Bronchiolitis Obliterans Syndrome after lung transplantation: biomarkers for inflammation and fibrogenesis

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Bronchiolitis Obliterans Syndrome after lung transplantation: biomarkers for inflammation and fibrogenesis

Het bronchiolitis obliterans syndroom na longtransplantatie:
biomarkers voor inflammatie en fibrogenese.

(met een samenvatting in het Nederlands)

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CHAPTER 1

GENERAL INTRODUCTION

1. Bronchiolitis obliterans syndrome after lung transplantation

Lung transplantation is a treatment for carefully selected patients with end-stage lung disease. For the majority of recipients, the procedure is intended to alleviate symptoms and to improve quality of life and survival. Rates of graft failure, rejection and mortality after lung transplantation, however, exceed most other solid organ transplants.[1] Especially, the development of bronchiolitis obliterans syndrome (BOS), a manifestation of chronic lung allograft rejection, remains the major limiting factor to long-term survival after lung transplantation.[2] In addition to its impact on long-term survival, BOS causes significant morbidity, impairs quality of life and increases costs.[3]

By the time BOS is diagnosed by a decline in lung function, for the majority of patients the process of inflammation, fibrosis and obliteration of the small airways is already at an advanced and mostly irreversible stage to effectively reverse this decline.

1.1 History

James Hardy and his team performed the first lung transplantation in a human in 1963 at the University of Mississippi Medical Center.[4] From 1963 until 1978, multiple attempts at lung transplantation failed because of rejection and problems with anastomotic bronchial healing. After the invention of the heart-lung machine and the development of immunosuppressive drugs, lungs could be transplanted with a reasonable chance of success.

In 1984, Burke et al, Stanford University, first described the development of obliterative bronchiolitis (OB) in recipients of heart-lung transplants who showed a progressive decline in forced expiratory volume in one second (FEV_1).[5] Lung biopsies of these patients revealed intraluminal polyps of granulation tissue that led to obliteration and fibrosis of the terminal bronchioles.

OB is difficult to document histologically, because of the patchy distribution. Therefore, a committee sponsored by the International Society of Heart and Lung Transplantation (ISHLT) formulated in 1993 the still actual definition of BOS. This committee aimed to provide a classification system for airway diseases after lung transplantation that did not rely on histopathologic findings because of the above mentioned difficulties. This classification system relied on tests that are available to all lung transplant physicians and that are relatively simple to understand and to apply. In this system the FEV_1 is the primary parameter.[6] BOS is defined by a decline in FEV_1 of ≥ 20% without the presence of other causes of functional decline such as infection and anastomotic problems (see also paragraph 3).[6]

1.2 Clinical features

The clinical onset of BOS is often nonspecific and consists of a gradual development of dyspnoea on exertion, often accompanied by a chronic productive cough. Few patients have an acute presentation with an initial episode of acute rejection or infection.

In the study by the Papworth group, 18% of 204 patients developed BOS following a smooth linear decline in FEV_1, whereas 56% showed a sudden drop in FEV_1.[7] Compared with those who had a slow linear decline in lung function, patients with a sudden drop in FEV_1 had a poor prognosis.[7] In Figure 1 the course of FEV_1 over time in two lung transplant recipients of our transplant centre is shown. The different BOS stages (BOS 1 to BOS 3) correspond with the magnitude of decrease in FEV_1.

When the disease progresses, permanent airway colonization with pathogens, such as Pseudomonas aeruginosa and Aspergillus fumigatus frequently develops, leading to increased sputum production and cough.[8]

The interval between transplantation and the onset of BOS may range from a few months to several years with a median time to diagnosis between 16 to 20 months.[9] BOS is unusual within the first three months after transplantation.[10] After the onset of BOS the median survival is three to four years.[3]

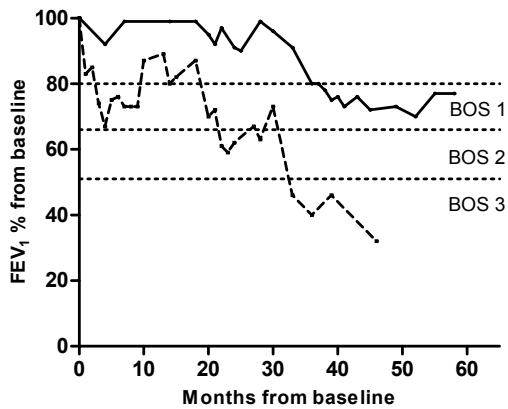

Figure 1. Changes in pulmonary function over time in two patients with BOS. The FEV_1 is presented as a percentage of the post-transplant baseline value.

