# On viral infections in lung transplant recipients

Jesper Magnusson

Respiratory Medicine

Internal Medicine and Clinical Nutrition
Institute of Medicine
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG
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jesper.magnusson@gu.se

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# On viral infections in lung transplant recipients

Jesper Magnusson

Respiratory Medicine, Institute of Medicine
Sahlgrenska Academy at University of Gothenburg
Sweden

## ABSTRACT

Viral infections are the most common type of infection in humans. Lung transplantation (LTx) recipients are exceptionally susceptible to infections in general, and the short- and long- term effects tend to be more detrimental. It is important to better determine the effects and outcomes of viral infections to improve survival and long-term quality of life after LTx. The following hypotheses were tested: that early viral respiratory tract infection (VRTI) has long term effect on outcome after lung transplantation (Papers I and III); that hepatitis E (HEV) antibodies are common in Swedish lung transplant recipients (Paper II); and that torque teno virus (TTV) and Epstein-Barr virus (EBV) may be potential biomarkers for monitoring of the net state of immunosuppression after LTx.

*Methods:* Bronchiolar lavage (BAL) samples from a retrospective cohort (Paper I) and from a prospective cohort, together with nasopharyngeal (NPH) samples (Paper III) were analyzed with a multiplex PCR for respiratory viruses. Prospectively collected blood samples were analyzed for HEV antibodies using two ELISA methods (Paper II) and for TTV and EBV using PCR (paper IV).

*Results:* VRTI during the first year was associated with a shortened time to chronic rejection but not to death in both the retrospective cohort and the prospective cohort (Paper I and III). Thirteen per cent of the patients had anti-HEV antibodies during follow-up. No association between TTV DNA nor EBV DNA and immunosuppression-related events could be found.

*Conclusions:* VRTI during the first year is an independent risk factor for chronic rejection. HEV antibodies are equally common in the LTx population and the general Swedish population. EBV DNA and TTV DNA have limited usefulness as biomarkers for monitoring of immunosuppression after lung transplantation.

**Keywords**: Lung transplantation, Respiratory infection, Respiratory virus, Hepatitis E, Torque teno virus, Epstein Barr virus, Chronic lung allograft dysfunction.

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# SAMMANFATTNING PÅ SVENSKA

Det övergripande syftet med denna avhandling är att studera effekten av virussjukdomar efter lungtransplantation samt vissa virus användbarhet som markör för immunsuppression och infektionsrisk efter lungtransplantation.
Avhandlingen består av fyra delarbeten där delarbete I testar hypotesen att virala luftvägsinfektioner efter lungtransplantation leder till kortare överlevnad och kortare tid till kronisk avstötning. För att testa denna hypotes gjordes en retrospektiv analys av bronkoskopiprover. Proverna analyserades för förekomst av luftvägsvirus med en multiplex PCR metod. Därefter jämfördes retrospektiva data för överlevnad och kronisk rejektion mellan gruppen med förekomst av luftvägsvirus med den utan. Resultatet visade ingen skillnad i överlevnad men väl en kortare tid till kronisk rejektion (p= 0,005). Delarbete II undersöker förekomsten av antikroppar mot Hepatit E virus bland svenska lungtransplanterade. För att ta reda på detta insamlades blodprover prospektivt från patienter. Blodproverna testades med två ELISA och hos patienter som uppvisade tecken till infektion med serokonversion testades proverna med PCR för Hepatit E. Proverna visade förekomst av antikroppar i paritet med tidigare studier av förekomst hos den svenska befolkningen. Endast en patient serokonverterade och inga patienter var positiva för Hepatit E i PCR. Delarbete III testar prospektivt hypotesen att virala luftvägsinfektioner tidigt efter lungtransplantation medför högre risk för kronisk avstötning. 98 patienter följdes prospektivt under ett år med regelbundna prover från luftvägar. Kliniska data registrerades såväl vid rutinbesök som vid akuta besök. Luftvägsproverna analyserades för förekomst av luftvägsvirus med multiplex PCR. Alla patienter följdes vidare minst fem år. Resultatet efter multivariatanalys visar en ökad risk för kronisk avstötning hos de pat. som uppvisar viral luftvägsinfektion (p=0,041). Delarbete IV testar hypotesen att EBV eller TTV DNA kan användas som biomarkör för immunsuppression hos lungtransplanterade. För att testa detta följdes en kohort prospektivt med regelbundna blodprover som sedan testades med PCR för förekomst av EBV respektive TTV DNA. Kliniska data om infektioner och avstötning insamlades också. Något tidsberoende samband mellan virusnivåer och infektioner/avstötning kunde inte återfinnas. Slutsatsen är att TTV- eller EBV-nivåer ej kan användas som biomarkör för monitorering av immunsuppression hos lungtransplanterade.

Slutsatsen är att tidig viral luftvägsinfektion ökar risken för kronisk avstötning men inte för död. Att hepatit E inte är vanligare bland lungtransplanterade och att EBV och TTV inte kan användas som biomarkörer för att styra immunsuppression hos lungtransplanterade.

# LIST OF PAPERS

This thesis is based on the following studies, which are referred to in the text by their Roman numerals.

I. Magnusson J, Westin J, Andersson LM, Brittain-Long R, Riise GC. The impact of viral respiratory tract infections on long-term morbidity and mortality following lung transplantation. Transplantation. 2013 Jan 27;95(2):383-8.

II. Magnusson J, Norder H, Riise GC, Andersson LM, Brittain-Long R., Westin J. Incidence of Hepatitis E antibodies in Swedish lung transplant recipients. Transplant Proc. 2015 Jul-Aug;47(6):1972-6.

III. Magnusson J, Westin J, Andersson LM, Lindh M, Brittain-Long R, Nordén R, Riise GC. Early Viral respiratory tract Infection is a risk factor for chronic rejection after lung transplantation. Submitted

IV. Nordén R, Magnusson J, Lundin A, Tang K, Nilsson S, Lindh M, Andersson LM, Riise CG, Westin J. Quantification of Torque teno virus and Epstein-Barr virus has limited potential as biomarkers for monitoring of immunosuppression after lung transplantation. Submitted.

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| **ARAD** | **Azithromycin-responsive allograft dysfunction** |
| **ATG** | **Anti-thymocyte globulin** |
| **AR** | **Acute rejection** |
| **BAL** | **Broncho-alveolar lavage** |
| **BOS** | **Bronchiolitis obliterans syndrome** |
| **CLAD** | **Chronic lung allograft dysfunction** |
| **CMV** | **Human cytomegalovirus** |
| **CNI** | **Calcineurin inhibitor** |
| **CoV** | **Human coronavirus** |
| **COPD** | **Chronic obstructive lung disease** |
| **CRF** | **Case report form** |
| **CS** | **Corticosteroids** |
| **CyA** | **Cyclosporine A** |
| **DNA** | **Deoxyribonucleic acid** |
| **dsDNA** | **Double-stranded DNA** |
| **EBV** | **Epstein-Barr virus** |
| **ELISA** | **Enzyme-linked immunosorbent assay** |
| **EVLP** | **Ex vivo lung perfusion** |
| **FEV1** | **Forced expiratory volume during the first second** |
| **FVC** | **Forced vital capacity** |

| | |
|---|---|
| **HA** | **Haemagglutinin** |
| **HLA** | **Human leukocyte antigen** |
| **HR** | **Hazard Ratio** |
| **HMPV** | **human Metapneumovirus** |
| **hPIV** | **Human parainfluenzavirus** |
| **hRV** | **Human rhinovirus** |
| **ILD** | **Interstitial lung disease** |
| **ICTV** | **International Committee on the Taxonomy of Viruses** |
| **ISHLT** | **the International Society for Heart and Lung Transplantation** |
| **LTx** | **Lung transplantation** |
| **MHC** | **Major histocompability complex** |
| **MERS-CoV** | **Middle-East respiratory syndrome coronavirus** |
| **MMF** | **Mycophenolate mofetil** |
| **NA** | **Neuraminidase** |
| **mTOR** | **Mechanistic target of rapamycin** |
| **NPH** | **Nasopharyngeal** |
| **OB** | **Obliterative Bronchiolitis** |
| **PCP** | ***Pneumocystis jirovecii* Pneumonia** |
| **PCR** | **Polymerase chain reaction** |
| **PTLD** | **Post-transplant lymphoproliferative disease** |

| | |
|---|---|
| **RAS** | **Restrictive allograft syndrome** |
| **REED** | **Repeated elevated EBV DNA** |
| **RNA** | **Ribonucleic acid** |
| **RSV** | **Human respiratory syncytial virus** |
| **SARSr-CoV** | **Severe acute respiratory syndrome-related coronavirus** |
| **TaC** | **Tacrolimus** |
| **TTV** | **Torque teno virus** |
| **TLC** | **Total lung capacity** |
| **VRTI** | **Viral respiratory tract infection** |

# 1 INTRODUCTION

## 1.1 A brief history of organ transplantation

In medicine, the noun transplantation is defined as "the process of taking an organ or living tissue and implanting it in another part of the body or another body" [1]. Already in 1883, Theodor Kocher successfully transplanted thyroid tissue [2] albeit to correct the mistake of removing it in the first place. The first well-documented successful procedure was the end-to-end anastomosis of blood vessels, performed by Alexis Carrel and published in "Lyon Médical", 1902 [3]. Later in his career, he devised a prototype machine for extracorporeal management of organs, together with the well-known aviator Charles Lindbergh. Dr Carrel also devised several methods for the transplantation of organs. In 1938, Carrel and Lindbergh published a book called "the culture of whole organs" [4], which became the foundation upon which further advancements in the field of transplantation were built [5].

The very first successful solid organ transplantation was a kidney transplantation performed by Dr Jean Hamburger in Paris, in 1952 [6]. This was two years prior to the procedure carried out by Joseph Murray [7], even though he is often merited as being the founding father of transplantation surgery. The first successful liver transplantation was performed on 1 March, 1963 by Dr Thomas Starzl [8], which was followed 11 June of the same year by the first successful lung transplantation [9]. This was performed by Dr James Hardy (Figure 1) at the University of Mississippi. The first heart transplant was carried out in South Africa on 3 December 1967, by Dr Christian Barnaard [10].

## 1.2 Lung transplantation

Lung transplantation (LTx) is a life-saving procedure for some patients with end- stage lung disease. Patients with short predicted survival who are in relatively good health except for the lung disease, are very likely to benefit from receiving a lung transplant. It is no simple solution; extensive intrathoracic surgery is followed by life-long immunosuppression, with associated complications. Even so, there has been good evidence of improvement of life quality in all patient groups. Evidence of prolonged survival is also good, except for recipients with COPD-where the evidence is fair but not conclusive.

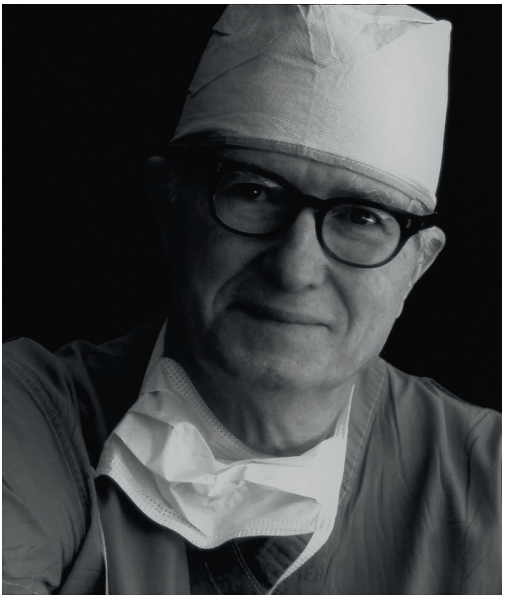


*Figure 1. Dr. James D. Hardy Reprinted from The Journal of Heart and Lung Transplantation, 2004. 23(11): p. 1307-1310. Giorgio et al. "James D. Hardy: A pioneer in surgery (1918 to 2003)" with permission from Elsevier*

### 1.2.1 History of LTx

Although Dr Hardy performed the first actual lung transplantation, the recipient, a man called John Richard Russel, only survived for 18 days. The autopsy determined the cause of death to be acute renal failure, however the lungs showed no signs of rejection. In the 10 years that followed, no less than 36 attempts were made with only two recipients surviving for more than a month [11]. The most successful of these was performed in Ghent where the recipient of a left lung survived for 10 months before succumbing to bronchopneumonia [12]. The pathologist looking at the graft post mortem concluded that no signs of acute rejection (AR) could be found; however, there were lesions compatible with chronic rejection.

The first successful lung transplant with long-term survival was a heart and lung transplant performed by Dr Norman Shumway and colleagues on 9 March 1981 at Stanford University [13]. The recipient was a 45-year-old woman with Eisenmenger's syndrome, and she lived for 5 years after the transplantation. The team performed two more transplantations in the same year. The success has been largely attributed to the introduction of

cyclosporine in the immunosuppression regimen. Both the first single lung transplant [14] (in 1983) and the first double lung transplant [15] (in 1986) were reported by the Toronto lung transplant group. The two procedures were led by Dr Joel Cooper and Dr Alexander Patterson, respectively. Toronto has since grown to become one of the world's largest lung transplant centers. The first really successful lobar transplantation was carried out by Vaughn Starnes in 1990 at Stanford [16]. Lobar transplantation is the only technique currently used to perform living donor LTx.

