for sampling, processing and bioinformatic analysis are lacking, especially regarding the respiratory microbiome. Regarding bacterial DNA extraction, gram-positive bacteria might be undervalued, as they present a thicker cell wall that requires additional steps for disruption (155). As mentioned, the 16S rRNA gene has different hypervariable regions that can be used for bacterial identification; however, it is unclear whether one of them, or even a combination, is the most suitable for assessing the respiratory microbiome. Different sequencing platforms can also be used to obtain bacterial reads, such as 454 from Roche or MiSeq and HiSeq from Illumina, and it has been observed that different results are obtained, probably because different primers are used and because the platforms produce amplicons of different lengths (156).

Traditionally, bioinformatic tools have used an approach to cluster sequencing reads at some level of similarity (threshold often set to 97%) under the general term of operational taxonomic units (OTUs) Recently, however, denoising methods have been introduced that produce amplicon sequence variants (ASVs) instead of clusters (157). The denoising methods generate an error model based on sequencing quality and use this model to distinguish between the predicted "true" biological variation and that likely generated by sequencing error. The remaining "true" sequences are defined as separate ASVs, which provide higher accuracy than OTUs (157–159).

The lack of a standardized protocol for microbiome studies represents an issue, as many sources of variability may be introduced throughout the process and, therefore, studies from different groups cannot be adequately compared.

1.7 THE MICROBIOME IN LUNG TRANSPLANTATION

As mentioned above, the use of metagenomic approaches has allowed the study of the respiratory microbiome in different diseases. In recent years, these advances have also been applied to the LT field. In particular, the role of the bacterial microbiome in LT recipient outcome has received growing interest. However, the role of the virome in this field has hardly been studied.

1.7.1. The bacterial microbiome in lung transplantation

The lung microbiome of LT recipients has been reported to contain a high bacterial load compared to healthy controls (160–163). Besides, compared to the healthy population, the lung microbiome after LT is mainly characterised by lower alpha diversity, although the opposite results were reported by Borewicz *et al.* (164).

In addition, after LT, the microbiome changes dynamically over time in its composition or beta-diversity, and is accompanied by a growth of specific taxa (160,162,163,165). For instance, the lungs of LT recipients are commonly enriched by the phylum Proteobacteria, especially the genus *Pseudomonas* (36,161,166,167) but other genera such as *Escherichia*

(161), *Prevotella*, *Veillonella* and *Rothia* (160) have also been detected. Nevertheless, alterations on microbiome composition usually occur early after LT, and microbiome composition tends to stabilise over time (162,163).

As stated previously, in the healthy population there is a balance between URT and LRT microbiomes, but apparently in LT, as well as in some respiratory diseases, this balance is disrupted. For instance, Charlson *et al.* (160) and Sharma *et al.* (10) observed dissimilarities when comparing the URT and LRT microbiomes in LT recipients.

Besides, the persistence of the donor microbiome in the allograft and its relation to the post-LT microbiome composition remains controversial, as similarities in the microbiome between LT donors and recipients have been described (169). Although different studies have shown opposite results (170,171), as well as the absence of a relationship between the bacteria that colonize the donor and the results of the LT (13).

However, different microbiome alterations have been associated with different outcomes after LT. Spence *et al.* reported that ACR episodes in LT recipients were associated with decreased alpha diversity of the lung microbiome compared to patients without ACR (163). In addition, Schneeberger *et al.* observed that *Prevotella* and *Veillonella* from the URT colonised the LRT microbiome of LT recipients with gastro-esophageal reflux disease (172).

Regarding the influence of antibiotic use on the microbiome in the LT field, there are numerous discrepancies in literature. For example, an early study showed that antibiotics had an impact on the lung microbiome, especially in the early post-LT period (166). Similarly, the antibiotics colistin and meropenem were found to alter microbial profiles in BAL in the early post-LT period (169). However, other studies did not associate azithromycin (163,173) or other antibiotics (161,173,174) with changes in microbial diversity either in early post-LT stages nor long-term phases (163,173). It should be noted that not all of the above studies were statistically powered to assess the effect of antibiotics on the microbiome (160,162,164,170,172,175) .

1.7.2. The virome in lung transplantation

Even with the rise of metagenomic methods, only a few studies have used this approach to assess the whole virome in LT recipients (123,176–178). Further, those that have done so have used different strategies: DNA virus only, RNA virus only, both types of viruses, enriching by viral particles, analysing only circulating cell-free DNA and thus losing information from encapsidated viruses (179), etc.

In fact, the first study of virome in LT that used a metagenomic approach focused solely on the study of DNA and found that there was a higher abundance of torque teno virus (TTV), viruses belonging to the family *Anelloviridae*, in the lungs of LT recipients compared to the healthy population (122). This finding was later confirmed in other body compartments, such as plasma (176). Indeed, replication of this virus, TTV, has previously been associated with the immune status of the patient for other pathologies such as IPF (180) or HIV infection (181) and it seems that TTV levels increase with escalating doses of immunosuppression. Thus, TTV viral load has been proposed as a potential non-invasive tool to monitor immunosuppressive therapy in LT recipients (182,183).

Regarding RNA viruses in LT population, CARV were found to be a common component of the lung virome and also to be transmitted by the transplanted organ at the time of transplantation (184). However, the only metagenomic study focusing on RNA viruses showed that the abundance and diversity of the lung RNA virome remained unchanged during the early stages after LT. In that study, Rhinovirus and Parainfluenza were detected as the most frequent genera (185).

Recent studies described relationships between virome and early post-LT complications. For example, smaller increases in TTV levels from pre- to post-LT stage were observed in LT recipients who developed PGD compared to those who did not (13,22). Furthermore, increases in TTV loads have been described to reduce the risk of ACR (19,23), although a

limitation of the predictive value of TTV for immune episodes during the first 1-2 months after LT has also been suggested, as the kinetics appear to differ between individuals (183).

1.7.3. The bacterial microbiome in chronic lung allograft dysfunction

Although the respiratory microbiome of LT recipients has been described, its role in CLAD pathogenesis is unclear. Current research focuses on identifying the microbial signature of CLAD, with discrepant results to date. Some studies have shown differences in microbiome composition between CLAD and CLAD-free patients (161,166,172–174), mainly attributed to an increase of *Pseudomonas* in CLAD recipients microbiome (161,172,174). For instance, Schott *et al.* found that Actinobacteria-dominated profiles in BAL were associated with better outcomes, whereas *Pseudomonas*-enriched microbiomes were more present in LT recipients that developed BOS (174). Similarly, Schneeberger *et al.* (172) observed that BAL of LT recipients early developing CLAD was enriched with *Pseudomonas* and *Staphylococcus* and Banday *et al.* (175) also reported a decrease of *Prevotella* along with an increase of *Pseudomonas* in CLAD LT recipients. However, this association of *Pseudomonas* with CLAD is not consistent in the literature. Dickson *et al.* reported that different species of *Pseudomonas* produced different symptoms and changes in microbiome diversity, suggesting that this may be the reason for the contradictory findings on the relationship between *Pseudomonas* and CLAD development (161). Thus, a microbiome pattern related to CLAD is not systematically found (162–164) or cannot always be attributed to a particular species (173).

1.7.4. The virome in chronic lung allograft dysfunction

Although associations have been described between the TTV viral species and early-stage complications such as PGD and ACR, little is known about the relationship that the virome may have with long-term complications, particularly in CLAD development. In fact, some of the previously mentioned studies followed LT recipients for short periods of time, so adequate associations between viroma and the development of CLAD could not be made

(177,185). Interestingly, Jaksch *et al.* (183) defined TTV levels below the cut-off point of 7 log_{10} copies/ml as being associated with an increased risk of developing CLAD. Subsequently, the same group proposed that a TTV level >9.5 log_{10} copies/ml was critical and possibly reflecting excessive immunosuppression, whereas a level <7 log_{10} copies/ml was considered to reflect potentially too low immunosuppression leading to a high risk of developing rejection (183).

Most virome studies in the LT field are only conducted until the first year after LT or are virus-specific studies, such as studies performed by quantifying plasma TTV levels for monitoring immunosuppression. Thus, since CMV or CARVs are well-described serious pathogens for LT recipients, viral infections, reactivations or colonisation are mainly monitored for these specific viruses, leaving the whole virome poorly studied. In fact, neither the long-term dynamics of the whole virome nor its relationship to LT recipients' prognosis has been clearly assessed.

2. HYPOTHESIS

HYPOTHESIS

Bacterial and viral infections represent one of the major complications in LT recipients, as well as being potentially related to the development of CLAD. In recent years, the rapid expansion of metagenomic methods have allowed the study of microbiome in LT recipients. Regarding bacterial microbiome, the relationship between bacterial dysbiosis and CLAD development is still not well understood. Besides, the dynamics of the plasma virome after LT have hardly been studied and might be associated with the clinical course of LT recipients at long-term.

Overall, the hypothesis of the present thesis is that bacterial microbiome and virome are modified after LT and that they will normalise in those patients who do not eventually develop CLAD after LT.

3. OBJECTIVES

MAIN OBJECTIVE

The final goal of this study is to characterise the bacterial microbiome and virome in LT recipients at different time points: prior and after LT, to provide a better understanding of their dynamics and to study their association with CLAD.

SECONDARY OBJECTIVES

1. To characterise the URT and LRT bacterial microbiome dynamics of LT recipients at long-term and determine its association with CLAD development.
2. To characterise the plasma virome dynamics of LT recipients at long-term and determine its association with CLAD development.

4. METHODS

4.1 Part 1. Respiratory tract bacterial microbiome modifications after lung transplantation and its impact on chronic lung allograft dysfunction

4.1.1 Study design and classification criteria

In this longitudinal, prospective, single-center study, 68 patients that received a lung transplantation between May 2017 and July 2018 were enrolled. The institutional ethics committee approved the study and all participants were provided with written informed consent (PR(AG)242/2016). Inclusion criteria required the collection of a lung tissue sample on the day of LT and at least one sample from the follow-up of the patient. Samples were collected from May 2017 to July 2019 and patients were clinically followed-up for 2 years. In addition, a group of 11 non-LT healthy controls, with no respiratory disease and not treated with immunosuppressors or antibiotics was included. Both groups were matched by age and gender.

4.1.2 Sample collection

On the day of LT, a nasopharyngeal (NP) swab was collected before surgery in the operating room. Once the explanted lung was removed, a bronchial swab and a piece of the lower lobe were obtained. Further, a bronchial swab was collected from the donor lung before its implantation. Tissue samples were snapped frozen within an hour from the surgery and then stored at -80ºC together with all the swabs. Longitudinally, bronchoalveolar lavage (BAL) and NP swabs were obtained under sterile conditions at three different time-points after LT: at hospital discharge after LT, between 2-5 months post-LT and one-year post-LT (figure 6). In addition, negative control samples were included by collecting sterile saline through the bronchoscope before bronchoscopy was performed. All samples were immediately stored at -80ºC.

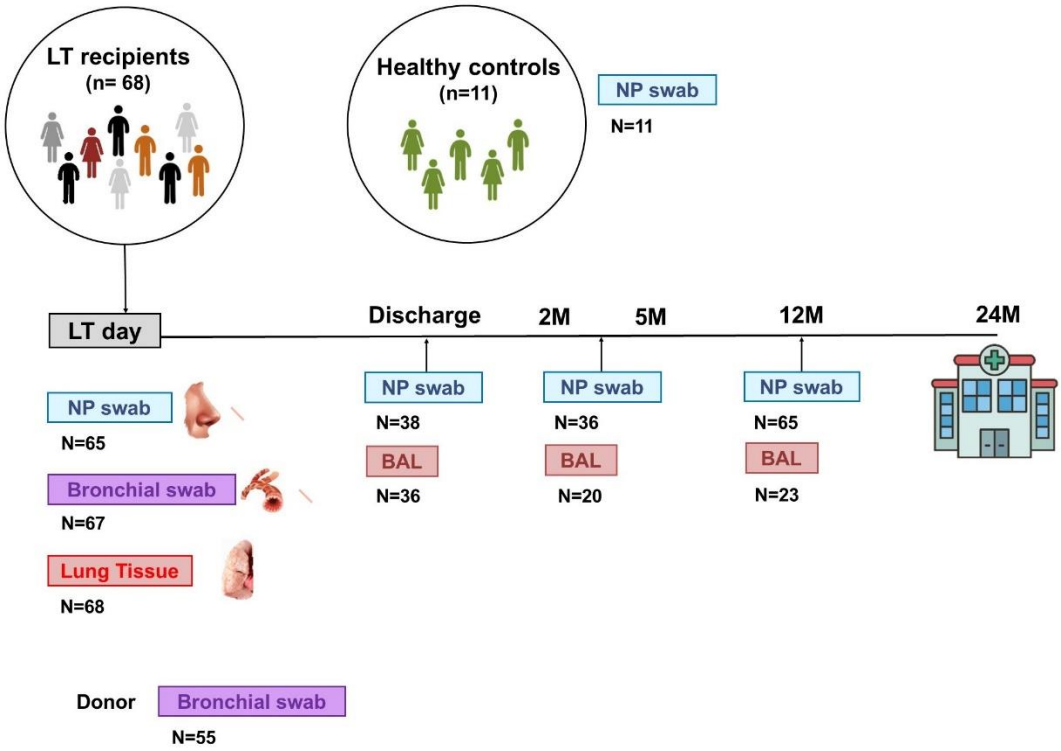

Figure 6. Diagram showing sample collection of LT recipients from LT day to 12 months after LT and clinical follow-up to 24 months after LT. Samples from healthy controls are also represented.

4.1.3 Samples pre-treatment

Lung tissue and BAL samples underwent a pre-treatment procedure before bacterial DNA extraction. Regarding tissue, 60-70 g of each tissue sample was digested following a protocol from Downward *et al.* (188). First it was suspended in ATL digestion buffer (Qiagen GmbH, Hilden, Germany) followed by a mechanical disruption using 1 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA). Then, proteinase K (Qiagen) was added and the mechanical disruption repeated. Samples were stored at -20ºC until extraction. BAL samples were thawed and first centrifuged at 11.000 g at 4ºC before starting the bacterial DNA extraction protocol.

4.1.4 Bacterial DNA extraction

The bacterial DNA extraction was performed using a modified protocol from Godon *et al.* (188) which we previously standardized for low-biomass respiratory samples. Briefly, samples were suspended in 250 µl of guanidine thiocyanate (Sigma Aldrich, Saint Louis,

MO, USA), 500 µl of 5% N-lauroyl sarcosine (Sigma Aldrich) and 40 µl of 10% N-lauroyl sarcosine. Then, 800 mg of 0.1 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA) were added to the sample to perform a mechanical disruption using the Mini-Beadbeater-24 (BioSpec Products). Then samples were washed using Poly Viny Poly-Pyrrolidone (Sigma Aldrich), Tris-HCl pH 8 1M, EDTA pH 8 0.5M, NaCl 5M and sterile water. After that, all nucleic acids were precipitated by adding 1 ml isopropanol. Then, the pellet was resuspended in phosphate buffer (pH 8) and potassium acetate and homogenised by pipetting up and down. RNA contamination was removed by adding RNase A (Qiagen GmbH, Hilden, Germany). Afterwards DNA was purified and recovered by several ethanol precipitations steps. Once the ethanol was dried, DNA was suspended in 30 µl of a TE buffer solution (Tris 10 mM, EDTA 1mM, pH 7) and stored at -20ºC. A negative control was included in all DNA extraction rounds.

4.1.5 Bacterial DNA amplification and libraries preparation

Standard PCR amplifications from bacterial DNA were performed using 0.75 units of Taq Polymerase (AmpliTaq Gold, Thermo Fischer, Waltham, MA, USA) and 20 pmol/µl of specific forward and reverse primers (IDT technologies) (see table 3) targeting the hypervariable region V4 from the 16S rRNA gene. Both primers were tagged with specific sequences for Illumina MiSeq Technology. Each unique Golay barcode (a 12 base paired sequence), downstream of the reverse primer, allowed the identification of each individual sample.

Table 3. Details of the primers used in the 16S rRNA gene amplification for Illumina sequencing

PRIMERS	Sequence 5'→3' Illumina Flowcell-Barcode-Adapter-Linker-V4 Region
Forward (V4F)	5′AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGT GTGCCAGCMGCCGCGGTAA3′
Reverse (V4R)	5′CAAGCAGAAGACGGCATACGAGAT{Golay barcode}AGTCAGTCAGCC GGACTACHVGGGTWTCTAAT3′

Three negative and one positive PCR controls were included in each amplification run. All samples and controls were amplified in duplicate and in a final volume of 50 µl. The PCR was performed in a Biometra T-Gradient thermocycler (Analytic-Jena, Göttingen, Germany) at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 sec, 56 °C for 60 sec, 72 °C for 90 sec, and a final cycle of 72 °C for 10 min. Three negative and one positive PCR controls were included in each amplification run. DNA integrity was examined by electrophoresis using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) with the DNA 12,000 kit.

4.1.6 Bacterial DNA purification and sequencing

Amplicons were purified with the QIAquick PCR Purification Kit (Qiagen) and quantified using a NanoDrop ND-1000 Spectrophotometer. Finally, samples were pooled in equal concentration. The pooled amplicons (2 nM) were then subjected to sequencing using Illumina MiSeq technology at the technical support unit of the Autonomous University of Barcelona (UAB, Spain), following standard Illumina platform protocols, and produce 1x300 bp single-end reads.

4.1.7 Bioinformatic and statistical analysis

Single-end reads were demultiplexed by using the *idemp* tool (189). The resulting single-end reads were processed using the DADA2 (190) (v. 1.16.0) pipeline obtaining an amplicon sequence variant (ASV) table to which taxonomy was assigned by using SILVA (191) 16s rRNA database (v. 132). Stringent parameters for filtering and trimming the reads using the filterAndTrim function (*truncLen=275, maxEE=8, truncQ=2, trimLeft=15*) were performed. As a result, filtered data included rather short reads, which prevented taxonomic assignment at the species level. Thus, all comparisons were performed at the genus or higher taxonomic levels.

