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Lymphangiogenesis in Bronchiolitis Obliterans Syndrome

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1. Abstract

Bronchiolitis obliterans syndrome (BOS) displays the most common form of chronic graft rejection in lung transplantation. It significantly affects long-term survival, causes substantial morbidity, impairs quality of life and increases costs. So far, the pathogenesis of BOS is not fully understood, however, it is suggested to be driven by both alloimmune and nonalloimmune mechanisms. Lymphangiogenesis, the growth of lymphatic vessels, has been shown to be altered in acute and chronic transplant rejection of several organs, however, the impact is still controversial. Previous investigations have shown an increase of lymphatic vessel density in acute graft rejection in lung transplantation. Heretofore, there has been no significant research on the impact of lymphangiogenesis in BOS.

We have performed immunohistochemical staining of podoplanin, a lymphendothelial marker, in patients suffering from BOS stage III (n = 23) and control subjects (n = 13). Lymphatic vessel density (LVD) has been compared between both groups, in addition subdivision for bronchiole with and without inflammatory infiltrates has been performed. Moreover, LVD has been correlated with time to BOS III diagnosis.

Neither LVD (lymphatic vessels per bronchiole, lymphatic vessels per mm bronchial epithelium and μm lymphatic endothelium per mm bronchial epithelium) has been altered in BOS nor could we detect a correlation with time to BOS III diagnosis.

Even though lymphangiogenesis seems to be involved in pathogenesis of acute and chronic transplant rejection in several organs, in chronic rejection of lung grafts it may be negligible. However, one must consider the inhibiting effect of immunosuppressive agents such as cyclosporine on the microvasculature system to be accountable for the equal distribution of LVD in patients suffering from BOS III and control subjects.
2. Zusammenfassung


Wir haben eine immunhistochemische Färbung für Podoplanin, ein Lymphendothelmarker, bei Patienten in BOS Stadium III (n = 23) und einer Kontrollgruppe (n = 13) durchgeführt. Die Lymphgefäßdichte wurde zwischen den beiden Gruppen verglichen, zusätzlich haben wir zwischen Bronchioli mit und ohne inflammatorischen Infiltraten unterschieden. Außerdem, wurde die Zeit bis zur Diagnose von BOS III mit der Lymphgefäßdichte korreliert.

Weder die Lymphgefäßdichte (Lymphgefäße per Bronchiolus, Lymphgefäße per mm Bronchialepithel und μm Lymphendothel per mm Bronchialepithel) war bei BOS Patienten verändert, noch konnten wir eine Korrelation mit der Zeit bis zu Diagnose von BOS III zeigen.

Obwohl Lymphangiogenese in der Pathogenese der akuten als auch chronischen Abstoßung von verschiedenen Organen eine Rolle spielen dürfte, scheint sie jedoch in der chronischen Abstoßung der transplantierten Lunge vernachlässigbar zu sein. Allerdings muss man hierbei auch den inhibierenden Effekt von immunsuppressiven Medikamenten wie Ciclosporin auf das mikrovaskuläre System berücksichtigen, da dieser für die gleichmäßige Verteilung der Lymphgefäßdichte bei BOS Stadium III Patienten und der Kontrollgruppe verantwortlich sein könnte.
3. **Introduction**

3.1. **Bronchiolitis Obliterans Syndrome**

Graft failure and mortality rates in lung transplantation exceed most other solid organ transplants\(^1\). According to the most recent report from the International Society for Heart and Lung Transplantation (ISHLT) registry, the median survival after lung transplantation is now 6.3 years whereas between 1998 and 2004 it was 5.8 years and before 1998 4.3 years\(^2\). However, in retransplantation survival rates are even lower, the median survival is now 3.0 years, but improved from 1.9 years between 1998 and 2004 and from 1.0 years before 1998\(^2\). Improved survival rates are mainly attributed to better operative and perioperative outcomes\(^1\). The main limitation of such poor survival rates is principally due to BOS, the most common form of chronic lung allograft dysfunction (CLAD)\(^1\). Other less common forms of CLAD are restrictive allograft syndrome and neutrophilic reversible allograft dysfunction\(^1\).

BOS has first been recognized in the mid-1980s as a form of CLAD\(^3\). It is the leading cause of death after the first year posttransplant, 50% and 76% of primary adult transplant recipients developed BOS after 5 and 10 years\(^2,4\). Besides from its impact on long-term survival, BOS causes significant morbidity, impairs quality of life and increases costs\(^5-7\).

Due to its nonuniform distribution of fibrosis and involvement of airways which are hardly sampled by transbronchial biopsy (TBB\(_x\)) BOS is defined as progressive airflow obstruction that cannot be explained by acute rejection, infection or any other confounding complication\(^8\).

3.1.1. **Pathogenesis**

3.1.1.1. **Risk Factors**

Probable risk factors for BOS are episodes of acute rejection, cytomegalovirus (CMV) pneumonitis, human leukocyte antigen (HLA) mismatching, lymphocytic bronchitis/bronchiolitis, noncompliance with medications and primary graft dysfunction (PGD)\(^9\). Potential risk factors may be colonisation with aspergillus of the lower airways, aspiration, CMV infection (without pneumonitis), donor antigen-specific activity, Epstein-Barr virus (EBV) reactivation, aetiology of native lung disease, gastrooesophageal reflux,
older donor age, pneumonia (gram negatives, gram positives, fungi), prolonged allograft ischemia and recurrent infection other than CMV. On the major risk factors will be elaborated in the following paragraphs.

**Acute Rejection**

Acute vascular (A grade) or airway (B grade) rejection are the main risk factors for BOS. Recurrent, late and severe episodes of acute rejection have been associated with an increased risk of developing BOS independent of other risk factors.

**Pneumonia/Airway Colonisation**

Pneumonia and airway colonisation are both independent causal factors of BOS. Kotsimbos et al. showed that serology to Chlamydia pneumonia in donors as well as recipients is associated with development of BOS. Exudative bronchiolitis has also been shown to be associated with an increased risk of BOS.

**Viral Infection**

Infections of lower respiratory tract with e.g. rhinovirus, coronavirus, respiratory syncytial virus, influenza A, parainfluenza, human metapneumovirus and human herpes virus-6 have been described to be risk factors for BOS. CMV and EBV infection have also been reported to be a potential risk factor.

**Primary Graft Dysfunction**

Daud et al. described PGD to be an independent risk factor for developing BOS. This increased risk was reported to be related to the severity of PGD.

**Gastrooesophageal Reflux**

The mechanism underlying the increment in risk to develop BOS by gastrooesophageal reflux is not clear. As bile acids and pepsin can be detected in bronchoalveolar lavage fluid aspiration may contributed to airway injury. Treatment with proton pump inhibitors did reduce acid reflux; however, non-acid reflux (including bile and pepsin) was not affected,
suggesting the presence of those to be associated with BOS. Early surgical treatment of gastroesophageal reflux reduces the rate of BOS and improves survival.

**Human leukocyte antigen mismatches**

The role of HLA mismatches as a risk factor for development of BOS remains controversial. The development of anti-HLA class I and II antibodies has been reported to be associated with BOS.

### 3.1.1.2. Natural History

BOS is not normally diagnosed less than 6 months posttransplant, diagnosis is most likely between 1.5 and 4 years after transplantation. The key symptom is airway obstruction with a reduction of forced expiratory volume in 1 second (FEV1), resulting in dyspnea, infections and loss of the grafted lung. The natural history of BOS is varying both in its timing of onset after transplantation and in the aggressiveness of its clinical course, it may be insidious with only a gradual decline in lung function over months to years, or abrupt with a severe decline in lung function over a few weeks. The median survival after BOS diagnosis is 2.5 years, however, early-onset BOS and high-grade-onset BOS usually predict worse survival.

### 3.1.1.3. Mechanisms of Pathogenesis

Pathogenesis of BOS is complex and not fully understood, however most evidence points towards a major role for the adaptive immune system and one hypothesises it to be driven by both alloimmune and nonalloimmune mechanisms. A possible process suggests that first lymphocytic infiltrates of the submucosa occurs, followed by epithelial cell injury, necrosis and ulcerations of the mucosa. Recruitment/proliferation of fibroblasts/myofibroblasts, epithelial cells, smooth muscle cells and mesenchymal precursor cells of the airway lumen is the result. Subtotal or total obliteration of airway lumen is caused by intraluminal polypoid granulation tissue.

**Effector Immune Responses**

All three effector immune responses (type 1, 2, 17) as well as regulatory T (Treg) immune response have been a subject to research lately, as a fragile balance between those is critical.
to airway wound repair. It is hypothesised that a disruption of this balance may lead to fibro-obliteration of allograft airways and BOS.

Type 1 immune response is associated with a cytotoxic T lymphocyte (CTL) and delayed type hypersensitivity (DTH) response. This type is characterised by production of interleukin (IL)-2, IL-12, interferon-γ (INF-γ) and lymphotoxin. On one hand, those cytokines have been associated with acute cellular rejection and on the other hand with BOS, although several studies draw contradictory conclusions. In animal tracheal transplantation models that reproduce the bronchiolitis seen in humans the initial alloimmune response is a type 1 immune response with INF-γ as a predominant cytokine. INF-γ induces expression of adhesion and costimulatory molecules in airway epithelial cells which results in augmentation of the alloimmune response via stimulation of lymphocyte infiltration and priming T cell responses. Hence, the activated airway epithelial cell renders a profibrotic milieu by production of growth factors, finally resulting in tracheal obliteration.

Type 2 immune response is characterised by production of IL-4, IL-5 and IL-13, which usually promote mucosal, allergic and humoral immunity. Although this type has been considered to favour acquisition of tolerance in several animal models, there is evidence that type 2 immune response plays a role in chronic rejection.