1.3 Epidemiology

1.3.1. Worldwide

In 2008, nearly 3000 lung transplantation procedures per year were reported worldwide. Although chronic obstructive pulmonary disease (COPD) is still the major indication for lung transplantation (35%), idiopathic pulmonary fibrosis (IPF) now accounts for 22% of the pre-transplant diagnoses, closely followed by cystic fibrosis (CF; 16%). Bilateral lung transplantation accounted for 71% of the transplant procedures.[2]

The survival after lung transplantation remains limited. Survival rates were reported to be 79% at 1 year, 52% at 5 years, and 29% at 10 years.[2] The major causes of death within the first year after transplantation are graft failure and non-cytomegalovirus (CMV) infections. After

the first year after transplantation, the most common causes of death are BOS (20-30%) and non-CMV infections (16-23%). In a large cohort of patients 49% developed BOS after 5 years and 75% after 10 years.[2]

1.3.2. The Netherlands – Utrecht/Nieuwegein

In September 1989, the first unilateral lung transplantation in The Netherlands was performed in the St Antonius Hospital, Nieuwegein, under supervision of professor J.M.M. van den Bosch.[11] This was followed by the first bilateral lung transplantation in 1990. In 1991, after a decision of the Dutch Health authorities, the University of Groningen started a lung transplantation program.[12] From 2001, the University Medical Centre of Utrecht (UMCU) in cooperation with the St Antonius Hospital and the Erasmus University Medical Centre, also started a lung transplantation program after permission was given by the Dutch Health authorities.

In the period from July 2001 until November 2010 184 patients (female:male = 93:91) were transplanted. The major indications for lung transplantation in this cohort were CF (31%) and COPD (30%) followed by alpha-1 antitrypsin deficiency (11%) and IPF (10%). The high percentage CF, as indication for lung transplantation, can be attributed to the CF Centre of Utrecht, which cares for a considerable number of CF patients with end-stage lung disease. Bilateral transplantation occurred in 79% of the procedures.

After 9 years of transplantation, the 1-year survival rate after lung transplantation is 84% and 69% at 5 years. The survival rate at 5 years is slightly higher compared with the survival rate worldwide, which may be attributed to the use of basiliximab as induction therapy, the intensive outpatient monitoring after transplantation and the regulatory measurement of the Epstein-Barr virus (EBV) and CMV viral load followed by treatment when necessary. Approximately 15% of the lung transplant recipients developed BOS with a mean BOS-free survival of two years.

2. Pathogenesis

The development of BOS is a multifactorial process and is considered to be the consequence of alloimmune-dependent and alloimmune-independent risk factors that cause injury to the airway epithelium (Table 1).

Table 1. Alloimmune-dependent and alloimmune-independent risk factors for the development of bronchiolitis obliterans syndrome.

Alloimmune-dependent risk factors	Alloimmune-independent risk factors
HLA incompatibility	Bacterial and viral (CMV) infections
Anti-HLA antibodies	Primary graft dysfunction
Acute rejection	Chronic ischemia
Lymphocytic bronchiolitis	Gastroesophageal reflux disease
	Medication noncompliance

2.1 Risk factors

2.1.1 Alloimmune-dependent

The presence of human leukocyte antigen (HLA) incompatibility, due to mismatches between donor and recipient, as a risk factor for BOS is controversial.[8] The largest series from the United Network for Organ Sharing/ISHLT registry, which included more than 3500 patients, found no association between HLA mismatch and the development of BOS.[8, 13] The lack of association is probably related to the fact that very few patients have two or less HLA mismatches because no attempt at HLA matching is made prior to lung transplantation due to the short ischemic time tolerated by lungs.[14]

Anti-HLA antibodies have been associated with a worse outcome in all solid-organ transplants. Anti-HLA antibodies preexisting to the transplant procedure may expose the patient to the risk of acute rejection.[8] Several studies have shown that development of anti-HLA antibodies after surgery is associated with the development of BOS.[15-17] Binding of these antibodies to the airway epithelium may induce injury and proliferation of the airway epithelial cells.[8]

Acute rejection (AR), characterized by perivascular infiltration which may extend into the alveolar space, has been identified in many studies as an important independent risk factor for BOS.[8, 18, 19] The risk for BOS increases when the acute rejection is histologically severe or when it persists or recurs after treatment.[14]

Lymphocytic bronchiolitis (LB), is a precursor of BOS and is characterized by lymphocyte infiltration and inflammation of the bronchioles but without luminal obliteration.[3] The presence of LB, and especially the severity, is also an important risk factor for the development of BOS.[20, 21]