Besides extraction and amplification negative controls were included (non-template samples) to assess contamination. Those taxa found uniquely in negative controls were

removed. For those taxa shared between samples and negative controls, the maximum number of reads in the negative controls was computed and used as a threshold to be removed from samples. Thus, for each of the samples if any of these particular taxa was present in a number of reads lower than in any control sample, a potential contamination was assumed and this taxon was filtered out from the sample. In addition, low abundant taxa (i.e. taxa with less than 50 reads and present in less than 2 samples) and samples with less than 50 reads in total were filtered out.

All ASV and taxonomy tables with the metadata were integrated, creating a phyloseq (192) (v.1.28.1) object. By using both Phyloseq and VEGAN (v. 2.5-6) R packages, we calculated alpha diversity metrics, including Observed and Shannon diversity indices, and beta diversity metrics such as the Bray Curtis and Aitchison distance indices. The Aitchison distance between the samples was calculated using the CodaSeq (193)(v.0.99.6) and zCompositions (194) (v. 1.3.4) R packages. Data was normalized by transforming the raw counts to centered log-ratios (clr).

The effect of the clinical variables on the overall microbiome composition was evaluated by performing a permutational multivariate analysis of variance (PERMANOVA) using the Adonis function from the Vegan R package (v. 2.5-6).

Differential abundance for CLAD was assessed by applying a mixed effect linear model implemented in the R package lme4 (v. 1.1–21) in which CLAD variable was considered a fixed effect and the sequencing run a possible source of batch effect. Tukey test implemented in the function glht from the multcomp R package (v.1.4-12) was applied to assess the differences between the groups and Bonferroni was used as multiple testing correction at this level by using the summary.glht function of the multcomp package. We ended up working with 450 samples, 14 phylum and 234 genera.

Categorical variables were expressed as number of cases and percentage and were compared using chi-square test or Fisher's exact test. Continuous variables were expressed

as median and interquartile range (IQR). Normality was evaluated in continuous variables using the Shapiro-Wilk test. For comparisons between patients, Mann-Whitney, unpaired t-test with Welch's correction and Kruskal-Wallis tests were used, as appropriate.

4.2 Part 2. Plasma virome dynamics after lung transplantation and its impact in chronic lung allograft dysfunction

4.2.1 Study design, sample collection and classification criteria

In this retrospective and longitudinal study 77 patients that had undergone lung transplant (LT) in Hospital Universitari Vall d'Hebron (Barcelona, Spain) from June 2015 to February 2017 were included. Plasma samples from all patients were collected at fixed time points, including pre-LT and at 6, 12 and 24 months post-LT (figure 11), and were processed by the Vall d'Hebron University Hospital Biobank as described in supplementary material from Part 2 with appropriate ethics approval. Eligibility criteria were that patients had a pre-transplant sample and at least one follow-up sample. Clinical data were recorded up to 3 years post-transplantation. In addition, a plasma sample was collected from 20 non-LT healthy controls without respiratory disease and not taking immunosuppressive treatment or antibiotic (figure 11). These controls were matched by age and gender to obtain comparable groups. The institutional ethics committee approved the study, and all participants were provided with written informed consent (PR(AG)518/2019).

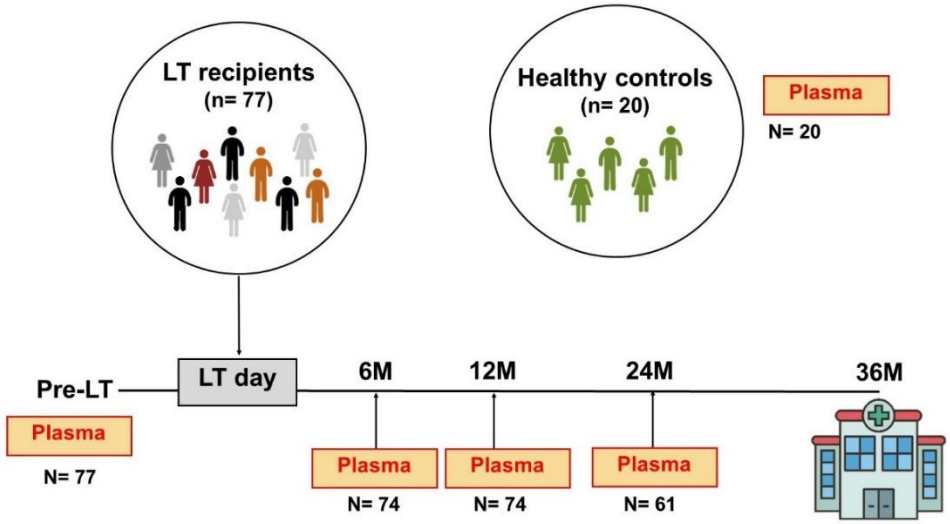

Figure 11. Diagram showing sample collection of LT recipients from pre-LT to 24 months after LT and clinical follow-up to 36 months after LT. Samples from healthy controls are also represented.

4.2.2 Viral particle enrichment

First, 3 ml of each plasma sample was filtered using a 0.45 µm Millex-HV filter (Merck) and then ultracentrifuged at 100.000g during 60 min at 4ºC using the microultracentrifuge Sorvall M120 (S100 AT6 rotor) (Thermo Fisher, Waltham, MA, USA). Ten negative controls were also included to assess contamination by filtering 3 ml of sterile 1x PBS. These controls then followed exactly the same process as the plasma samples.

The pellet obtained was resuspended with 300 µl of sterile 1x PBS. Samples were treated with a 20x digestion buffer [1M Tris, 100 mM $CaCl_2$ and 30 mM pH 8 $MgCl_2$), 250 U/µl Benzonase (Sigma Aldrich, Saint Louis, MO, USA) and 100 U/µl Micrococcal nuclease (New England Biolabs) and incubated for 2 h at 37ºC. The nucleases reaction was stopped by placing the samples on ice and adding 15 mM EDTA. The samples were incubated for 10 min at 75ºC.

4.2.3 Viral DNA and RNA extraction

Viral DNA and RNA was extracted using the High Pure Viral Nucleic Acid kit (Roche) following the manufacturer's instructions.

4.2.4 Viral genetic material amplification

Viral genetic material was amplified using a modified sequence-independent single-primer amplification (SISPA) method (105,195). First, viral RNA genomes were retrotranscribed into cDNA with SuperScript IV Reverse Transcriptase (Invitrogen, Thermo Fisher, Waltham, MA, USA) and tagged with random primer A (5'-GTT TCC CAG TCA CGA TCN NNN NNN NN-3') (Biomers). DNA-RNA hybrids were denatured with RNaseH (Thermo Fisher).
Next, the complementary cDNA strand was synthesized with Sequenase DNA polymerase version 2.0 (Applied biosystems). Finally, PCR was performed with primer B (5'-GTT TCC CAG TCA CGA tc-3') (complementary to random primer A) (Biomers) and AmpliTaq® Gold DNA Polymerase (Applied biosystems, Thermo Fisher, Waltham, MA, USA) to amplify the viral dsDNA sequences. This PCR was performed on a 96-well fast Veriti™ thermal cycler (Applied biosystems, Thermo Fisher) using the following protocol: 95°C for 10 min, 30 cycles (94°C for 30 s, 40°C for 30 s, 50°C for 30 s, 72°C for 1 min), 72°C for 10 min and holding at 4°C.

4.2.5 Purification and quantification of DNA

To purify the amplified viral ds cDNA, the DNA Clean & Concentrator-5 kit (Zymo Research) was used following the manufacturer's instructions. The DNA was quantified with Qubit 2.0 fluorometer by using Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, Carlsbad, CA, USA).

4.2.6 Libraries preparation and NGS sequencing

One ng of each sample (0.2 ng/µl) was used to prepare shot gun libraries for high-throughput sequencing, using the Nextera XT DNA Library Prep kit (Illumina, CA, USA) according to the manufacturer's protocol. Libraries were quantified and equimolar pools of about 70 samples per run were prepared. Sequencing was performed on a NextSeq 500 sequencer (Illumina) and produced 2x150 bp paired-end reads.

4.2.7 Bioinformatic and statistical analysis

Paired-end reads were filtered and trimmed for quality with the Fastp application (version 0.23.2) (196). The filtered reads were mapped to the Homo sapiens genome (GRCh38.p14) using Bowtie2 (version 2.4.5) (197), with the very-sensitive-local preset. With the help of Samtools (version 1.16) (198), aligned reads were discarded (pairs corresponding to primary alignments with both pairs unmapped are extracted from the SAM file). Using Kaiju (version 1.9.0) (199), taxonomic assignment of non-human reads against the NCBI nr+euk (2022/03/10) reference database was performed, with a maximum of 5 mismatches allowed and a minimum match length of 20 amino acids. Using the R package (version 4.1.1) (200), the best match for each query read was selected and query reads matching the same viral taxon for each sample were counted and used to construct a read count abundance table.
In addition, the reads were subjected to further manual and stringent filters. First, non-viral reads and taxa that accounted for less than three reads in the sample set were removed. In addition, taxa shared between negative controls and samples were removed as potential contamination.
To avoid bias caused by differences in the number of reads between samples, a random subset containing the same number of reads per sample was generated using QIIME version 1.9 (201) by rarefying each sample 1000 times at a depth of 100 reads. Thus, we obtained abundance tables unnormalized and normalized to 100 reads per sample. Integrating the taxonomic tables with the clinical metadata, we calculated alpha diversity metrics with the Shannon diversity index (script "alpha_rarefaction.py" and "compare_categories.py") and by a two-tailed non-parametric t-test using Monte Carlo permutations with false discovery rate (FDR) correction (202). In addition, we obtained the corresponding abundance bar charts using the script "summarize-taxa-through_plots.py".
To test for differences between viral communities, beta diversity metrics were calculated. Absolute abundance data were modelled using “Analysis of Microbiome Composition with Bias Correction” (ANCOM-BC) (203), which estimates unknown sampling fractions and

corrects for bias introduced by their differences between samples. ANCOM-BC was implemented using the R package to calculate p-values corrected for FDR, which were the values taken into account to consider the results significant. Normalized tables were used to calculate Bray Curtis dissimilarity index matrices and a non-parametric permutational multivariate analysis of variance (PERMANOVA) called Adonis tests. Samples were represented in cluster trees with principal coordinate analysis (PCoA). Categorical variables were expressed as number of cases and percentage and compared using the chi-squared test or Fisher's exact test. Continuous variables were expressed as median and interquartile range (IQR). Normality was assessed for continuous variables using the Shapiro-Wilk test. Mann-Whitney test, unpaired t-test with Welch's correction and Kruskal-Wallis were used for between-groups comparisons, as appropriate.

5. RESULTS

5.1 Part 1. Respiratory tract bacterial microbiome modifications after lung transplantation and its impact on chronic lung allograft dysfunction

After 2 years of follow-up, 12 out of 68 LT recipients were diagnosed with CLAD. Demographic and clinical characteristics of CLAD-free, CLAD LT recipients, and healthy controls (HC) are described in Table 4. The only difference found was that double-LT was more frequent in CLAD LT recipients (p= 0.03).

Table 4. Demographic and clinical characteristics of the population

	CLAD-free (n=56)	**CLAD (n=12)**	**HC (n=11)**	***p***
Age, median (IQR)	58 (51.25-61)	55.5 (51-58.5)	55 (40-60)	0.39
Gender (males), n (%)	33 (58.9)	6 (50)	7 (63.6)	0.79
Aetiology, n (%)				0.27
ILD	27 (48.2)	3 (25)		
COPD	15 (26.8)	6 (50)		
CF/Bronchiectasis	5 (8.9)	2 (16.7)		
Other aetiologies*	9 (16.1)	1 (8.3)		
Smoking, n (%)	39 (69.6)	10 (83.3)		0.49
Bacterial colonization recipient, n (%)	22 (39.3)	8 (66.7)		0.11
Bacterial colonization donor, n (%)	31 (55.4)	8 (72.7)		0.38
CMV serostatus pre LT, n (%)	48 (80.4)	9 (75)		0.39
Antibiotic pre LT, n (%)	11 (19.6)	0 (0)		0.19
Bilateral lung transplant, n (%)	39 (69.6)	12 (100)		**0.03**
Minutes ischemia⁺, (median, IQR)	307 (246-364)	302 (271-373)		0.66
Primary graft dysfunction, n (%)	27 (48.2)	10 (83.3)		0.05
ACR, n (%)	40 (71.4)	7 (58.4)		0.49
Days ICU, (median, IQR)	12 (6-33.5)	27.5 (13.3-35.8)		0.11
Days invasive MCV, (median, IQR)	3.5 (1-26.5)	17.5 (10-25.5)		0.15
Days hospitalization, (median, IQR)	15 (10.25-17)	12.5 (6-14)		0.05
Months from LT to CLAD diagnosis		13.5 (3-24)		

CLAD: Chronic Lung Allograft Dysfunction. HC: Healthy controls. ILD: Interstitial lung disease. COPD: Chronic obstructive pulmonary disease. CF: Cystic fibrosis. CMV: Cytomegalovirus. ACR: Acute cellular rejection. ICU: Intensive Care Unit. MCV: Mechanical ventilation. *Other aetiologies: cystic disease, bronchiolitis obliterans, pulmonary hypertension. ⁺Minutes of ischemia calculated using the longest time. **Bold value**: statistically significant result

Supplementary table 1 shows the pulmonary function variables at follow-up visits, which were made at discharge, 2-5, 12 and 24 months after LT. In addition, immunosuppression

treatments from each time-points are described. Briefly, when CLAD is diagnosed the following protocol is used in our hospital: MMF is retired, doses of azithromycin are increased and rapamycin is incorporated. Besides, detailed information on infections during follow-up is included in supplementary table 2. CLAD LT recipients presented more infection episodes than CLAD-free patients did at 6-12 months (p= 0.04) and at 12-18 months after LT (p= 0.03), with a predominance of bacterial infections.

On LT day, a significant increased diversity was observed in NP swab compared to both lung tissue and bronchial swab (p= $6.6x10^{-8}$) (figure 7a). Besides, bacterial microbiome composition was also different between URT and LRT samples: while bronchial swabs and lung tissue had a similar bacterial composition, NP swabs had a different composition of both (p= 0.001) (figure 7b). Actinobacteria was the predominant phylum at the URT, and Proteobacteria, Firmicutes and Bacteroidetes phyla at the LRT (supplementary figure 1).

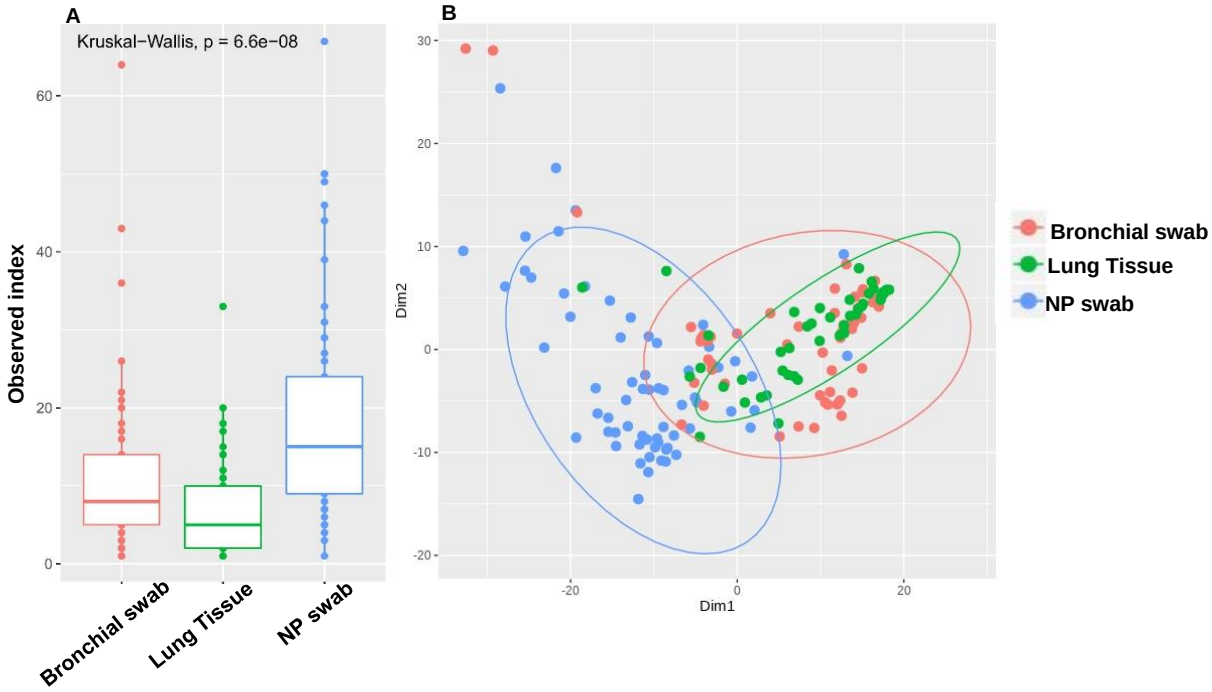

Figure 7. Alpha and beta diversity of lung tissue (green), bronchial swabs (orange) and nasopharyngeal swabs (blue) specimens at LT day**. A)** Boxplot comparing bacterial alpha diversity (Observed index) between the three sample types (Kruskal-Wallis, p= 6.6x10-8). **B)** Multidimensional scaling (MDS) plot summarizing beta diversity of bacterial composition between samples based on Bray-Curtis distance matrix (Adonis test, p= 0.001, R^2= 0.171).