Type 17 immune response is characterised by production of IL-17 and IL-23 and is associated with autoimmunity. Recent studies suggest self-antigens to be unveiled throughout the transplantation process and furthermore promote allograft dysfunction. This leading to an increase in attention towards type 17 immune response in allo-/autoimmunity in lung transplantation. Burlingham et al. could show that autoimmunity mediated by col(V)-specific T(H)17 cells increases risk for development of BOS. Furthermore, type 17 skewing cytokines such as transforming growth factor-β (TGF-β), IL-1β, IL-6 and IL-23 and the type 17 effector cytokine IL-17 were elevated in the bronchoalveolar lavage fluid (BALF) of patients suffering from BOS.

Treg immune response has the ability to alleviate effector type immune responses via Treg cells, CD4+CD25+ T helper cells which constitutively express the transcription factor Forkhead-Box-Protein P3 (FoxP3). The important role of Tregs in prevention of chronic allograft rejection in lung transplantation has been supported by Bhorade et al. who could
show that stable graft recipients can be distinguished from patients suffering from BOS by a higher percentage of FoxP3⁺ cells in BALF. This suggests that prevention of BOS posttransplant depends on alleviation of all three effector immune responses and augmentation of the regulatory immune response.

**Alloimmune Reactivity**

Both acute and chronic allograft rejection highly depend on major histocompatibility complex (MHC) molecules. In humans, there are class I MHC molecules, usually referred to as HLA A, B and C which are constitutively expressed on most nucleated cells, and class II MHC molecules, also known as HLA DR, DP and DQ which are expressed only by bone marrow-derived antigen-presenting cells. These molecules play an important role in the immune system as they present peptides to T cells. Allograft rejection is finally achieved through cytotoxicity caused by CD4⁺ or CD8⁺ T cells. In lung transplantation HLA mismatch between donor and recipient correlate with an increased risk of BOS.

Moreover, acute cellular rejection (ACR) depicts a crucial risk factor for BOS. For both A-grade ACR (perivascular inflammatory cell infiltrates) and B-grade ACR (lymphocytic bronchiolitis) association with development of BOS has been shown.

**Humoral Immunity**

There is no conclusive evidence concerning antibody-mediated rejection in lung transplantation. However, presence of serum anti-HLA-antibodies and deposition of complement in alveolar tissue after lung transplantation was shown. Suggesting a role for humoral immunity in acute and chronic rejection of lung grafts, even though the presence of anti-HLA-antibodies is undoubtedly a marker of indirect allorecognition one can only speculate whether or not a specific pathology in lung allografts is directly attributable to donor-specific alloantibodies. However, development of anti-HLA-antibodies posttransplant has been shown to correlate with an increased risk for BOS and worse overall survival. It has been shown in vitro that anti-HLA-antibodies induce activation of airway epithelial cells, stimulation of fibroblast proliferation which ultimately undergo apoptosis. In an in vivo system instillation of anti-HLA-antibodies into the trachea
induces epithelial cell hyperplasia, fibrosis and obliteration of the small airways, a phenotype similar to bronchiolitis obliterans (BO)\textsuperscript{27}.

\textit{Autoimmunity}

Immunologic response to cryptic self-antigens has been reported in lung transplantation\textsuperscript{28}. A concept implying chronic rejection to be biphasic has been suggested\textsuperscript{41}. In the first phase tissue injury occurs and the second phase represents autoimmunity\textsuperscript{41}. Hidden epitopes are exposed resulting from ischemia and reperfusion injury or other insults that damage respiratory epithelium\textsuperscript{41}. Those released self-antigens display triggers for autoreactive T cell proliferation and autoantibody production\textsuperscript{41}. Patients who develop antibodies to the self-antigens col (V) and K-\(\alpha\)1 tubulin are more likely to develop BOS\textsuperscript{28}. Col (V) is expressed in perivascular/-bronchial connective tissue beneath the basement membrane, however, physiologically it is incorporated within collagen I fibrils that protect it from immunological responses\textsuperscript{1,20}.

\textit{Innate Immunity}

Any insult such as infection, aspiration and ischemia-reperfusion injury leads to the propagation of danger signals that activate professional antigen-presenting cells (e.g. dendritic cells) via Toll-like receptors (TLRs)\textsuperscript{42}. Additionally, immune activation via TLRs alleviates transplant tolerance in animal models\textsuperscript{43}. Moreover, TLR signalling is involved in the innate immune response fundamental to PGD and PGD displays an independent risk factor for development of BOS\textsuperscript{1,13}.

3.1.2. \textbf{Diagnosis}

Diagnosis is usually made by clinical, physiological and radiographic parameters\textsuperscript{9}.

3.1.2.1. \textbf{Lung Function}

In 1993 the ISHLT developed a classification based on FEV\textsubscript{1}, as this parameter was considered the most reliable and consistent indicator of chronic transplant rejection\textsuperscript{9}. BOS is characterised by a sustained (≥ 3 weeks) decline of forced expiratory volume in the first second of expiration (FEV\textsubscript{1}), given that other possible causes of pulmonary dysfunction such as acute allograft rejection, anastomotic complications/stricture, disease recurrence or
infection have been excluded. The original classification system from 1993 has been revised and extended in 2001. Baseline FEV₁ is defined as the average of the two highest posttransplant measurements without the use of a bronchodilatator, at least 3 weeks in between. A decline of 20% or more is defined as BOS and progressive BOS stages (I – III) reflect worsening of airflow obstruction. An FEV₁ between 81 and 90% of baseline and/or forced expiratory flow rates (FEF) 25 – 75% less than or equal to 75% of baseline are defined as BOS 0-p (potential BOS) (Table 3-1).

<table>
<thead>
<tr>
<th>2001 Classification according to the ISHLT</th>
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<tbody>
<tr>
<td>BOS 0</td>
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<tr>
<td>BOS 0-p</td>
</tr>
<tr>
<td>BOS I</td>
</tr>
<tr>
<td>BOS II</td>
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<tr>
<td>BOS III</td>
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</tbody>
</table>

Table 3-1: Bronchiolitis obliterans syndrome classification system (adapted from Estenne et al. 2002)

3.1.2.2. Histology

The histological hallmark of BOS is OB. OB describes an inflammatory/fibrotic process affecting small noncartilagenous airways characterised by subepithelial fibrosis causing partial (Figure 3-2) or complete (Figure 3-1) luminal occlusion. Early lesions display lymphocytic inflammation and disruption of the epithelium. Fibro-obliteration is either concentric or eccentric and often associated with atrophy of smooth muscle and destruction of elastic part of the airway wall. In some cases, the only histologic evidence of BOS may be a ring of circumferential elastin around an undetectable airway (“vanishing airways disease”). However, transbronchial biopsy (TBBx) is a rather insensitive method for detecting BOS due to the sporadic and inconsistent involvement of OB, even though mucostasis and/or foamy histiocytes which are typically associated with OB may be seen in TBBx.
3.1.3. Treatment

Treatment options for BOS are generally disappointing\(^1\). Studies usually favour a stabilisation or reduction in the rate of decline of FEV\(_1\), however, an improvement was rarely documented\(^1\). A proven therapy for treatment or prevention of BOS has not been found\(^1\).

3.1.3.1. Immunosuppressive Therapy

An adjustment in immunosuppressant therapy has proven to be beneficial\(^9\). Cairn et al. demonstrated that a switch from cyclosporine to tacrolimus stabilises lung function in patients with BOS\(^{44}\). In a small study Whyte et al. showed that therapy with mycophenolate mofetil induced stabilisation of lung function parameters\(^{45}\).

3.1.3.2. Azithromycin

Azithromycin, a macrolide antibiotic, displays an anti-inflammatory effect, bacterial and nonbacterial antimicrobial beneficial effects and may be beneficial in gastrooesophageal reflux disease\(^{46}\). In a large study Gottlieb et al. demonstrated that therapy with azithromycin improved FEV\(_1\) and reduced airway neutrophilia in patients with BOS\(^{46}\).
3.1.3.3. Extracorporeal Photopheresis

Extracorporeal photopheresis limns a method to treat T-cell dependent inflammatory diseases, including BOS\(^9\). In the late 1990’s two studies reported a stabilisation of airway obstruction in patients suffering from BOS treated with extracorporeal photopheresis\(^{47,48}\).

3.1.3.4. Retransplantation

Lung transplantation depicts an option for selected patients suffering from end-stage pulmonary or pulmonary-vascular disease, such as chronic obstructive pulmonary disease, cystic fibrosis, idiopathic pulmonary fibrosis and idiopathic pulmonary arterial hypertension\(^1,49\). Intention of this treatment option is alleviation of symptoms, improvement of quality of life and survival\(^1\).

Retransplantation displays the only definitive treatment for BOS\(^9\). However, lung transplantation in those patients is regarded controversial as survival rates are lower that of initial transplantation\(^2,9,50\). The incidence of BOS after retransplantation is higher than after initial transplantation\(^50\). Moreover, limited organ availability needs to be considered\(^9\). Nevertheless, survival after retransplantation in BOS is better than survival after retransplantation for early causes of graft failure\(^50\). As survival rates in retransplanted patients with BOS have improved over the last decade, it should be regarded a possible treatment option\(^9\).

3.1.3.5. Prevention

As by the time BOS is diagnosed irreversible damage to the airways may have occurred, one is in need for strategies to prevent BOS\(^1\). Initial prevention and treatment of associated factors depict an important therapeutic concept\(^9\). As mentioned above PGD displays a crucial risk factor for developing BOS, reducing the rate of PGD by decreasing mechanical ventilation time in donors, reducing allograft ischemia time, limiting cardiopulmonary bypass and blood product transfusion during transplantation may affect the risk of development of BOS in later course positively\(^9\). Moreover, monitoring of transplant recipients in order to detect chronic rejection early by routinely performed lung function testing is important, as there seems to be a therapeutic window for several treatment options, e.g. administration
of azithromycin before development of BOS II is associated with a reduction in the risk of death\textsuperscript{9,51}. 
3.2. **Lymphangiogenesis**

The term lymphangiogenesis describes the process of the growth of lymphatic vessels\(^5^2\). In adult individuals it is involved in several pathological processes such as tissue inflammation, acute and chronic transplant rejection and tumour metastasis, however, also occurs in physiological processes e.g. wound healing and during development of the corpus luteum\(^5^2\). In adults lymphangiogenesis happens primarily by sprouting from pre-existing lymphatic vessels, but bone-marrow-derived cells such as macrophages may transdifferentiate into lymphatic endothelial cells as well\(^5^2\).