2.1.2 Alloimmune-independent

The transplanted lung is a vulnerable organ because it is consistently exposed to exterior factors, such as microorganisms and its toxins, gastroesophageal reflux and processes related to organ procurement and transplantation, which may all cause local inflammation and activation of the innate immune system. The innate immunity appears to be an important co-factor linking alloimmune-independent mechanisms of lung injury to alloimmune responses.[1]

CMV infections have demonstrated to be associated with BOS development.[18, 22, 23] A regimen of prolonged ganciclovir or valganciclovir prophylaxis decreased the rate of active CMV infection and disease, reduced the incidence of BOS, and improved the survival rate.[24]

Bacterial and viral infections have shown to play a role in the development of BOS. Recent evidence suggests that pneumonias with gram-positive, gram-negative and fungal pathogens occurring prior to BOS were independent determinants of chronic allograft dysfunction.[25]

Pulmonary ischemia and cold-ischemic storage, that occurs during the time interval between organ procurement and transplantation and allograft reperfusion, incite multiple

inflammatory pathways that promote primary graft dysfunction (PGD).[14, 26] Although, the onset of PGD occurs within 48 hours after surgery with resolution in most patients by the first week, it is an independent risk factor for the development of BOS.[27, 28] A mechanism for this association might be the increased pro-inflammatory chemokines and cytokines that were noted in patients with PGD at variable time points up to six months after transplantation.[29]

Chronic ischemia due to interruption of the bronchial artery supply after re-implantation of the graft is a potential facilitator of subsequent small airway injury. However, the fact that bronchial artery revascularization at the time of surgery has not significantly reduced the development of BOS argues against the role of chronic airway ischemia as risk factor for the development of BOS.[30]

The incidence of gastroesophageal reflux disease (GERD) is increased by the transplant procedure. Along with a denervated lung, GERD likely increases the risk of aspiration and airway injury, as shown by the presence of increased levels of bile acids and pepsin in bronchoalveolar lavage (BAL) samples.[31, 32] A retrospective study about gastric fundoplication after lung transplantation showed greater freedom of BOS which supports the role of GERD in the development of BOS.[33]

Medication noncompliance may also represent an important, but often underestimated, risk factor for chronic rejection.[14] Several additional factors have been proposed as risk factors for BOS, but convincing data to support their role is lacking. These factors include a history of smoking or asthma of the donor, increased donor age and primary pulmonary hypertension as recipient primary disease.[14]

2.2 Mediators of the pathogenesis of BOS

The development of BOS represents a final common pathway, in which various insults (alloimmune-dependent and alloimmune-independent) can lead to a similar histological result.[34]

Activation of the innate immune system, via Toll-like receptors, leads to the release of cytokines that are able to activate antigen-presenting cells leading to optimized antigen presentation to T-lymphocytes. This process leads to an increased alloantigen expression by the graft and to the potential triggering of the adaptive immunity (Figure 2).[35]

Critical to airway wound repair is a delicate balance between pro- and anti-inflammatory cytokines. Changes in this balance can influence allograft airway repair and remodeling. The specific mechanisms that lead to the fibro-obliteration of allograft airways during BOS may involve an imbalance between T-helper (Th)1 and Th2 cytokines.[3] Th1 cytokines, such as interleukin (IL)-2, IL-12, interferon (IFN)-gamma, are mainly associated with cell-mediated immunity. These Th1 cytokines have been associated with rejection and BOS.[1]

While evidence suggests that rejection is strictly a Th1 response, others indicate that Th2 responses also promote rejection, especially chronic rejection.[36, 37] Th2 cytokines, IL-4, IL-5, and

IL-13 are involved in mucosal, allergic and humoral immunity, but also have distinct roles in the regulation of tissue remodeling and fibrosis.[38]

Profibrotic cytokines, such as transforming growth factor (TGF)-β, platelet derived growth factor (PDGF) and insulin-like growth factor (IGF), elicit attraction and proliferation of fibroblasts, leading to extracellular matrix (ECM) deposition, proliferation of smooth muscle cells and fibrosis. Especially, TGF-β is known for its role in the development of fibrosis.[38]