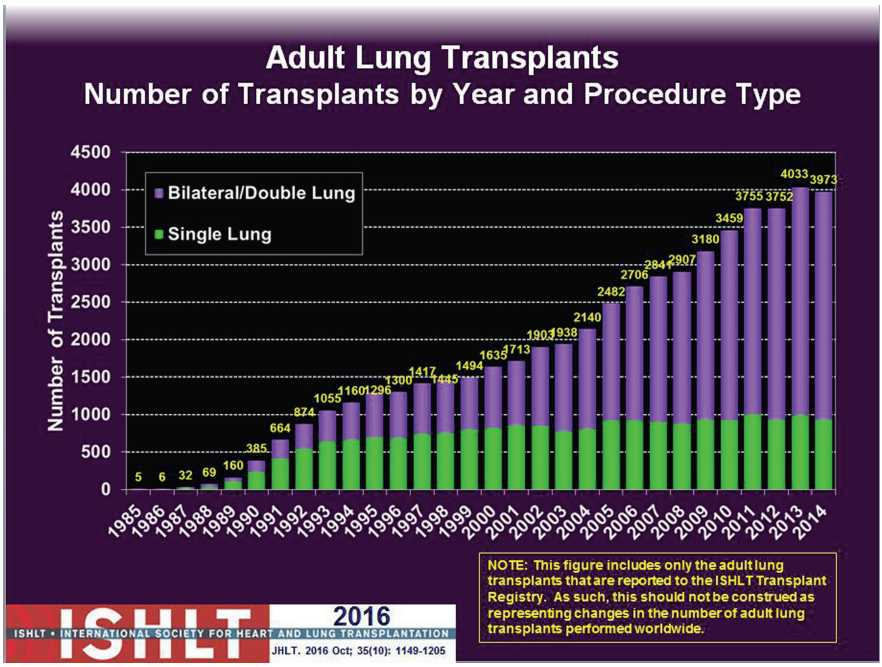



*Figure 2. Number of reported adult lung transplants by year and procedure type. As reported to the ISHLT registry 1985-2015. Reprinted with the permission of ISHLT*

## 1.2.2 Current status of LTx

More than 60,000 transplantations were recorded in the international society for heart and lung transplantation (ISHLT) registry up to June 2016 [17]. During 2015, 4,122 procedures on adults were reported from 140 centers worldwide. About a quarter of the procedures were single lung transplants while the rest were bilateral lung transplants (Figure 2). Pediatric lung transplants are still a very uncommon procedure with only 138 cases being reported between 2015 and June 2016.

The majority of the recipients suffer from either chronic obstructive lung disease (COPD), interstitial lung disease (ILD), or cystic fibrosis (CF). Patients with one of these three diagnoses constitute around 80% of all transplant recipients reported to the ISHLT. The remaining 20% are less common diagnoses that are possible to treat by transplantation, such as sarcoidosis and pulmonary artery hypertension. About 4% of the total amount of procedures are re-transplantations.

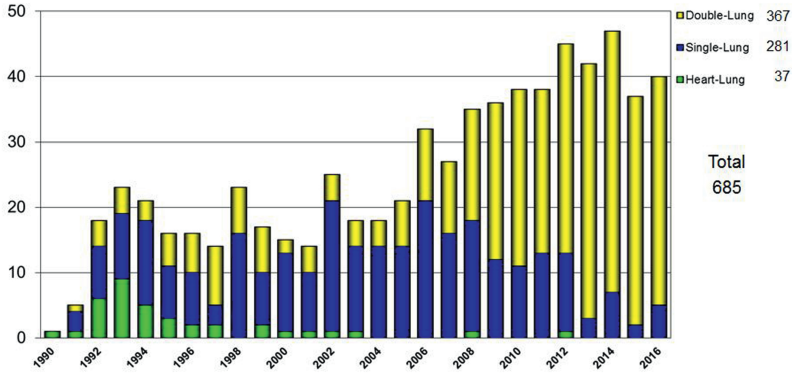
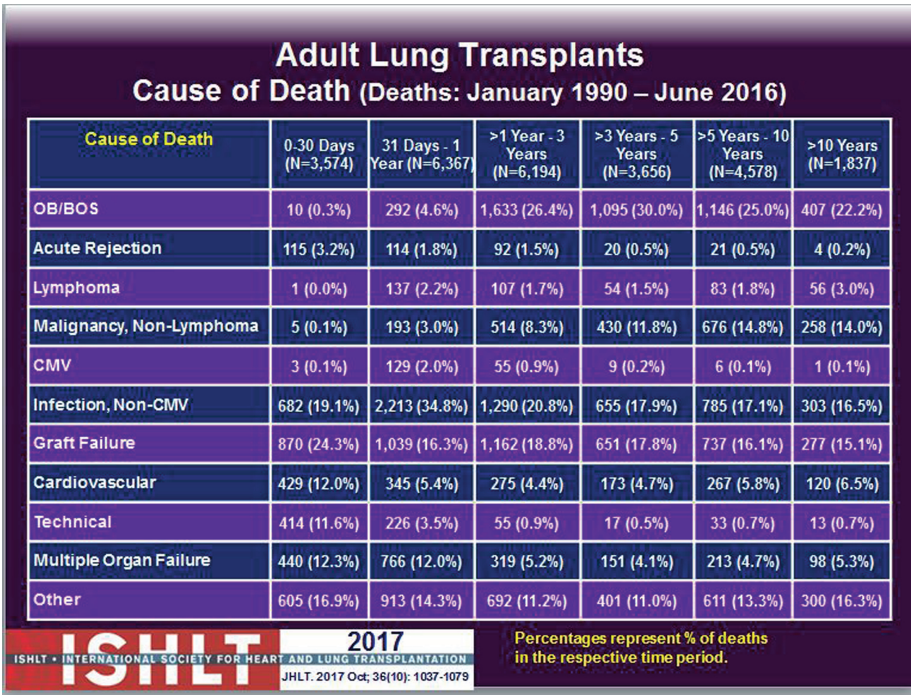



*Figure 3. Lung transplantations at Sahlgrenska, since 1990*

The Sahlgrenska lung transplant program started in 1990, and over 700 procedures have been performed since then. In the last few years, more than 40 patients per year have been transplanted (Figure 3). The demographics reflect the international registry quite well and the results are good by comparison with a 5-year survival of 70%.



**Adult Lung Transplants**
**Cause of Death (Deaths: January 1990 – June 2016)**

| Cause of Death | 0-30 Days (N=3,574) | 31 Days - 1 Year (N=6,367) | >1 Year - 3 Years (N=6,194) | >3 Years - 5 Years (N=3,656) | >5 Years - 10 Years (N=4,578) | >10 Years (N=1,837) |
|---|---|---|---|---|---|---|
| OB/BOS | 10 (0.3%) | 292 (4.6%) | 1,633 (26.4%) | 1,095 (30.0%) | 1,146 (25.0%) | 407 (22.2%) |
| Acute Rejection | 115 (3.2%) | 114 (1.8%) | 92 (1.5%) | 20 (0.5%) | 21 (0.5%) | 4 (0.2%) |
| Lymphoma | 1 (0.0%) | 137 (2.2%) | 107 (1.7%) | 54 (1.5%) | 83 (1.8%) | 56 (3.0%) |
| Malignancy, Non-Lymphoma | 5 (0.1%) | 193 (3.0%) | 514 (8.3%) | 430 (11.8%) | 676 (14.8%) | 258 (14.0%) |
| CMV | 3 (0.1%) | 129 (2.0%) | 55 (0.9%) | 9 (0.2%) | 6 (0.1%) | 1 (0.1%) |
| Infection, Non-CMV | 682 (19.1%) | 2,213 (34.8%) | 1,290 (20.8%) | 655 (17.9%) | 785 (17.1%) | 303 (16.5%) |
| Graft Failure | 870 (24.3%) | 1,039 (16.3%) | 1,162 (18.8%) | 651 (17.8%) | 737 (16.1%) | 277 (15.1%) |
| Cardiovascular | 429 (12.0%) | 345 (5.4%) | 275 (4.4%) | 173 (4.7%) | 267 (5.8%) | 120 (6.5%) |
| Technical | 414 (11.6%) | 226 (3.5%) | 55 (0.9%) | 17 (0.5%) | 33 (0.7%) | 13 (0.7%) |
| Multiple Organ Failure | 440 (12.3%) | 766 (12.0%) | 319 (5.2%) | 151 (4.1%) | 213 (4.7%) | 98 (5.3%) |
| Other | 605 (16.9%) | 913 (14.3%) | 692 (11.2%) | 401 (11.0%) | 611 (13.3%) | 300 (16.3%) |

ISHLT • INTERNATIONAL SOCIETY FOR HEART AND LUNG TRANSPLANTATION
2017
JHLT. 2017 Oct; 36(10): 1037-1079

Percentages represent % of deaths in the respective time period.

*Figure 4. Causes of death after lung transplantation, according to the ISHLT registry, from January 1990 up to June 2016. Reprinted with the permission of ISHLT*

### 1.2.3 Limitations in survival after LTx

Even though there has been much progress in short term survival, the long-term survival after lung transplantation is still unsatisfactory. The median survival has increased by about two years in the last two and a half decades, and the international 5-year survival is now 59% [17]. The causes of death differ between the very early period (0-30 days), the early period (30 days to 1 year), and the late period (1>year) after transplantation (Figure 4). The very early period is dominated by primary graft failure and infections, of which primary graft failure is the most common. The early period has the same two major causes but is dominated by infections. After the first year, even though infections are still an issue, the major cause of death is obliterative bronchiolitis (OB), a form of chronic rejection. One-year survival is 82%, so chronic rejection is the major limiting factor for long-term survival even though infections always play a detrimental role in an immunosuppressed population. The causes of death after LTx are similar at Sahlgrenska (Figure 5).

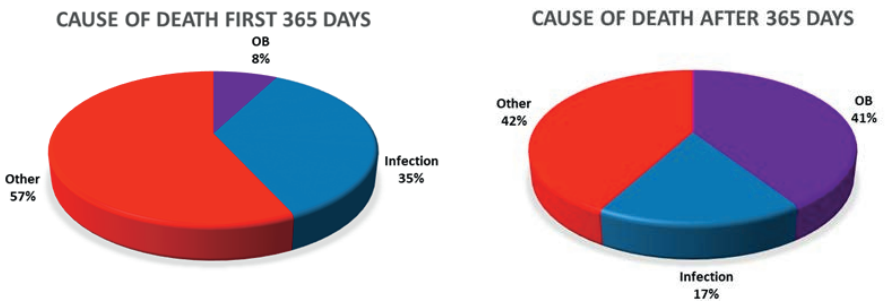



*Figure 5. Cause of death after lung transplantation at Sahlgrenska up to January 2017 during and after the first 365 days, post-transplant. OB, Obliterative bronchiolitis.*

### 1.2.4 Exposure to infectious agents after LTx

The lung is normally exposed to huge amounts of airborne, potentially infectious agents since it is in direct contact with the surrounding environment.

In relation to the sheer amount of exposure, infections rarely occur in an individual with a non-suppressed immune system. In the healthy airway, there are three levels of defense against infectious agents. Firstly, there is the mechanical defense consisting of the mucociliary clearance and the tight adherence between respiratory epithelial cells through apico-lateral junctions [18]. Secondly, the airway has a multitude of innate antimicrobial defense mechanisms that immediately react to potentially harmful organisms. The innate immunity consists of several antimicrobial enzymes secreted by the airway epithelium and also immediately reactive, lymphoid progenitor cells [19]. The antimicrobial enzymes have a direct toxic effect on pathogens. The lymphoid progenitor cells differentiate to innate lymphoid cells of three groups (1, 2, and 3), which produce cytokines and transcription factors [20]. Of these, Group 2 might be the most interesting from an antiviral standpoint since it contains - amongst other cell lines - natural killer cells that do not require major histocompability complex (MHC) antigens or targeted antibodies to recognize stressed cells. Lastly, there is the adaptive immune system consisting of B and T cells [21]. The adaptive immunity can distinguish self from non-self, antigens. Once non-self antigens are identified it can produce antibodies via B Cells or directly destroy foreign microorganisms via T cells. The adaptive immune system also forms memory cells that recognize the foreign microorganism if there is another exposure. The physical barriers and the innate immunity are immediate and usually

effective obstacles to infection by microbial organisms. The adaptive immunity is developed over the course of weeks, but T and B memory cells mediate for a much more rapid response on the next exposure.

In the lung-transplanted, patient, the situation, is somewhat different. The T and B cell functions are deliberately suppressed; even though immunosuppression varies over time, it is always present. Furthermore, these patients have lost the cough reflexes in the transplanted lung [22] severely hampering the function of the mucociliary clearance. There is some evidence that this reflex may be regained at a later stage [23], but it is not present at the initial stages when immunosuppression levels are at its highest. There is also the issue of the anastomosis between donor and recipient lung, which is a locus for infections (mostly fungal) [24]. The adherence of the apical junctions in transplanted patients is not well investigated, but hypothetically their efficacy could also be reduced. The sum of these deficiencies in the antimicrobial defense leaves the lung transplant recipient much more susceptible to all types of airway infections.

## 1.3 Immunosuppression after Lung transplantation

Before discussing the different aspects of infections, it is important to have an understanding of the immunosuppressive agents used after lung transplantation. Immunosuppression is needed to prevent the body from rejecting the transplanted organ, by lowering the activity of the immune response. Unfortunately, this also makes the transplant host more susceptible to infections which—as already mentioned—jeopardizes the long-term survival. A balance between the risk of infections and the risk of rejection is always strived for in immunosuppressive therapy.

The most common strategy for immunosuppression after lung transplantation is an induction therapy to reduce the risk of AR, followed by a life-long triple maintenance therapy consisting of a calcineurin inhibitor (CNI), a proliferation inhibitor, and a corticosteroid. The dosage of the CNI is adjusted to maintain specific serum levels that are gradually reduced after transplantation. The dosage of corticosteroids is also lowered at regular intervals, but for the proliferation inhibitor the aim is to keep the area under the curve at a constant target value.