However, one can find insufficient lymphangiogenesis in patients suffering from lymphedema due to several conditions\(^5^2\).

3.2.1. **Lymphatic System**

The lymphatic system consists of a network of thin-walled vessels and the lymphoid organs\(^5^3,5^4\). Lymph nodes, tonsils, Peyer’s patches, spleen and thymus are referred to as lymphoid organs\(^5^4,5^5\). Prenodal collector vessels drain the capillary networks and transport lymph to regional lymph nodes or directly into lymphatic ducts\(^5^3\).

Lymphatic vessels are referred to as the afferent arm of the immune reflex arc, regional lymph nodes are the central processing unit and blood vessels are named the efferent arm of the immune reflex arc\(^5^6\).

3.2.1.1. **Lymphatic Vessels**

The lymphatic and blood vascular system share many similarities\(^5^4\). They run parallel, but the lymphatic vascular system develops secondarily to the blood vascular system, however, both consist of endothelial cells\(^5^4\). Contrary to the blood vascular system, the lymphatic vascular system is a low flow and low pressure system\(^5^7\).

Lymphatic vessels are found in all vascularised tissues except for bone marrow, the retina and the central nervous system yet there exist some connections between the cerebrospinal fluid and lymphatic vascular system, perivascular spaces in the brain are considered to have a quasi-lymphatic function as they connect to cervical lymph nodes\(^5^2,5^3,5^8\). Especially rich in
lymphatic vessels are the fat-absorbing small intestine and tissues that frequently are in contact with foreign antigens, e.g., the skin and mucous membranes.

Embryology

The “centrifugal” theory implies that lymphatic vessels develop after the cardiovascular system, in humans at about embryonic week 6-7, being equivalent to E9.5-E10.5 in mice, and have their origin from embryonic veins, as the American anatomist Florence Sabin has already stated in 1902. Even though embryonic veins are present at E8.5 lymphatic vessel development starts much later, at about E9.75. This indicates that the presence of veins is necessary, but not sufficient to start lymphatic endothelial cell development. Embryonic veins express vascular endothelial growth factor receptor-3 (VEGFR-3) highly, however, a certain subpopulation of those endothelial cells in large central veins upregulate lymphatic vessel hyaluronan receptor-1 (LYVE-1). Although LYVE-1 is involved in such an early step in the genesis of lymphatic vessels, gene targeting in mice indicates a rather dispensable role in lymphatic development and function. In those LYVE-1 positive cells the transcription factor sex-determining region Y-related high mobility group box 18 (SOX18) is induced which subsequently induces the expression of Prospero-related homeobox protein 1 (Prox1). While Prox1 expression is upregulated in future lymphatic endothelial cells, VEGFR-3 expression is downregulated in blood vessels, an important indication of an blood vascular origin of the lymphatic system. Besides from Prox-1 and VEGFR-3 lymphatic endothelial cells begin to express neuropilin-2 which renders them more responsive to vascular endothelial growth factor-C (VEGF-C), released from lateral mesenchyme. These signals are necessary for sprouting of lymphatic endothelial cells, which form lymph sacs. Prox1 knockout embryos do not form lymph sacs or lymphatic veins, merely budding and unpolarized sprouting from the cardinal vein occurs. Lymphatic endothelial cells begin to express podoplanin which leads to platelet aggregation via activation of spleen tyrosine kinase (Syk) tyrosine kinase in platelets. Subsequently, platelet aggregation leads to separation of the blood and lymphatic vascular system. Further centrifugal growth is driven via the VEGF-C/VEGFR-3 axis (Figure 3-3).

The peripheral lymphatic system develops exclusively from the lymph sacs by sprouting of lymphatic endothelial cells and invasion into surrounding tissues and organs.
Function

Blood vessels act as a circulatory system for distribution of oxygen, nutrients, hormones and cells to tissues as well as to collect carbon dioxide and further metabolic waste products\textsuperscript{52}. As blood pressure causes 20 – 30 l plasma to filtrate from the arterial side of the capillary bed into the interstitial space, but only 90% of the extravasated water will be reabsorbed at the venous side, 10% remain there and need to be returned by lymphatic vessels\textsuperscript{52,53}. Altogether 50% of total plasma protein passes through lymphatics\textsuperscript{53}. This process, also named regulation of tissue fluid, depicts the main function of lymphatic vessels\textsuperscript{52}. Interstitial fluid, macromolecules and cells which are absorbed by lymphatic capillaries are commonly called lymph once they are in the lymphatic vessels\textsuperscript{52}. Lymph consists of most of the
components plasma consists of, although the concentration of high molecular weight components is lower and total protein concentration is about one half that of serum.53

Moreover, lymphatics are crucial in immune defence.53 One the one hand, lymphatic vessels serve as a conduit for extravasated leukocytes and activated antigen-presenting cells, on the other hand, pathogens which invade the body may be taken up together with the interstitial fluid.52,53 As lymph passes one or several lymph nodes before entering the venous system, T and B lymphocytes recognise those pathogens and initiate a specific immune response.53,60 Activated B cells start to proliferate in the lymph nodes and produce antibodies.53 Antibodies and activated cells are delivered into the blood vascular system via efferent lymphatic vessels.53

Another important function of lymphatic vessels is the intestinal absorption and transport of long-chain dietary triglycerides and lipophilic compounds, e.g. fat-soluble vitamins (A, D, E, K), in the form of chylomicrons.53,57,60 This is due to the fact that high molecular weight molecules and colloids are usually absorbed by lymphatics.53

Structure

In contrast to the blood vascular system the lymphatic vascular system is not a circulatory system, lymphatic capillaries start blind-ended and are thin-walled vessels of 30-80 µm in diameter.52,53 They are composed of a single layer of non-fenestrated lymphatic endothelial cells and in contrast to blood vessels lack pericytes and smooth muscle cells and have little or no basement membrane.52,53,58 Because lymphatic capillaries lack smooth muscle cells they have no intrinsic contractility and depend completely on extrinsic forces such as respiratory movement, contraction of muscles and pulsation of adjacent arteries.53 Moreover, they feature an individual gene expression pattern.52 Lymphatic endothelial cells are interconnected by specialised discontinuous button-like junctions that contain few intercellular tight junctions or adherend junctions. As those junctions are loose and display large interendothelial pores lymphatic capillaries represent sites of leukocyte entry.52,54 Moreover, lymphatic capillaries are connected to the extracellular matrix by anchoring filaments, mainly composed of the elastic microfibril-associated protein emilin-1 and fibrillin, which attach to lymphatic endothelial cells via adhesion molecules e.g. αvβ3 integrin.52 Those anchoring filaments attach to collagen fibers, which become tight in case of tissue
swelling, eventuating in opening of the lymphatic vessel lumen, decreased intralumenal pressure and increased uptake of tissue fluid\textsuperscript{52}. Both large interendothelial pores and anchoring filaments render lymphatic capillaries highly permeable\textsuperscript{54}.

Subsequently, smaller lymphatic capillaries drain into pre-collecting lymphatics\textsuperscript{54}. Those eventually merge into larger secondary-collecting lymphatic vessels\textsuperscript{54}. However, lymphatic secondary-collecting vessels possess a layer of smooth muscle cells, so that they can contract, a continuous basal membrane and lymphatic endothelial cells that display continuous zipper-like junctions\textsuperscript{53,54}. Furthermore, directed lymph flow is maintained by valves\textsuperscript{53}. Both, valves and a layer of smooth muscle cells facilitate a flow of lymph against a pressure gradient\textsuperscript{53}. Finally, larger collecting vessels merge into the thoracic duct and right lymphatic duct, the two largest collecting lymphatic vessels which both enter the venous network\textsuperscript{53,54}.

**Lymphatic Vessels in Diseases**

Afferent lymphatic vessels serve as a conduit for soluble antigens and antigen-presenting cells and conduct them from peripheral tissues to lymph nodes or other secondary lymphoid organs\textsuperscript{52}. Lymphatic endothelial cells attract leukocytes expressing C-C chemokine receptor type 7 (CCR7) via producing of its ligand chemokine (C-C motif) ligand (CCL21)\textsuperscript{52}. Lymphangiogenesis typically occurs at sites of tissue inflammation\textsuperscript{52}. It is a crucial mechanism in tissue inflammation as it alleviates tissue edema and enhances immune responses by advancing macrophage and dendritic cell mobilization\textsuperscript{52}. VEGF-C is induced in response to proinflammatory cytokines (e.g. tumour necrosis factor $\alpha$, TNF-$\alpha$) by macrophages and granulocytes\textsuperscript{52}. This mechanism and hence lymphangiogenesis can be blocked with the soluble VEGFR-3-Ig ligand trap\textsuperscript{52}.