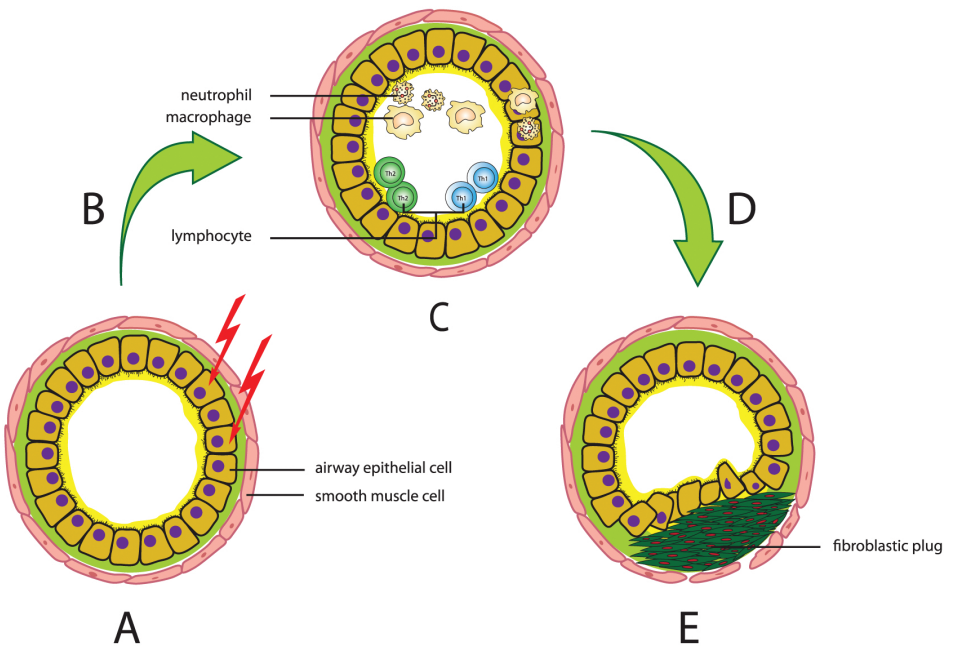

Figure 2. Primary damage to airway epithelium by alloimmune-dependent and -independent mechanisms (A) leads to activation of the innate immune system i.e. via Toll-like receptors with release of cytokines (Th1 and Th2) and chemokines. This is followed by interaction of antigen-presenting cells and T-lymphocytes activation of the adaptive immune system (B). The inflammatory response and injury of the airway epithelium (C) is followed by an aberrant repair response with fibroblast proliferation and extracellular matrix deposition mediated by metalloproteinases and several growth factors (TGF-β, IGF, PDGF) (D). The last step in this process is fibrosis of the airway epithelium with obliteration of the lumen (E).

2.3. Fibrosis

In contrast to acute inflammatory reactions, which are characterized by vascular changes, edema and neutrophilic inflammation, the development of fibrosis typically results from chronic inflammation, which is defined as an immune response that persists for several months and in which inflammation, tissue remodeling and repair processes occur simultaneously.[38, 39]

In lung transplant recipients the initial process consists of lymphocytic infiltrates of the mucosa due to migration of lymphocytes through the basement membrane into the epithelium (lymphocytic bronchiolitis).[39] At this place epithelial cell injury occurs with necrosis and ulcerations of the mucosa. A secondary cascade of non-specific pro-inflammatory mediators, cytokines and other chemotactic inflammatory mediators attracts other cells, including neutrophils.[6] Subsequently, the persistent inflammatory reaction and remodeling of the airways result in recruitment and proliferation of fibroblasts.[6, 39]

Fibroblasts contribute to the chronic repair process in several ways. They induce the production of ECM and the formation of intraluminal plugs of granulation tissue that may lead to (sub)total obliteration of the airway lumen.[39-41] Moreover, fibroblasts are the main producers of collagen and proteoglycans that contribute to the development of fibrosis.[40]

Besides resident airway fibroblasts, other cells may contribute to the fibroblast population in OB. There is a recruitment of circulating progenitors, such as fibrocytes, to the lung, where they differentiate into fibroblasts.[40] Another potential source of fibroblasts is epithelial-mesenchymal transition (EMT), in which epithelial cells differentiate into cells with a mesenchymal phenotype such as fibroblasts.[41]

Although activation of fibroblasts and accumulation of ECM is an important process in normal wound repair, the repair process in transplanted lungs appears to be excessive, which leads to the formation of a permanent fibrotic scar.[38, 42]

In addition, an increased expression of matrix metalloproteinases (MMPs) is part of the process of repair and remodeling.[43] MMPs represent a family of enzymes that are secreted or anchored to the cell surface and are responsible for the turnover and degradation of the ECM. Their ability to break down major structural proteins in the lung, such as collagen and elastin, give MMPs a major role in pulmonary ECM formation.[43, 44] Recent evidence suggests that an imbalance in MMPs and its anti-proteases is a critical factor involved in the fibroproliferation seen in BOS.[45]

Thus, after an initial epithelial injury of the lungs, caused by alloimmune-dependent and -independent mechanisms, which activate the innate and adaptive immunity, inflammation and ineffective epithelial regeneration eventually result in aberrant tissue repair with scar tissue obliterating the airway lumen (Figure 2).[1, 9, 14]

3. Diagnosis

The diagnosis BOS is defined by using spirometry, however, histological and radiological findings might contribute to this diagnostic process as well. Early and accurate diagnosis of BOS is necessary since this may enable the stabilization of pulmonary function at higher levels.