NP microbiome diversity decreased when compared with healthy controls in the early stages after LT, including at discharge (Wilcoxon-test, p = 0.0024) and 2-5 months after LT (Wilcoxon-test, p = 0.032) (Figure 8a). At 12 months visit, diversity tends to recover and gets closer to that of the healthy individuals. Similarly, BAL microbiome diversity increased significantly over time (Kruskal-Wallis, p = 0.027) (figure 8b). As BAL samples of healthy controls were not available, due to ethical reasons, a comparison with this group was not possible.

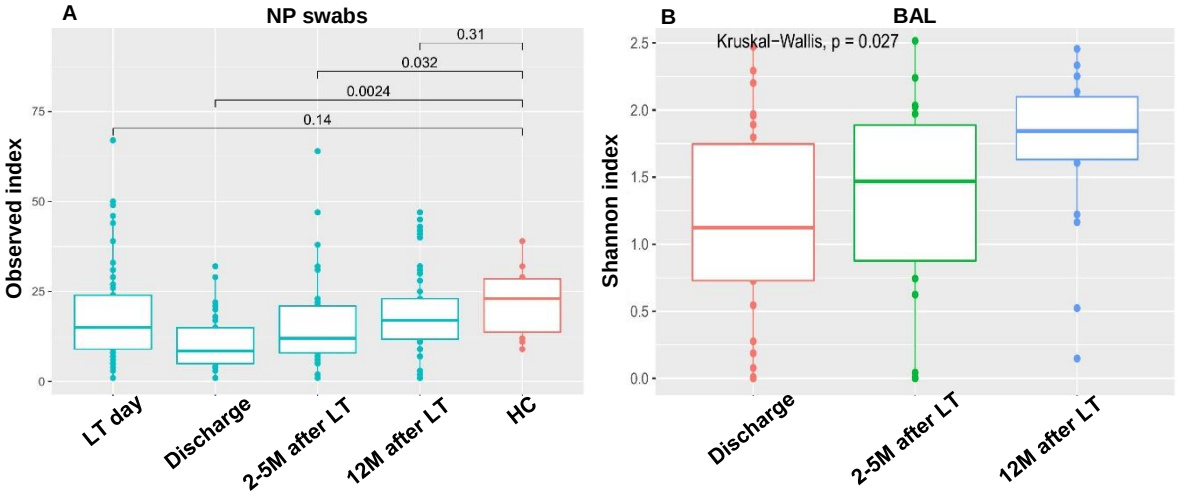

Figure 8. Dynamics of alpha diversity after LT. **A)** Boxplot representing the variation of bacterial alpha diversity (Observed index) of NP samples from LT day to 12 months after LT and comparison with healthy controls (HC). **B)** Boxplot representing the variation of bacterial alpha diversity (Shannon index) of BAL samples from discharge to 12 months after LT.

At LT day, NP microbiome composition at genus level was similar between CLAD and CLAD-free recipients and healthy controls when measured by Aitchison distance distributions (Adonis test, p= 0.62) (figure 9a). Same result was observed at discharge (Adonis test, p= 0.88) (figure 9b). At 2-5 months visit, different compositions were found between CLAD recipients and healthy controls (Adonis test, p= 0.013) and between CLAD and CLAD-free recipients (Adonis test, p= 0.089), but healthy controls and CLAD-free recipients microbiomes clustered together (Adonis test, p= 0.97) (figure 9c). One year after LT, these findings persisted with higher distances between CLAD recipients and healthy controls (Adonis test, p= $2x10^{-4}$) and CLAD-free recipients (Adonis test, p= $6.5x10^{-5}$).

Healthy controls and CLAD-free recipients microbiomes remained similar (Adonis test, *p*= 0.94) (figure 9d). Further, one year after LT, *Streptococcus* and *Staphylococcus* were the most abundant genera in CLAD recipients and *Corynebacterium* and *Staphylococcus* in both CLAD-free recipients and healthy controls (supplementary figure 5).

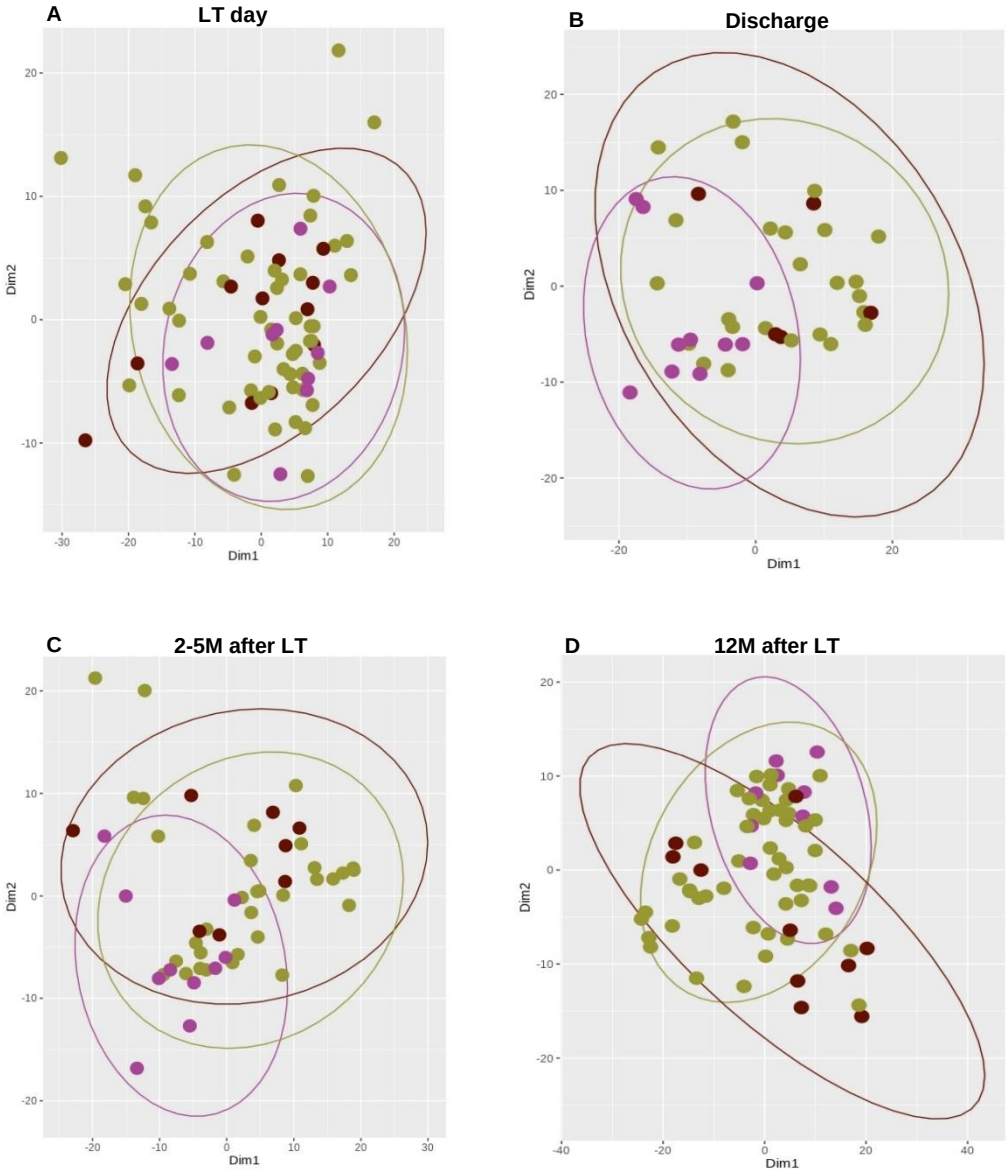

Figure 9. Multidimensional scaling (MDS) plot representing beta diversity of bacterial composition between CLAD-free (green) and CLAD (brown) recipients and healthy controls (pink) from LT day to 12 months after LT based on Bray-Curtis distance matrix. **A)** LT day (Adonis test, *p*= 0.62), **B)** discharge (Adonis test, *p*= 0.88), **C)** 2-5 months after LT (Adonis test, *p*= 0.35) and **D)** 12 months after LT (Adonis test, *p*= 0.02).

The genera *Capnocytophaga*, *Lachnoanaerobaculum*, *Selenomonas*, *Solobacterium* and *Peptostreptococcus* were more abundant in the NP samples from CLAD than CLAD-free

recipients (figure 10a). *Capnocytophaga* and *Lachnoanaerobaculum* were also significantly more abundant in CLAD recipients than in healthy controls, together with *Rothia* genus (figure 10B). On the other hand, *Lawsonella*, *Massilia*, *Stenotrophomonas*, *Rheinheimera* and *Aureimonas* were more abundant in healthy controls than in CLAD and CLAD-free recipients (figure 10b and 10c). Detailed of differentially abundant taxa at genus level is represented in supplementary table 2.

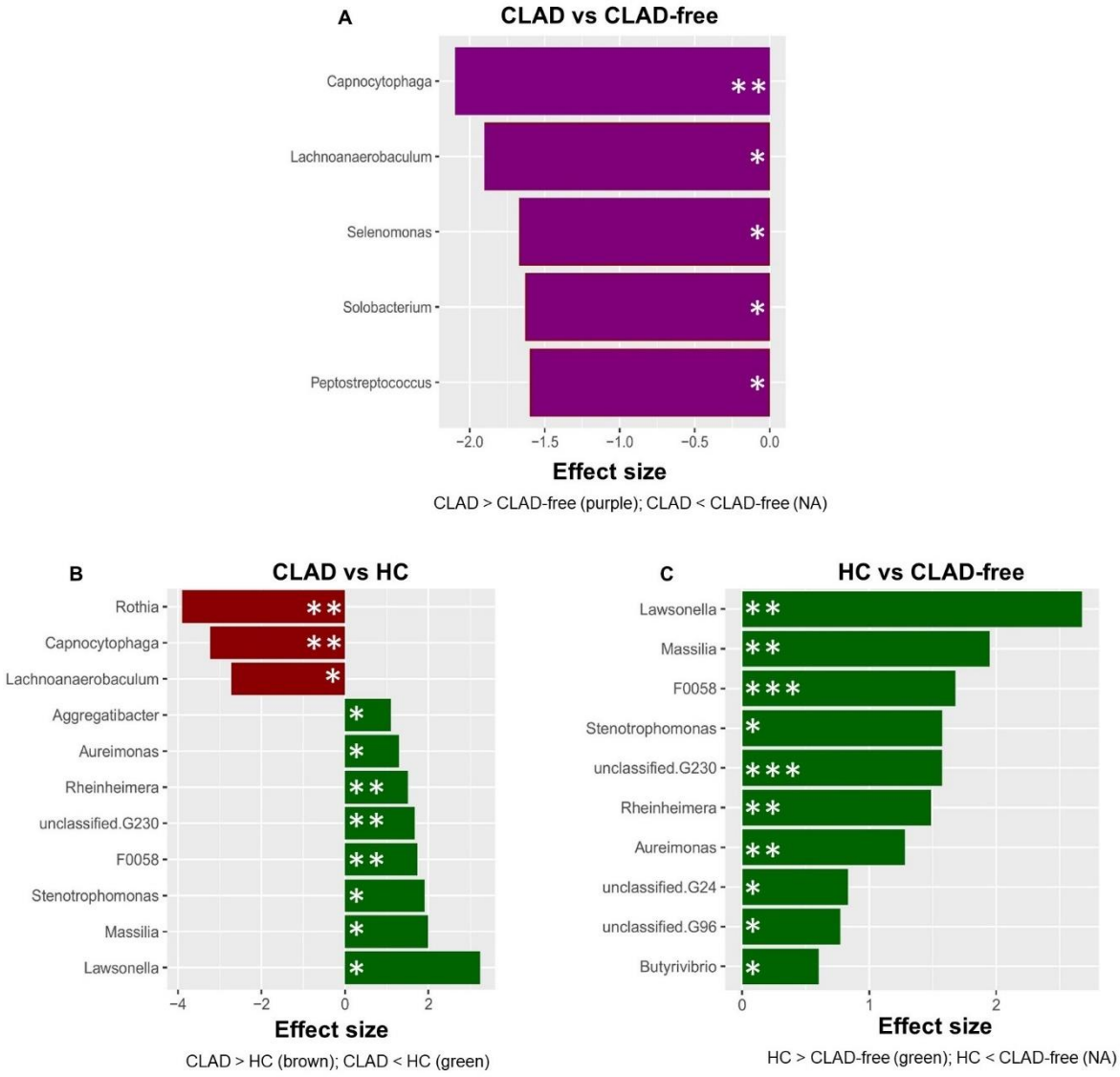

Figure 10. Differentially abundant bacteria between CLAD and CLAD-free recipients and healthy controls (HC). The horizontal bar plots show the effect size of those bacterial genera significantly more abundant in CLAD than CLAD-free LT recipients (purple bars) **(A)**, those more abundant in HC than CLAD recipients (green bars) and those more abundant in CLAD recipients than HC (brown

bars) **(B)** and those more abundant in HC than CLAD-free LT recipients **(C)**. *** p<0.0001; ** *p*= <0.009-0.0009; * *p*= 0.05-0.01

In addition, we confirmed that the composition of the donor microbiome of patients who ultimately developed CLAD did not differ from that of those who remained CLAD-free (*p*= 0.38, R^2= 0.018) (data not shown). Therefore, in this cohort, the composition of the donor microbiome does not confer risk of developing CLAD.

Supplementary table 1 describes the use of immunosuppressors and antibiotic prophylaxis during the follow-up. The effect on NP microbiome composition of those drugs that were not administered uniformly to the entire cohort (including MMF, cotrimoxazole, colistin and tobramycin) was assessed at 12 months after LT. After comparing, those patients that were treated with each drug and those who were not, no differences were found.

5.2 Part 2. Plasma virome dynamics after lung transplantation and its impact in chronic lung allograft dysfunction

A total of 306 plasma samples were obtained and processed, 286 from 77 LT patients and 20 from 20 healthy controls, together with 10 negative controls. Illumina sequencing generated 2.485.405.356 reads, 43.42% (1.079.178.868 reads) of which were viral reads. This value was reduced to 853.483.902 reads after applying filters that involved the removal of those reads that were only present in the negative controls, those reads shared between the samples and the negative controls and those that had less than 3 reads among all samples. Afterwards, 205 different viral genera were identified in plasma samples of LT patients and healthy controls.

The plasma virome of LT recipients presented lower alpha diversity compared to healthy controls (p= 0.003) (figure 12a) and the virome composition was also different between both (p=0.0017) (figure 12b). Different genera of the *Anelloviridae* family consisting of Alphatorquevirus, Gammatorquevirus, as well as a group of unclassified anelloviruses were the most frequently identified in both groups (figure 12c). Interestingly, Pegivirus (also one of the most abundant viral genera), Betatorquevirus and Circovirus genera were only identified in LT recipients (figure 12d). Further, Alphatorquevirus, Betatorquevirus and unclassified anelloviruses, together with Pegivirus and other unclassified flaviviruses genera, both from the *Flaviviridae* family were significantly more abundant in LT recipients comparing to healthy controls (figure 12e).

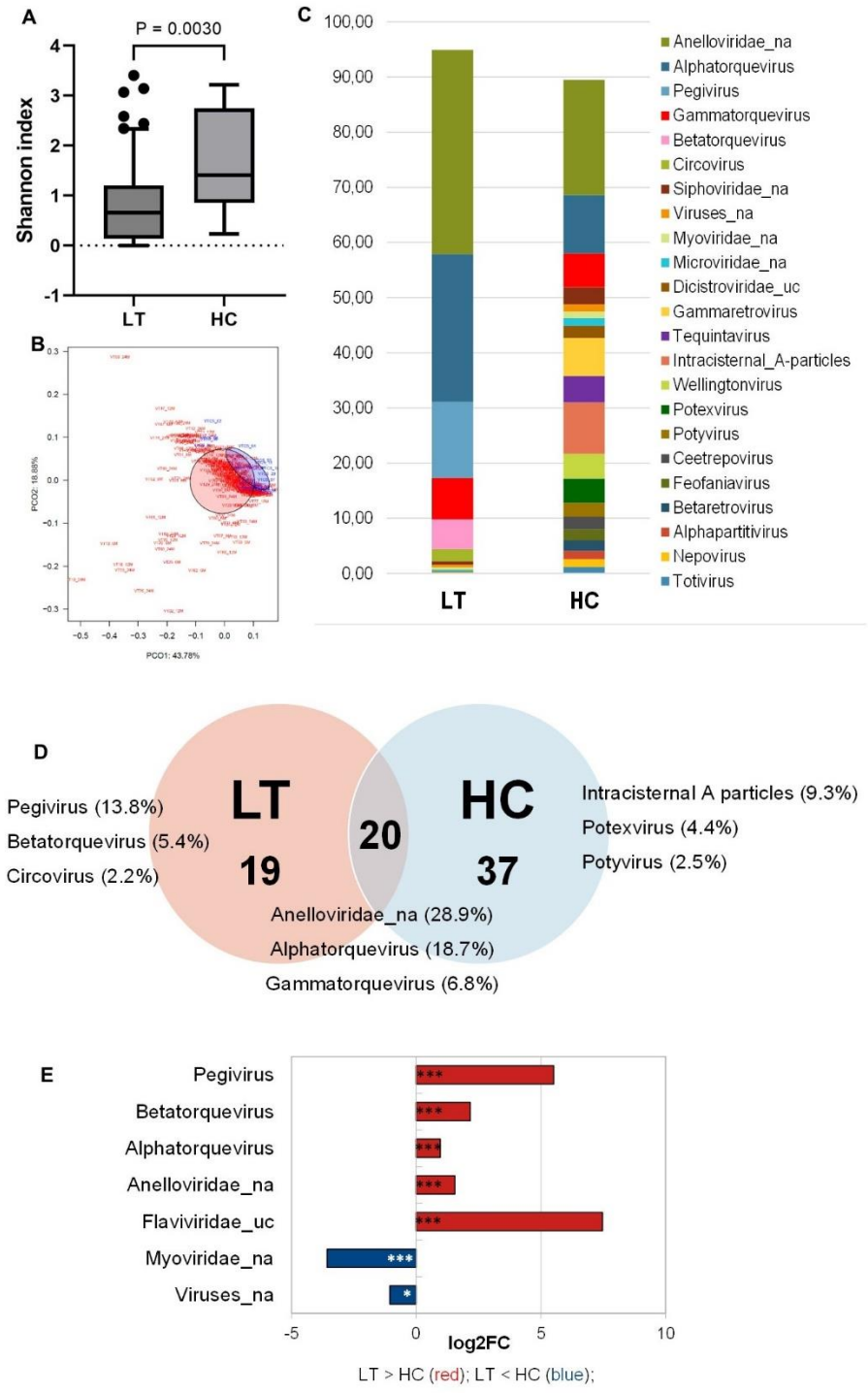

Figure 12. Virome composition, alpha and beta diversity and differentially abundant virus between LT recipients and healthy controls. **A)** Boxplot comparing viral normalized alpha diversity (Shannon index) between plasma samples from LT recipients and healthy controls (HC) (p=0.003). Rarefaction was performed at a depth of 100 reads per sample. **B)** Principal Coordinates Analysis (PCoA) summarizing beta diversity of viral community composition between LT recipients (red) and healthy controls (blue) based on a Bray-Curtis distance matrix at the genus level (p=0.0017, R^2=0.034). **C)** Barplots of the relative abundances of the viral genera comparing LT and HC. Viruses detected in <1% in both groups have not been represented. **D)** Venn diagram with the most abundant viral genera detected only in LT (red), only in HC (blue) and commonly detected in both groups

(intersection). Viruses detected in <0.1% have not been represented. **E)** Horizontal bar plot showing log2-Fold change (log2FC) of those viral genera significantly more abundant in LT than HC (red bars) and those more abundant in HC than LT (blue bars). *** *p*= <0.00001; * *p*= 0.05-0.01.