Regional lymph node metastasis depicts the first sign of tumour dissemination\textsuperscript{52}. The lymphatic system serves as the primary way for metastatic spread to regional lymph nodes and potentially to distant organs\textsuperscript{53}. The concept of lymphangiogenesis within the tumour or adjacent to it already became widely accepted\textsuperscript{53}. Nevertheless, it is not clear if tumour cells enter the lymphatic system via pre-existing or newly formed lymphatic vessels\textsuperscript{52}. However, lymphangiogenic growth factors produced by tumour cells and tumour-associated macrophages stimulate growth of lymphatic vessels\textsuperscript{52}. 
In opposition to increased lymphangiogenesis in inflammation and tumour metastasis there are several conditions in which the lymphatic transport capacity is impaired due to either abnormal vessel development or obstruction or obliteration of lymphatic vessels. Lymphatic insufficiency results in lymphedema (imbalance of lymph absorption), a disfiguring and disabling disorder which results in swelling of extremities, as proteins and associated water cannot be cart away, resulting in fibrosis, impaired immune responses and accumulation of subcutaneous fat, as the protein-rich interstitial fluid initiates a persistent inflammatory response. On one hand there is primary lymphedema, mostly caused by genetic mutations or of unknown origin, such as Milroy disease, lymphedema-distichiasis or hypotrichosis-lymphedema-telangiectasia syndrome. On the other hand there is secondary lymphedema, responsible for over 99% of all lymphedema cases worldwide, due to damage to the lymphatic vessels caused by filariasis, trauma or bacterial infections. Especially lymphedema due to mastectomy represents probably the most common lymphatic condition in developed countries. However, filariasis, caused by an infection by *Wuchereria bancrofti* or *Brugia malayi*, depicts the most common cause of lymphedema worldwide.

### 3.2.1.2. Lympathic Endothelial Cells

Lymphatic endothelial cells are terminally differentiated cells which differ from blood vascular endothelial cells in particular molecules. This has enabled identification of lymphatic vessels in tissues as well as depicts a target for specific induction or inhibition of lymphatic vessel growth. Such lymphatic specific markers are Prox1, the membrane glycoprotein podoplanin, VEGFR-3 and LYVE-1.

### 3.2.2. Molecular Mechanisms

Whereas angiogenesis, the growth of blood vessels, is a well explored field, molecular mechanisms of lymphangiogenesis have received less attention so far. Nevertheless, the knowledge of molecular mechanisms controlling lymphangiogenesis has improved over the past years and several key players such as Prox1, Sox18, VEGFR-3/VEGF-C, Forkhead-Box-Protein C2 (FoxC2), podoplanin, Syk/lymphocyte cytosolic protein 2 (Slp76 or LCP2), Angiopoietin 2 (Ang2) and ephrin B2 have been identified during the last ten years.
VEGFR-3/VEGF-C/-D Axis

VEGFR-3 is a receptor tyrosine kinase and was one of the first lymphatic endothelial markers to be discovered\textsuperscript{52}. VEGFR-3 is activated by the two lymphangiogenic growth factors VEGF-C and VEGF-D, members of the VEGF family, and is expressed in all endothelia during early development\textsuperscript{52,53}. Mice deficient in VEGFR-3 die from cardiovascular failure, as impaired remodelling and maturation of large vessels occurs\textsuperscript{53}. Endothelial cells which commit to lymphatic endothelial fate express VEGFR-3 highly and as soon as the lymphatic vascular system starts to develop VEGFR-3 expression is restricted to lymphatic endothelial cells, besides from endocrine organs such as the thyroid, the adrenal glands and pancreas\textsuperscript{52,62}.

The two isoforms VEGF-C and VEGF-D are a result of proteolytic splicing\textsuperscript{52}. Both differ from other VEGF family members by the presence of long N- and C- terminal extensions\textsuperscript{53}. The VEGF homology domain of VEGF-C and VEGF-D are 60% identical\textsuperscript{53}. Additional to high affinity binding to VEGFR-3 VEGF-C and VEGF-D can also bind to VEGFR-2 and induce growth of blood vessels\textsuperscript{53,60}. Both VEGF-C and VEGF-D induce proliferation, migration and survival of primary lymphatic endothelial cells\textsuperscript{52,62,63}. Moreover, both induce proliferation of lymphatic endothelial cells in transgenic mice and the chorioallantoic membrane\textsuperscript{53}. During development VEGF-C is expressed in regions of lymphatic vessel development, as previous findings in mice, Xenopus tadpoles and zebrafish have shown VEGF-C seems to be crucial for migration of lymphatic endothelial cells and forming of primary lymph sacs\textsuperscript{64-66}. VEGF-C is also expressed in vascular smooth muscle cells and mesenchyme cells near veins, indicating that VEGF-C controls migration of Prox1 positive lymphatic endothelial cells\textsuperscript{54}. Karkkainen et al. showed that homozygous deletion of Vegfc in mouse embryos lead to a complete absence of a lymphatic vascular system and even Vegfc heterozygous mouse embryos feature cutaneous lymphatic hypoplasia\textsuperscript{64}. Whereas VEGF-C-overexpressing transgenic mice develop lymphatic vessel hyperplasia\textsuperscript{67}. However, absence of VEGF-D does not affect lymphatic development, although overexpression of VEGF-D rescues the phenotype of VEGF-C knockout mice\textsuperscript{64,68}.

Overexpression of the so called “VEGF-C/D Trap” (a soluble VEGFR-3-immunoglobulin G Fc-domain fusion protein) after formation of lymph sacs leads to severe hypoplasia of the lymphatic vessels which indicates strongly that the VEGF-C/VEGFR-3 signalling plays a key
role in further development of the lymphatic vascular tree\textsuperscript{52,69}. The VEGF-C/D Trap or VEGFR-3 blocking by monoclonal antibodies induces regression of already developed lymphatic vessels during the first two postnatal weeks, however, vessel regrow at four weeks of age despite ongoing VEGFR-3 pathway inhibition\textsuperscript{70}. Nonetheless, prolonged inhibition of the VEGF-C/D-VEGFR-3 pathway does not affect adult lymphatic vessels\textsuperscript{52}.

In adult tissue overexpression of VEGF-C or VEGF-D stimulates lymphangiogenesis\textsuperscript{66}. Several growth factors and inflammatory cytokines induce expression of VEGF-C, whereas steroid hormones inhibit its expression\textsuperscript{53}. The expression of VEGF-D may be influenced by hypoxia and cell-cell-contacts\textsuperscript{53}.

3.2.2.2. Prox1

The transcription factor Prox1 is essential for the lymphatic endothelial phenotype, in case of Prox1 knockout or conditional downregulation in embryonic, postnatal or adult stages lymphatic endothelial cells retain their blood vascular endothelial phenotype\textsuperscript{52,71,72}. Moreover, functional knockout of Prox1 in mice the first animal model that completely lacked a lymphatic vascular system\textsuperscript{72}. On the other hand, overexpression of Prox1 in human blood vascular endothelial cells upregulates lymphatic endothelial cell-specific gene expression\textsuperscript{52,71}. This may suggest Prox1 to be a master switch in determination of lymphatic endothelial cell fate\textsuperscript{52}. However, the signals leading the expression of Prox-1 and its target genes in lymphatic endothelial cell are obscure\textsuperscript{60}. Prox-1 is found on embryonic and adult lymphatic endothelial cells, heart, CNS, lens, pancreas and retina\textsuperscript{55}. It is the most specific lineage marker for lymphatic endothelium\textsuperscript{73}.

3.2.2.3. Podoplanin

Podoplanin is a 43 kDa O- and N-glycosylated transmembrane protein being mainly expressed in osteoblastic cells, lung alveolar cells, kidney podocytes, cells of the choroid plexus, keratinocytes and on lymphatic endothelium\textsuperscript{52,74,75}. Podoplanin is critically involved in separation of lymphatic vessels from blood vessels by platelet activation via the tyrosine kinase Syk and adaptor protein Slp78\textsuperscript{76,77}. Platelet aggregation occurs at the separation zone of podoplanin positive lymph sacs, however, in \textit{podoplanin} knockout mouse embryos or in case of pharmacological platelet inhibition separation of the two vascular systems is impaired\textsuperscript{77}. Moreover, podoplanin seems to be involved in connecting deep and superficial
lymphatic vessels, as knockout of podoplanin leads to impaired connections\textsuperscript{75}. Eventually, all lymph sacs lose their connection with the blood vascular system, merely at the jugulo-subclavian junction they re-establish a connection\textsuperscript{53}.

This membrane mucoprotein serves as a highly specific marker for lymphatic endothelial cells\textsuperscript{78}.

3.2.2.4. **LYVE-1**

LYVE-1 is a CD44-related only to the lymphatic endothelium restricted hyaluronate receptor\textsuperscript{79,80}. It is found on embryonic and adult lymphatic endothelium (downregulated in collecting lymphatic vessels), liver, spleen and macrophages\textsuperscript{19,55,81,82}. LYVE-1 might be involved in transporting hyaluronate across the lymphatic vessel wall and in regulation of leukocyte migration throughout the lymphatics\textsuperscript{80}.
3.3. Previous Findings on Lymphangiogenesis in Transplantation

The lymphatic system plays a key role in immune cell trafficking, inflammatory and immune response, this in mind it renders it plausible to expect an effect of the immunologic response and acute or chronic transplant rejection on the lymphatic endothelium\textsuperscript{83,84}. In patients who received an organ graft lymphatic vessels transport antigen presenting cells (APCs) with bound foreign antigens and soluble antigens to regional lymph nodes\textsuperscript{56}.

3.3.1. Lung Transplantation

Dashkevich et al. explored lymphatic vascular density (LVD) in tissue specimens of lung transplants\textsuperscript{83}. LVD was significantly higher in specimens of patients suffering from acute rejection (ISHLT A1/A2), at 14 days after lung transplantation as well as at 90 days after lung transplantation\textsuperscript{83}. Operative procedure disconnects all lymphatic connections of the transplanted lung\textsuperscript{83}. After lymphatic vessels have been connected to regional lymph nodes donor antigens can be presented to the host immune system\textsuperscript{83}. The extent of lymphatic reconnection may therefore represent the immunologic response against the donor organ, indicating a possible explanation of the correlation of LVD and acute graft rejection\textsuperscript{83}. Moreover, edema is associated with acute organ rejection, lymphatic vascular reconnection may be essential for maintenance of fluid homeostasis\textsuperscript{83}.