3.1 Spirometry

Spirometry is the gold standard in the diagnosis of BOS. The diagnosis BOS is made when a decline in $FEV_1 \geq 20\%$ from the baseline value occurs, which fall is determined by the average of two values measured at least three weeks apart in the absence of known other causes of declining FEV_1, such as acute rejection and infection.[6] The baseline value, to which subsequent measures are referred, is defined as the average of the two highest (not necessarily consecutive) post-transplant measurements obtained at least three weeks apart. Such measurements need to be made without the use of an inhaled bronchodilator.[6]

For each lung transplant recipient, a stable post transplant baseline FEV_1 is defined as BOS stage 0. In patients who experience a decrease in FEV_1, progressive stages of BOS (stage 1 to 3) are defined according to the magnitude of the decrease (Table 2).[6]

Spirometry to assess a decline in FEV_1 is widely available, noninvasive, reproducible and inexpensive. However, one of the limitations of this grading system was that it is not sensitive enough to pick up early small changes in pulmonary function. Several reports have shown that the mid-expiratory flow rate (FEF_{25}-FEF_{75}) is more sensitive than FEV_1 for early detection of airflow obstruction in BOS. These observations have led to an updated classification proposed by the ISHLT in 2002 in which the current classification of BOS is described and a potential BOS (BOS 0-p) stage is added to detect early, but potentially important, changes in pulmonary function (Table 2).[6]

Table 2. Classification of BOS.[6]

BOS stage	Lung function
BOS 0	$FEV_1 > 90\%$ of baseline and $FEF_{25-75} > 75\%$ of baseline
BOS 0-p	FEV_1 81% to 90% of baseline and/or $FEF_{25-75} \leq 75\%$ of baseline
BOS 1	FEV_1 66% to 80% of baseline
BOS 2	FEV_1 51% to 65% of baseline
BOS 3	FEV_1 50% or less of baseline

3.2 Histology

Transbronchial biopsies (TBB) have an important role to assess the presence or absence of AR and infections.[1] However, TBB is an insensitive method for detecting OB because of the patchy distribution of the lesions through the lungs.[39, 46]

OB is characterized by the presence of fibrosis in the submucosa of the respiratory bronchioles, which results in partial or complete luminal occlusion.[39] This scar tissue can be concentric or eccentric and may be associated with fragmentation and destruction of the smooth muscle wall and may extend to the peribronchiolar interstitium.[47] In advanced OB a spectrum from partial to complete acellular fibrotic obliteration can be found.[39]

3.3 High resolution computed tomography

The role of high resolution computed tomography (HRCT) in the diagnostic pathway of BOS is controversial. One study showed that an airtrapping threshold of 32% on CT is sensitive, specific, and accurate for diagnosing BOS. Patients with air trapping below 32% were unlikely to have BOS.[48] On the other hand, several authors did not confirm that the features of the HRCT corresponded with the development of BOS.[49, 50] Notably, the HRCT is complementary to bronchoscopy to help to establish other causes of a declining FEV_1 such as anastomotic abnormalities, infection and recurrence or progression of the native disease.

4. Current insights into the molecular aspects of BOS

Several proteins produced by the epithelium of the respiratory tract are normally present in the serum of healthy individuals. In some lung disorders, such as BOS, changes in the concentrations of these proteins can be attributed either to loss of integrity of the bronchoalveolar blood barrier or to changes of protein secretion into the respiratory tract. As for proteins, this also counts for cytokines, chemokines and growth factors that are involved in the process of inflammation, remodeling, repair and fibrosis. An overview of proteins, cytokines, chemokines and growth factors in serum (Table 3), exhaled breath condensate and BAL fluid which have been investigated in relation to the development of BOS and genetic polymorphisms playing a role in the susceptibility of a lung transplant recipient to develop BOS (Table 4) has been given below.