Plasma virome alpha diversity decreased from pre-LT to early stages after LT, including both at six (*p*= 0.0003) and 12 months (*p*= 0.0001) (figure 13a). This decrease is also significant at both 6 and 12 months when compared to healthy controls (*p*= 0.01 and *p*= 0.007, respectively) (figure 13a). At the 24-months visit, diversity tends to recover and approaches that of healthy individuals (figure 13a). Conversely, viral load (i.e. nucleic acid concentration after SISPA and purification) increased at both six (*p*= < 0.0001) and 13 months (*p*= < 0.0001) after LT compared to healthy controls and decreased to normal after two years (figure 13b).

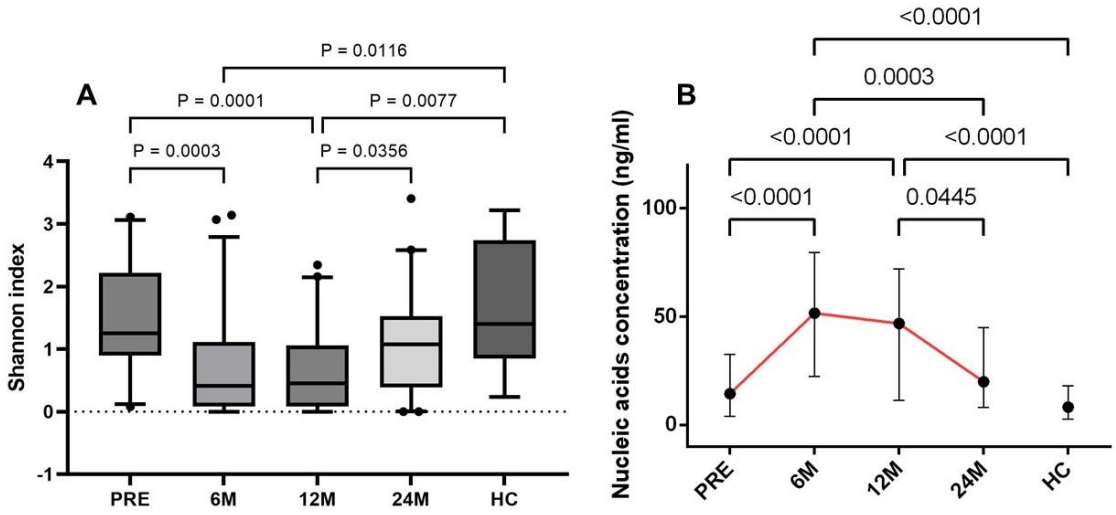

Figure 13. Dynamics of alpha diversity and viral load after lung transplantation. **A)** Boxplot showing the variation of virome alpha diversity (Shannon index) from pre-LT to 24 months post-LT on rarefied data and comparison with healthy controls (HC). **B)** Lineplot depicting median virome load (i.e. nucleic acid concentration after SISPA and purification) from pre-LT to 24 months post-LT and comparison with healthy controls.

Further, we confirmed that global virome composition, β-diversity, differed between LT recipients and healthy controls both before transplantation (*p*= 0.03) and at all follow-up times (*p*= 0.0017 for 6, 12 and 24 months after LT) (supplementary figure 3). Again, the viral component is mainly composed of unclassified anelloviruses (30.4%) and Alphatorquevirus

(20.5%) at all time points and in healthy controls, followed by Pegivirus (12.5%) which are detected both prior to LT and during all follow-up (figure 14a), but not in healthy controls. Interestingly, anelloviruses were significantly more abundant at all time points after transplantation than before (figure 14b) and there was a significant change in Alphatorquevirus and Gammatorquevirus between 6 and 12 months (figure 14b) being higher at 6 months, suggesting a relationship of these viruses to the immunosuppressed state. Surprisingly, after LT, the global virome composition (β-diversity) only differed between 6 and 24 months (p= 0.02) (supplementary figure 4). In particular, at 24 months compared to 6 months, there was a significant decrease mainly in three bacteriophages: Tequintavirus (p= 0.0001), Tunavirus (p= 0.0002) and unclassified Microviridae (p= 0.0001), as well as Ourmiavirus (p= 0.0002) (figure 3b) and conversely, an increase in the unclassified Orthomyxoviridae (p= 0.0005) and Hepacivirus genera (p= 0.003) and the unclassified Siphoviridae (p= 0.0002) and Narnaviridae (p= 0.0096) (figure 14b) bacteriophages.

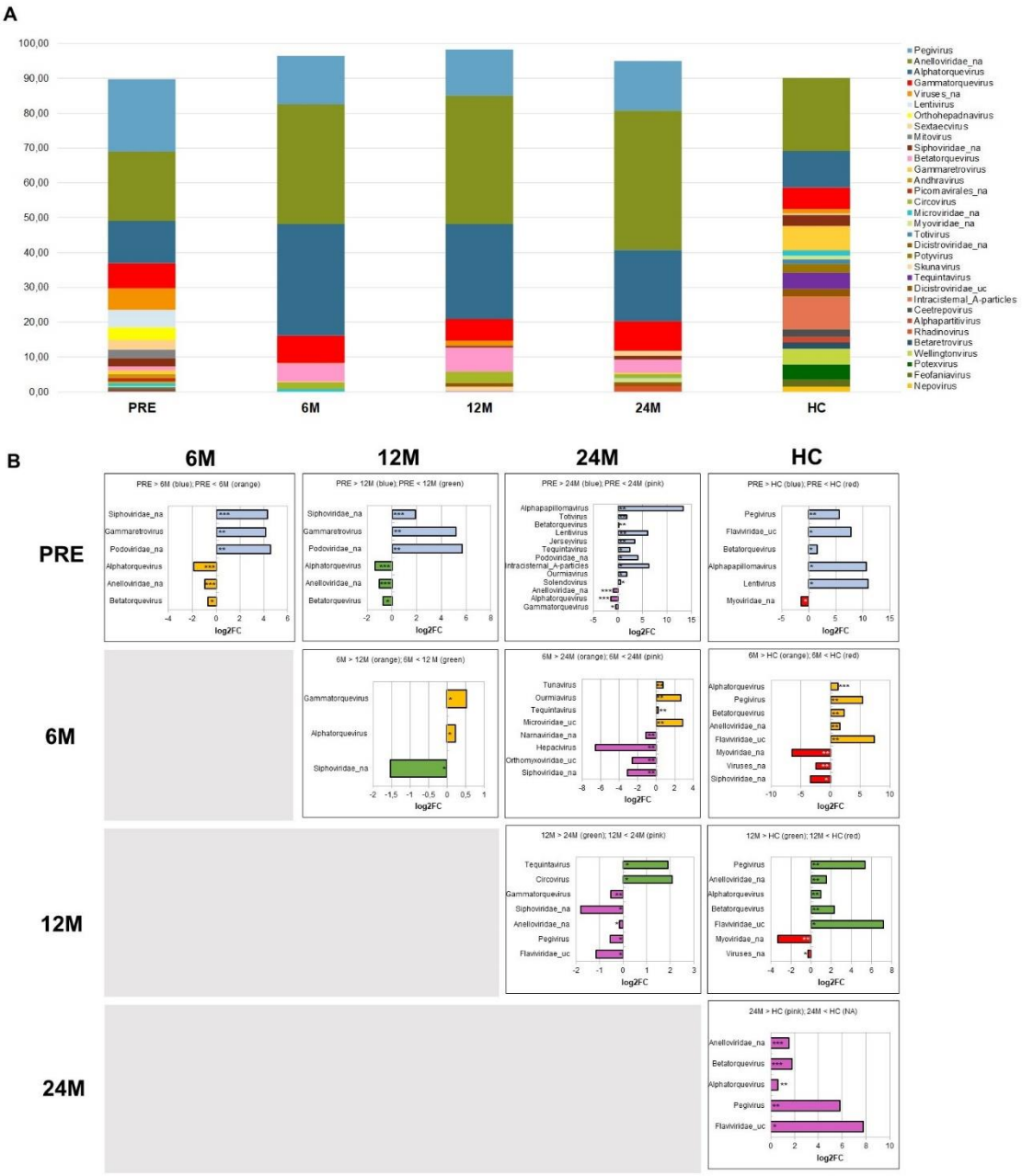

Figure 14. Dynamics of the relative abundance of viral genera and significant changes over time. **A)** Bar plots of the relative abundances of viral genera comparing different time points before and after lung transplantation and also with healthy controls. Viruses detected in <1% in all groups are not represented. **B)** Horizontal bar plot showing log2-Fold change (log2FC) of those viral genera significantly more abundant on the right and less abundant on the left (using the vertical group as a reference) at different time points. *** p<0.0001; ** *p*= <0.009-0.0009; * *p*= 0.05-0.01.

Interestingly, before transplantation, ILD patients had a different global viral composition (β-diversity) than healthy controls (supplementary figure 5). Lung recipients with ILD were taking more corticosteroids and MMF prior to LT than patients with COPD/emphysema/A1ATD and other aetiologies (supplementary table 4). Although not significant, before LT we detected twice the viral load in those patients taking corticosteroids and MMF (23.65 ng/ml) compared to those not receiving immunosuppressive treatments (10.6 ng/ml) (supplementary figure 6). Further, we assessed whether aetiology influenced virome dynamics after LT. In all three aetiological groups, alpha diversity decreased in the early phases after LT and tended to normalise after 2 years, although this trend only reached statistical significance in ILD patients (figure 15a, 15c and 15e), but probably could not reach significance in the other groups due to the low sample size after normalisation. In terms of relative abundances, Alphatorqueviruses and unclassified Anelloviridae were the most frequent in all three aetiologies during all follow-up after LT: on average, 37.3% in ILD, 29.9% in COPD/emphysema/A1ATD and 21.2% in other aetiologies. Interestingly, ILD patients showed less Pegiviruses (5%) than the rest of groups (21.4% in COPD/emphysema/A1ATD, 25.7% in other aetiologies) (figure 15b, 15d and 15f).

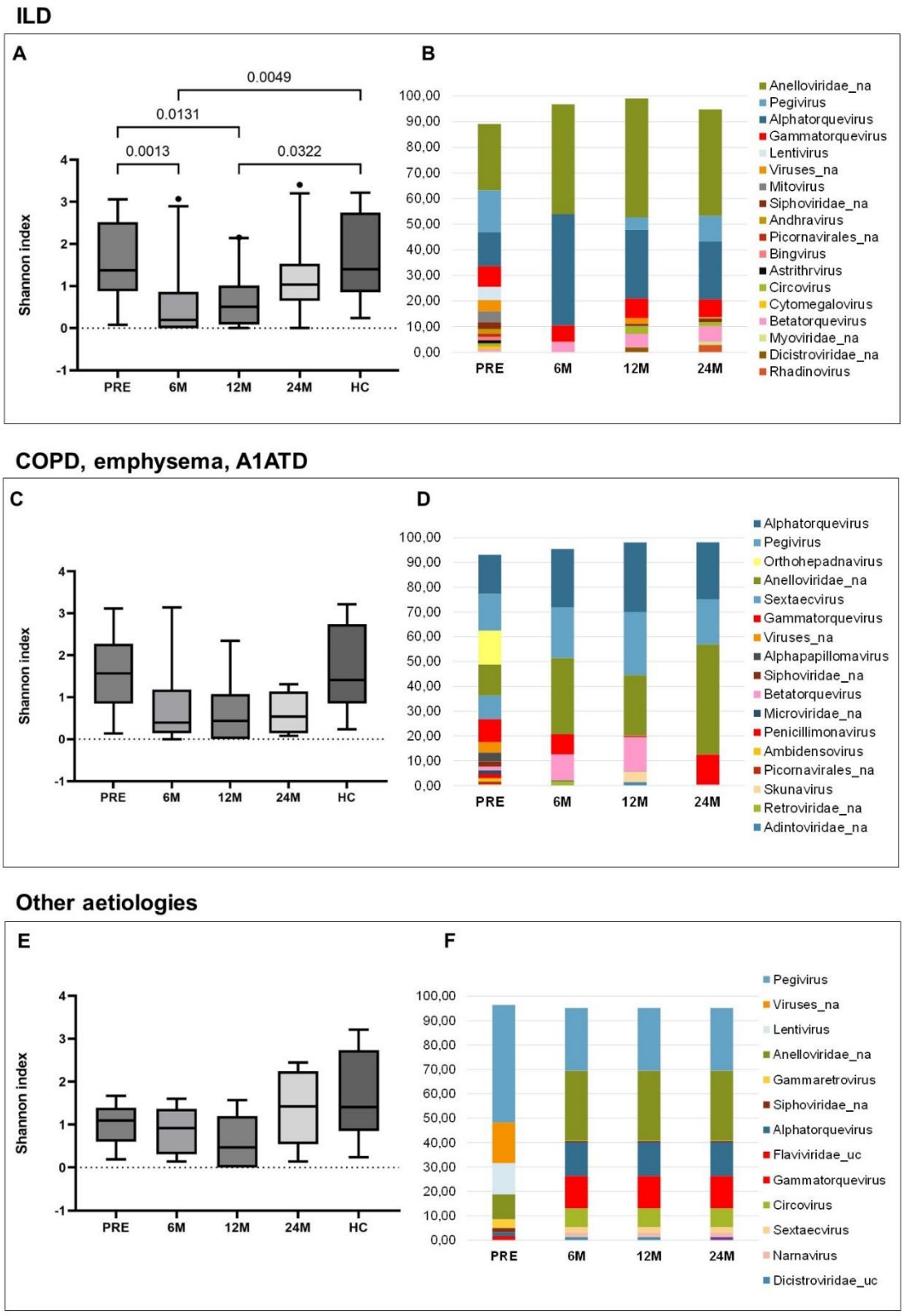

Figure 15. Dynamics of alpha diversity and relative abundance of virus genera by aetiology. **A)** Boxplot showing variation in virome alpha diversity (Shannon index) of ILD recipients from pre-LT to 24 months post-LT in rarefied data and comparison with healthy controls (HC). **B)** Bar plots of the relative abundances of viral genera comparing different time points before and after lung transplantation of ILD patients and also with healthy controls. Viruses detected in <1% in all time points are not represented. **C)** Boxplot showing variation in virome alpha diversity (Shannon index)

of COPD, emphysema and Alpha-1-antitrypsin recipients from pre-LT to 24 months post-LT in rarefied data and comparison with healthy controls (HC). **D)** Bar plots of the relative abundances of viral genera comparing different time points before and after lung transplantation of COPD, emphysema and Alpha-1-antitrypsin patients and also with healthy controls. Viruses detected in <1% in all time points are not represented. **E)** Boxplot showing variation in virome alpha diversity (Shannon index) of recipients with other aetiologies from pre-LT to 24 months post-LT in rarefied data and comparison with healthy controls (HC). **F)** Bar plots of the relative abundances of viral genera comparing different time points before and after lung transplantation of patients with other aetiologies and also with healthy controls. Viruses detected in <1% in all time points are not represented.

Besides, at 6 months, ILD patients presented a different virome composition compared to the other two groups (p= 0.03, R^2= 0.05) (figure 16a), although this difference was not maintained over time (p= 0.1 at 12 and p= 0.8 at 24 months after LT). In depth at 6 months, ILD patients tended to have lower alpha diversity compared to COPD/emphysema/A1ATD and other aetiologies, yet this was not significant (figure 16b). Five genera were only found in ILD patients, albeit at low frequencies, such as Sinsheimervirus and unclassified Genomoviridae, while the genera Pegivirus, Retroviridae and unclassified Flaviviridae were found in patients with COPD/emphysema/A1ATD and other etiologies, but not in ILD patients at 6 months after LT (figure 16c). However, almost all the entire viral component of ILD patients at 6 months was composed of Alphatorquevirus (43.4%) and unclassified Anelloviridae (42.9%) (figure 16b).