### 1.3.1 Induction therapy

The induction therapy currently used at our center is anti-thymocyte globulin (ATG). ATG is a polyclonal antibody preparation isolated from rabbit sera, which contains antibodies to human thymocytes and has a T-cell depleting effect [25]. In other centers, the anti-IL-2 compounds, basiliximab and daclizumab are also used [26]. There has only been one prospective study comparing one of these drugs after lung transplantation with ATG. The randomized controlled trial by Mullen et al. in 2007 comparing induction with ATG versus Daclizumab showed no difference in survival acute or in chronic rejection [27].

### 1.3.2 Calcineurin inhibitors (CNI)

Calcineurin is a protein phosphatase that activates T cells through a pathway that upregulates interleukin-2 (IL-2) expression [28]. The two drugs most commonly used are cyclosporine A (CyA) and tacrolimus (TaC).

CyA was the first CNI available for use, and a breakthrough for long-term survival after transplantation. It forms an intracellular complex that prevents transcription of IL-2, thus preventing upregulation of T cells [29].

The second CNI available was TaC (also known as FK506). The potency of this drug is 10-100 times that of CyA. It binds to the intracellular protein FKBP 12. In doing so, it prevents the transcription of several cytokines, including IL-2 [30].

To date, there have been five prospective randomized studies comparing the efficacies of CyA and TaC after lung transplantation. The results are mixed and difficult to compare, because of the heterogeneity in endpoints but no study has shown any difference in survival depending on choice of CNI [31-35]. The largest of these studies included 249 patients and showed a difference in the incidence of chronic rejection in the form of grade 1 bronchiolitis obliterans syndrome (BOS) after 3 years ($p = 0.037$) in favor of tacrolimus. However, there were many exceptions from the randomization procedure in this study that could possibly have made the TaC group biased towards having a lower risk of BOS development.

### 1.3.3 Antimetabolites

Today, mycophenolate mofetil (MMF) is the most common antimetabolite used internationally after lung transplantation [17]. This agent inhibits inosine monophosphate dehydrogenase, which is an enzyme that stimulates proliferation of both T and B lymphocyte proliferation [36]. Historically,

Azathioprine has been used to achieve this but its use is now completely marginalized after lung transplantation [17], which is due more to issues with side effects than improved outcomes [37].

### 1.3.4 Corticosteroids (CS)

CS have been used since the inception of organ transplantation [7] and are still a linchpin both in induction therapy and in maintenance therapy in almost all lung transplant centers [17]. CS has a multitude of effects on the immune system, including reduced macrophage activation, alteration of lymphocyte migration, cytokine inhibition, to mention a few [38]. There is little evidence for using steroid-free maintenance therapy after lung transplantation [39], and it is generally avoided due to the risk of graft failure, but the dosage is lowered as fast as is reasonably safe with the aim of reaching the lowest possible dosage that can maintain a stable lung function. There is no international consensus on the pace of reduction of CS and it is most often adapted to the response in the individual patient.

### 1.3.5 Mechanistic target of rapamycin (mTOR)

These drugs inhibit a serine/threonine-specific kinase. The protein was identified as the target of the older immunosuppressive drug rapamycin, and over the years has been identified as a major player in the governance of cell proliferation and cell growth [40]. It mainly functions in its immunosuppressive capacity by inhibiting activation of conventional T cells and proliferation of regulatory T cells. It also diminishes B cell proliferation and differentiation to antibody secreting cells, through inhibition of the IL2 pathway. The drug also has some anti-neoplastic properties. In lung transplantation, the drug is most often used in conjunction with a CNI in trying to reduce the nephrotoxic effect of that agent. Delayed wound healing has also been reported, which makes the use of mTOR agents dubious in the early postoperative phase. It is possible that the next generation of mTOR drugs, would not have this side effect, which would make them much more attractive from a lung transplant point of view.

## 1.4 Non-viral respiratory infections after lung transplantation

Bacterial and fungal infections are common after lung transplantation. Knowledge of non-viral infections is essential if one is to discuss the implications of the viral infections. Bacterial and fungal culture remains the gold standard for diagnosing these infections and, thus a considerable amount of data is available on their effect on outcome after lung transplantation. This contrasts with, viral infections where virus culture is time-consuming and is no longer used for diagnostic purposes [41]. Polymerase chain reaction (PCR) for viral detection has been used for a shorter period of time, so the documentation on the effects of viral infections on outcomes after lung transplantation is less extensive.

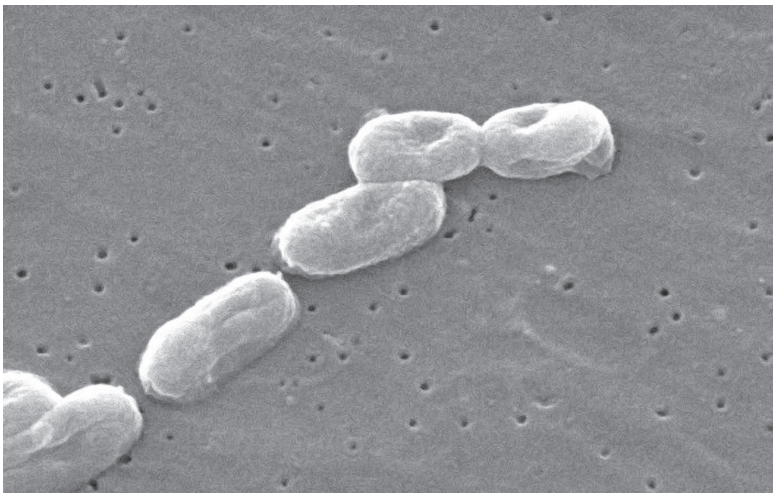


*Figure 6. Burkhordelia cepacia complex. Reprinted with a creative commons license*

### 1.4.1 Respiratory Bacterial infections in lung transplant patients

It has been estimated that between 60% and 80% of symptomatic infections after lung transplantation are of bacterial origin. Gram-negative bacteria such as *Moraxella catarrhalis*, *Escherichia coli, and Haemophilus influenzae* cause the most common infections. Of the Gram- positive species, *Staphylococcus aureus* appears to be over-represented, although

*Pneumococcus pneumoniae* is still common [42, 43]. Many uncommon and rare bacterial agents that are usually harmless to the immunocompetent patient can cause serious infections in the transplanted lung. Although there are many such species, some deserve special mention. *Pseudomonas aeruginosa is* a Gram-negative facultative aerobic bacterium that is mostly opportunistic and has an intrinsic resistance to antibiotics. Cystic fibrosis patients are very susceptible to this infection, but lung transplant recipients are also especially at risk [44]. *Acinetobacter* is another Gram-negative aerobic bacterium that is commonly found in soil that survives well on dry surfaces. Even though it is prevalent as a pathogen in all wards where ventilator care is used, lung transplant recipients are especially at risk [45]. *Burkholderia* (Figure 6) is a genus of Gram-negative aerobic bacteria with 48 named species that vary greatly in virulence. Of the species with respiratory pathogenicity *Burkholderia cenocepacia* is considered the most threatening because of its extreme innate resistance to antibiotics and ability to survive in otherwise sterile environments such as medical devices and even antiseptics. When treated it is seldom completely eradicated but may be suppressed [46, 47]. Among the Gram- positive bacteria *Corynebacterium* is a genus of aerobic bacteria that—except for the well-known *Corynebacterium diphtheria*—is mostly harmless to healthy patients. However, immunocompromised patients, especially lung transplant recipients, are at risk of infection [48].

### 1.4.2 Respiratory fungal infections after lung transplantation

The most common fungi that cause infection after lung transplantation are *Aspergillus* and *Candida* [49]. Internationally, *Scedosporum* is also reported to be a possibly harmful fungal agent [50, 51]. However, it is not seen after the lung transplantations that are performed in Sweden. Historically, *Pneumocystis jirovecii* was a high-risk agent for all patients with a low CD4+ T Cell count. For solid organ recipients, this threat has diminished after the introduction of prophylactic treatment with trimethoprim-sulfamethoxazole [52], and almost no *Pneumocystis jirovecii* infections are reported for lung transplant recipients [53]. Fungal infections are manageable with modern antifungal compounds, but interactions with immunosuppressive agents and toxicity remain problematic. A positive fungal culture is not necessarily a sign of an invasive fungal infection, since fungi may be part of the normal flora. Currently, there are two major classifications for the probability/severity of fungal infections. One is from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections and the National Institute of allergy and Infectious Diseases

Mycoses Study Group [54]. The other classification that should preferably be used for thoracic transplant recipients has been defined by ISHLT [55]. The classification systems may be helpful in the clinical situation when assessing specific patients, but they do present a challenge when comparing studies with different definitions of fungal disease.

*Aspergillus*: *Aspergillus fumigatus* and *Aspergillus niger* are the most common species to cause infection after lung transplantation [49]. *Aspergillus* is found in the surrounding environment, including soil [56]. It grows—as all moulds—as multicellular filaments called hyphae. The incidence of *Aspergillus* infections after lung transplantation vary from 8% to 31% [49, 57, 58] The wide range is due to differences in definition, the lower end of the interval being more probable if one considers verified invasive fungal disease instead of just colonization. Both *Aspergillus fumigatus* and *Aspergillus niger* are able to form airborne spores that are inhaled by humans on a regular basis [59]. In the immunocompetent host, the innate immune system of the airways will take care of the spores, but it is difficult for an immunocompromised host to overcome an established aspergillosis without the help of antimycotics.

*Candida: Candida* species are yeasts that grow as single-cell organisms capable of forming colonies of attached cells. This is the most common fungal infection in humans [60]. Though *Candida* is often isolated, it is less likely than *Aspergillus* species to cause invasive mycosis [49], and it is also more easily treated. A positive culture of *Candida* does not necessarily indicate infection, even in lung-transplanted patients. In contrast to *Aspergillus*, there are few reports of candida infections with lethal outcome after lung transplantation.

## 1.5 Viral infections after lung transplantation

There is a vast variety of viruses; they are among the smallest of all organisms and procreate through infection of living cells. As a pathogen, it was first conceptualized in 1898 by a Dutch microbiologist and first proven to exist in humans in 1901 by Dr Walter Reed through his research on yellow fever [61]. Today, there are more than 5,400 viruses described in the database kept by the International Committee on the Taxonomy of Viruses (ICTV) [62]. For obvious reasons viruses that cause airway infection are especially important after kung transplantation. There is also interest in common viruses that are of low pathogenicity in the immunocompetent host, since their

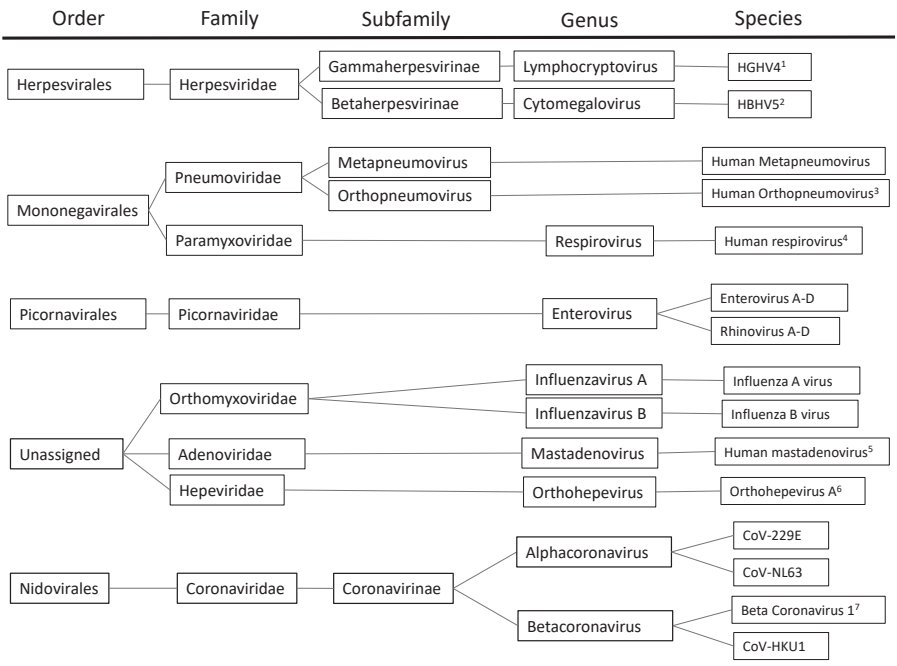



*Figure 7. Taxonomy of viruses in this thesis according to ICTV 2017.* ***Abbreviations:*** *HBHV5, Human Betaherpesvirus5; HGHV4, Human Gammaherpes4; CoV, Coronavrius.* ***Common names of viruses with changed taxonomy since studies were performed:*** *1.Epstein-Barr Virus. 2.Cytomegalovirus. 3.Respiratory syncytial virus. 4.Metapneumovirus. 5.Adenovirus. 6.Hepatitis E Virus. 7.Coronavirus Oc43.*

behavior can change drastically when not controlled by an efficient immune response.

In this thesis, I will focus mainly on viral airway pathogens, ubiquitous intracellular viruses and one, often overlooked, hepatotropic virus. It is of some importance to know that virus taxonomy as defined by the ICTV, has changed slightly since the studies were designed. The changes are a result of our improved understanding of the viral genome and its expression [63]. Even though some of the common names have been changed taxonomically, for all practical purposes the names remain the same. (Figure 7).

A basic understanding of the transmission and pathogenesis is necessary to further understand their implications for the transplanted lung and its recipient.

### 1.5.1 Viral respiratory pathogens

*Adenovirus:* There are currently 57 accepted types of human adenovirus in seven species (A-G) where types B and C are those that most commonly cause respiratory disease. Adenovirus is a non-enveloped virus with a non-segmented double-stranded DNA (dsDNA) virus. The particle is resilient and can survive for long periods of time outside of a host. The infection is usually transmitted by respiratory droplets, but gastroenteritis caused by certain adenovirus can also be spread via the fecal route. The symptoms differ between the different virus types. They most commonly infect the respiratory system and cause symptoms consistent with the common cold, but adenovirus can also cause bronchitis and even pneumonia. Specific adenoviruses can also cause gastroenteritis, conjunctivitis, tonsillitis and rash [64]. The infection is usually self-limiting, though on very rare occasions it can progress and cause severe or even fatal infections even in immunocompetent patients [65].