4.1 Biomarkers in serum

Clara cell 16 (CC16) secretory protein is the main product of the bronchiolar Clara cells. It has potent immunosuppressive properties and is believed to be an important down-regulator of airway inflammation. It is decreased in patients developing BOS but it remains controversial whether it is useful as an early marker for BOS.[51, 52]

Krebs von den Lungen-6 (KL-6) is a glycoprotein that is mainly expressed in the lungs on the epithelial surface of type II alveolar cells. It is chemotactic for fibroblasts which indicates a possible role in the development of fibrosis.[53] Two independent studies demonstrated that serum KL-6 was elevated in lung transplant recipients with BOS (BOS^{pos}) compared with recipients without BOS (BOS^{neg}) and healthy controls.[54, 55]

Two other lung epithelium specific proteins are surfactant protein (SP)-A and SP-D. Paantjens et al. showed that SP-D levels after lung transplantation were not different between BOS^{pos} and BOS^{neg} patients.[52] However, low SP-D levels before allogeneic hematopoietic stem cell transplantation may be a useful, noninvasive predictor for the development of BOS after allogeneic hematopoietic stem cell transplantation.[56] SP-A in serum was never measured in

lung transplant recipients, but SP-A levels in BAL fluid could predict patients who are at higher risk to develop BOS.[57]

Matrix metalloproteinases (MMPs) are key enzymes in the regulation, turnover and degradation of the extracellular matrix.[44, 58] MMP-9 is primarily involved in matrix degradation. In lung transplantation, the role of MMP-9 in the development of BOS remains controversial.[45, 59-61] MMP-9 in serum was described in only one study, in which no significant difference in BOS^{pos} and BOS^{neg} patients was observed.[59]

Soluble CD30 (sCD30) is a marker for Th2 cytokine-producing T cells. In two studies sCD30 was significantly elevated during the development of BOS compared with BOS^{neg} patients.[62, 63] This is in contradiction with the findings of Kwakkel et al. who demonstrated that after lung transplantation, sCD30 levels were consistently low, but BOS is not prevented, indicating that sCD30 cannot be used as a biomarker to predict BOS after lung transplantation.[64]

As mentioned earlier, critical to repair of the airway epithelium is a balance between pro- and anti-inflammatory cytokines and chemokines. Bharat et al. found an early post-transplant elevation of basal serum levels of pro-inflammatory chemokines induced protein (IP)-10 and monocyte chemoattractant protein (MCP)-1, and Th1 cytokines IL-1β, IL-2, IL-12 and IL-15 in BOS^{pos} patients compared with BOS^{neg} patients and controls. In addition, a threefold decline in IL-10 levels was found during BOS development.[65] Hodge et al. found significantly lower levels of T-cell IFN-γ and tumor necrosis factor (TNF)-α in the blood of stable patients compared with BOS^{pos} patients and they reported that an increase in these pro-inflammatory cytokines was associated with a decrease in FEV_1.[66, 67] However, the precise role of cytokines in the development of BOS remains a field of interest.

Chemokines and their receptors direct the cell trafficking between the sites of antigen presentation, lymphocyte activation, and inflammation and thus incorporate adaptive, innate, humoral and autoimmune activation. There are ~50 chemokines identified. Many of these chemokines and their receptors have been identified in the allograft, though their role in developing rejection is unclear.[42] Paantjens et al. investigated the relation between the development of BOS and Th2 chemoattractant thymus and activation regulated chemokine (TARC/CCL17). They found that median serum TARC levels after transplantation in BOS^{pos} patients were significantly lower than those of the matched BOS^{neg} patients.[68]

Several growth factors, such as PDGF, TGF-β, hepatocyte growth factor and IGF-1, may contribute to the proliferation of epithelial cells, fibroblasts and smooth muscle cells and have each been implicated in the development of BOS.[1, 69-71]

Table 3. Biomarkers in serum which are investigated in lung transplant recipients and are increased, decreased or equal in BOS^{pos} patients compared with BOS^{neg} patients after lung transplantation.

Increased in BOS	Decreased in BOS	Equal
KL-6[54,55], sCD30[62,63], IP-10[65], MCP-10[65], IL-1β[65], IL-2[65], IL-12[65], IL-15[65], IFN-γ[66], TNF-α[66]	CC16[51,52], IL-10[65], TARC/CCL17[68]	SP-D[52], MMP-9[59], sCD30[64]

CC16: Clara cell 16; IFN: interferon; IL: interleukin; IP-10: induced protein-10; KL-6: Krebs von den Lungen-6; MCP: monocyte chemoattractant protein-1; SP-D: surfactant protein-D; TARC: thymus and activation regulated chemokine; TNF: tumor necrosis factor.

4.2 Biomarkers in exhaled breath condensate

Volatile and nonvolatile markers originating from the respiratory tract can be measured in exhaled breath condensate (EBC).[72] Exhaled nitrogen oxide (eNO) and exhaled carbon monoxide (eCO) are well-recognized biomarkers of airway inflammation especially in patients with COPD and asthma.[72-75] Patients with BOS had a lower EBC pH and had an increased eNO and eCO compared with stable lung transplant patients and healthy volunteers.[76-79] However, a recent study suggested that the assessment of EBC pH is of limited value for the diagnosis of BOS.[80]

In a study of Antus et al. the cytokines IL-10, IL-13, and IFN-γ in EBC were increased in BOS^{pos} compared with BOS^{neg} patients.[81]

4.3 Biomarkers in bronchoalveolar lavage

The lung allograft is suited to study by bronchoalveolar lavage (BAL), which allows sampling of cellular components and proteins of the lower respiratory tract. There are many studies describing markers in BAL fluid and their correlation with the development of BOS. Because this thesis is about markers in serum, EBC and DNA, only a very short overview will be described below.