In a rat model of tracheal transplant the transfer of the VEGF gene increased the number of lymphatic vessels in allograft airway walls\textsuperscript{85}. Administration of a VEGFR protein tyrosine kinase inhibitor resulted in a decrease of lymphatic vessels\textsuperscript{85}. Additionally, a decrease in the number of CD4 and CD8 T cells in airway walls was shown\textsuperscript{85}. This may suggest lymphangiogenesis to enhance antigen presentation of donor tissue to the host immune system, thus increasing alloimmune sensitization, hence leading to rejection or graft injury\textsuperscript{19}.

In a rat model of obliterative airway disease (experimental obliterative bronchiolitis) in rat tracheal allografts induction of lymphangiogenesis in the airway walls by staining for LYVE-1 has already been shown\textsuperscript{86}. In syngrafts a transient increase in lymphangiogenesis was observed, however, in allografts, where persistent alloantigen presentation occurs, lymphangiogenesis was enhanced much more\textsuperscript{86}. The induced lymphangiogenesis was dependent on VEGF-C. Overexpression of VEGF-C induced obliterative airway disease and
lymphangiogenesis, however, blocking of this molecule by administration of VEGFR-3-Ig inhibited both obliterative airway disease and lymphangiogenesis\textsuperscript{86}. Moreover, the induction of lymphangiogenesis could be inhibited by Cyclosporine A in a dose-dependent fashion\textsuperscript{86}.

### 3.3.2. Kidney Transplantation

Acute rejection of kidney grafts is characterised by massive invasion of alloreactive mononuclear recipient cells\textsuperscript{87}. Disappearance of this rejection infiltrate may be due to apoptosis\textsuperscript{87}. However, even such a high apoptosis rate guarantees only insufficient clearance, suggesting lymphatic vessels to serve as exit routes\textsuperscript{87,88}.

Kerjaschki et al. showed massive increase of lymphatic vessels in human kidney grafts\textsuperscript{87}. Lymphatic vascular density increased in patients with nodular and non-nodular infiltrates\textsuperscript{87,89}. In a 28 weeks follow up no difference in median lymph vessel density was observed in renal protocol biopsies\textsuperscript{89}. The presence of lymphatic vessels in infiltrated areas of renal grafts seems to be a weak indicator of superior outcome as lymph vessel density inversely correlated with serum creatinine\textsuperscript{89}.

However, in acute rejection the role of lymphatic vessels is controversial\textsuperscript{89,90}. Whereas Yamamoto et al. could show a four- to ninefold increase in lymphatic vessel density, Stuht et al. did not show a difference\textsuperscript{89,90}. Mononuclear cells were frequently found in lymphatic vessels, reinforcing the hypothesis that lymphatic vessels serve as an exit route for mononuclear cells in acute transplant rejection\textsuperscript{87}. Lymphatic neoangiogenesis may have been promoted by CD68\textsuperscript{+}CD23\textsuperscript{+} macrophages that produce VEGF-C and VEGF-D\textsuperscript{87}. However, it was shown that lymphangiogenesis in renal transplants is driven by division of pre-existent endothelial cells as well as recipient-derived cells that function as lymphatic endothelial progenitor cells\textsuperscript{91}. The origin of these progenitor cells is not clear, yet\textsuperscript{91}. A subpopulation of CD133\textsuperscript{+}VEGFR-3\textsuperscript{+}CD34\textsuperscript{+} cells and CD14\textsuperscript{+}VEGFR-3\textsuperscript{+}CD34\textsuperscript{+}CD31\textsuperscript{+}VEGFR-2\textsuperscript{+} monocytes may contribute to de novo lymphangiogenesis\textsuperscript{91}.

Disturbed lymphatic function may be a contributing factor in development of fibrosis in renal allografts\textsuperscript{92}. Lymphatic vessels have been shown to be increased within the tubulointerstitium in chronically rejected renal grafts characterized by interstitial fibrosis and tubular atrophy\textsuperscript{93}.
3.3.3. **Corneal Transplantation**

Experimental corneal transplantation serves as a model for allogenic transplantation. As the cornea is an avascular and therefore immunologically privileged tissue, it allows the analysis of the impact of lymphatic neoangiogenesis in graft survival.

It was shown that inhibition of hem- as well as lymphangiogenesis promotes corneal graft survival in a murine model. However, inhibition of lymphatic neoangiogenesis is suggested to be more important in alleviation of graft rejection.

3.3.4. **Liver Transplantation**

Lymphangiogenesis is induced by inflammation in acute cellular rejection of allografts in a rat model. As expanded lymphatic vessels are filled with lymphocytes the authors suggested that lymphangiogenesis may be involved in resolution of acute cellular rejection and reduction of inflammation. However, in grafts with severe acute cellular rejection and acute antibody mediated rejection the number of lymphatic vessels decreased in severely rejected areas, but increased in only moderately rejected areas.

3.3.5. **Heart Transplantation**

Disruption of lymphatic vascular structure due to heart transplantation may be crucially attributable to acute graft failure by promoting myocardial fibrosis.

Geissler et al. showed a decrease of VEGFR-3+ lymphatic vessels in patients who had suffered from an episode of acute rejection higher than ISHLT grade IIIa 0.5 months after transplantation. However, at any other point of time no difference was observed.

3.3.6. **Implication of Lymphangiogenesis in Transplantation**

It is suggested that lymphatic vessels are involved in the pathogenesis of transplantation in order to present antigens, traffic immune response and regulate fluid homeostasis and tissue edema. Increased lymphangiogenesis may influence graft survival either in a positive way by promoting inflammatory cell clearance or negatively by promoting antigen presentation in draining lymph nodes and hence facilitating alloimmune response.

Lymphangiogenesis early after transplantation may be beneficial as reconnection of the graft
leads to improved homeostatic removal of interstitial fluid and infiltrating cells. However, later in transplant lifetime lymphangiogenesis may promote formation of lymphoid follicles and chronic injury. Nevertheless, these results emphasise the importance of the lymphatic system in organ transplantation, however, the exact role of lymphangiogenesis – if beneficial or detrimental - in acute and chronic organ rejection remains to be determined.
3.4. **Aims of the Study**

Previous investigations clearly indicate a role for the lymphatic system in chronic lung allograft rejection. However, heretofore there has not been sufficient research. The objective of this study was to investigate if lymphangiogenesis occurs in patients suffering from BOS compared to a control group. If lymphangiogenesis can be detected in BOS patients, we depicted if there was a difference between those groups mentioned above. Additionally, we subdivided bronchiole in sites with or without inflammatory infiltrates. Moreover, we investigated if peribronchial lymph vessel density correlates with the time to BOS III diagnosis.
4. Materials and Methods

4.1. Description of the Study Collective

The ethics committee of the Medical University of Vienna approved the diploma thesis (vote: 1269/2014). Overall 36 patients have been included in this retrospective study, 23 have been assigned to the BOS group and 13 to the control group. Patients who underwent retransplantation due to BOS III at the Department of Thoracic Surgery, Medical University of Vienna, Austria between 2003 and 2013 have been included. Diagnosis of BOS III has been secured by analysis of lung function testing. Clinical data regarding primary transplantation such as type of lung transplantation (LuTX), time from first transplantation to retransplantation (m), mean ischemic time, extracorporeal membrane oxygenation (ECMO) bridging to transplantation, intraoperative ECMO support, time of mechanical ventilation (d), duration of intensive care unit (ICU) stay (d), duration of hospital stay (d), immunosuppression protocol, date of BOS III diagnosis and lung function parameters at the date of BOS III diagnosis have been gathered by examination of patient charts. Patients who underwent surgery (lobectomy to remove N. bronchi) at the Department of Thoracic Surgery, Medical University of Vienna, Austria due to various reasons and of whom healthy lung tissue could be obtained served as a control group for immunohistochemical staining and the evaluation of those. Lung tissue specimens have been provided from the archives of the University Clinic of Pathology, Medical University of Vienna, Austria.

4.2. Immunohistochemical Staining

Formalin-fixed, paraffin-embedded lung tissue specimens have been assessed using the Avidin-Biotin-Peroxidase complex technique. Briefly, paraffin-embedded blocks have been cut in 4µm thick sections with a microtome and melted on slides at 60°C for at least 60 min. Sections have been deparaffinized in HistoSAV, following alcohol in decreasing concentrations and distilled water for re-hydration. For demasking of the antigens, the slides have been put into plastic jars with citrate-buffer pH=6 and have been heated in a microwave oven (Miele Supratronic M752, Wals, Austria). Afterwards the slides have been allowed to cool down for 20 min. After washing them in phosphate buffered saline (PBS) (without the addition of calcium and magnesium), endogenous peroxidase activity has been
quenched with 0.3% H$_2$O$_2$ for about 30 min. The sections have been washed in PBS (without the addition of calcium and magnesium) again. As secondary step, an Anti-IgG mouse Kit (Vector Laboratories, Burlingame, California) has been used, including the components for preparing the normal serum buffer, a biotinylated secondary antibody and the Avidin-Biotin-Peroxidase Complex. Firstly, the slides have been incubated with normal serum, diluted in buffer. After blotting the excess serum from the slides, they have been treated with a podoplanin primary antibody (clone D2-40, Cell Marque Corporation, Rocklin, CA, USA) in a dilution of 1:50. Later, the sections have been washed in PBS (without the addition of calcium and magnesium) and incubated with the biotinylated secondary antibody for 30 min. After washing them again in PBS (without the addition of calcium and magnesium) the Avidin-Biotin-Peroxidase complex reagent have been added and allowed to bind for 30 min. Following a further washing-step with PBS (without the addition of calcium and magnesium), the DAB substrate (Vector Laboratories, Burlingame, California) has been added and the reaction has been visualized under a microscope. After washing in PBS (without the addition of calcium and magnesium) and tap water, the sections have been counterstained with haemalum solution (Mayer’s Haemalum Solution, Merck, Germany), washed with tap water, put in alcohol in increasing concentrations and mounted with a non-aqueous mounting medium by an automated glass coverslipper (Thermo Fisher Scientific, Waltham, MA, USA).