*Human coronavirus:* Coronavirus (CoV) is the largest family within the order Nidovirales. CoV is an enveloped virus with a single- stranded ribonucleic acid (RNA) genome. The strains are subdivided into alpha, beta, and gamma CoV. The well-known human CoVs are the two alpha HCoVs (229E and NL63) as well as the two beta CoVs (OC43 and HKU1). These most often cause a mild respiratory symptoms in the immunocompetent patient [66]. There are also two almost categorically harmful coronaviruses, the severe acute respiratory syndrome-related coronavirus (SARSr-CoV) and the Middle-East respiratory syndrome coronavirus (MERS-CoV), both are zoonotic viruses with some capacity to spread from person to person.

*Human enterovirus:* Enterovirus belongs to the family Picornaviridae of the order Picornavirales and has a single-stranded non-enveloped RNA. Coxsackievirus A and B and polioviruses are examples of enteroviruses. Human enteroviruses are grouped into four species (A-D). Enteroviruses have the ability to infect different human tissues including the nervous system, lungs and cardiac muscle. They can also cause pancreatitis and has been implicated in the development of type-1 diabetes [67]. It spreads through the fecal-oral route and in respiratory droplets. Enterovirus is considered to be the primary cause of myocarditis [68], but it rarely causes severe disease in the respiratory system —except for pleurodynia, in the form of Bornholm disease [69]. Interestingly, enteroviruses are resistant to many forms of common disinfectants such as 70% ethanol and isopropanol [70].

*Human metapneumovirus:* Human metapneumovirus *(HMPV)* is an enveloped virus with a single-stranded RNA genome. It belongs to the family Paramyxoviridae. Humans are the only natural host for HMPV, and the infection rate among adults is 1-9%, with a variety of symptoms from being fully asymptomatic to severe respiratory disease [71]. High viral load in the nasopharyngeal tract has been associated with worse disease severity [72]. Although the virus has a high affinity for lung tissue, it has been found in blood in non-immunocompetent individuals, with a very high viral load in the respiratory tract [73].

*Human rhinovirus:* There are three genotypes of human rhinoviruses (hRVs) (A-C) and they belong to the genus enterovirus in the family Picornaviridae. The virus is a non-enveloped single-stranded RNA virus with a lot of heterogeneity and over 100 different types. Their virulence differs and there are also previously documented asymptomatic infections [74]. Rhinoviruses spread via droplets or by direct contact [75]. The virus is often present in the lower airways when detected by PCR in the upper airways [76]. hRV infections are selective for airway epithelium, and have only been found to cause viremia in very few cases [77]. The symptoms are those of the classic "common cold", but they can also cause otitis media and have been associated with a persistent increase in bronchoreactivity [78]. The virus can cause severe lower respiratory tract infections [79] and is also the most common infectious cause of exacerbation in both asthma [80] and COPD [81, 82].

*Influenzavirus:* This is an enveloped, single-stranded RNA virus with three distinct types (A, B, and C). Influenzavirus has a well-known ability to cause an annual outbreak globally. This must be distinguished from the very large outbreaks called pandemics, which have occurred at least four times in the last 100 years. The Spanish flu in 1918, Asian influenza in 1957, the Hong-Kong influenza in 1968, and the H1N1 influenza in 2009 [83]. Influenza A has two major surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). There are 16 known known types of HA t (H1–H16) and nine types NA (N1–N9). These undergo minor changes over time through point mutations called antigenic drift. If a whole new gene segment has been acquired this is called antigenic shift and it will cause changes in both the HA and NA antigens — to which the human population has poor or no immunity. Antigenic shift does not occur in influenza B and antigenic drift is less frequent. Influenzavirus mostly causes symptoms in the respiratory tract, but it can also cause muscle pain, vomiting, diarrhea and even encephalopathy [84], though this is rare. Changes in antigen expression,

together with a declining immune response to the vaccine, is what necessitates vaccinations on an annual basis or risk groups.

*Parainfluenza virus*: This is an enveloped, double-stranded RNA virus that belonging to the family Paramyxoviridae. There are four serotypes that can infect humans, human parainfluenzavirus (hPIV) 1-4. In addition, there are several zoonotic viruses. Person-to-person contact is required for virus propagation, since it does not last long in the environment. It usually causes mild respiratory symptoms; also, involvement of mucous membranes of the sinuses and ears can cause sinusitis and otitis media. In children parainfluenzavirus can also cause severe acute laryngotracheobronchitis (viral croup) [85]. This is associated with bronchial hyperactivity later in life, but there is no evidence that the association is causative [86].

*Respiratory syncytial virus:* This is an enveloped RNA virus belonging to the family Paramyxoviridae. Respiratory syncytial virus (RSV) spreads mainly through contact with infected individuals and subsequent contact with nasal and conjunctival mucosa. It can also spread through aerosolization, but this does not appear to be as important for virus propagation [87]. Infections are most common during infancy and childhood, causing up to 20% of all hospitalizations in this age group [88]. In adults, it may be the second most significant cause of respiratory tract illness after influenza [89]. Infected individuals have symptoms from the nasopharyngeal tract and airways. Interesting this virus is capable of reinfection—even of an immunized patient. It has the ability to inhibit signaling from interferon gamma in macrophages [90], and the capacity to hinder migration into the lung of CX3CR1 protein- bearing leukocytes means that it can inhibit both the innate and the adaptive immune responses [91]. There are indications of persistent bronchospastic symptoms after RSV infection, but there is no clear evidence of a causative association.

### 1.5.2 Hepatitis E

Hepatitis E virus is a non-enveloped, single positive-stranded RNA virus belonging to the family Hepeviridae. The hepatitis E virus was discovered in 1983 [92]. Today it is considered by the WHO to be a major cause of acute symptomatic viral hepatitis, especially in resource-limited settings [93]. It is estimated to cause about 20 million HEV infections globally of which about 16.7 million are asymptomatic however, 44,000 are estimated to have a fatal outcome [93]. Especially at risk of death are pregnant women in third trimester, and infants [94, 95]. There are four genotypes infection humans (HEV1-HEV4). HEV1 and HEV2 are mostly found in developing countries

and cause epidemics. HEV3 and HEV4 are found in industrialized countries where HEV3 is more widespread. Both HEV3 and HEV4 have a zoonotic reservoir amongst domestic animals (e.g. pigs and game like deer or wild boar) [96]. These animals are sources of transmission and blood products have been identified as another source [97]. There are case reports of transmission through organ donation [98], but should probably be considered to be very rare. In a Swedish population of healthy blood donors, the seroprevalence of anti-HEV IgG was 16% [99]. Anti-HEV IgM is seen in acute infection, while anti-HEV IgG can be seen both in acute infection and in resolved hepatitis. PCR can be used as a marker for virus replication in serum. The incubation period varies between four and six weeks. Common symptoms are fever, anorexia, vomiting, jaundice, and a rise in liver enzymes. Asymptomatic infection is common, but some retrospective studies have suggested that HEV may be a cause of acute liver failure [100, 101]. Most of these cases had previously been misdiagnosed as drug-induced liver injury. Acute HEV infection resolves spontaneously in most cases, but HEV may cause chronic hepatitis in solid organ transplant recipients. In 2008, eight immunocompromised patients were verified as being carriers of a chronic infection defined by elevated liver enzymes and detectable HEV RNA lasting more than 6 months, as well as liver biopsy findings consistent with chronic hepatitis [102]. However fatalities among transplant recipients are still limited to case reports [98]. The treatment options are reduction of immunosuppression [103], ribavirin monotherapy [104], and possibly interferon injections [105]. However, there is a risk of triggering a rejection of the transplanted graft when using interferon, so this is unlikely to become a preferred therapeutic option despite positive results in case reports [106].

### 1.5.3 Ubiquitous viruses

*Epstein-Barr virus:* Epstein-Barr virus (EBV) is an enveloped DNA virus belonging to the family Herpesviridae. It is also called human gamma herpesvirus 4. Ninety per cent of adults are infected with this virus, and primary infection is most common between the age of 2-4 years and close to the age of 15. The symptoms of primary infection vary in, children but in teenagers it commonly presents as mononucleosis, including cervical lymphadenopathy, hepatomegaly, splenomegaly, fever, and fatigue. The symptoms resolve within 2-4 weeks, although post-viral fatigue may persist for 6 months or more. The virus causes a latent infection in B cells and epithelial cells. It spreads to previously uninfected individuals through close contact with, for example, saliva, and it appears to be more liable to infect B cells even though the virus leaving the host seems to emerge from epithelial cells [107].

In the B cells, EBV binds to the CD21 receptor and internalizes through endocytosis. This mechanism is not available in epithelial cells, and thus cellular entry is mediated through a different mechanism, which is less well characterized. In B cells EBV has the ability to establish a latent infection with a low expression of viral genes [108]. The virus has been associated with human cancers such as Burkitt's and Hodgkin's lymphoma. Interestingly, in immunosuppressed patients, the virus has also been detected in T cells when the EBV levels in blood have risen [109]. EBV infection triggers innate immune responses, including interferon and NK-cell response which are important for early control of infection. The initial B-cell response to acute infection is a heterogenic release of non-specific antibodies, and later with specific antibodies targeting viral proteins. Thus, the chronic life-long infection is kept at bay by the adaptive immunity [110]. There is also an increase in CD8+ T cells during EBV infection [111], with varying targets depending on which stage the infection is at. In immunosuppressed patients, EBV may trigger EBV lymphoproliferative disorder. Amongst transplant recipients this is called post-transplant lymphoproliferative disease (PTLD). The underlying cause of this is reduced T cell efficiency, and thus inability to control the EBV-infected B cells. It is more common in lung transplant recipients (2-5%) than in recipients of other solid organs, except for small intestine transplant recipients, where the infection rate can possibly reach about 20% [112]. The disease presents differently, but many patients have an enlargement of tonsils, fever, and fatigue similar to that of mononucleosis. PTLD often presents as solid lesions, most commonly in the gastrointestinal tract or the transplanted organ. EBV-negative recipients are also at risk of developing EBV-positive Hodgkin lymphoma, even though it is very rare. A reduction of immunosuppression is most often sufficient to treat PTLD, but in some cases regular treatment with chemotherapeutic regiments might be necessary. Since EBV is a herpesvirus, Acyclovir and Ganciclovir would have a theoretical effect on EBV proliferation, but this effect seems to be very modest in healthy subjects [113]. The ubiquity of the virus together with the balance between infection and immune response makes the virus interesting as a possible biomarker of net immunosuppression [114, 115].

*Cytomegalovirus:* Human cytomegalovirus (CMV) is an enveloped, double-stranded DNA virus. The infection is spread through contact with infected bodily fluids. If the virus can overcome the host's innate and adaptive immune system, a sustained replication follows with possible findings of virus in urine and saliva for prolonged periods. Most often, this is asymptomatic, but it can be accompanied by brief mononucleosis-like symptoms. The virus can also be transferred via the placenta [116] and lead to congenital CMV infection, which might cause several different

sensorineural deficiencies including deafness in the fetus. The virus can replicate in many different cell types, but it is most concentrated in myeloid cells. It is transported via the bloodstream to all organs, and is therefore transferrable via transplantation. It is usually a latent infection but in immunosuppressed individuals it can be an overt infection., This may also, but less commonly, occur after trauma, surgery, and autografting [117]. The primary effective response to primary CMV infection seems to be T cell-mediated [118]. However the ability to control the chronic infection is dependent on humoral immunity, as exemplified by the high risk for a patient without CMV antibodies before transplantation, developing early and late CMV- associated complications after lung transplantation [119]. The immune response is unable to completely clear CMV infection from a host, but rather works to control viremia. These systems are usually very efficient but in the case of the critically ill patient they can temporarily fail [117]. The risk of end-organ disease increases with the levels of viral markers in blood [120]. CMV can cause end-organ disease [121] such as pneumonitis [122], gastrointestinal lesions [123], hepatitis [124], pancreatitis [124], and myocarditis [125]. Pneumonitis in particular can—and often does—lead to dysfunction of the transplanted organ [126]. There are several drugs with proven effect against CMV. Many of them have a high risk of toxicity for the user, and must be carefully monitored. Of the available drugs valganciclovir is most often used after lung transplantation. Other drugs that are available are ganciclovir, forscarnet, and cidofovir. In most lung transplant centers, recipients receive CMV prophylaxis for 6–12 months after transplantation, which, locally at Sahlgrenska, has reduced the incidence of CMV infections compared to previous regimens with a shorter period of prophylaxis. As in most other centers, we follow the patients with sampling at regular intervals, and in the case of CMV DNAemia, they are treated pre-emptively with valganciclovir.

*Torque teno virus:* The torque teno virus (TTV) is a fairly recent addition to the pool of known viruses. It belongs to the genus Alphatorque virus within the family Anelloviruses that was discovered in 1997. It was first found in a patient with transfusion-associated hepatitis [127] and has since shown great diversity with at least 29 species making up the Alphatorquevirus genus [128]. Even so, there has not been any association with any specific human illness [129, 130]. TTV resides in peripheral blood mononuclear cells, and hematopoietic stem cells appear to play an important role in maintaining the viral DNA in plasma [131]. There is evidence that reduced T cell-mediated immunity leads to increased TTV levels [132]. The virus has since been detected in many mammalian hosts [133]. TTV virus is most commonly a latent infection not causing any detectable pathology [127, 129, 130, 134].