BAL cellularity may be a signal for complications after lung transplantation.[82] Evidence of an increase in percentage of neutrophils in stable lung recipients in BAL fluid might be an indication of subclinical alloimmune stimulation and the development of BOS.[83-85] Molecular markers in BAL fluid that have been shown to be altered in BOS include IL-8, antioxidants, TGF-β, MCP-1, vascular endothelial growth factor, IFN-γ and MMP-8 and -9.[45, 59, 86]

4.4 Genetics

Several investigators evaluated genetic polymorphisms as potential independent risk factors for post-transplant outcomes. For several single nucleotide polymorphisms (SNPs) in innate immunity and cytokine genes the association with the development of BOS was evaluated. SNPs are considered the most common type of variant, in which only one nucleotide is substituted by another.[87]

The presence for either of the two functional polymorphisms (Asp299Gly or Thr399Ile) in Toll-like receptor 4 (*TLR4*) was associated with endotoxin hyporesponsiveness.[88] Lung transplant

recipients carrying the minor allele for either Asp299Gly or Thr399Ile had significantly reduced frequency and incidence of acute rejection sustained over three years after transplant, but no differences were observed in the overall onset of BOS. A trend, however, towards reduced onset of BOS grade 2 or 3 was observed in patients carrying one of the minor alleles for *TLR4*.[89, 90]

Mannose binding lectin (MBL), a recognition molecule of innate immunity, has been associated with transplant outcome in other solid organ transplantation. The donor X allele, which corresponds to the LXPA haplotype, is associated with superior lung transplant outcome, including a better BOS-free survival. Recipient *MBL* genotype was not associated with transplant outcome.[91]

Another study investigated whether the effect of a common promotor polymorphism of the lipopolysaccharide receptor *CD14* was associated with increased transcriptional activity upon the development of post-transplant rejection and graft survival. They found an earlier onset of BOS and worse post-transplant graft survival in patients with the *CD14* -159 TT genotype. Furthermore, TT patients had significantly higher levels of soluble CD14, TNF-α and IFN-γ in the peripheral blood implying a heightened state of innate immune activation that drives the development of rejection.[92]

Snyder et al. found that genetic polymorphisms in the genes of tumor necrosis factor alpha (*TNFA)*, interferon gamma (*IFN*G), transforming growth factor beta1 (*TGFB1)*, interleukin (*IL*)6 and *IL10* do not appear to influence the onset of BOS or graft survival in lung transplant recipients.[93] Conversely, Lu et al. found that the presence of genetic polymorphisms in the *IL6* and the *IFNG* gene significantly increases the risk for BOS development after lung transplantation.[94] Awad et al. suggested that genetic polymorphisms in the *IFNG* gene can influence the development of fibrosis in lung allografts.[95] Besides, this group also found an association in the *TGFB1* gene and the development of fibrosis in lung allografts.[96]

The results of studies on genetic polymorphisms in innate immunity and cytokine genes implicate that they have a role in the susceptibility of lung transplant recipients to develop BOS, however, more research is needed.

Table 4. Different genetic polymorphisms in innate immunity genes and cytokine genes that are investigated for their association with the development of bronchiolitis obliterans syndrome.

Gene	Association with BOS
Toll-like receptor 4	No, trend with BOS[89,90]
Mannose binding lectin	Yes, in donor[91]
CD14	Yes[92]
Tumor necrosis factor-α	No[93, 94]
Interferon-γ	Controversial [93, 94]
Transforming growth factor-β1	No[93, 94]
Interleukin 6	Controversial[93, 94]
Interleukin 10	No[93, 94]

Transplanted lungs have the genetic profile of the donor. In most of the aforementioned studies the genetic background of the recipient was determined. Chimerism, the presence of cells with a different genetic background within the graft, was detected in different solid organ transplants.[97] An integration of recipient-derived cells into the parenchyma was observed for hepatocytes and cholangiocytes in the liver, for tubular epithelial and endothelial cells in the kidney and for cardiomyocytes in the heart.[98-100] In lung transplant recipients, recipient-derived cells in the bronchi, pneumocytes and glandular epithelium were found with an engraftment range from 2-30%.[97] Besides, the epithelium of the adult human lung has the capacity to renew itself, using cells recruited from extrapulmonary sources, including the bone marrow.[101, 102]
In lung transplant recipients with chronic rejection increased engraftment rates of recipient cells were found.[97] This suggests that chimerism after transplantation is increased in a process with elevated cell turnover, which is present in lung transplant recipients who develop BOS. Although consequences of chimerism are outside the scope of this thesis, it is important to take into account the relevance of this phenomenon.