Negative controls have been processed. Stained tissue samples have been digitised by an automated scanning microscope, TissueFAXs (TissueGnostics, Vienna, Austria) provided by the Core Facility Imaging (Priv.-Doz. Marion Gröger, PhD, Medical University of Vienna, Austria) at a magnification level of 20x. Regions of interests have been selected after digitisation using TissueFAXs Viewer version 4.2 software (TissueGnostics, Vienna, Austria).

4.3. **Evaluation of Immunohistological Stainings**

Lymphatic vessels (podoplanin positive) in the peribronchiolar region have been counted and measured as described before\(^99\). The number of lymphatic vessels has been referred to the number of bronchioli and per mm bronchial epithelium (measured lumen-sided). Moreover, cumulative length of all lymphatic vessels in the peribronchiolar region have been measured and referred to mm bronchial epithelium. Infrastructure has been provided by the surgical research facility of the Medical University of Vienna and the Christian Doppler
Laboratory for Cardiac and Thoracic Diagnosis and Regeneration. All measurements have been performed using ImageJ version 1.46r software.

4.4. Statistics

All data are expressed as median, 1st and 3rd quartile or mean ± SD and displayed as box plots (whiskers depict minimum and maximum). As data did not meet requirements for parametric comparison, Mann-Whitney test was applied for comparison of lymphatic vessels per bronchiole, lymphatic vessels per mm bronchial epithelium and μm lymphatic epithelium per mm bronchial epithelium. Correlation of lymphatic vessels per bronchiole with time to BOS III diagnosis was evaluated using Spearman’s rank correlation coefficient. All analyses were performed using IBM SPSS Statistics version 21 software (IBM, Armonk, NY, USA). Graphical representation was performed using GraphPad Prism6 software (GraphPad Software, LA Jolla, CA, USA). A two-sided corrected p-value < 0.05 was regarded as statistically significant.
5. Results

5.1. Initial Evaluation of Demographical Data

In Table 5-1 demographical data including diagnosis, perioperative parameters of primary transplantation, time to BOS III diagnosis and time to retransplantation are shown. Comparing the BOS group to the control group, no significant difference could be detected.

<table>
<thead>
<tr>
<th></th>
<th>BOS</th>
<th>Control</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of infiltrates (with/without)</td>
<td>15/20</td>
<td>3/13</td>
<td>0.0946</td>
</tr>
<tr>
<td>Sex (ma/fe) (%)</td>
<td>12 (52) / 11 (48)</td>
<td>7 (54) / 6 (46)</td>
<td>0.923</td>
</tr>
<tr>
<td>Diagnosis (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1-AT deficiency</td>
<td>1 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>1 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>COPD</td>
<td>5 (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTEPH</td>
<td>1 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>8 (35)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAM</td>
<td>1 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>1 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pulmonary fibrosis</td>
<td>5 (22)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LuTX type (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLuTX</td>
<td>17 (74)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLuTX</td>
<td>6 (26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ischemic time, min (mean ± SD)</td>
<td>303 ± 74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECMO bridging to transplantation (y/n) (%)</td>
<td>2 (11) / 16 (89)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraoperative ECMO support (y/n) (%)</td>
<td>10 (59) / 7 (41)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanical ventilation, d (mean ± SD)</td>
<td>3 ± 2</td>
<td></td>
<td></td>
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<tr>
<td>ICU stay, d (mean ± SD)</td>
<td>8 ± 5</td>
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<tr>
<td>Hospital stay, d (mean ± SD)</td>
<td>24 ± 11</td>
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<tr>
<td>Immunosuppression</td>
<td></td>
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</tr>
<tr>
<td>Cyclosporine A (y/n)</td>
<td>8/13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycophenolic acid (y/n)</td>
<td>19/2</td>
<td></td>
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</tr>
<tr>
<td>Tacrolimus (y/n)</td>
<td>13/8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azathioprine (y/n)</td>
<td>2/19</td>
<td></td>
<td></td>
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<tr>
<td>Time to ReTX, m (mean ± SD)</td>
<td>84.13 ± 51.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to BOS III diagnosis, m (mean ± SD)</td>
<td>62.65 ± 45.28</td>
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</tr>
</tbody>
</table>

Table 5-1: Characteristics of both groups investigated. Abbreviations: ma = male; fe = female; y = yes; n = no; α1-AT deficiency = α1-antitrypsin deficiency; COPD = chronic obstructive pulmonary disease; CTEPH = chronic thromboembolic pulmonary hypertension; LAM = lymphangioleiomyomatosis; DLuTX = double lung transplantation; SLuTX = single lung transplantation; ReTX = retransplantation
5.2. **Lymphatic Vessel Density**

Figure 5-1 and Figure 5-2 depict representative images of peribronchial lymphatic vessels in a patient suffering from BOS (Figure 5-1) and a control subject (Figure 5-2).

Figure 5-1: Lymphatic vessels in the peribronchiolar region of a patient suffering from BOS. Mayer’s haemalum stain. Brown: podoplanin positive lymphatic vessels. 200x magnification.

Figure 5-2: Lymphatic vessels in the peribronchiolar region of a control subject. Mayer’s haemalum stain. Brown: podoplanin positive lymphatic vessels. 200x magnification.
The amount of peribronchiolar lymphatic vessel was statistically equal in patients suffering from BOS as well as in control subjects (median = 4.50, 1<sup>st</sup> quartile = 1.00, 3<sup>rd</sup> quartile = 9.00 vs. median = 4.25, 1<sup>st</sup> quartile = 4, 3<sup>rd</sup> quartile = 5; * p = n.s.) (Figure 5-3).

![Figure 5-3: Lymphatic vessels per bronchiole in peribronchiolar region (p = n.s.). Abbreviations: LVs = lymphatic vessels](image)

Furthermore, we subdivided both groups in bronchiole with inflammatory infiltrates and without inflammatory infiltrates. However, we could not detect a difference between BOS patients and the control group (with infiltrates: median = 7.00, 1<sup>st</sup> quartile = 1.00, 3<sup>rd</sup> quartile = 10.00 vs. median = 7.00, 1<sup>st</sup> quartile = 2.00, 3<sup>rd</sup> quartile = 8.00, p = n.s.; without infiltrates: median = 4.11, 1<sup>st</sup> quartile = 1.40, 3<sup>rd</sup> quartile = 10.00 vs. median = 4.25, 1<sup>st</sup> quartile = 2.67, 3<sup>rd</sup> quartile = 5.00, p = n.s.) (Figure 5-4).

![Figure 5-4: Lymphatic vessels per bronchiole in peribronchiolar region, additional subdivision between bronchiole with and without infiltrates (p = n.s.). Abbreviations: LVs = lymphatic vessels, w/ = with, w/o = without, BOS = bronchiolitis obliterans syndrome](image)
If the number of lymphatic vessels is relativised to mm bronchial epithelium in peribronchial region again no difference between the BOS and control group could be detected (median = 2.302, 1st quartile = 0.417, 3rd quartile 4.980 vs. median = 1.975, 1st quartile = 1.453, 3rd quartile = 2.494; p = n.s.) (Figure 5-5).

Again, we split both groups in sites with or without inflammatory infiltrates and could not detect a difference between the BOS and control group in the number of lymphatic vessels per mm bronchial epithelium (with infiltrates: median = 2.616, 1st quartile = 0.533, 3rd quartile = 5.746 vs. median = 3.020, 1st quartile = 0.456, 3rd quartile = 5.477, p = n.s.; without infiltrates: median = 2.865, 1st quartile = 0.866, 3rd quartile = 4.172 vs. median = 1.722, 1st quartile = 1.113, 3rd quartile = 1.981, p = n.s.) (Figure 5-6).
Additionally, we evaluated the difference of μm lymphatic endothelium per mm bronchial epithelium in peribronchiolar region and could not detect a difference between patients suffering from BOS and the control group (median = 484,984, 1st quartile 176,752, 3rd quartile = 889.536 vs. median = 435.166, 1st quartile = 341.326, 3rd quartile = 752.629; p = n.s.) (Figure 5-7).

Figure 5-7 μm lymphatic endothelium per mm bronchial epithelium in peribronchiolar region (p = n.s.).

Dividing of both groups investigated has been performed here as well. However, μm lymphatic endothelium per mm bronchial epithelium is equal regardless the presence of infiltrates in both groups (with infiltrates: median = 384.717, 1st quartile = 172.353, 3rd quartile = 902.628 vs. median = 496.994, 1st quartile = 226.149, 3rd quartile = 1247.689, p = n.s.; without infiltrates: median = 494.568, 1st quartile = 174.286, 3rd quartile = 1061.667 vs. median = 491.602, 1st quartile = 245.719, 3rd quartile = 657.031, p = n.s.) (Figure 5-8).

Figure 5-8: μm lymphatic endothelium per mm bronchial epithelium in peribronchiolar region, additional subdivision between bronchiole with and without infiltrates (p = n.s.). Abbreviations: w/ = with, w/o = without, BOS = bronchiolitis obliterans syndrome
5.3. **Correlation of Lymphatic Vessel Density with Time to BOS III Diagnosis**

Moreover we investigated if the number of lymphatic vessels in the peribronchiolar region correlates with the time to BOS III diagnosis, however we could not detect a significant correlation ($r = 0.1748, p = \text{n.s.}$) (Figure 5-9).