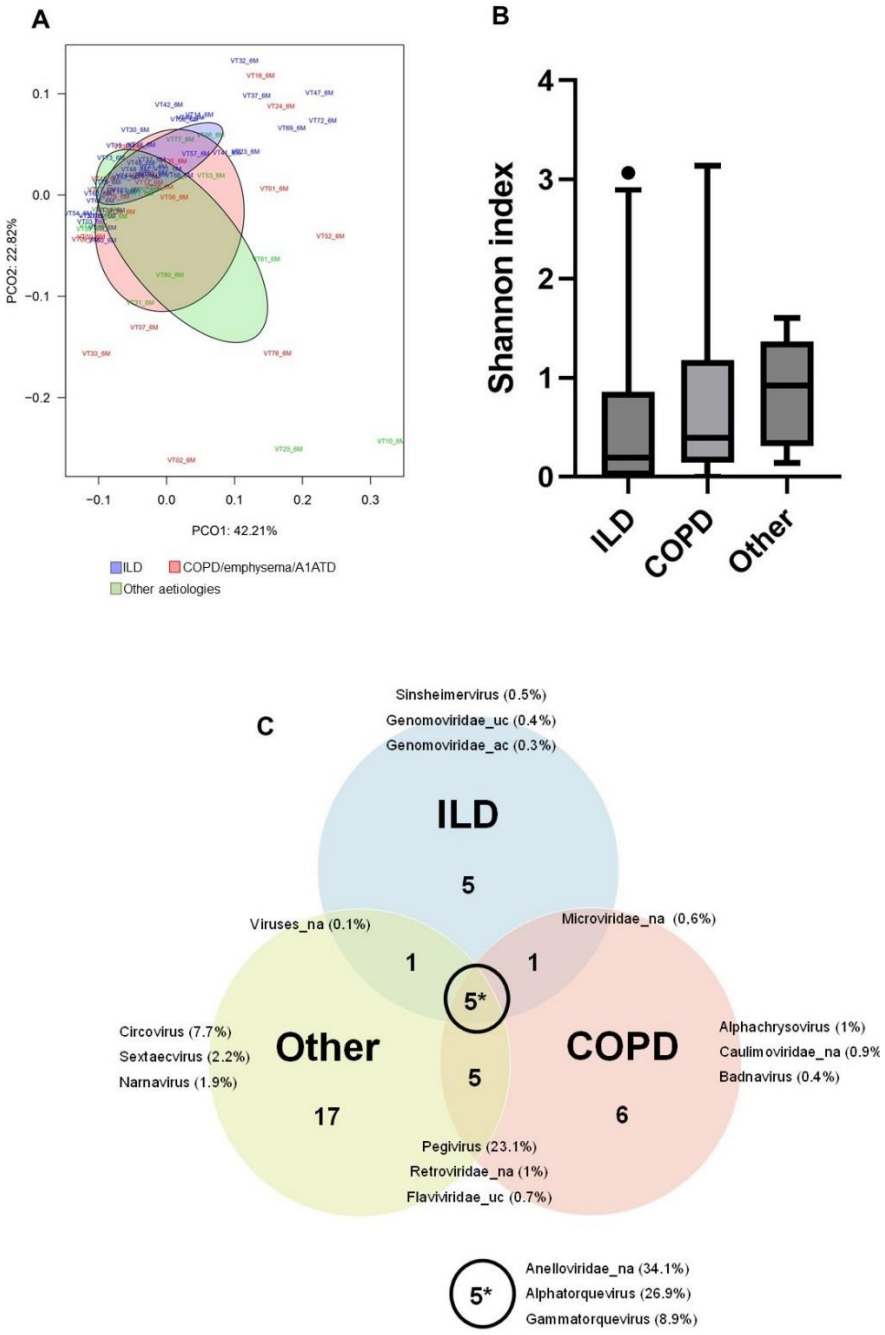

Figure 16. Virome composition, alpha and beta diversities and differentially abundant virus between aetiologies at 6 months post-LT. **A)** Principal Coordinates Analysis (PCoA) summarizing beta diversity of viral community composition between ILD recipients (blue), patients with COPD/emphysema/A1ATD (red) and patients with other aetiologies (green) based on a Bray-Curtis distance matrix at the genus level at 6 months post-LT (p= 0.03, R2= 0.05). At 12 (p> 0.05) and 24 (p> 0.05) months after LT was not significant (plot not shown). **B)** Boxplot comparing viral normalized alpha diversity (Shannon index) between plasma samples from LT recipients with different aetoiologies (*p* >0.05). **C)** Venn diagram with the most abundant viral genera detected only in ILD (blue), only in patients with COPD/emphysema/A1ATD (red), only patients with other aetiologies (green) and commonly detected in all groups or by pairs (intersections). Viruses detected in <0.1% have not been represented.

As expected, ILD patients underwent more single-LT than the other aetiological groups (supplementary table 4). Therefore, we assessed virome composition according to LT type to evaluate whether it could influence the above result. In both double and single-LT recipients, alpha diversity normalises 2 years after transplantation (figure 17a and 17c). Regarding relative abundances, unclassified Anelloviridae and Alphatorquevirus were the most frequent in both types of LT at all follow-up. Strikingly, Pegivirus is absent in single-LT recipients and ILD patients at 6 months after LT and accounting to all the follow-up, its relative abundance represents in average 2.9% and 7.8% for single-LT and ILD patients, respectively, suggesting that both findings are analogous (figure 17b and 17d).

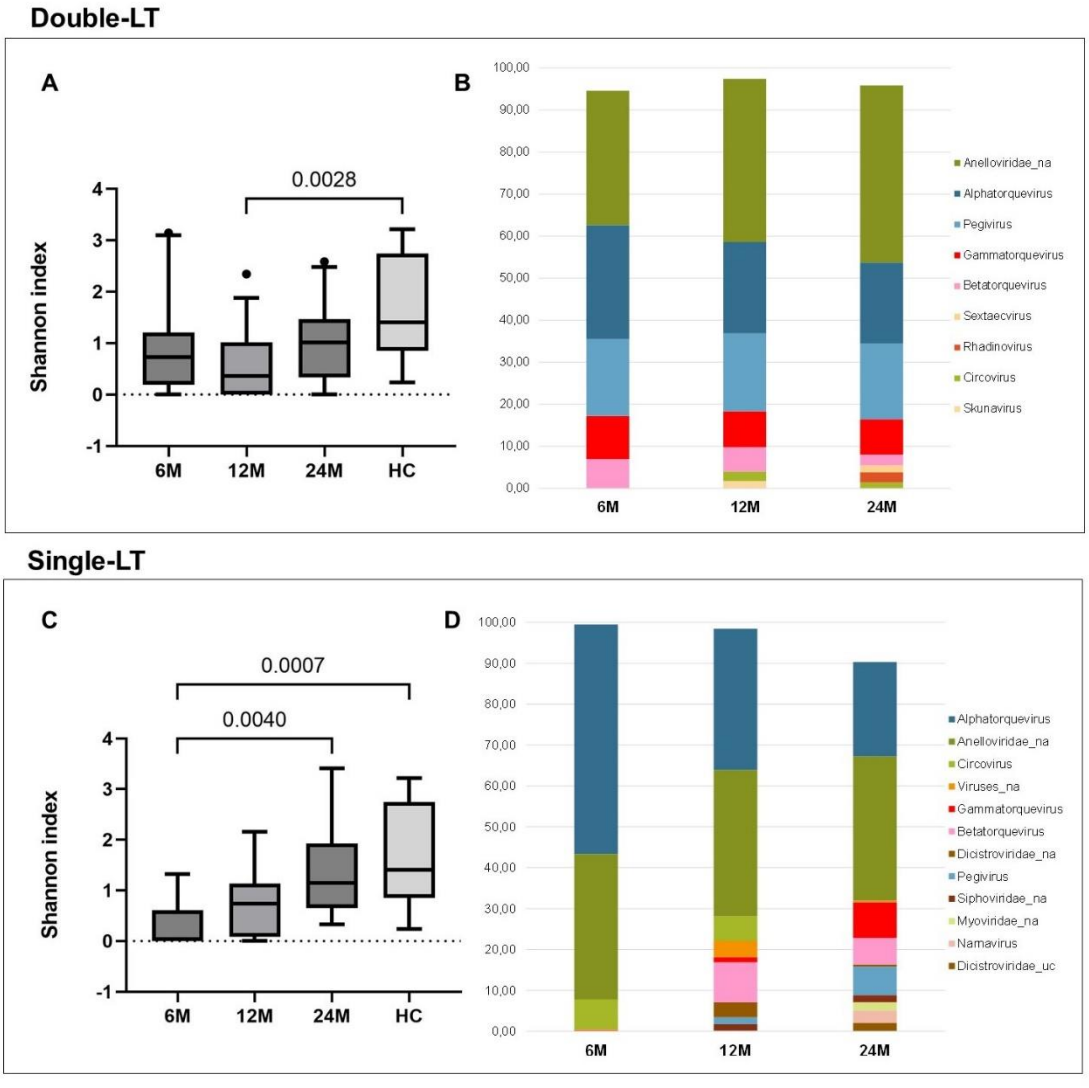

Figure 17. Dynamics of alpha diversity and relative abundance of virus genera by type of trasplant. **A)** Boxplot showing variation in virome alpha diversity (Shannon index) of double-LT recipients from 6 to 24 months post-LT in rarefied data and comparison with healthy controls (HC). **B)** Bar plots of the relative abundances of viral genera comparing different time points after lung transplantation of double-LT recipients and also with healthy controls. Viruses detected in <1% in all time points are not represented. **C)** Boxplot showing variation in virome alpha diversity (Shannon index) of single-LT recipients from 6 to 24 months post-LT in rarefied data and comparison with healthy controls (HC). **D)** Bar plots of the relative abundances of viral genera comparing different time points after lung transplantation of single-LT recipients and also with healthy controls. Viruses detected in <1% in all time points are not represented.

Notably, again at 6 months post-LT, single and double-LT recipients had different global virome compositions (p= 0.01, R^2= 0.05) (figure 18a), and as in the case of the aetiological analysis, did not persist over time (p= 0.6 at 12 and p= 0.4 at 24 months after LT). At this time point, double-LT recipients had higher alpha diversity compared to single-LT recipients (p= 0.005) (figure 18b). Also at 6 months, both LT types had unique viruses in their composition, especially Pegivirus in double-LT (18.3%) and Circovirus in single-LT (7.3%) (figure 18c). Deeper, Pegivirus, Gammatorquevirus, Betatorquevirus and unclassified Flaviviridae genera were significantly more frequent in double than single-LT patients at 6 months after LT (figure 18d).

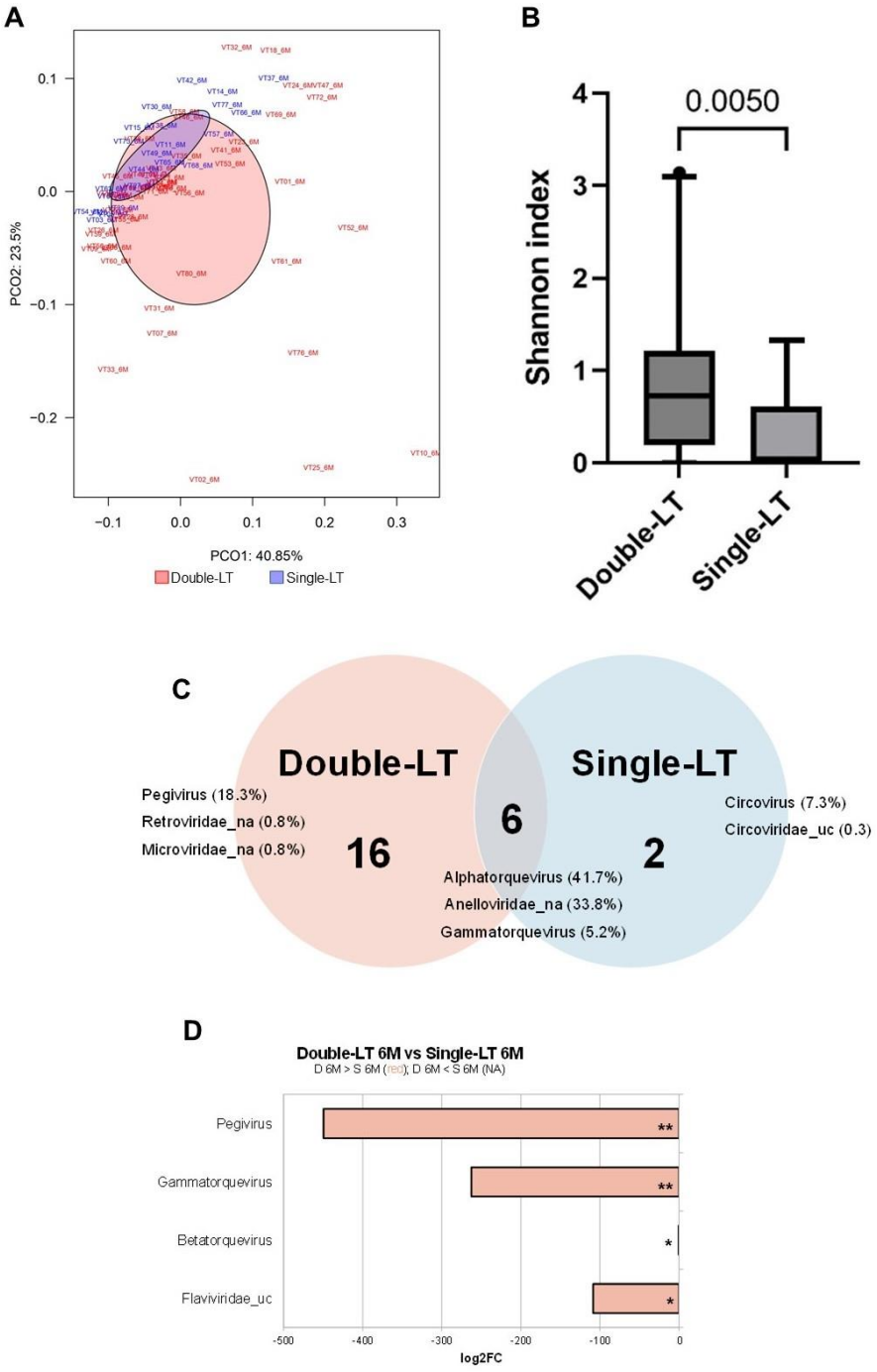

Figure 18. Virome composition, alpha and beta diversities and differentially abundant virus between aetiologies at 6 months post-LT. **A)** Principal Coordinates Analysis (PCoA) summarizing beta diversity of viral community composition between double-LT recipients (red) and single-LT recipients (blue) based on a Bray-Curtis distance matrix at the genus level at 6 months post-LT (p= 0.01, R^2= 0.05). At 12 (p> 0.05) and 24 (p> 0.05) months after LT was not significant (plot not shown). **B)** Boxplot comparing viral normalized alpha diversity (Shannon index) between double and single-LT plasma samples at 6 months after LT (p >0.05). **C)** Venn diagram with the most abundant viral genera detected only in double-LT (red), only in single-LT (blue) and commonly detected in both groups (intersection). Viruses detected in <0.1% have not been represented. **D)** Horizontal bar plot showing

log2-Fold change (log2FC) of those viral genera significantly more abundant in double-LT than single-LT recipients (red bars). *** *p*= <0.00001; * *p*= 0.05-0.01. ** *p*= <0,009-0,0009; * *p*= 0,05-0,01.

LT patients were followed-up clinically for 3 years after LT. After that time, 47 LT patients remained CLAD-free and 30 developed CLAD. The main clinical characteristics of the population are summarized in supplementary table 5. LT recipients with CLAD suffered more episodes of CMV replication (*p*= 0.02) and other episodes of viral (*p*= <0.0001), bacterial (*p*= 0.04) and fungal replication (*p*= 0.03) than CLAD-free LT patients. Further, CLAD LT patients suffered more ACR episodes (*p*= 0.0007) and the percentage of exitus was higher compared to CLAD-free LT patients (*p*= 0.009).

Supplementary table 6 shows the pulmonary function variables at the follow-up visits, which were performed at 6, 12, 24 and 36 months after LT. In addition, antibiotics, immunosuppression and antiviral treatments at each time point are reported.

To assess virome composition, all samples taken over time from patients who eventually developed CLAD were considered as CLAD samples. No differences in alpha diversity or virome composition were observed between CLAD and CLAD-free recipients at 6, 12 and 24 months after LT. However, the virome composition among both groups tended to diverge over time (supplementary figure 7). Surprisingly, 2 years after LT, a higher relative abundance of Gammatorquevirus genera was observed in those patients who eventually developed CLAD (14.9%) compared to CLAD-free recipients (4.7%), although not statistically significant (figure 18a), and we found 18 genera unique to CLAD patients (figure 19b).