Even though there are some epidemiological data associating TTV with disease [135], there is no clear evidence of TTV in itself being causative. Theoretically, when the T cell count is low and TTV DNA replicates to high levels, it might signal a higher risk of infections resulting from a reduced immune system efficacy. Thus, it has also been suggested that TTV DNA could reflect the net state of immunosuppression in transplanted individuals [136]. Görzer et al. suggested that TTV DNA levels reach a steady-state post-lung transplant, after which the TTV DNA can possibly be used as a biomarker. This was suggested because the virus is ubiquitous and non-pathogenic and virus levels were steady before transplant in that particular study. However, a recent study has indicated that TTV can be transmitted from swine to humans, which opens up the possibility of further zoonotic, transmission and thus possibly de novo infection after transplantation [137] which might confound any findings.

## 1.6 Chronic Lung Allograft Dysfunction (CLAD)

As previously stated, chronic rejection is the most important factor limiting long- term survival after lung transplantation. For many years, this was used synonymously with bronchiolitis obliterans syndrome (BOS), but over time the condition has been split into three subgroups with the collective name CLAD. The common denominator is that there is a persistent 20% decrease of FEV1 from post-transplant baseline that is not better explained by another condition. Except for BOS, there is also a restrictive form of rejection, most commonly called restrictive allograft syndrome (RAS) but is has also been called R-CLAD or R-BOS. The common denominator for these three descriptions is that the predominant finding is a reduction in volume of the transplanted lung, i.e. That the total lung capacity (TLC) or forced vital capacity (FVC) is reduced by more than the forced expired volume during one second (FEV1). However, the details of the definition for RAS or its equivalent term, are still under some debate whereas the definition of BOS is universally agreed upon. Recently the CLAD phenotype, Azithromycin-responsive allograft dysfunction (ARAD) was introduced. What is specific to this phenotype is that it is defined by the responsiveness to the only widely available drug with a convincing effect on development of CLAD. It is hypothesized that there are, yet undiscovered immunological properties amongst the ARAD patients that differentiate them from the other phenotypes. A few patients do not fit into these general categories, and they most probably belong to yet uncharacterized subtypes (Figure 8).

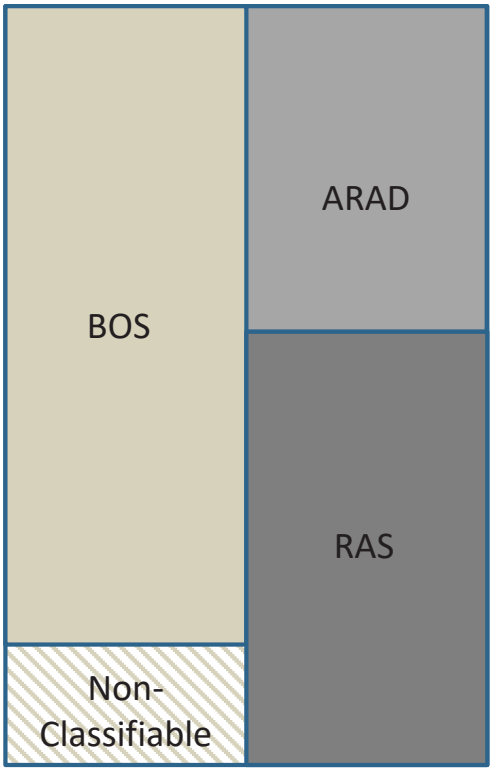



*Figure 8. An illustration of the approximate distribution between different CLAD phenotypes. BOS, Bronchiolitis obliterans syndrome; RAS, Restrictive allograft syndrome; ARAD, Azithromycin-responsive allograft dysfunction.*

### 1.6.1 Bronchiolitis obliterans syndrome (BOS)

The classic form of chronic rejection was first defined by a pathologically confirmed OB [138]. Microscopically, the condition has some degree of bronchiolar obliteration and fibroproliferation and an increased presence of monocytes (Figure 9) [139]. However, the development of OB is known to be patchy and one or several transbronchial biopsies might very well miss those areas that are affected. A more practical approach was suggested in 1993, in the form of BOS [140]. This is a clinical correlation to previous findings of OB, and is defined as an obstructive and persistent decline in pulmonary function. It is calculated as a decline in FEV1 of at least 20% of the average maximum value of consecutive measurements at least 30 days apart during the first postoperative year, without a better alternative diagnosis. There is also a grading of "possible" BOS when there is a loss of

10-19% in FEV1 with a concomitant reduction in forced expiratory flow at 25-75% of the pulmonary volume to 75% predicted or less. Further grading is possible, with BOS grade 1 being 66-80 % of baseline, BOS grade 2 being 51-65% of baseline, and BOS grade 3 being 50% or less. The condition is not easily treatable, and the most well-documented form of treatment is azithromycin which will be further elaborated upon under section 1.6.3 (ARAD). There has been some evidence that adding a mTOR inhibitor to the

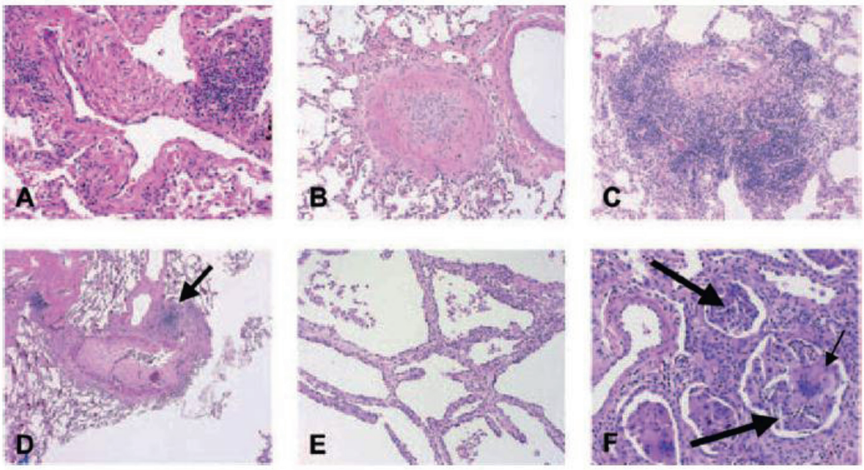



*Figure 9. Photomicrographs of histological samples of explanted allografts. A. Severe bronchiolar-epithelial atrophy. B. Total bronchiolar obliteration with fibrous tissue. C. BO with severe infiltration of mononuclear inflammatory cells. D. Lesion with perivascular lymphocytes found proximal to a bronchiole E. Interstitial fibrosis. F: Cholesterol Clefts and multinucleated giant cells. Reprinted from Chest, 2006.* ***129****(4): p. 1016-23. Martinu, T., et al., "Pathologic correlates of bronchiolitis obliterans syndrome in pulmonary retransplant recipients" with permission from Elsevier.*

immunosuppression might slow down the progress [141] also some suggest that a switch from cyclosporine to tacrolimus might be beneficial [142]. There have been some small studies with a slightly improved outcome after the use of extracorporeal photopheresis when all other options have been unsuccessful [143]. There are studies on the effect of antifibrotics on BOS however they are ongoing as this is being written and no preliminary results are available.

### 1.6.2 Restrictive allograft syndrome (RAS)

Restrictive allograft syndrome is a phenotype of CLAD, first proposed by Sato et al. [144] and the Toronto lung transplant team. It was first defined as a loss of 10% of the baseline TLC. This also forced a redefinition of BOS to only involve patients free of RAS. In the first estimate about one-third of the patients previously classified as BOS were in fact RAS patients with a prognosis that differed from BOS. Distinct radiological patterns were also found that were consistent with those in interstitial lung disease. The first study, however, did not include single lung transplants; nor did it consider the possibility of using radiography for identifying the subgroup. Furthermore, the use of TLC is impractical, since the patient cannot always do this test properly, so the simpler FVC has been proposed as a marker [145] as it is associated with TLC. Although the lung transplant community is in accordance on this being an actual phenotype, there is currently a lot of debate on the best and most efficient way to define it. Inclusion of a possible radiographic criterion has also been also discussed [146]. There is no official consensus document, but most centers adhere to a previous proposal [147]. It is not clear whether the risk factors for RAS are the same as for BOS, but this will surely become clear in time.

### 1.6.3 Azithromycin-responsive allograft dysfunction (ARAD)

Azithromycin-responsive allograft dysfunction is a retrospective diagnosis based on the responsiveness to treatment with azithromycin [147]. Studies have shown that up to 40% of the patients with a BOS diagnosis respond in some way to treatment with azithromycin, bearing in mind that this figure might be higher since the RAS cohort is not readily defined in these studies [148, 149]. Previously, it was thought that responders to azithromycin could be predicted by their predominance of neutrophils in BAL fluid but, since this is not universally true [150] the condition has been retrospectively defined as BOS that responds to azithromycin with an increase in FEV1 of ≥ 10% after 2–3 months of treatment. The defining article suggested that those patients with BOS that are non-responders represent the classical fibrotic OB, thus indirectly suggesting that the obstructive pulmonary decline in ARAD patients is predominantly driven by inflammation. Whether the effect of azithromycin is temporary or whether it permanently inhibits further pulmonary deterioration is not known, but the latter is probably more likely considering published data and my own clinical experience.

## 1.7 Acute rejection (AR)

AR is common in lung transplant recipients. Up to 50% of lung transplant recipients are treated for acute allograft during the first year post transplant [17]. The mechanism leading to AR is the most basic way of identifying and fending off foreign organisms. This response stems from every living organism's ability to differentiate self from non-self, which is absolutely necessary for survival. This is called alloimmunity, and is predominantly driven by T cells and their ability to recognize foreign MHC antigens. In humans, this is also called the human leukocyte antigen (HLA). This very basic and effective immune response does, however, become a problem when the ambition is to put a foreign organ into a recipient. Unless immunosuppression is applied, a massive T cell response will ensue when the foreign HLA is introduced into the body. This is most likely further enhanced regarding lung transplants; since the innate immunity of the lung is very active, it has also been suggested that cryptic self-epitopes are exposed during lung damage at the time of transplantation [151]. This would result in allograft injury and loss of function, and is the probable cause reason for the success in lung transplantation being unattainable before introduction of calcineurin inhibitors. The diagnosis of AR relies on obtaining a representative transbronchial sample with lymphocytic perivascular or peribronchiolar infiltrates. The rejection is graded A1-A3 based, on the severity of infiltrates in the transbronchial sample [152]. Spirometry data can be useful, but they have only been found to have a sensitivity of 60% for detecting a rejection of grade A2 or higher [153] neither can it differentiate between an acute rejection and an infectious episode. Today, the treatment for acute rejection is high-dose prednisone; this is based on studies from the 1990s (37,78). There is previous evidence showing an increased risk of BOS after acute rejection already after a grade A1 rejection [154, 155]. Our Study III, further supports this claim.

# 2 AIMS

Our aim was to expand or knowledge on the incidence and long-term effects of viral infections after lung transplantation, as well as investigating the possibility of using viruses as biomarkers for immunosuppression. The research questions were as individual hypotheses that were tested separately in each study.

Hypotheses tested:

Paper I: In the first study, the hypothesis tested was that respiratory viral infections would have a long-term effect on development of bronchiolitis obliterans syndrome and survival in a retrospective cohort.

Paper II: In the second study, the hypothesis tested was that hepatitis E virus antibodies are commonly found in lung transplant recipients.

Paper III: In the third study, the hypothesis tested was that viral infections would have a long-term effect on development of BOS and survival in a prospective cohort.

Paper IV: In the fourth study, the hypothesis was that Epstein-Barr virus and/or torque teno virus would be potential biomarkers for monitoring of the net state of immunosuppression after lung transplantation.

# 3 PATIENTS AND METHODS

## 3.1 Patients and ethics

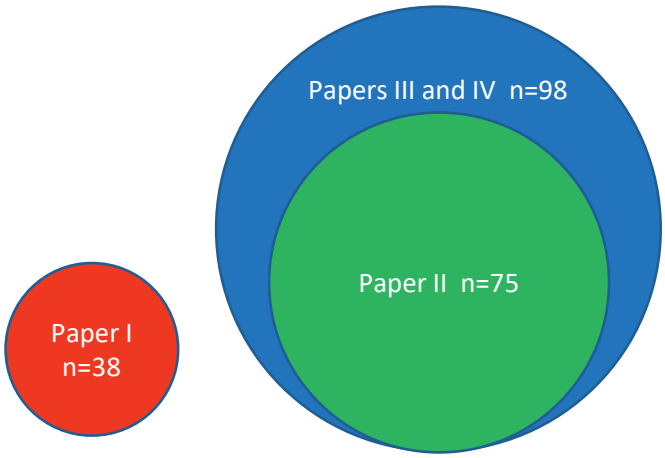



*Figure 10. The relative sizes of - and relationship between - the populations in Papers I–IV*

Two different study populations were used in this dissertation (Figure 10). The first study was retrospective. The population used for study 1 was transplant recipients operated between 26 February 1998 and 10 October 2000 at Sahlgrenska University Hospital. This population was chosen since they had systematically collected BAL samples in a biobank. The participants were informed and consented according to regulations at the time. The second, third and fourth study used the same population or parts of it. All patients were recruited prospectively between 23 February 2009 and 11 April 2012. They were asked to participate after the first postoperative intensive care period, and gave written and oral consent to participate.

Approval for all studies were given by the regional ethical review board in Gothenburg (Dnr791-08).