![Figure 5-9: Correlation of peribronchiolar lymphatic vessels with time to BOS III diagnosis ($r = 0.1748, p = \text{n.s.}$). Abbreviations: LVs = lymphatic vessels, m = months](image)
6. Discussion

Recent findings have indicated a role for lymphangiogenesis in acute and chronic transplant rejection in kidney, cornea, liver, heart and lung transplantation\textsuperscript{56,83,85-87,89,91,95-98}. In experimental corneal transplantation, a common model for allogenic transplantation, it was shown that inhibition of lymphneoangiogenesis promotes graft survival\textsuperscript{56}. Furthermore, in kidney transplantation the role of lymphangiogenesis concerning acute graft rejection is still controversial\textsuperscript{87,89,90}. There has already been research concerning lymphangiogenesis in acute and chronic rejection of lung transplants\textsuperscript{83,85}. Dashkevich et al. observed an increased lymphatic vessel density in patients suffering from acute rejection\textsuperscript{83}. Lymphangiogenesis in chronic lung allograft rejection has only been studied in a rat obliterative airway disease model\textsuperscript{86}. Consequently, one can conclude from previous work that there has not been sufficient investigations on lymphangiogenesis in human chronic lung allograft rejection. Even though it might display a potential target in prognosis, diagnosis and therapy of organ rejection.

Hence, in this study we investigated the relationship between lymphangiogenesis, assessed by immunohistochemical staining for podoplanin, a reliable marker for lymphatic endothelial cells, and by lung function testing verified BOS stage III\textsuperscript{87}. The results show that neither the amount of peribronchiolar lymphatic vessels per bronchiole or mm bronchial epithelium nor the length of peribronchiolar lymphatic endothelium per mm bronchial epithelium differs in patients suffering from BOS III compared to control subjects. Since Stuht et al. have observed an increased lymphatic vessel density in renal transplants at sites of inflammatory infiltrates\textsuperscript{89}, we have performed subdivision of both groups in bronchiole with and without infiltrates. Nonetheless, in neither of the parameters investigated we could observe a change in lymphangiogenesis accompanied by inflammatory infiltrates. As the lymphatic system plays a key role in immune cell trafficking and immune response we hypothesised that the time to BOS III diagnosis may correlate with LVD, however, this is not the case\textsuperscript{84}.

All lymphatic connections of the transplanted lung are disrupted due to the operative procedure\textsuperscript{83}. It takes up to 7 to 14 days to re-establish new connections after transplantation between graft lymphatics and regional lymph nodes which may be an explanation for an
increased LVD in acute rejection. However, we received specimens from the date of retransplantation (mean ± SD: 84.13 ± 61.76 months after primary transplantation), this may be the reason for an equal LVD in both BOS patients and control subjects.

Sato et al. have demonstrated increased de novo lymphoid tissue in OB. This may indicate increased lymphangiogenesis in BOS. Dashkevich et al. showed an increment of lymph vessels 14 days after transplantation in patients suffering from acute rejection, however, the number of lymph vessels did not further increase by day 90. A possible explanation for increased lymphoid neogenesis, however, constant lymphangiogenesis in BOS may be that lymph vessels formed early are sufficient to support development of lymphoid later in graft lifetime.

Besides from its role in immune defence lymphatic vessels are important in tissue homeostasis. Tissue oedema displays a common finding in acute lung allograft rejection, however not in BOS. The lack of tissue oedema in chronic lung allograft rejection may explain why we could not find an alteration in lymphangiogenesis in patients suffering from BOS stage III.

The influence of macrophages on adult lymphangiogenesis is a widely accepted theory. Lymphangiogenesis is associated with chronic inflammation, a process particularly mediated by macrophages. Many established pro-lymphangiogenic factors are either inflammatory cytokines themselves or downstream products of inflammatory pathways which are activated by transcription factors of the nuclear factor-kappaB (NF-κB) family. VEGF-C, a NF-κB-transcribed gene stimulates lymphangiogenesis in a direct way. Macrophages themselves can release VEGF-C and other products that contribute to the formation of new lymphatic vessels. Additionally, an inflammation-mediated increase in VEGFR-3 on existing lymphatic vessels is required for responsiveness to VEGF-C. Besides from contribution by release of paracrine factors macrophages can transdifferentiate into lymphatic endothelial cells themselves and incorporate into the growing lymphatic vasculature. As in this study the distribution of bronchiole with and without inflammatory infiltrates is equal in patients suffering from BOS as well as in control subjects, it may be suggested that the inflammatory situation is similar in both groups which could explain the equal distribution of LVD in both groups. Tissue samples in the control group have been
obtained from patients who have been operated to resect lung cancer, as obtaining tissue from healthy subjects was not possible. Their underlying disease may be a confounder by attributing to lymphangiogenesis, even though the evaluated sample was free of potential pathological characteristics usually featured by the patients’s primary disease.

Furthermore, it is important to take into account that the interaction between the immune response and the microvasculature systems (both capillaries and lymphatic vessels) may be altered by an immunosuppressive therapy regime\textsuperscript{103}. Cyclosporine, a calcineurin inhibitor and commonly used maintenance immunosuppressive agent, features an inhibitory effect on VEGF through the calcineurin-pathway\textsuperscript{103,104}. Even though Krebs et al. showed an increase of LYVE-1\textsuperscript{+} cells in a rat model of obliterative airway disease, this effect is reversed by administration of Cyclosporine A in a dose dependent fashion\textsuperscript{86}. A possible explanation for this effect may be the inhibition of nuclear factor of activated T cells 1 (NFATc1), which is known to regulate lymphangiogenesis, by Cyclosporine A\textsuperscript{105}. As the inductive immunosuppressive therapy in eight of our patients in the BOS group contained Cyclosporine A a biasing effect of this therapy cannot be excluded. Information on the ongoing immunosuppressive therapy regime is missing which clearly depicts a limitation on the impact of the results we obtained. Moreover, one needs to keep the limitation of the rat obliterative airway disease in mind\textsuperscript{86}. The investigated tissue is the trachea and not non cartilaginous bronchioles which are affected in BOS\textsuperscript{86}. Additionally, there is more lymphoid tissue in a lung allograft than in the trachea\textsuperscript{86}. Both arguments need to be considered when this study is assessed in synopsis with the results obtained in this work. Nevertheless, these contradictory outcomes suggest extended research should be performed.

In summary, the lymphatic system plays an important role in acute and chronic rejection of several solid organs, however, in patients suffering from BOS we could not detect a difference compared to control subjects. Alterations in lymphangiogenesis in acute as well as in chronic transplant rejection have been thought to display on one hand a new aspect in diagnosis and prognosis of transplant recipients and on the other hand a novel therapeutic target\textsuperscript{87,89}. Undoubtedly, this study features several limitations. The number of patients is rather small which is attributable to the low prevalence of BOS. Moreover, the retrospective study design leads to incomplete clinical data. Nevertheless, this study displays a proof of concept. Although the underlying pathophysiologic mechanisms of lymphangiogenesis in
acute and chronic transplant rejection remain to be further investigated, we hypothesize the strict obligatory immunosuppression therapy in lung transplant patients to be at least partially responsible for equal LVD in both groups investigated.
7. References


## 8. Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>α1-ATdeficiency</td>
<td>α1-antitrypsin deficiency</td>
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<td>ACR</td>
<td>acute cellular rejection</td>
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<tr>
<td>Ang2</td>
<td>angiopoitin 2</td>
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<td>APC</td>
<td>antigen presenting cells</td>
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<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
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<td>BOS</td>
<td>bronchiolitis obliterans syndrome</td>
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<tr>
<td>CCL21</td>
<td>chemokine (C-C motif) ligand 21</td>
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<td>CCR7</td>
<td>C-C chemokine receptor type 7</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CLAD</td>
<td>chronic lung allograft dysfunction</td>
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<td>CMV</td>
<td>cytomegalovirus</td>
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<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
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<td>CTEPH</td>
<td>chronic thromboembolic pulmonary hypertension</td>
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<tr>
<td>CTL</td>
<td>cytotoxic T-lymphocyte</td>
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<td>d</td>
<td>day</td>
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<td>DLuTX</td>
<td>double lung transplantation</td>
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<td>DTH</td>
<td>delayed type hypersensitivity</td>
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<td>EBV</td>
<td>Epstein-Barr virus</td>
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<td>ECMO</td>
<td>extracorporeal membrane oxygenation</td>
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<td>fe</td>
<td>female</td>
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<tr>
<td>FEF</td>
<td>forced expiratory flow rates</td>
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<td>FEV₁</td>
<td>forced expiratory volume in the first second of expiration</td>
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<td>FoxC2</td>
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<td>h</td>
<td>hour</td>
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<td>HLA</td>
<td>human leukocyte antigen</td>
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<td>ICU</td>
<td>intensive care unit</td>
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<td>IL</td>
<td>interleukin</td>
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<td>interferon-γ</td>
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<tr>
<td>ISHLT</td>
<td>International Society for Heart and Lung Transplantation</td>
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y  yes
9. Register of Illustrations and Tables

9.1. Tables

Table 3.1-1: Bronchiolitis obliterans syndrome classification system (adapted from Estenne et al. 2002)

Table 5.1-1: Characteristics of both groups investigated. Abbreviations: ma = male; fe = female; y = yes; n = no; α1-AT deficiency = α1-antitrypsin deficiency; COPD = chronic obstructive pulmonary disease; CTEPH = chronic thromboembolic pulmonary hypertension; LAM = lymphangioleiomyomatosis; DLuTX = double lung transplantation; SLuTX = single lung transplantation; ReTX = retransplantation

9.2. Figures

Figure 3-1: Complete fibrous obliteration of small bronchiole with residual elastic layer and atrophied smooth muscle (hematoxylin and eosin stain; original magnification x400) (adapted from Weigt SS et al. 2013)

Figure 3-2: Partial obliteration of bronchiole with mononuclear cell infiltration in subepithelial fibrosis (combined Masson trichrome and elastic van Gieson stain; original magnification x40). (adapted from Weigt SS et al. 2013)

Figure 3-3: Development of the Mammalian Lymphatic Vasculature (adapted from Tammela et al. 2010)

Figure 5-1: Lymphatic vessels in the peribronchiolar region of a patient suffering from BOS. Mayer’s haemalum stain. Brown: podoplanin positive lymphatic vessels. 200x magnification.