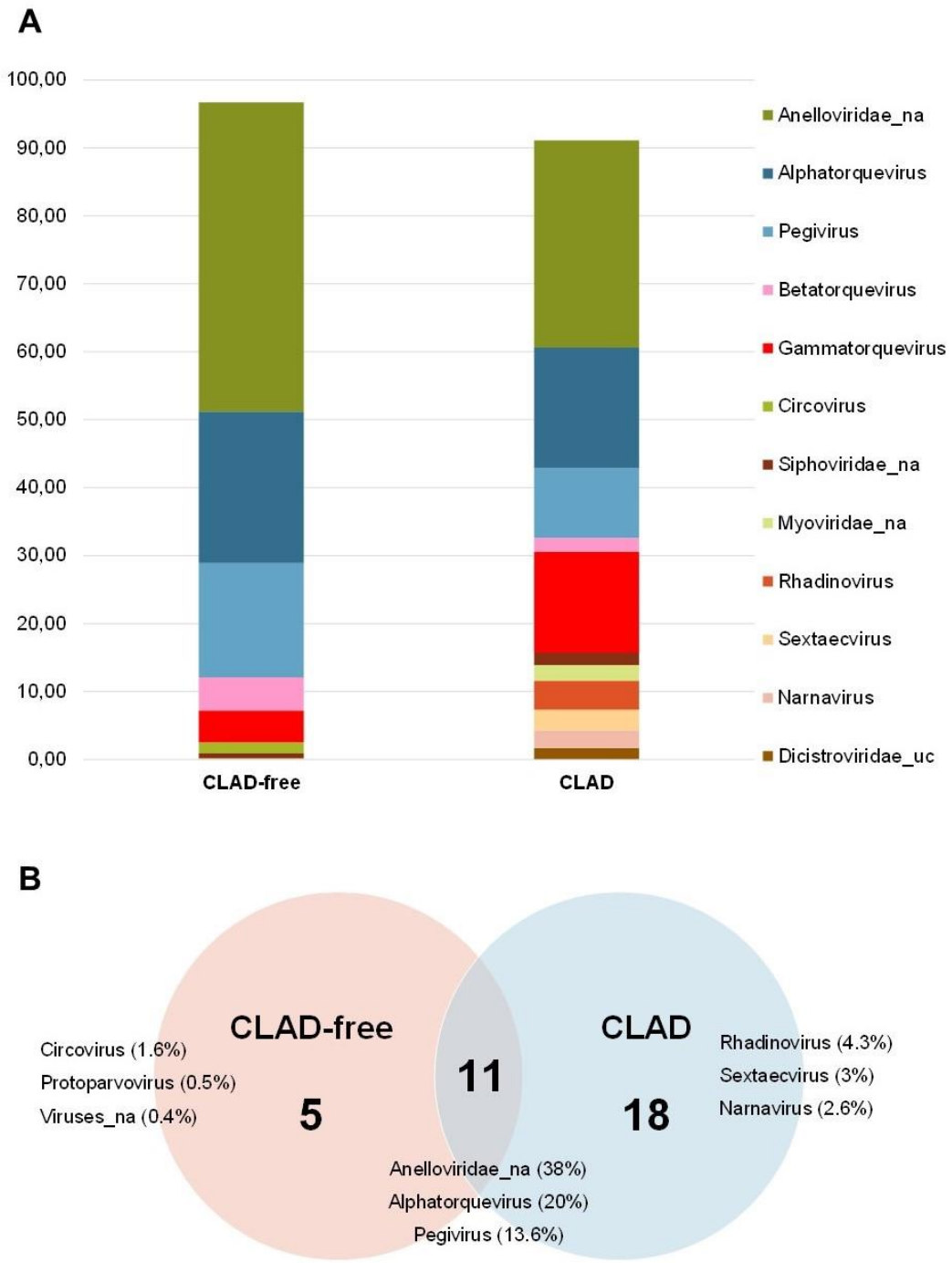

Figure 19. Taxonomic analyses of viral metagenomic reads on the genus level between CLAD and CLAD-free recipients at 24 months post-LT. **A)** Bar plots of the relative abundances of viral genera comparing CLAD and CLAD-free recipients. Viruses detected in <1% are not represented. **B)** Venn diagram with the most abundant viral genera detected only in CLAD (blue), only in CLAD-free patients (red) and commonly detected in both groups (intersection). Viruses detected in <0.1% have not been represented.

6. DISCUSSION

LT represents the last therapeutic option for patients suffering from various end-stage respiratory diseases. Despite all the advances in recent years in surgical techniques, immunosuppressive therapies and other treatments, the life expectancy of LT remains the lowest of all solid organ transplants. This is conditioned by the occurrence of infections, which account for 37% of deaths in the first year after LT (18) and CLAD, which is the main limitation for long-term survival, with a 25-30% mortality rate between 3-5 years after LT, since no effective treatment is available. Besides, both complications are related, as infections in LT recipients are a well-known risk factor for CLAD development (18,29).

In recent years, metagenomic methods have made it possible to characterise the respiratory bacterial microbiome of LT recipients and to describe its relationship with long-term outcomes (160,173,174), although its role in CLAD pathogenesis is unclear. Further, it should be noted that most evidence in this field is based on LRT samples obtained by invasive procedures. However, in other respiratory diseases, such as CF (204,205), COPD or asthma (206), it has been suggested that the URT microbiome might also play a pathogenic role in these diseases. Thus, URT sampling could be an effective and less invasive alternative to study the respiratory microbiome.

In the case of the viral component, information is even scarcer, as the entire virome, including DNA and RNA viruses, has not been studied in depth. In fact, only a few studies have used the metagenomic approach to evaluate the whole virome in LT recipients (123,176–178) and they have done so following very different strategies, in some of them even studying only the circulating cell-free DNA and thus losing information from encapsidated viruses (179). Moreover, neither the virome long-term dynamics nor its relationship with the prognosis of LT recipients has been clearly assessed.

Therefore, the present thesis was designed to evaluate in detail the respiratory bacterial microbiome and the complete plasma virome composition in LT recipients using metagenomic approaches to assess the temporal dynamics, its eventual restoration and the potential impact on long-term prognosis and CLAD development.

In our study of the bacterial microbiome, the genera *Corynebacterium*, *Staphylococcus* and *Streptococcus* were the most abundant in NP swabs from LT recipients and healthy controls. *Corynebacterium* and *Staphylococcus* have been described as frequent colonisers of the nares in healthy population (119,207,208). On the other hand, genera *Peptostreptococcus*, *Solobacterium* and *Selenomonas* (all belonging to the phylum Firmicutes) were only found in LT recipients. Interestingly, all three genera were overrepresented in CLAD recipients and, although not frequently reported, they have also been detected in BAL of LT recipients (169). Thus, it remains to be elucidated whether these bacterial genera really play a role in the LT population. Besides, although they were represented in lower abundances, the genera *Lawsonella*, *Massilia*, *Stenotrophomonas*, *Rheinheimera* and *Aureimonas* were significantly more abundant in NP swabs from healthy controls compared to LT recipients. Most of these genera belong to the phylum Proteobacteria (except *Lawsonella*, which is an Actinobacteria). Borewicz *et al.* showed that healthy lung microbiome was generally composed of Proteobacteria and also Firmicutes (164), however, other studies found in BAL samples that the most common genera in the microbiome of healthy lungs were *Prevotella* (Bacteroidetes), *Streptococcus* and *Veillonella* (Firmicutes) (116,119,120,209) and Actinobacteria in the nasal microbiome (208).

In our study of the plasma virome, Alphatorquevirus and other unclassifield genera of Anelloviridae, all belonging to the *Anelloviridae* family, were the most prevalent in both LT recipients and healthy controls, although they were overrepresented in recipients. Although *Anelloviridae* have not been described to cause disease in humans (210), this virus family, and in particular TTV species of the genus Alphatorquevirus, has gained interest in recent years as a tool to monitor the immunosuppression status of LT recipient (182,183). In this regard, the aim of this study was not to assess the specific load of a virus family, but to evaluate the overall viral composition, so Anelloviridae loads were not quantified. As a result, we were able to analyse the frequencies of the relative abundances of all the Anellovirus genera identified and observed a significant increase from the pre-transplant to

all subsequent times points, similar to when TTV loads are quantified (183) and to that observed after kidney and liver transplantation (211,212). Interestingly, the Pegivirus genus, belonging to *Flaviviridae* family, was only found in patients, but not in healthy controls. Although human Pegivirus can cause persistent infections (213), no association was observed between Pegivirus and clinical outcomes in LT recipients, nor was it found in a recent study by Graninger *et al*. in which they found that Human pegivirus 1 prevalence in LT recipients was 18.2%, but its detection was not associated with graft rejection or other microbial infections (214). Therefore, it remains to be elucidated whether Pegivirus plays a role in this population. Another viral genus detected only in LT recipients was Circovirus, which has been commonly associated with swine infections (215,216). However, in 2022, a new Circovirus species capable of infecting humans was discovered as the causative agent of hepatitis contracted by a cardiopulmonary transplant patient (217). In this thesis, because of the clinical importance of CMV in LT, we wanted to evaluate its detection in the metagenomic sequencing analysis performed. However, CMV was not detected in the metagenomic analyses of the follow-up even though 21 plasma samples were positive for CMV by routine PCR assays, suggesting limited sensitivity of metagenomic methods for CMV detection. This low sensitivity may be due to the filtering step, as it has been reported to reduce the recovery of Herpesvirus but is more efficient in detecting Anellovirus (178).

To date, in the LT field, BAL is the preferred specimen for assessing the bacterial microbiome (160,164,169,173,174) and virome (122–124,176–178,185). In the LT population, the lung bacterial microbiome has been reported to be distinct from the oral microbiome (160,168). Regarding NP and BAL bacterial microbiomes, we could not find significant differences in composition in our cohort from LT recipients (data not shown). However, it should be noted that the sample size was not sufficiently representative for BAL, as routine bronchoscopy is only performed in our center before patients' discharge. In the follow-up period, bronchoscopy is only performed when clinically necessary due to the aforementioned disadvantages. As for virome, the correlation between BAL and our sample

choice, plasma, has only been evaluated twice in LT recipients, observing decreased diversity in BAL samples (176,177) and different virome compositions, suggesting the presence of site-specific viral communities (177). Unfortunately, due to the retrospective design of the virome study, BAL was not available. However, it should be noted that BAL is an invasive procedure that is not free of risks and causes discomfort to patients. Therefore, its collection is not always feasible and is limited in longitudinal studies. The limitations in obtaining BAL samples will be even more pronounced in the near future, as noninvasive tools are under development to monitor LT recipients.

Interestingly, the alpha diversity of the NP bacterial microbiome and plasma virome decreased in the early stages after LT compared to healthy controls. This diversity increased over time, tending towards normal one year after LT for the NP bacterial microbiome and normalising after 2 years for the plasma virome.

The longitudinal increase in diversity was also observed when analysing bacterial microbiome in BAL samples, although in this case the comparison with healthy controls was not possible due to the lack of BAL samples from this population. Similarly, Simon-Soro *et al.* observed greater alpha diversity in the URT microbiome of healthy controls when compared with LT recipients, although in this case the oropharyngeal tract was analysed (218). In addition, several studies have also noted decreased alpha diversity of the BAL microbiome in LT recipients compared to healthy controls (160,161,166), but a single study, which also analysed BAL, found that alpha diversity was increased among LT recipients compared with controls, although this was a small study in which only 4 patients were sampled (164).

In relation to the plasma virome, the decrease in diversity in the early phases after LT is accompanied by an increase in total viral relative abundance. In parallel, once diversity normalises 2 years after LT, viral abundance declines. We speculate that the high-intensity immunosuppressive and antiviral prophylaxis that recipients undergo in the first months after LT may lead to an increase in the viral load of specific species, which in turn may

displace others, thus causing a decrease in diversity. The only study that performed a similar analysis in a LT population was unable to relate plasma virome variations to time after LT, but the sample collection time intervals were quite wide (177).

Although the most abundant genera coincide between all times, the overall virome composition differed between 6 and 24 months after LT. Specifically, at 6 months, the virome composition had a higher abundance of bacteriophages compared to 24 months: namely the genera Tequintavirus and Tunavirus, belonging to the order Caudovirales, and the unclassified Microviridae, whereas at 24 months the genera with a significantly different abundance when compared with 6 months were the unclassified Orthomyxoviridae and Hepaciviridae. Although the order Caudovirales has been reported to be very abundant after LT (176,179), the clinical impact of these and other bacteriophages in LT recipients remains unclear. In contrast, the unclassified genera Orthomyxoviridae and Hepaciviridae, found at 24 months, are not usually reported in LT recipients. In this immunosuppressed population, the most frequently reported genera, besides Anellovirus, are Coronavirus and Herpesvirus (122,176,177), although the studies are very diverse in design and protocol and their results are difficult to compare. Some of them evaluate the virome at an early stage after LT, with different sample types such as BAL (122) and with very different viral enrichment protocols, nucleic acid extraction and sequencing strategies. Besides, most of them have small sample size.

In our first cohort of patients, when comparing CLAD, CLAD-free recipients and healthy controls, no differences in the NP bacterial microbiome composition were observed in the early post-LT period. However, one year after LT, the bacterial genera *Capnocytophaga* and *Lachnoanaerobaculum* were significantly more abundant in CLAD recipients compared to CLAD-free and the healthy population. Both genera have been found in oral or oropharyngeal tracts. Species belonging to both genera have been reported as pathogens (219,220), unfortunately, our analysis could not reach the species level. Different studies pointed out that the predominance of *Pseudomonas* in BAL microbiome of LT recipients is

a risk factor for CLAD development (161,172,174). For instance, Schott *et al* observed that the predominance of the Proteobacteria phylum, including *Pseudomonas*, increased BOS susceptibility (174). However, this is not conclusive, as in other studies these findings could not be confirmed (166,173). In our cohort, an association between *Pseudomonas* and CLAD could not be assessed in relation to BAL samples, as sample size was limited. Interestingly, a normalisation of the microbiome composition was observed in CLAD-free recipients after two years of post-LT follow-up.

Regarding the virome study population, although specimen collection in this cohort extended up to 2 years after LT and the clinical follow-up to 3 years, we were unable to detect changes in virome composition associated with CLAD. Nevertheless, 2 years after LT we observed higher relative abundances of Gammatorquevirus genera in patients who eventually developed CLAD, although it was not statistically significant. However, these findings could be because after the diagnosis of CLAD, immunosuppressive treatment is increased and could be a reflection of the immunosuppressed state. Blatter *et al.* correlated an early post-LT decrease in Betatorquevirus with a later development of BOS in pediatric LT recipients (221) and Frye *et al.* observed that those LT recipients diagnosed with ACR showed a prior decrease in their TTV levels (187). Perhaps these findings show that those patients who are less immunosuppressed post-transplantation would have a higher risk of developing rejection in its different forms. Thus, immunosuppression could play a role in virome alterations and, in this sense, in the field of lung transplantation it remains to be clarified whether infections are in themselves a risk factor for the development of CLAD or whether the dysfunction is induced by the immunosuppression adjustments made in the presence of infection.

From the results, it appears that the composition of the donor bacterial microbiome was not related to an increased risk of developing CLAD. However, we were unable to analyse whether the donor microbiome remained in the LRT of recipients after LT, as BAL samples from donors were not available for comparison. In this regard, few studies have reported

that donor bacteria do not influence post-LT outcomes (169,171). As for virome donor plasma samples were not available, so its impact on recipients after LT could not be analysed either. Other studies have quantified only specific viruses, such as TTV (123) or CARVs (184), to assess their transmission from donors to recipients, but there is no information on the influence of the donor virome on LT recipients. Consequently, the influence of the donor microbiome and virome on LT recipient outcomes needs further study.

Besides, both studies provide the opportunity to analyse the bacterial microbiome or virome in patients with advanced respiratory diseases, as in both cohorts we collected samples before transplantation. In particular, we demonstrated that, prior to LT; bacterial microbiome composition differed between URT (NP samples) and LRT (lung tissue and bronchial swabs). Similar findings have been reported in two of the main aetiologies that could lead to LT, CF (204,205) and COPD (206,222), where differences between URT and LRT microbiomes have been described. For instance, *Streptococcus*, *Microbacterium* and *Ralstonia* genera are more abundant in the URT of CF patients while *Pseudomonas* and *Prevotella* are more abundant in the LRT (204). As for COPD, *Lactobacillus* and *Pseudomonas* have been found in the URT (206) whereas *Veillonella*, *Fusobacterium* and *Prevotella* are mostly described in the LRT (222). However, no differences in bacterial diversity and composition were observed when compared between aetiologies (data not shown).

Similarly, we also found no differences in plasma virome composition between etiologies; however, we did detect differences between patients with ILD and healthy controls. Importantly, of these patients, 70.7% were receiving immunosuppressive therapy prior to LT, which affects CD4 T lymphocytes, which could be causing changes in virome composition, as has been described to occur in HIV patients (223,224). Surprisingly, at 6 months post-LT, patients transplanted for ILD and single-LT patients had a different viral composition compared to patients transplanted for other indications and double-LT

recipients, respectively. Despite the fact that, after LT, all recipients followed the same prophylaxis, regardless of their pre-LT aetiology or the type of LT they underwent. This leads us to propose two hypotheses. In relation to the underlying pathology, those patients with ILD may be carrying over the observed variations in virome composition prior to LT until at least 6 months after LT. In the case of transplant type, that in those patients with a single-LT the native lung may contribute to modifying virome composition up to 6 months after LT. Although it is most likely that both findings are a mere reflection of each other as the vast majority of single-lung recipients have ILD as their underlying aetiology. The first hypothesis is more plausible as the native organ is maintained throughout the post-transplant period and the effect is transient. Contrary to our findings, Widder *et al.* (177) reported that patients transplanted for COPD or fibrosis had a different plasma virome than CF patients after LT, although this difference did not correlate with time after LT. These findings could also be due to the diversity of the transplant populations compared and the frequency of occurrence of each of the indications leading to transplantation.

Regarding the influence of treatments on results, one year after LT, the overall bacterial composition of the bacterial microbiome did not differ between patients treated or not treated with MMF, cotrimoxazole, colistin, or tobramycin. Similar results have been reported previously (163,173,174), and only one study found variations in the microbiome attributable to meropenem, colistin or cotrimoxazole in early stages after LT (169). Since azithromycin was administered to the majority of our population to prevent the occurrence of CLAD (64,65), the effect of this drug on the microbiome could not be fully explored. In the case of virome, no changes in its global composition were found when comparing high versus low doses of corticosteroids (>12 or <12 mg, respectively) or high versus low tacrolimus blood levels (>10 or <10 ng/ml respectively) at any time during follow-up (data not shown). However, virome composition differed from pre-TL to all post-TL stages, suggesting an impact of immunosuppression on these alterations, as before LT only some patients were

taking corticosteroids and/or MMF (especially those with ILD), but once transplanted, all recipients take these treatments uniformly.