## 3.2 PCR

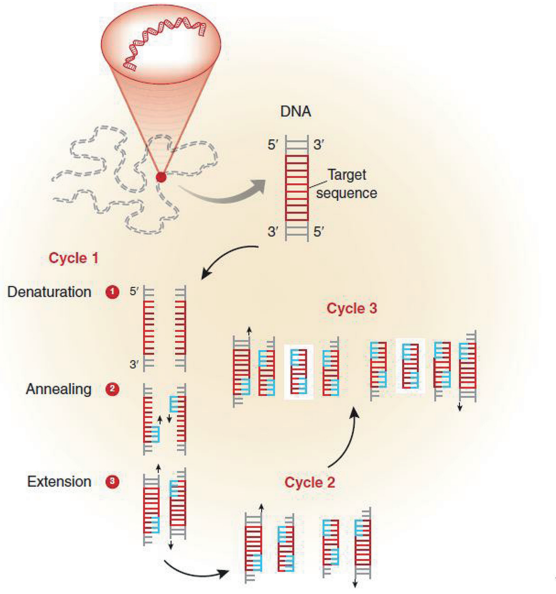



*Figure 11. The PCR amplification process as described below, with a repeated sequence of denaturation, annealing and extension. Reprinted from The Journal of investigative dermatology 133.3 (2013): e6, Garibyan et al. "Research Techniques Made Simple: Polymerase Chain Reaction (PCR)"*

PCR is a process that can amplify DNA to millions of copies of a specific DNA sequence. It was first described in 1983 [156], and is now a universally used method for diagnosis of microbial infections and for other purposes. Briefly, the process follows the steps A-C below (Figure 11).

A. Extraction of genetic material from the sample.
B. Transformation of RNA to complimentary DNA (cDNA) by the enzyme reverse transcriptase. (This step is omitted if DNA is the sample material).
C. Repeated amplification cycles:
    1. Denaturation of double dsDNA to single-stranded DNA.
    2. Hybridization/primer annealing.
    3. Elongation/polymerase copying, extension.

Real-time PCR, is a method whereby the detection of DNA can be measured continuously during the amplification process, enabling quantification of the target gene. Multiplex real-time PCR refers to a process where several agents can be analyzed in the same test run. DPCR or digital droplet PCR (ddPCR) is an improvement on conventional PCR methods and allows a more exact and reliable measurement of the amount of DNA/RNA in the sample. In our studies, we used a method that was initially set up at the Department of Clinical Virology at Sahlgrenska University Hospital, Gothenburg, Sweden, between 2004 and 2005. It has been described in detail previously [157]. In brief a Magnapure LC robot (Roche Molecular Systems, Mannheim, Germany) with a nucleic acid protocol was used with an ABI real-time PCR system (Applied Biosystems, Foster City, CA) for detection of viral nucleic acids. The system is designed to detect adenovirus, bocavirus, *Chlamydophila pneumoniae*, CoV NL63, HKU1, OC43 and 229E, human enterovirus, HMPV, hRV influenza A and B, *Mycoplasma pneumoniae*, hPIV (1, 2 and 3) and RSV.

## 3.3 Enzyme-linked immunosorbent assay (ELISA)

For the second study, ELISA was used for detection of HEV antibodies in serum. Two commercially available assays from Mikrogen and Diagnostics system respectively, were used. Briefly, recombinant HEV antigens form a coating on the surface of reaction plates, in specific and well-quantified numbers. The test material is applied, and then all unbound material is washed away. Mouse antibodies with an enzyme connected to them, specific for human antibodies, are added and the surplus is again washed away. A substrate is then added to the plates and the enzyme converts the substrate to a color that can be measured using a spectrophotometer. The intensity of color is measured and thus the presence and the quantity of any HEV antibody can be determined.

## 3.4 Bronchoscopy

Bronchoscopy was performed in a standardized manner. After sedating the patient, the bronchoscope was inserted via an either a laryngeal tube or a tracheal tube. The lung was inspected, and afterwards BAL was performed by

infusion of 3 sterile 50-mL pyrogen-free phosphate-buffered saline aliquots at 37° C into a segmental middle lobe or lingula bronchus, with the bronchoscope in a wedged position. On clinical indication, transbronchial biopsies were performed under continuous X-ray guidance. Part of the fluid was transferred to a separate container in a sterile manner and frozen at −80°C until analysis.

## 3.5 Nasopharyngeal swabs

NPH samples were obtained in a standardized manner. A specially trained nurse obtained these samples by inserting the swab (E-Swab; Nordic Biolabs, Täby, Sweden) deep into the nasopharyngeal cavity and rotating 360°. The swab was then put in a container with 1 mL of Amies medium and immediately transported to the laboratory, or frozen at −80°C until analysis.

## 3.6 Paper I

In Paper I, we retrospectively analyzed BAL samples that had been previously collected in a sterile manner and frozen at −70°C until analysis. The samples were collected from 38 consecutive lung transplant recipients transplanted between 26 February 1998 and 10 October 2000. They were sampled at every regular outpatient visit. All clinical data were collected retrospectively from patient charts. VRTI was detected in BAL fluid, using multiplex PCR as described under 3.2, except that the system could not yet detect bocavirus nor CoV-HKU1. The following were identified and recorded: CMV infection, bacterial or fungal infection, and AR. Also, time in ventilator and time in intensive care unit were recorded, together with possible symptoms of airway infection at the time of bronchoscopy.

BOS as endpoint was defined as described in the Introduction under section 1.6.1. Graft loss as endpoint was defined as either death or re-transplantation.

## 3.7 Paper II

In Paper II, we followed 75 patients in a prospective cohort with the aim of using the sera after 12 months for detection of antibodies. At the time of the study, only 62 patients were able to leave a sample. 11 patients had died before 12 months and two patients were temporarily lost to follow-up at the time. Blood products received during surgery were recorded and the patients were followed at scheduled outpatient visits 1, 2, 3, 4.5, 6, 9, and 12 months after surgery. Liver enzyme levels were tested every other week during follow- up. At one year, none of the patients had reported having severe liver complications. ELISA was used to test for antibodies, as described previously (under 3.3). For patients who tested positive for anti-HEV IgG antibodies, pre-transplant samples were acquired and tested. Since there had been no reports of liver complications, all the available documentation for these patients was retrospectively re-examined for symptoms or laboratory tests showing signs of acute hepatitis. For the single patient who seroconverted, all available samples were tested for the presence of HEV IgM (using ELISA) and HEV RNA (using PCR). The cohort was a subgroup of the population used in Papers III and IV.

## 3.8 Paper III

In Paper III, we followed 98 patients for a minimum of 5 years after lung transplantation. Clinical information, including immunosuppression, was recorded in a case report form (CRF) at baseline and at every scheduled outpatient visit during the first year. Also, extra visits with signs of acute rejection or suspected viral respiratory tract infections were recorded in the same manner. Non-viral respiratory infections were also recorded, as were CMV infections. After the initial follow-up, all patients were subsequently followed with spirometry at least twice a year until death or for at least five years.

At every outpatient visit, the patients were sampled with nasopharyngeal (NPH) swabs. Blood samples were also collected. On the visits 1, 3, and 12 months after transplantation, a mandatory bronchoscopy with BAL was performed. BAL samples were also collected on clinical indication. VRTI was detected using multiplex PCR on BAL and NPH samples. All BAL samples were also routinely cultured for bacteria and fungi; they were also routinely tested with real-time PCR for detection of *Legionella pneumophila*, *Pneumocystis jirovecii,* and CMV. Patients who had been diagnosed with

cystic fibrosis were also routinely cultured for *Mycobacterium tuberculosis* and atypical mycobacteria.

BAL samples were collected in a sterile siliconized container and immediately transported on ice to the laboratory. Part of the fluid was transferred to a separate container in a sterile manner and frozen to −80°C until analysis.

The endpoints were CLAD or organ loss, which was defined as death or re-transplantation. CLAD was defined as previously presented in the introduction. To minimize the risk of subjectivity, two experienced lung-transplant physicians reviewed each patient separately for development of CLAD. The results were compared, and a consensus was reached on any discrepancies.

## 3.9 Paper IV

In the cohort presented in study III, extra serum samples were collected at every visit. These samples were analyzed for the presence of TVV. Isolation of nucleic acids was performed using a MagNA Pure LC instrument with a MagNA Pure LC total nucleic acid or DNA isolation kit for serum or whole blood, using a standardized protocol, according to the instructions (Roche Diagnostics, Mannheim, Germany). The levels of TTV-DNA were determined using a 7300 real-time PCR system (Applied Biosystems, Foster City, CA). Quantification was obtained from a plot of Ct values for quantification standards. The quantification range of the assay was determined by serial dilution of plasmids with an insert of a synthesized sequence corresponding to that used in the TTV PCR.

The date of all infectious events was recorded. Infectious events were defined as being either bacterial infection or fungal infection. Any elevated CMV DNA levels that were treated pre-emptively with valganciclovir were also considered an infectious event, as well as all VRTI. Acute rejections were also recorded.

TTV levels were then plotted against infectious events as well as EBV levels to display possible correlations graphically. The population was subdivided into two groups depending on which calcineurin inhibitor was used.

## 3.10 Statistics

Comparisons at the group level for numerical variables were made using the Mann Whitney U-test. Ordinal variables were compared using the Chi-square test in paper I and two-sided Fisher exact test in paper III. The probability of event-free survival in Papers I and III was calculated using Kaplan-Meier analysis, and differences in the distributions were analyzed with the log-rank test. Associations between proposed risk factors and time to BOS (Paper I) /CLAD (Paper III) development and graft loss respectively, were assessed using Cox proportional hazards. In Paper III, a combination of time-dependent and time-independent co-factors were used. For Paper IV, Pearson´s correlation and linear regression was used to evaluate the relationship between the real-time PCR and the ddPCR measurements. Cox regression in the time frame 3 to 24 months with TTV or EBV as time dependent covariates were used to analyze the relationship between the two biomarkers and the outcomes AR, VRTI, fungal infection, bacterial infection, CMV viremia or any infectious event. Each patient was treated as a cluster to handle multiple infections occurring in a single individual. Univariable logistic regression was used in each of four periods q1 (1-3 months), q2 (3-6), q3 (6-12) and q4 (12-24) with the outcomes VRTI, fungal infection, Bacterial infection, CMV viremia infection or acute rejection and the predictors age, treatment, CMV mismatch, log TTV (beginning of period) or log EBV (beginning of the period). Any p-value of 0.05 or less was uniformly considered to be significant.

In Papers I-III the SPSS (IBM, Armonk, NY) software package was used. The versions were 17.0.3 for Paper I and 22.0.0 for Papers II and III. For paper IV we used the R software version 3.3.1 (R core team (2016), R Foundation for Statistical Computing, Vienna, Austria (URL http://www.R-project.org/)) was used for all statistical analyses except for the Pearson´s correlation analysis that was performed using the GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA).

# 4 RESULTS

## 4.1 Results from Paper I

The groups are defined by whether the patient had at least once, at least one virus detectable by qPCR of BAL fluid, at least once during the first year after transplant. The groups were subsequently called “viral” and “non-viral”. In the population of 38 patients, 14 belonged to the ”viral” group and 24 were “non-viral”. We found that time to BOS was significantly shortened (p=0.005) in the viral group, as shown in the Kaplan-Meier plot below (Figure 12). However, we did not find a significantly shorter time to organ loss (p=0.79). The attempt to model according to Cox proportional hazards was unsuccessful since no other covariate was close to being included in the model.

Stratification into severity of BOS showed that the significance in the viral group, for shortened time to BOS 1 (p=0,027) remained but not for BOS grades 2 or 3. Subdividing the viral group into respective viral species did not give any significant differences in time to BOS or time to graft loss. There were only five patients in whom symptoms of airway infection were present at the same time as a respiratory virus in BAL. Symptomatic viral patients did not show significant associations with any endpoint.

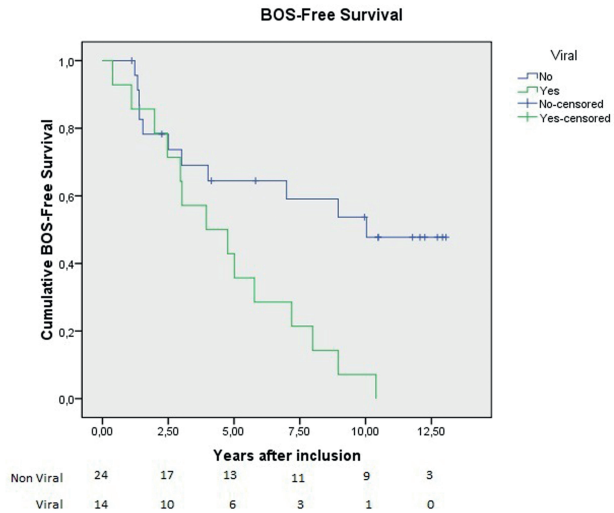



*Figure 12. Kaplan-Meier plot showing BOS-free survival, stratified into viral (n=14) and non-viral (n= 24) population (p=0.005). Patients at risk are shown beneath the x-axis for both populations.*

## 4.2 Results from Paper II

Sixty-two patients had a serum sample available for analysis after 12 months. Of these, only 8 (13%) of the patients had detectable levels of anti-HEV IgG and there were only two patients with anti-HEV IgM antibodies (Table 1). However, all patients with anti-HEV IgM antibodies as well as seven of the eight patients with anti-HEV IgG antibodies were positive in the pre-transplant samples that had been taken. None of the patients showed any symptoms of acute hepatitis during follow-up. For the seroconverting patient, the recorded values of liver enzymes showed a slight elevation for a few days during the first postoperative month, for no apparent reason. There were 11 prospectively collected samples, between transplantation and 1-year post-transplant and no detectable anti-HEV IgG or IgM antibodies at the time of the slight elevation of enzymes for this patient. The anti-HEV IgG antibodies first appeared at some time between week 28 to week 39 after lung transplantation. In the patient who seroconverted, no test was positive for anti-HEV IgM at any time, nor was HEV RNA detectable by PCR in any sample,

| Subject No | HEV IgG Pre-LTx | HEV IgM Pre-LTx | HEV IgG 12 Months | HEV IgM 12 Months |
|---|---|---|---|---|
| 1 | 5.8 | 5.5 | 5.9 | 5.5 |
| 2 | 2 | 1.3 | 3 | 0.6 |
| 3 | 4.5 | 0 | 3.2 | 0 |
| 4 | 5.5 | 2.5 | 2.7 | 5.8 |
| 5 | 4.6 | 0 | 1.8 | 0 |
| 6* | 0.4 | 0 | 3.2 | 0.1 |
| 7 | 11.1 | 0 | 10.5 | 0 |
| 8 | 1.8 | 0.2 | 1.7 | 0 |

*Table 1. Antibody levels pre LTx and 1year post-transplant for HEV-IgG pos. subjects, N=8; HEV, Hepatitis E virus; IgG, Immunoglobulin G; IgM, Immunoglobulin M; LTx, lung transplantation. The table shows the optical density of the sample. The subject who seroconverted is marked with an asterisk.*

## 4.3 Results from Paper III

Ninety-eight patients were followed for 5-8 years. During the first year, 629 follow-up visits were recorded. The patients were sub grouped depending on whether there were viruses detected by qPCR in NPH or BAL samples at any point during the first year post-transplant. The group with detectable viruses was called VRTI-positive. There were no significant differences between the two groups at baseline.