Figure 5-2: Lymphatic vessels in the peribronchiolar region of a control subject. Mayer’s haemalum stain. Brown: podoplanin positive lymphatic vessels. 200x magnification.

Figure 5-3: Lymphatic vessels per bronchiole in peribronchiolar region (p = n.s.). Abbreviations: LVs = lymphatic vessels
Figure 5-4: Lymphatic vessels per bronchiole in peribronchiolar region, additional subdivision between bronchiole with and without infiltrates (p = n.s.). Abbreviations: LVs = lymphatic vessels, w/ = with, w/o = without, BOS = bronchiolitis obliterans syndrome

Figure 5-5: Number of lymphatic vessels per mm bronchiolar epithelium in peribronchiolar region (p = n.s.). Abbreviations: LVs = lymphatic vessels

Figure 5-6: Number of lymphatic vessels per mm bronchiolar epithelium in peribronchiolar region, additional subdivision between bronchiole with and without infiltrates (p = n.s.). Abbreviations: LVs = lymphatic vessels, w/ = with, w/o = without, BOS = bronchiolitis obliterans syndrome

Figure 5-7 μm lymphatic endothelium per mm bronchial epithelium in peribronchiolar region (p = n.s.).

Figure 5-8: μm lymphatic endothelium per mm bronchial epithelium in peribronchiolar region, additional subdivision between bronchiole with and without infiltrates (p = n.s.). Abbreviations: w/ = with, w/o = without, BOS = bronchiolitis obliterans syndrome

Figure 5-9: Correlation of peribronchiolar lymphatic vessels with time to BOS III diagnosis (r = 0.1748, p = n.s.). Abbreviations: LVs = lymphatic vessels, m = months
10. Curriculum vitae

Personal Background

Nationality: Austrian
Date of birth: 25th of May, 1993
Family status: Single

Education

1999 – 2003 Primary School, Vienna, Austria
2003 – 2007 Sportreagymnasium, Vienna, Austria
2007 – 2011 Sir-Karl-Popper-Schule at the Wiedner Gymnasium, Vienna, Austria
(High School)
2011/06 High School Graduation
2011/10 - present Medical Student at the Medical University of Vienna, Austria
2011/12 - present Student Research Fellow at the Christian Doppler Laboratory for Diagnosis and Regeneration of Cardiac and Thoracic Diseases, Medical University of Vienna, Austria

Clinical Training

2012/07 Clinical Clerkship at the Department of Cardiac Surgery, Medical University of Vienna, Austria (4 weeks)
2013/08 Clinical Clerkship at the Department of Internal Medicine, Wilhelminenspital, Vienna, Austria (4 weeks)
2013/09 Clinical Clerkship at the Department of Thoracic Surgery, Medical University of Vienna, Austria (4 weeks)
2014/07  Clinical Clerkship at the Department of Surgery, Diakonissen Linz, Austria (1 week)

2015/04  Clinical Clerkship at the Department of Trauma Surgery, Medical University of Vienna, Austria (2 weeks)

**Continuing Education**

2011/10  Breaking Bad News Kommunikationstraining
Breaking Bad News Communication Training, Medical University of Vienna, Austria

2011/10  Wissenschaftliche Methodenlehre in der immunologisch chirurgischen Forschung
Scientific Methodology in Immunological Surgical Research, Medical University of Vienna, Austria

2011/10  PhD and Thesis Seminar in Cardiac and Thoracic Surgery Research, Medical University of Vienna, Austria

since 2011/10 JC Current Topics in Applied Immunology, Medical University of Vienna, Austria

since 2011/10 TS Applied Immunology and Tissue Regeneration, Medical University of Vienna, Austria

2013/02  Biometrie I: Beschreibung und Visualisierung medizinischer Daten
Biometry I: Description and Visualization of medical data, Medical University of Vienna, Austria

2013/11  Biometrie II: Statistische Tests und Lebensdaueranalyse bei Medizinischen Fragestellungen
Biometry II: Statistical Tests and Analysis of Survival in Medical Research, Medical University of Vienna, Austria
2014/10  Anästhesiologie und Intensivtherapie bei Lebertransplantation
Anaesthesiology and Intensiv-Care Medicine in Liver Transplantation, Medical University of Vienna, Austria

2014/10  Dissertantenseminar: Durchflusszytometrie - Methodik und immunologische Anwendungsmöglichkeiten
Thesis Seminar: Flow Cytometry – Methods and Immunological Applications, Medical University of Vienna, Austria

2015/04  Einführung in die Molekular- und Zellbiologischen Arbeitsmethoden der Dermatologie
Introduction into the Molecular and Cellbiological Work Methods in Dermatology, Medical University of Vienna, Austria

2015/04  Methodenseminar: Klinische Epidemiologie: Systematischer Review und Metaanalyse
Methods Seminar: Clinical Epidemiology: Systematic Review and Metaanalysis, Medical University of Vienna, Austria

Awards and Grants

since 2013/01 Research Scholarship of the Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Medical University of Vienna, Austria

2013/05  Research Scholarship of the Medical University of Vienna, Austria

2014/05  Research Scholarship of the Medical University of Vienna, Austria

2014/10  Best Poster Price in 23rd Annual Conference of the German Society of Thoracic Surgery

Teaching Activity

2013/09 - present  Teaching Assistant at the Department of Administration, Problem-based learning, Medical University of Vienna, Austria
2014/09 - present  Teaching Assistant at the Department of Anaesthesiology, Intensive Care and Pain Management, Medical University of Vienna, Austria

**Congress and Meetings**

2013/06  53rd Annual Meeting of the Austrian for Surgery, Salzburg, Austria

2014/03  Symposium Management of Laryngotracheal Problems, Vienna, Austria

2014/03  4th EACTS Meeting on Cardiac and Pulmonary Regeneration and Stem Cell Technology, Bern, Switzerland

**Original Articles (peer-reviewed)**


**Stromal Expression of Heat-Shock Protein 27 Is Associated with Worse Clinical Outcome in Patients with Colorectal Cancer Lung Metastases**


**Secretomes of apoptotic mononuclear cells ameliorate neurological damage in rats with focal ischemia**


**Antimicrobial peptides are highly abundant and active in postoperative pleural drainage fluids.**

*Ann Thorac Surg.* 2014 Sep  

IF: 3.534

IF: 3.631

**Carbonic anhydrase IX is associated with early pulmonary spreading of primary colorectal carcinoma and tobacco smoking.**

*Eur J Cardiothorac Surg. 2014 Jul*  
IF: 2.674

**Abstracts**

Hoetzenecker K, Hochdanninger M, Traxler D, Mitterbauer A, Schweiger T, Hegedüs B, Klepetko W, Ankersmit HJ, Mildner M

**Antimicrobial peptides are highly abundant and active in postoperative pleural drainage fluids**

*Meeting of the Austrian, German and Swiss Society of Thoracic Surgery, Basel, Switzerland*


**Heat-shock proteins 27 and 70 in primary colorectal cancer and corresponding pulmonary metastases**

*Meeting of the Austrian, German and Swiss Society of Thoracic Surgery, Basel, Switzerland*

T Schweiger, D Kollmann, C Nikolowsky, D Traxler, E Guenova, G Lang, P Birner, W Klepetko, HJ Ankersmit, K Hoetzenecker

**Early pulmonary spreading of primary colorectal carcinoma is associated with carbonic anhydrase IX expression and tobacco smoking**

*Meeting of the Austrian, German and Swiss Society of Thoracic Surgery, Basel, Switzerland*


**Heat-shock proteins 27 and 70 in primary colorectal cancer and corresponding pulmonary metastases**

*ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria*

**Pulmonary Metastasectomy at the Department of Thoracic Surgery, MUV, 2009-2013 – A computer-based prospective database**

*ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria*


**Impact of pulmonary metastasectomy on lung function parameters**

*ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria*

T Schweiger, D Kollmann, C Nikolowsky, D Traxler, E Guenova, G Lang, P Birner, W Klepetko, HJ Ankersmit, K Hoetzenecker

**Early pulmonary spreading of primary colorectal carcinoma is associated with carbonic anhydrase IX expression and tobacco smoking**

*ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria*

T Schweiger, D Kollmann, C Nikolowsky, D Traxler, E Guenova, G Lang, P Birner, W Klepetko, HJ Ankersmit, K Hoetzenecker

**Early pulmonary spreading of primary colorectal carcinoma is associated with carbonic anhydrase IX expression and tobacco smoking**

*130th Annual Congress of the German Society of Surgery, Munich, Germany*  
*Abstract published in: Langebeck’s Archives of Surgery, Volume 398(4), 2013*

T Schweiger, D Kollmann, C Nikolowsky, D Traxler, E Guenova, G Lang, P Birner, W Klepetko, HJ Ankersmit, K Hoetzenecker

**Early pulmonary spreading of primary colorectal carcinoma is associated with carbonic anhydrase IX expression and tobacco smoking**

*36th Seminar of the Austrian Society for Surgical Research, Vienna, Austria*  
K Hoetzeenecker, T Schweiger, M Mildner, C Plass, D Traxler, M Lichtenauer, M Gyöngyösi, HJ Ankersmit

**Secretome of apoptotic peripheral blood cells (APOSEC) induces coronary vasodilation:**
**Impact on microvascular obstruction during acute myocardial infarction**
53. Österreichischer Chirurgenkongress 2012, Salzburg, Austria

**Scientific Excursions**

2013/07/10-12 Kaposvar, Hungary, APOSEC study

**Methods**

Cultivation of human cell lines
Immunohistochemistry
ELISA