Only De Vlaminck *et al.* (179) have evaluated the effect of treatments on virome composition, they found alterations mainly due to corticosteroids, which at low doses increased Herpesvirus and Caudovirales, whereas at high doses, Anellovirus increased. However, most of their plasma samples were collected at early stages after LT and they only analysed cell-free DNA, so information on encapsidated viruses is lacking.

Finally, it should be noted that in early 2023, the International Committee on Taxonomy of Viruses abolished the families Myoviridae, Podoviridae and Siphoviridae and the order Caudovirales. Viral members within these families and order were placed in the class Caudoviricetes, leaving some taxa as "unclassified". The new taxonomy release can be found on the ICTV website (https://ictv.global/). However, old terms may continue to be used in order to retain their historical reference and to facilitate comparisons with earlier publications (225).

The main common limitation of both studies is that samples were only obtained at fixed time points and not when an event occurred. This fact has not allowed correlating alterations in the bacterial microbiome and virome with infection episodes or at the onset of CLAD. Methodologically, we only amplified a single hypervariable region of the 16s rRNA gene (V4) and the reads generated after sequencing were single-end. Consequently, we did not achieve sufficient sensitivity to reach the taxonomic level of species. On the other hand, virome analysis was perform by SMS with a higher taxonomic resolution, but in this case, the main limitation lies in the subsequent bioinformatic analysis, as the sequencing alignment tools are very incomplete for viruses.

In our center, bronchoscopies are performed as required by the clinic or routinely when the patient is discharge. Thus, only a few BAL samples were available to study the bacterial microbiome and no direct comparisons with other studies of microbiome in BAL could be made. In this line, BAL samples are not routinely collected in Vall d'Hebron University

Hospital Biobank, so, due to the retrospective design of the virome study, BAL samples were not available for analysis. Therefore, we cannot currently confirm that the viral composition found in plasma samples reflects what occurs at the respiratory level.

A final limitation, especially in the study of the bacterial microbiome, is that clinical follow-up ended 2 years after LT, resulting in the diagnosis of only 12 CLAD. Thus, the relatively small size of this group of patients makes comparisons between groups difficult and limits the possibility of drawing conclusions about the NP microbiome as a potential predictor of CLAD.

On the other side, the following strengths are noteworthy: both studies have a longitudinal design and include 68 LT recipients and 450 respiratory samples in the bacterial microbiome study and 77 patients and 306 plasma samples in the virome one.

In addition, the first study of this thesis is one of the largest based on the URT microbiome, offering an innovative approach and a non-invasive alternative to monitor the respiratory microbiome in LT recipients. Finally, the second study is the largest to evaluate the dynamics of the entire plasma virome, DNA and RNA viruses, using a metagenomic approach including samples up to 2 years post-LT and a clinical follow-up up to 3 years, allowing to assess its relationship with CLAD development.

7. CONCLUSIONS

This thesis provides valuable data to better understand both the characteristics and dynamics of the upper tract bacterial microbiome and plasma virome in lung-transplanted recipients, and the following conclusions are derived:

1. The bacterial genera *Corynebacterium*, *Staphylococcus* and *Streptococcus* were the most prevalent in the upper tract of lung transplanted recipients and healthy controls. However, the genera *Lawsonella*, *Massilia*, *Stenotrophomonas*, *Rheinheimera* and *Aureimonas* were significantly more abundant in the upper tract from healthy controls compared to lung-transplanted recipients.

2. The viral genera Alphatorquevirus and other unclassifield Anelloviridae were the most prevalent in the plasma of both lung transplant recipients and healthy controls, being over-represented in recipients. Most strikingly, the Pegivirus genus was only present in transplant recipients and in non-negligible relative abundance. In particular, double-lung transplant recipients contributed this genus.

3. The alpha diversity of the upper respiratory tract bacterial microbiome and plasma virome decreases in the early stages after lung transplantation, tending to normalise one year after transplantation in the case of the bacterial microbiome and normalising two years after transplantation in the case of the plasma virome.

4. The composition (beta diversity) of the upper respiratory tract bacterial microbiome of patients without chronic lung allograft dysfunction is normalised with respect to that of healthy controls one year after lung transplantation, however this finding is not observed in the plasma virome.

5. The bacterial genera *Capnocytophaga* and *Lachnoanaerobaculum* were more abundant in CLAD recipients than in CLAD-free recipients and healthy controls, while no differentiating viral genus was observed between CLAD and CLAD-free recipients.

8. FUTURE PERSPECTIVES

The main objective of this thesis was to analyse bacterial microbiome and virome in LT recipients. The results provided could contribute to a better understanding of the dynamics of respiratory bacterial microbiome and plasma virome after LT and their involvement in CLAD, showing also the potential of NP samples to non-invasively monitor the microbiome in LT population. In turn, these results open new perspectives for future research.

The first objective, in order to facilitate comparison between studies, should be to reach a consensus on all aspects related to microbiome and virome research, including sampling, nucleic acids extraction protocols, sequencing platform and bioinformatic methodologies. In addition, it is also essential to determine the most informative hypervariable region of the 16S rRNA gene for bacterial microbiome studies and the most efficient amplification method for virome studies. In addition, we should raise awareness of contamination from reagents or the environment as a potential confounding factor.

Although the results of the present thesis are of potential relevance, longitudinal studies involving longer follow-up, sampling when a relevant clinical event occurs, such as CLAD onset, and more in-depth methodological approaches are needed to better understand the role of microbiome in the mechanisms involved in CLAD development after LT. These studies could be performed with NP samples that would allow a more continuous follow-up than with invasive sampling such as BAL.

For instance, longer follow-ups would increase incidence of CLAD, helping to understand whether pathophysiological changes in the lung when CLAD occurs may lead to microbial dysbiosis or whether this dysbiosis may lead to upregulation of inflammatory signals in response to pathogens. Thus, the role of the microbiome as consequence or cause for CLAD, respectively, could be established.

Other more in-depth approaches could be the study of the interactions of the microbiome with inflammatory mediators or metatranscriptomics and metabolomics analyses. Metatranscriptomics would allow not only to recognise the bacteria or viruses detected in

the samples, but also what their functions are, based on gene expression, while metabolomics would allow to identify small molecules produced by the microbiota and to understand their functional role.

Finally, another interesting line of research would be to perform studies characterising the entire microbiome, which means including the analysis of interactions between bacteria, viruses, and fungi in a single cohort of LT recipients.

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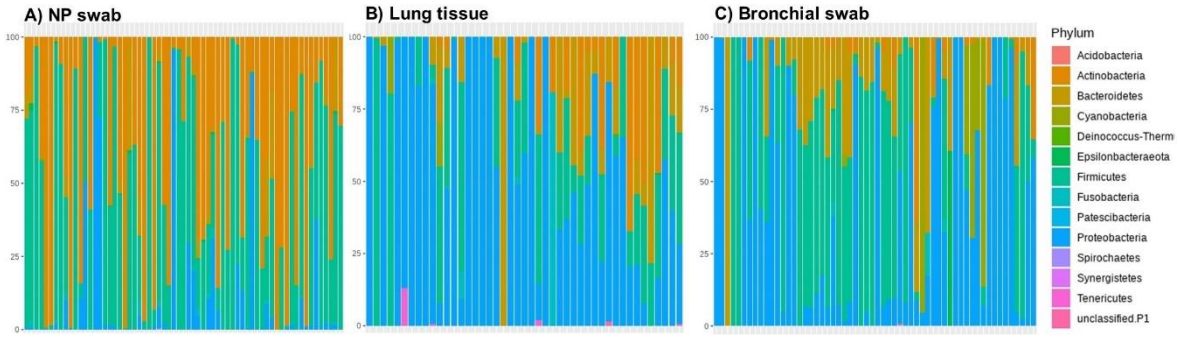
10. ANNEXES

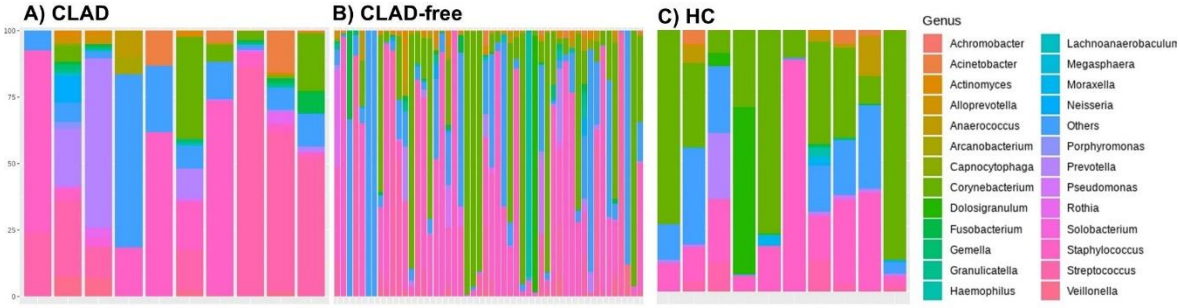
10.1. ANNEX 1. Supplementary Material from Part 1

Supplementary figure 1



Supplementary figure 1. Barplots of the relative abundances of the bacterial phyla in nasopharyngeal swabs **(A)**, lung tissue **(B)** and bronchial swabs **(C)** at LT day.

Supplementary figure 2



Supplementary figure 2. Barplots of the relative abundances of the bacterial genera in CLAD **(A)** and CLAD-free **(B)** recipients one year after LT and from healthy controls **(C)**.

Supplementary table 1. Longitudinal characteristics of the population

	Discharge			2-5 months post-LT			12 months post-LT			24 months post-LT		
	CLAD-free	**CLAD**	***p***	**CLAD-free**	**CLAD**	***p***	**CLAD-free**	**CLAD**	***p***	**CLAD-free**	**CLAD**	***p***
FVC %, median (IQR)	59.8 (50.7-68.7)	67 (58.1-71)	0.39	72 (63.4-84)	67.3 (62.3-84.2)	0.65	74.4 (61.8-85.5)	71.8 (59-84.5)	0.8	76.5 (65.5-89.2)	68.7 (48.9-81.9)	0.22
FEV_1 %, median (IQR)	63.5 (49.8-73.8)	60 (56-69)	0.6	73.3 (59.8-85)	69 (46.5-90.2)	0.53	74.6 (60.6-87.4)	60 (43.2-88.1)	0.53	74.8 (65.3-93.3)	40.5 (38.5-69.2)	**0.005**
Immunosupression												
*Tacrolimus, median (IQR)	11.3 (7.9-13.2)	7.8 (6.1-13.8)	0.25	10.3 (8.4-14-4)	12.7 (8.9-14.6)	0.34	9.3 (7.7-11.5)	9.2 (6.4-14.3)	0.74	8.4 (6.7-11.4)	10 (6.5-12.8)	0.53
MMF, n (%)	55 (98.2)	12 (100)	1	53 (94,6)	12 (100)	1	49 (92.4)	8 (72.7)	0.09	45 (90)	6 (54.5)	**0.01**
Corticosteroids, n (%)	53 (94.6)	12 (100)	1	56 (100)	12 (100)	1	53 (100)	11 (100)	1	50 (100)	9 (81.8)	**0.03**
Rapamycin, n (%)	--	--	--	--	--	--	--	3 (27.3)	--	--	5 (45.5)	--
Antibiotic prophylaxis, n (%)												
Azithromycin	2 (3.6)	1 (8.3)	0.45	26 (46.4)	6 (50)	1	46 (86.8)	11 (100)	0.34	43 (86)	11 (100)	0.33
Cotrimoxazole	56 (100)	12 (100)	1	55 (98.2)	12 (100)	1	50 (94.3)	11 (100)	1	46 (92)	4 (90.9)	1
L-AmB	56 (100)	12 (100)	1	56 (100)	12 (100)	1	52 (98.1)	11 (100)	1	49 (98)	10 (90.9)	0.33
Colistin	15 (26.8)	2 (16.7)	0.72	15 (26.8)	2 (16.7)	0.72	21 (39.6)	2 (18.2)	0.3	17 (34)	3 (27.3)	1
Tobramycin	31 (55.4)	8 (66.7)	0.54	29 (51.8)	8 (66.7)	0.52	6 (11.3)	4 (36.4)	0.06	2 (4)	0 (0)	1

FVC: Forced vital capacity. FEV_1: Forced expiratory volume in 1 second. MMF: Mycophenolate mofetil. L-AmB: Liposomal Amphotericin B

*All LT recipients were taking tacrolimus during all the follow-up. Tacrolimus levels in ng/ml. **Bold value**: statistically significant result

Supplementary table 2. Frequency of LT recipients with infection episodes during the follow-up

% LT recipients with infection	CLAD-free	CLAD	*p*
Hospitalization			
Bacterial	42.9	58.3	0.36
Viral	1.8	0	1
Fungal	5.4	0	1
Total	50	58.3	0.75
2-5 months post-LT			
Bacterial	19.6	33.3	0.44
Viral	5.4	0	1
Fungal	8.9	16.7	0.6
Total	33.9	50	0.33
6-12 months post-LT			
Bacterial	20.8	45.5	0.12
Viral	11.3	18.2	0.62
Fungal	3.7	9.1	0.44
Total	35.8	72.7	**0.04**
12-18 months post-LT			
Bacterial	3.4	27.3	**0.03**
Viral	1.9	9.1	0.33
Fungal	1.9	0	1
Total	7.8	36.7	**0.03**
18-24 months post-LT			
Bacterial	12	9.1	1
Viral	2	9.1	0.33
Fungal	2	0	1
Total	16	18.2	1

Infection episodes include pneumonia, bronchitis, tracheobronchitis and upper respiratory tract infections. Frequency of LT recipients with 1 or >1 infection episode (maximum 3/recipient) calculated with Fisher's exact test. **Bold value**: statistically significant result

Supplementary table 3. Differentially abundant genera between healthy controls and CLAD-free and CLAD recipients in NP samples at one year after LT.

	HC vs. CLAD	CLAD-free vs. CLAD	CLAD-free vs. HC
Lawsonella	3.23 *p*= 0.018	NA	-2.68 *p*= 0.009
Rothia	-3.9 *p*= 0.007	NA	NA
F0058	1.73 *p*= 0.0004	NA	-1.68 *p*= 5.4e-06
Capnocytophaga	-3.22 *p*= 0.001	-2.09 *p*= 0.007	NA
Unclassified.G230	1.67 *p*= 0.0003	NA	-1.57 *p*= 7.3e-06
Butyrivibrio	NA	NA	-0.601 0.04
Lachnoanaerobaculum	-2.72 *p*= 0.02	-1.9 *p*= 0.038	NA
Peptostreptococcus	NA	-1.6 *p*= 0.01	NA
Solobacterium	NA	-1.63 *p*= 0.026	NA
Selenomonas	NA	-1.67 *p*= 0.026	NA
Unclassified.G24	NA	NA	-0.83 0.022
Aureimonas	1.29 *p*= 0.01	NA	-1.28 *p*= 0.0008
Rheinheimera	1.51 *p*= 0.006	NA	-1.49 *p*= 0.0003
Massilia	1.98 *p*= 0.023	NA	-1.95 *p*= 0.002
Aggregatibacter	1.09 *p*= 0.042	NA	NA
Unclassified.G96	NA	NA	-0.77 *p*= 0.028
Stenotrophomonas	1.9 *p*= 0.021	NA	-1.58 *p*= 0.012

CLAD: Chronic lung allograft dysfunction. HC: Healthy controls. The value at the top of each box represents the effect size. Positive values indicate that the first group of those compared is the one with the highest abundance of the corresponding bacterial genus. The value at the bottom of each box represents the p-value.

10.2. ANNEX 2. Supplementary Material from Part 2

Plasma preparation

EDTA (Ethylenediaminetetraacetic acid) tubes were first centrifuged at 1300 g for 10 min at room temperature. A second centrifuged was performed with the supernatant at 2500 g for 15 min at room temperature. Plasma samples were stored at -80ºC until use.

Supplementary table 4. Demographic and clinical characteristics of the population regarding aetiologies

	ILD (n=41)	**COPD, emphysema, A1ATD (n=22)**	**Others* (n=14)**	***p***
Age, median (IQR)	59 (51-62)	57 (55-62.3)	33.5 (27.8-50)	**0.0002**
Gender (males), n (%)	26 (63.4)	14 (63.6)	7 (50)	0.64
Smoking, n (%)	22 (53.7)	22 (100)	5 (35.7)	**< 0,0001**
Pre-LT immunosup, n (%)				
Tacrolimus	2 (4.9)	0 (0)	0 (0)	--
MMF	12 (29.3)	0 (0)	0 (0)	**0.002**
Corticosterioids	26 (63.4)	2 (9.1)	4 (28.6)	**< 0,0001**
mTOR inhibitors	0 (0)	0 (0)	2 (14.3)	--
Pre-LT FVC %, (median, IQR)	41.1 (31.8-52.2)	39.7 (31.3-42.9)	37.9 (26.1-62.1)	0.67
Pre-LT FEV_1 %, (median, IQR)	47.5 (36.2-61.1)	20 (16.7-23.6)	23.4 (17.9-37)	**< 0,0001**
Pre-LT FEV_1/FVC, (median, IQR)	85.1 (80-88.6)	41.4 (35.9-46.6)	52.7 (41.3-71.5)	**< 0,0001**
Single-LT, n (%)	22 (53.7)	0 (0)	2 (14.3)	**< 0,0001**
CLAD development, n (%)	15 (36.6)	8 (36.4)	7 (50)	0.64

ILD: Interstitial lung disease. COPD: Chronic obstructive pulmonary disease. A1ATD: Alpha-1 antitrypsin deficiency. Immunosup: immunosuppressive treatments. MMF: Mycophenolate mofetil. FVC: Forced Vital Capacity. FEV1: Forced Expiratory Volume in 1 second. IQR: Interquartile range. CLAD: Chronic Lung Allograft Dysfunction.