We did not find any association between VRTI and graft loss. There was a significant association between VRTI as a time-dependent covariate and CLAD development in univariate analysis (p = 0.046; HR = 1.83(1.01–3.31). In the Cox multivariate model, age at transplant, single lung transplant, cyclosporine treatment, COPD as transplant diagnosis, and CF as transplant diagnosis were included as static covariates and VRTI acute rejection, fungal infection, and REED were included as time-dependent covariates except In the final model acute rejection (p = 0.002; 2.85 (1.44–5.61)), cyclosporine treatment (p = 0.021, 4.36 (1.25-15.16) and VRTI (p=0.041; 1.94 (1.03–3.66)) remained significant risk factors for development of CLAD.

Stratification of the material into symptomatic or asymptomatic VRTI did not provide further insights; nor did stratifying for upper or lower respiratory tract infections. However, when stratifying for viral species, we found that corona virus infection was significant in univariate and multivariate analysis using the same covariates as previously. (p = 0.007; 2.93 (1.33–6.45)). No individual species was associated with a shortened time to graft loss. The numbers were too few to analyze respective subspecies of virus. The incidence of respective virus is described below (Figure 13).

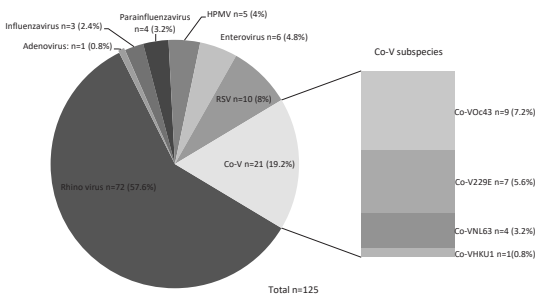



*Figure 13. Part of the total number of detected viruses, divided into viral species. RSV, respiratory syncytial virus; HMPV, human metapneumovirus; Co-V, coronavirus.*

## 4.4 Results from Paper IV

Eight hundred and thirty-seven samples were collected, including pre-transplant samples, from 98 patients over 24 months. Most patients had detectable TTV DNA levels before transplantation that continued to increase until 3 months post-transplantation, after which they reached a steady state. TTV DNA was detectable for the full length of the follow-up of most patients. For EBV, very few patients did not have detectable EBV DNA before transplantation and only a small peak was observed during the first and second months after transplantation; after this, the levels were consistently low. Only a few patients had detectable EBV DNA over the full length of the follow-up. All TTV results above 8 $\log_{10}$ between 6 and 12 months were assessed with digital droplet PCR, to verify the results of previous PCR testing. The results correlated well with each other (Pearson r = 0.67, p= 0.009). (Figure 14)

Choice of CNI affected the mean levels of TTV DNA in serum. From 6 months and until the end of follow-up, the mean TTV DNA levels were significantly lower in the group treated with cyclosporine than in the group treated with tacrolimus. No such same pattern could be found for EBV DNA, where there was no statistically significant difference at any time point.

The frequency of infectious events was highest at the beginning of the follow-up, especially with viral infections. No CMV DNAemia at all was present during the first three months, and most of the fungal and bacterial infections happened during the first nine months. The acute rejections mostly happened during the first 6 months.

The frequency of infectious events was compared to the mean TTV-DNA or EBV-DNA levels during each period (q1-q4) and the during the total FU period, respectively. No statistically significant association was found. With logistic regression log TTV-DNA or log EBV-DNA levels in the beginning of the period did not predict any infectious event in any of the periods.

Cox regression of infection, any infectious event or CMV viremia. TTV and EBV levels were added as time varying covariates to provide the most accurate statistical model possible. No significant associations were found.

Most acute rejection events (AR) occurred within the first 6 months post LTx. (Logistic regression with initial TTV-DNA or EBV-DNA for each period respectively did not predict whether an acute rejection event would occur during that period.

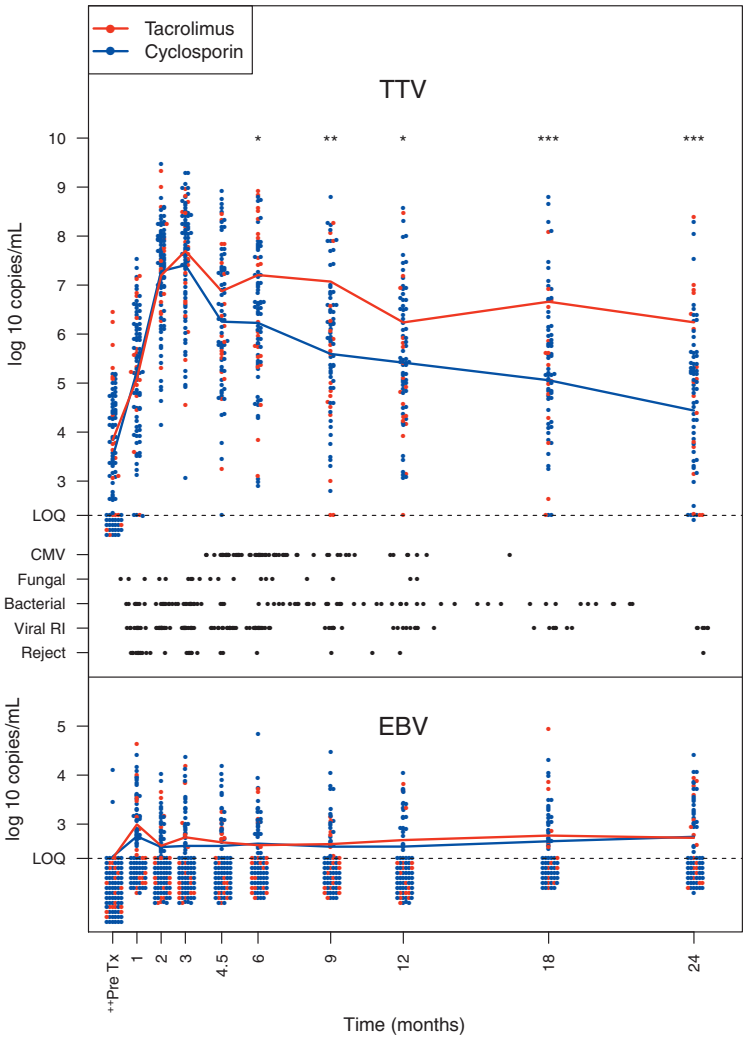



*Figure 14. Kinetics of TTV- and EBV-DNA levels, in serum and whole blood respectively, before and during the follow-up period after lung transplantation. TTV-DNA levels in serum starting pre-LTx were determined by real-time PCR. Individual TTV levels are indicated by dots. EBV-DNA levels in whole blood post LTx were determined by real-time PCR and individual EBV levels are indicated by dots. Patients receiving either Tacrolimus treatment (n=19) and Cyclosporine treatment (n=79) are indicated by red and blue dots respectively. The mean level of TTV and EBV in Tacrolimus treated patients is indicated by a red line in respective graph. The mean level of TTV and EBV in Cyclosporine treated patients is indicated by a blue line in respective graph. Infectious events and acute rejections are indicated by black dots. Statistic calculations were done using Mann Whitney U (p-values are indicated by * <0.05, ** <0.01 and *** <0.001). ++ The levels of EBV-DNA pre-LTx was determined in serum samples. Viral RI: viral respiratory tract infections, CMV: cytomegalovirus, reject: acute rejection of which all but three were biopsy verified, LOQ: level of quantification.*

# 5 DISCUSSION

## 5.1 VRTI

VRTI has been discussed as a possible cause of chronic rejection in lung transplant recipients for more than two decades. The theory has an easily understandable rationale, since free circulating viral agents have access to the transplanted organ directly through the airways. That respiratory viruses trigger inflammation and damage pulmonary tissue is no new knowledge, but as described in the introduction, we now know much more about the innate immunity and its possible local and systemic effects. Our findings in Paper I and Paper III suggest that VRTI during the first year is associated with a shortened time to CLAD development.

### 5.1.1 Previous publications on VRTI after LTx

A systematic review of the area was presented by Vu et al. in 2011 [158], with the conclusion that the methodology and design of previous studies made previous results too disparate to draw any conclusion about any association. Two of the studies published before Paper I, were prospective cohort studies that used multiplex PCR to assess the risk of BOS after VRTI. In 2006, Milstone et al. [159] presented a study in which they had recruited 50 outpatients who had previously received a lung transplant. The patients were sampled with nasopharyngeal swabs and BAL during the winter season and followed for a year. Multiplex PCR was used but also cell cultures. No association was found between VRTI and BOS. Notably the methods used were not capable of hRV which is arguably the most common find in both Paper I and Paper III. In 2009, Gottlieb et al. presented a single-season study [160], where they prospectively followed a cross-sectional cohort of all outpatients, irrespective of time after transplantation, for one year. Both cell cultures and multiplex PCR was used for detection of viruses. By multivariate analysis, there was an association between VRTI with fourteen out of fourteen possible symptoms of infection, and BOS. For both studies the numbers of detected VRTI were small. Paper I also had a small number of VRTI-positive patients, but the length of follow-up is still uniquely long. We did not perform a multivariate analysis in Paper I, since the covariates consisted of very few events and no covariate reached a significance of $p=0.2$ or lower. The data were retrospectively collected from paper charts over a decade old which is a reasonable explanation for this outcome. The most interesting study published in the field, between Papers I and III is a large study by Allyn et al., published in 2016 [161] where a prospectively gathered

material, between 2000 and 2009, was analyzed. The bulk of the viral detection was culture based but multiplex PCR was also used. They found that viral pneumonia, defined as a symptomatic viral infection and a radiographic infiltrate was associated with a shorter time to CLAD onset. Paper III found a similar association even though the material was numerically smaller, although we did not find that severity of infection contributed. Possibly this is a result of multiplex PCR being a more sensitive method for viral detection than viral cultures.

### 5.1.2 Representativeness of the cohorts

The patients in both Papers I and paper III are relatively representative for their respective time period, compared to ISHLT data, except that the relative portion of COPD/emphysema patients were higher in the cohort of Paper I. The number of yearly procedures a little more doubled between the two papers [17]. In Paper I there were a dominance of single lung transplants and in Paper III there were a dominance of double lung transplants. Cumulative evidence for better survival after double lung transplantation prompted this move towards the latter procedure [162].

### 5.1.3 CLAD and graft survival.

Both Papers I and III showed an association of VRTI with development of BOS/CLAD. However, neither paper showed an association with a shortened time to graft loss. This is somewhat surprising considering that we know OB to be the most common cause of death amongst lung transplanted patients [17]. For Paper I, one could hypothesize that this could be due to the later accepted subdivision of chronic rejection into BOS and RAS [144] with RAS, having a shorter survival being more prominent in the non-viral group [146]. However re-examining the material from Paper I, show that there are two possible RAS candidates in each group which makes this hypothesis unlikely to be true. Testing the same hypothesis on Paper III we find that out of the 11 RAS patients; seven were in the VRTI group but there was still no significant difference in graft survival. Another possible hypothesis is that the reason for there being less CLAD development in the non-VRTI group is that they die slightly earlier before having the possibility to develop CLAD, which leads to a similar survival. But since CLAD is more common in the VRTI-positive group among the long-time survivors, this is not likely to be the explanation. A third hypothesis, could be that CLAD, triggered by VRTI, constitutes a less aggressive phenotype than CLAD triggered by other risk factors. We still do not know the exact mechanism of CLAD development, but we know of a multitude of risk factors [147]. It is possible that there are

not one but several trigger mechanisms for CLAD leading to diverging outcomes for the patient.