*Other etiologies: cystic fibrosis, bronchiolitis obliterans, pulmonary hypertension, systemic lupus erythematosus, lymphangioleiomyomatosis, Erasmus syndrome.

Bold value: statistically significant result

Supplementary table 5. Demographic and clinical characteristics of the population regarding CLAD development

	CLAD-free (n=47)	**CLAD (n=30)**	**HC (n=20)**	***p***
Age, median (IQR)	56 (51-61)	57 (46.5-63)	55 (42.3-60.8)	0.6
Gender (males), n (%)	32 (68)	15 (50)	12 (60)	0.3
Aetiology, n (%)				0.65
ILD	26 (63.4)	15 (36.6)		
COPD, emphysema, A1ATD	14 (63.6)	8 (36.4)		
Other aetiologies*	7 (50)	7 (50)		
Smoking, n (%)	27 (57.4)	22 (73.4)		0.22
LAS Score, median (IQR)	37.3 (34.6-43.9)	35.8 (33.5-41.5)		0.28
Single-LT, n (%)	14 (29.8)	10 (33.3)		0.8
Ischemia time (min)⁺, (median, IQR)	312.5 (260-421)	317.5 (271-350)		0.36
Days hospitalization, (median, IQR)	31 (22-60)	42.5 (31-58)		0.12
PGD, n (%)	9 (19.1)	11 (36.7)		0.11
CMV replication episodes, (median, IQR)	2 (1-4)	4 (2-6)		**0.02**
Other viral replication episodes, (median, IQR)	0 (0-1)	1 (2-2.3)		**< 0.0001**
Bacterial replication episodes, (median, IQR)	1 (1-3)	1 (3-4.3)		**0.04**
Fungal replication episodes, (median, IQR)	0 (0-1)	0 (1-1)		**0.03**
ACR episodes, (median, IQR)	0 (0-0)	0 (0.5-1)		**0.0007**
Exitus, n (%)	5 (10.7)	11 (36.7)		**0.009**
Months from LT to CLAD diagnosis		21.7 (3-36)		

CLAD: Chronic Lung Allograft Dysfunction. HC: Healthy controls. ILD: Interstitial lung disease. COPD: Chronic obstructive pulmonary disease. A1ATD: Alpha-1 antitrypsin deficiency. LAS score: lung allocation score. Single-LT: Single-lung transplantation. CMV: Cytomegalovirus. IQR: Interquartile range. ACR: Acute cellular rejection.

*Other aetiologies: cystic fibrosis, bronchiolitis obliterans, pulmonary hypertension, systemic lupus erythematosus, lymphangioleiomyomatosis, Erasmus syndrome.

⁺Minutes of ischemia calculated using the highest time.

Bold value: statistically significant result.

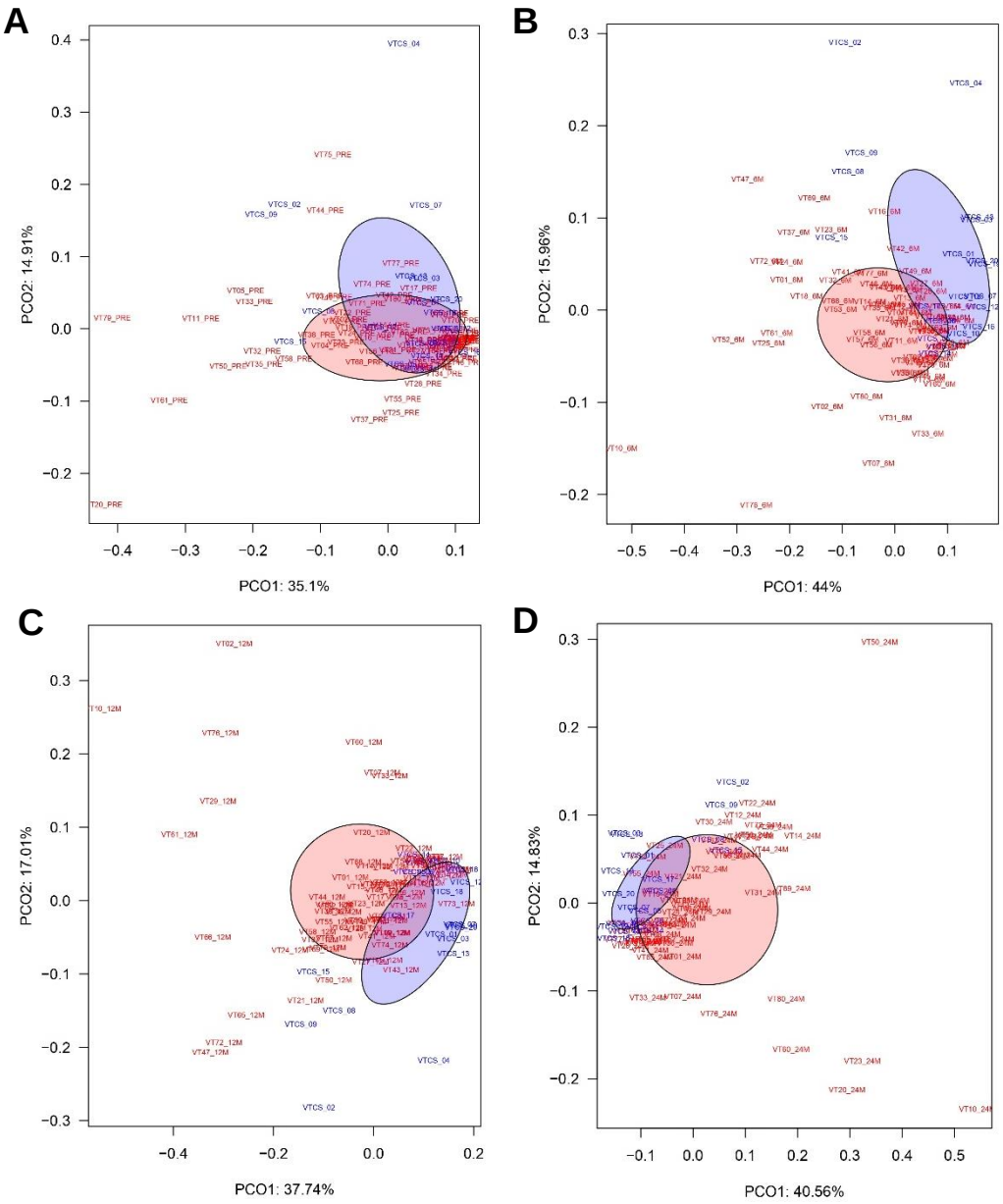
Supplementary table 6. Longitudinal medication and pulmonary function data of the population

	6 months post-LT			12 months post-LT			24 months post-LT			36 months post-LT		
	CLAD-free (n=47)	**CLAD (n=30)**	***p***	**CLAD-free (n=47)**	**CLAD (n=29)**	***p***	**CLAD-free (n=43)**	**CLAD (n=25)**	***p***	**CLAD-free (n=42)**	**CLAD (n=19)**	***p***
FVC %, median (IQR)	61.1 (51.6-77.3)	64.9 (50.8-70.8)	0.88	68 (17.6)*	64.8 (15.9)*	0.42	74.4 (17.9)*	65.8 (16.9)*	0.07	74.8 (58.3-82.5)	41.5 (37.2-67.5)	**< 0.0001**
FEV_1 %, median (IQR)	68.1 (19)*	63.4 (18)*	0.29	70.6 (19.4)*	60.1 (17.2)*	**0.02**	74.5 (19.2)*	54.3 (20.9)*	**0.0007**	74.8 (19)*	44.7 (12.3)*	**< 0.0001**
Immunosupression												
Tacro ng/ml, median (IQR)	11.3 (9.3-13.7)	10.8 (8.1-14.7)	0.82	10.3 (8.2-12.2)	8.1 (6-9.8)	**0.04**	8.5 (6.3-9.8)	7.1 (5.5-10.9)	0.39	7.3 (5.9-9.2)	7.7 (3.9-10.7)	0.91
MMF, n (%)	43 (91.5)	27 (90)	1	43 (91.5)	24 (82.7)	0.29	31 (72.1)	17 (68)	0.78	32 (76.2)	11 (57.9)	0.22
Cortis mg, median (IQR)	16 (15-20)	16 (14.3-24)	0.62	12 (10-15)	12 (10-16)	0.37	6 (4-8)	10 (6-13.5)	**0.005**	4 (4-6.4)	6 (4-10)	**0.007**
mTOR inhibitors, n (%)	1 (2.1)	3 (10)	0.3	3 (6.4)	4 (13.8)	0.41	8 (18.6)	5 (20)	1	5 (11.9)	7 (36.8)	**0.03**
Antibiotics, n (%)												
Azithromycin	44 (93.6)	28 (93.3)	1	46 (97.9)	28 (96.6)	1	42 (97.7)	25 (100)	1	41 (97.6)	19 (100)	1
Cotrimoxazole	47 (100)	30 (100)	1	47 (100)	29 (100)	1	40 (93)	25 (100)	0.29	39 (92.8)	18 (94.7)	1
L-AmB	47 (100)	30 (100)	1	47 (100)	29 (100)	1	42 (97.7)	25 (100)	1	40 (95.2)	19 (100)	1
Colistin	16 (34)	6 (20)	0.21	17 (36.2)	6 (20.7)	0.2	18 (41.9)	7 (28)	0.3	15 (35.7)	4 (21.1)	0.37
Tobramycin	7 (14.9)	3 (10)	0.73	5 (10.6)	3 (10.3)	1	1 (2.3)	1 (4)	1	1 (2.4)	2 (10.5)	0.23
Valganciclovir, n (%)	36 (76.6)	25 (83.3)	0.57	9 (19.1)	7 (24.1)	0.77	3 (7)	0 (0)	0.29	1 (2.4)	1 (5.3)	0.53

*Data showed as media (standard deviation). CLAD: Chronic Lung Allograft Dysfunction. FVC: Forced vital capacity. IQR: Interquartile range. FEV1: Forced expiratory volume in 1 second. Tacro: Tacrolimus. MMF: Mycophenolate mofetil. Cortis: Corticosteroids. L-AmB: Liposomal Amphotericin B.

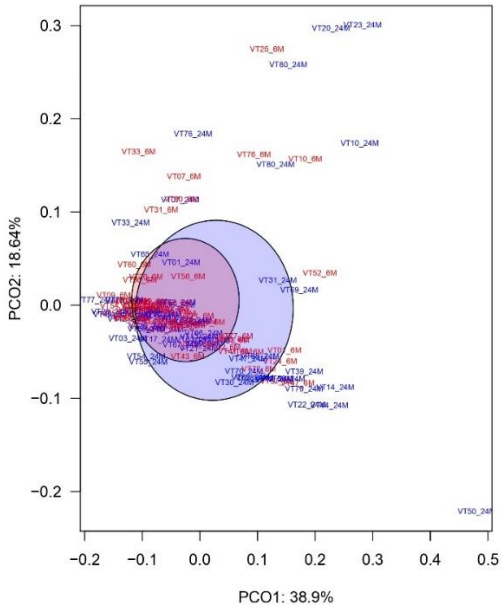
Bold value: statistically significant result.

Supplementary figure 3



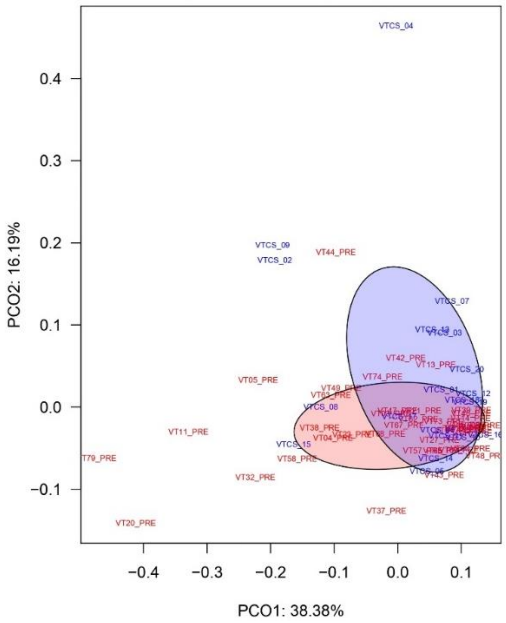
Supplementary figure 3: Principal coordinates analysis (PCoA) summarizing beta diversity of viral community composition between time points (red) and healthy controls (HC) (blue) based on a Bray-Curtis distance matrix at the genus level. **A)** Before LT vs HC (Adonis, *p*= 0.03, R^2= 0.02), **B)** 6 months after LT vs HC (Adonis, *p*= 0.0017, R^2= 0.09), **C)** 12 months after LT vs HC (Adonis, *p*= 0.0017, R^2= 0.06) and **D)** 24 months after LT vs HC (*p*= 0.0017, R^2= 0.07)

Supplementary figure 4



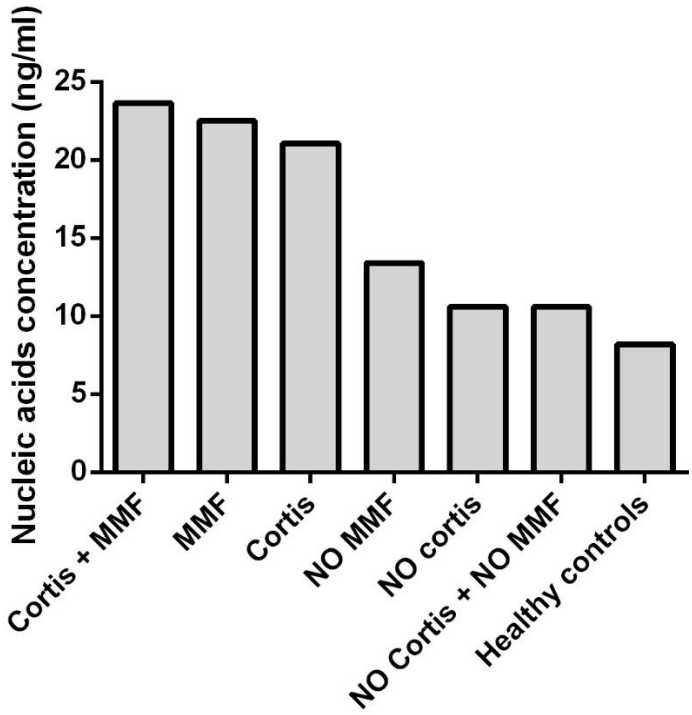
Supplementary figure 4: Principal coordinate analysis (PCoA) summarizing beta diversity of viral community composition between 6 (red) and 24 months (blue) after LT based on a Bray-Curtis distance matrix at the genus level (Adonis, p= 0.02, R^2= 0.026).

Supplementary figure 5



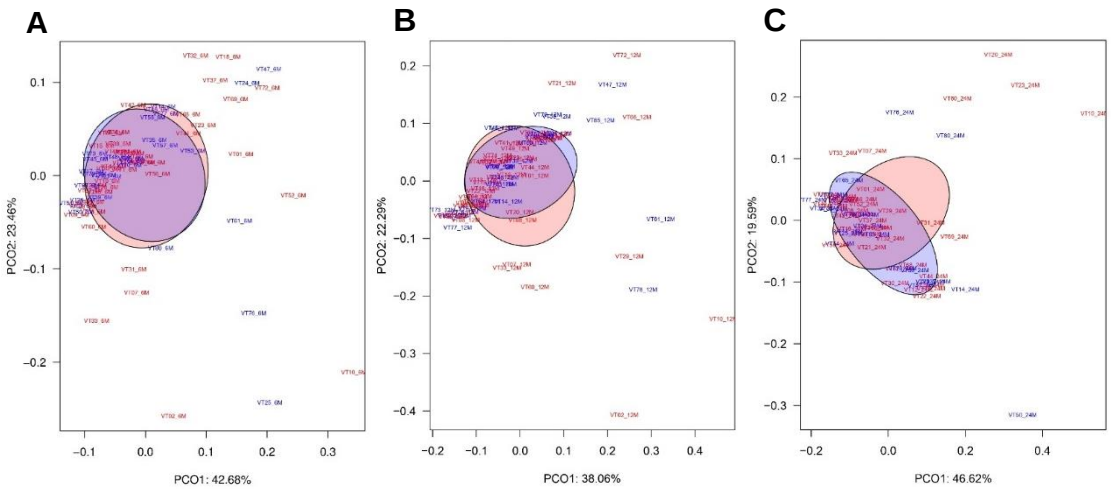
Supplementary figure 5: Principal coordinate analysis (PCoA) summarizing beta diversity of viral community composition between ILD patients (red) and healthy controls (HC) (blue) based on a Bray-Curtis distance matrix at the genus level (Adonis, p= 0.043, R^2= 0.037).

Supplementary figure 6



Supplementary figure 6: Bar plot depicting median viral load (i.e. nucleic acid concentration after SISPA and purification) before LT by immunosuppressive regimens: 23.65 (with corticosteroids and MMF), 22.5 (MMF only), 21.05 (corticosteroids only), 13.4 (without MMF), 10.6 (without corticosteroids) and 10.6 (without corticosteroids and MMF) ng/ml. Healthy controls had a median viral concentration of 8.2 ng/ml.

Supplementary figure 7



Supplementary figure 7: Principal coordinates analysis (PCoA) summarizing beta diversity of viral community composition between CLAD-free (red) and CLAD (blue) recipients based on a Bray-Curtis distance matrix at the genus level at different time points. A) at 6 months post-LT (Adonis, p= 0.9, R^2= 0.0002), B) at 12 months post-LT (Adonis, p= 0.7, R^2= 0.008) and C) at 24 months post-LT (Adonis, p= 0.6, R^2= 0.01).