### 5.1.4 Possible Mechanisms

Paper I and III add to a now cumulatively large body of indicating that there is an association between VRTI and CLAD development. The mechanism by which a VRTI could lead to CLAD is still unknown. BOS, as described under heading 1.6.1, is characterized by mononuclear infiltrates and fibrotic transformation [139]. This is very similar to the proliferative phase of diffuse alveolar damage (DAD) [163], where proliferation of type-2 pneumocytes is also seen. DAD can be seen after viral infections, especially aggressive influenza and coronavirus infections [164]. CoV-HKU1 has shown an affinity for type- 2 pneumocytes [165] which could possibly lead to a reduced amount of fully functioning cells. One of the local effects of type-2 pneumocytes is to produce VGEF [166]. Locally produced VGEF is an important factor in contributing to the acute inflammatory response and pulmonary edema on the one hand, but it is also a protective factor for the alveolar epithelial barrier. In mice, reduction of local VGEF in the lung, has shown increasing perpetuation of lung damage [166]. In Paper III, CoV in particular seem to be associated with a shortened time to CLAD. It is possible that the immunosuppressed patient has a reduced capacity to compensate for damage conferred to type 2- pneumocytes, especially by CoVs. thus, developing a slowly progressive DAD, such a theory would need, to be tested with an appropriate model. Another previously presented theory is that of the CXCR3/ligand being the mediator of cellular damage. CXCR3 is a chemokine receptor and ligands to this receptor especially CXCL10 and CXCL11 has been of some interest [167] since very elevated levels of these have predicted persistent decrease in FEV1 in lung transplant recipients after VRTI [168]. It has been suggested that dysregulation of these ligands leads to persistent fibroblast recruitment/proliferation and subsequent development of DAD. This theory implicates RSV as an especially dangerous viral agent, due to its ability to affect the CXCR3 receptor. There is no support for the harmfulness of RSV in Papers I or III but admittedly few instances of this virus type were detected.

The findings in this thesis suggest that VRTI during the first year could be an important risk factor for long term CLAD development. Further mechanistic studies of viral interaction in transplanted lungs are warranted.

## 5.2 Hepatitis E

Although there was found a prevalence of anti-HEV IgG of 13% among the cohort of lung transplant recipients 1 year after transplantation no patient developed a chronic infection and only one seroconversion was detected compared to pre-transplant samples.

### 5.2.1 Previous publications on HEV after LTx

We know that the four different genotypes is of HEV unevenly distributed around the world [169], where genotype 1 predominates in Asia and is found in Africa. Genotype 2 is found in Africa and Mexico, while genotype 3 and 4 predominate in western world. We also know that Incidence varies greatly between European countries, thus results from studies from other part of Europe might not be directly applicable to the Swedish lung transplant population.

However, the largest retrospective study of Hepatitis E, so far, was performed by Riezebos-Brilman et al. [170], and published in 2013. They retrospectively examined a large cohort of lung transplant recipients from Vienna and the Netherlands, retrospectively with previously sampled blood tests. They did this on the patients who showed liver elevation at any time during the post-transplant period and found that 2.1% of the population had HEV RNA in blood, suggesting active infection. No patients, with no liver enzyme elevation was tested; nor was the prevalence of HEV antibodies. Pischke et al. analyzed blood from 95 LTx recipients for anti-HEV antibodies HEV RNA[171]. The patients were most likely from Hannover, although this is not clearly stated in the article. They found five patients with chronic HEV as proven by persistent HEV viremia by >3 months. Interestingly they only detected anti-HEV antibodies in 5.3% (5/95). Seemingly only one patient developed antibodies without also developing chronic HEV infection. One possible cause for this difference might be the usage of other ELISA kits (MP biomedical and Wantai diagnostics) Also 50% of the patients had persistent elevated alanine aminotransferase. Since the time for being ‘persistent’ was not defined it is hard to properly compare the results with Paper II. However, given that persistent was more than 2 weeks the same number in the cohort of paper II would be 1,5%, suggesting either major differences in either analysis range/method or population. A different population would explain why there are more chronic HEV in the Hannover cohort.

### 5.2.2 The impact of immunoassays

The difference in sensitivity and specificity between different immunoassays has been the subject of previous debate [172-174]. Our approach to this was to use two systems in parallel (DSI and Mikrogen). Pischke et al. did something similar, however they only tested the patients presenting with HEV RNA with the kit from Wantai. Norder et al, published a paper in 2016 reviewing performance five commercially available anti-HEV immunoassays [99] in large cohort. They found that one of the tests (Mikrogen) used in Paper II had a fairly low sensitivity for anti-HEV IgM. Another kit had better sensitivity, albeit slightly worse specificity (Dia Pro) and this kit is now use as standard at the Virology department at Sahlgrenska. Neither of the tests used by Psichke et al. was examined. It is possible that if the tests from Paper II been re-analyzed with the kit from Dia-pro, the incidence of anti-HEV IgG had been higher. However, the kit from Mikrogen used in our study have also been used in previous analysis in a heathy Swedish cohort [175] and these results should be comparable.

### 5.2.3 Patients positive for anti-HEV antibodies.

The age among patients who tested positive for HEV IgG was higher than for patients with negative HEV IgG which may reflect a higher cumulative risk of being exposed to HEV. After having analyzed all pre-transplant samples we could only find one single patient seroconverting after transplantation. This patient had a primary infection somewhere during the first 28 weeks after transplantation. The infection seems to have been transient without any signs of acute hepatitis, without any permanent damage to the liver and with a viral shedding phase of less than 6 weeks. Blood transfusion has previously been suggested as a source of transmission for HEV [176-178] however the seroconverting patient did not receive blood products, peri- or post-operatively up until the time when seroconversion was detected. Both transfusion related infections and passive transfer of anti-HEV IgG can be ruled out. Ingestion of pork is a possible source of infection.[179], intense and or prolonged contact with animals is another [180].

HEV RNA was analyzed in all available samples of the seroconverting patient without detection of a positive sample. The incidence of anti HEV antibodies amongst lung transplants is similar to results in the general population in previous publications using the same immunoassays. Even if awareness of the disease is now much higher than when Paper II was published, there is still to be a confirmed case of chronic HEV in the Swedish lung transplant population.

## 5.3 TTV and EBV

Studies of both TTV and EBV have previously been published with concluding remarks suggesting a relationship with net immunosuppression in lung transplant recipients. In Paper IV, these associations are somewhat less convincing.

### 5.3.1 TTV

We found that TTV DNA levels increased rapidly, already one month after LTx the levels were markedly elevated, presumably due to ablation of the functional effector cells of the immune system, that normally control the TTV infection [131, 132]. At three months post LTx, the mean TTV DNA level peaked and then gradually declined, reflecting the gradual moderation of immunosuppressive therapy. Interestingly, the TTV DNA level was lower in patients who received cyclosporine treatment, possibly reflecting a different immune modulatory mechanism for Cyclosporine compared to Tacrolimus. Görzer *et al.* [136] previously suggested that this might be due to Cyclosporine being less efficient as an immunosuppressant. However, we found no association between type of immunosuppressive regimen and acute rejection or other events that would indicate a difference in net immunosuppressive effect. Moreover, high levels of TTV-DNA are probably not a clinical problem per se since the virus is never been found to cause disease, even in immunosuppressed patients [127, 181-185]. It remains to be clarified in which cell types TTV replication occur but CD4+ T cells and CD8+57+ T cells appear to be important for controlling the infection, whereas EBV reside within the B cell pool and is controlled by CD4+ and CD8+ T cells [131, 132, 186-192]. In this work, we show that the choice of calcineurin inhibitor affects the TTV- but not the EBV-DNA levels, possibly reflecting separate mechanism for viral replication rather than merely ablation of the T cell pool.

### 5.3.2 Previous publications on TTV DNA after LTx

Görzer et al. have produced several articles about TTV DNA in an LTx population. Firstly, TTV DNA dynamics were described in a retrospective cohort of 31 patients who were followed for up to 720 days [136]. It showed a pattern very similar to ours albeit with higher TTV DNA levels and less of a decrease after the initial peak. It is possible that these two differences in mean TTV might be due to the differences in induction therapy between the two studies. Regarding the difference in mean TTV it is also possible that analyzing plasma, as Görzer et al., renders different results for TTV DNA, than analyzing serum, as used in Paper IV. However, there are no apparent reasons for such a hypothetical difference. Görzer et al. reported in the same

article, that tests preceding infectious events had a statistically higher mean TTV level. These tests were taken up to 76 days before the event and no mean or median was reported. We did not find the same pattern; nor could we find any tendencies towards this. However, like Görzer et al. we found a statistically significant difference in mean TTV dynamics, depending on the choice of CNI. A second article describes the initial kinetics of the TTV DNA after LTx. A steep initial rise in levels after transplantation and also, a difference in dynamics between TTV levels for basiliximab and ATG during the same time period [193] is presented. There were no patients induced with basiliximab in Paper IV, and nor did we have any samples close to transplantation for comparison of dynamics. However, looking at the similarities of the slopes between 0-1 month, one can conclude that results cannot be radically different. Recently, TTV DNA has been associated with chronic lung allograft dysfunction after lung transplantation [194] but in our study this was not included as an outcome.

### 5.3.3 EBV

In Paper IV, mean EBV DNA levels did not increase as markedly as TTV DNA levels after transplantation, even though EBV specific T-cells most likely are subverted by the immunosuppressive therapy [195]. The seroprevalence for EBV pre LTx was 97 % and a similar number of patients were positive for EBV-DNA at any time during FU compared to previous studies [114, 115, 196]. One possible confounder, when determining EBV DNA load, is the use of valganciclovir for preventing reactivation or primary infection of CMV. All patients received 3 months of valganciclovir treatment initially after LTx and there are a few studies showing that valganciclovir treatment may reduce EBV load which might have affected the results in Paper IV [197, 198]. This might also, possibly in part explain why mean EBV-DNA levels did not increase as markedly as TTV-DNA levels after transplantation, even though EBV specific T-cells most likely are subverted by the immunosuppressive therapy [195].

### 5.3.4 Previous publications on EBV after LTx

In 2007 Ahya et al. published a paper where they found an association between elevated EBV RNA and a reduced risk of acute rejection [199] and EBV is discussed as a biomarker for immunosuppression. They also reported no association between EBV RNA and infectious events. However, these results are not directly comparable to those in Paper IV since we measured. In contrast to this, Engelmann et al. published a paper where EBV DNA in peripheral blood was associated with development of BOS [115] the implication being that it was related to net immunosuppression. However, in

the article, it was stated that developed BOS at the time of the first sample was excluded but not that BOS diagnose under the initial 6-month follow up, was excluded. Consequently, it is not certain that elevated EBV DNA preceded development of BOS for patients with more than one blood sample. This constituted about half of the EBV positive cohort. These results are also difficult to compare with the results in Paper IV since we did not look at the same variables. Bakker et al. presented an article where EBV was suggested to be a biomarker of the degree of immunosuppression [114]. Seventy-five patients at a median of 4.25 years after lung transplantation were included and followed for 5.5 years, or until death. Observed elevations in EBV were treated with a reduction in the antiproliferative immunosuppressive drug. Also, it was treated with an antiviral agent for an undisclosed amount of time. They concluded that EBV reactivation was normalized and not associated with BOS progression or acute rejection if treated in this manner. However, since an antiviral agent with proposed effect on EBV [113, 200-202] was introduced at the same time as the reduction of immunosuppression, it is difficult to know what caused the normalization of EBV DNA. There was no description from what levels the immunosuppressive agents were reduced and we also know that acute rejection is very rare after the first two years [17] and can appear regardless of dosage of antiproliferative agent. There was no control group to verify the proposed effect.

Even if there are previous published articles on the association between EBV DNA and immunosuppression after lung transplantation, the collective results of these studies on EBV DNA presents a weaker case for its use as a biomarker than the fairly recent publications on the association between TTV DNA and immunosuppression. In Paper IV we found no association between any of the proposed biomarkers and infections or acute rejection. We did find a statistically significant difference in TTV DNA levels depending on choice of CNI for immunosuppression. No similar difference could be found for EBV DNA. These results must be interpreted with caution since choice of immunosuppression was not randomized but the findings still suggest that there might be some association between TTV DNA load but not EBV DNA load and the net immunosuppressive state after LTx. However, the direct practical usefulness of this finding may be limited Further studies are warranted in order to define the effect of immunomodulation on TTV and EBV replication in relation to various immunosuppressive regimens.

# 6 CONCLUSIONS

## 6.1 PAPER I

Lower VRTI during the first year was associated with an increased development of BOS but had no effect on overall survival. The material was not large enough to allow subgroup analysis.

## 6.2 PAPER II

The prevalence of anti-HEV IgG antibodies in our series was comparable with that in general population of Sweden. HEV did not appear to be a large unrecognized contributor to morbidity in the lung transplant population at our center.

## 6.3 PAPER III

VRTI during the first post-operative year, was found to be an independent risk factor for long-term development of CLAD in lung transplant recipients. Coronaviruses appear to be an important risk factor.

## 6.4 PAPER IV

TTV and EBV has limited usefulness as biomarkers for monitoring of the net state of immunosuppression in lung transplant recipients. The choice of immunosuppression appears to affect TTV levels over time. The same pattern cannot be found for EBV.

# 7 FUTURE PERSPECTIVES

Although this dissertation adds significantly to the present body of knowledge on viral infections after lung transplantation, the area is still underexplored. Further mechanistic studies could be of special interest, since they would not only provide insight into the underlying effect of the virus but might also provide more insight into the general immunology after lung transplantation. Three promising areas for future studies are listed below.

## 7.1 VRTI

The results of Papers I and III have had clinical impact on the screening for, and treatment of VRTI in LTx recipients at Sahlgrenska. Although we can present evidence that speaks in favor of early VRTI as an important risk factor for CLAD development, the subject warrants further exploration. A multicenter cohort to would help to reach higher numbers of the respective viral species to allow us to better understand if there are particular virus families that are associated with particular risks or other families that do not contribute to CLAD development. Also, the issue of prevention and/or effect of treatment present several options for future complimentary research.

## 7.2 HEPATITIS E

The conflicting results on the situation regarding the incidence and prevalence of HEV in Swedish lung transplant recipients present further options for exploration.

## 7.3 IMMUNOSUPRESSION BIOMARKERS

Even though we could not verify a practically applicable way of using either TTV or EBV as biomarkers we, could still see a divergent response for TTV especially, depending on the choice of calcineurin inhibitor. What we know of the mechanisms of these drugs does not explain this difference. Thus, it is possible that there is another application for measuring TTV DNA after lung transplantation, and the subject should be approached with new endpoints.

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