5. Treatment

The treatment options for BOS are limited. By the time BOS is diagnosed with pulmonary function testing, airway pathology is too advanced for treatment to effectively reverse the process.

5.1 Medication

Treatment of lung transplant recipients with medication has two main aims. First, to prevent the development of all forms of rejection, including BOS, by administrating maintenance immunosuppressive therapy after lung transplantation. Second, to halt the decline in FEV_1 after the diagnosis of BOS is made, by augmenting or switching or addition of immunosuppressive therapy.[8] Most immunosuppressive therapies aim to suppress lymphocyte function and inflammatory responses. Therefore, they are likely to be more effective in the early stages of BOS than in later stages, that are characterized by fibrosis.[103]

Actually, long-term azithromycin treatment seems to be the most promising therapeutic option for BOS treatment. Azithromycin is a macrolide antibiotic with a broad spectrum of anti-inflammatory and immunomodulatory activities. Long-term oral azithromycin therapy significantly improves FEV_1 in a subgroup of patients with established BOS.[104] Moreover, reduced neutrophilia, chemokine release and bacterial exacerbations have been demonstrated, which suggest that the drug may down-regulate pulmonary inflammation.[105] Several studies confirmed the effect of azithromycin in the treatment of BOS.[106, 107] Recently, a randomized placebo-controlled trial revealed that azithromycin prophylaxis in lung transplant recipients

attenuates inflammation, improves FEV_1 and reduces the occurrence of BOS two years after lung transplantation.[108]

5.2. Retransplantation

Because advanced BOS is largely refractory to any therapeutic modality, retransplantation is the last option for patients with BOS.[109] Patients who underwent a retransplant had a 30% higher risk of death compared with patients who underwent a first lung transplant procedure, and moreover, these patients have an increased risk to develop BOS again compared with initial transplant recipients.[110] Therefore, retransplantation for BOS has been controversial in light of the very limited availability of donor lungs, the recurrence of BOS and ethical principles.[8, 82]

6. Aim and outline of the thesis

Increased understanding of the pathogenesis of BOS after lung transplantation continues to be a priority for the lung transplantation physician worldwide. The development of fibrosis and end-stage BOS is considered to be the final common pathway after alloimmune-dependent and alloimmune-independent mediated injury of the epithelial structures of the lungs. In the process of chronic injury of the airway epithelium, which is followed by repair, it appears to be important to what extent a lung transplant recipient is able to repair the injured epithelium and to slow down the fibrogenesis.

When the diagnosis BOS is made, the process of fibrosis and obliterative bronchiolitis is already at an advanced and mostly irreversible stage which limits the treatment options. This emphasizes the need for identification of biomarkers that could detect the development of subclinical BOS, before loss of lung function and functional impairment occurs.

The aim of this thesis is to investigate whether biomarkers, in serum, in EBC or in DNA, can be used as risk factor for the development of BOS in lung transplant recipients before the deterioration in lung function is established. The biomarkers described in this thesis are selected on their possible involvement in processes that characterize the development of BOS, such as inflammation, repair and fibrosis. Some of the investigated biomarkers have already been described in literature for their role in other pulmonary diseases in humans and animal models.

We hypothesize that the levels of the investigated biomarkers are increased or decreased in serum or EBC of BOS^{pos} patients compared with BOS^{neg} patients and that the genotype distribution is different between these two groups, which suggest that these biomarkers might be potential risk factors for BOS.

In lung transplant recipients there is evidence of increased stimulation of the innate immune system. Activation of the innate immune system via the genetically heterogeneous Toll-like receptors (TLRs) may play a role in transplant tolerance. Genetic polymorphisms in the *TLR* genes are related to interindividual differences in immune response and might contribute to susceptibility to BOS. In **chapter 2** the distribution of 64 single nucleotide polymorphisms (SNPs) in the genes encoding for *TLR1* to *TLR10* in BOS^{pos} patients, BOS^{neg} patients and healthy controls is investigated.

Beneficial to airway repair is a balance between pro-inflammatory and anti-inflammatory cytokines and chemokines. Especially, the imbalance between T-helper (Th)1 and Th2 cytokines might lead to fibrosis and obliteration of allograft airways and finally to BOS. In **chapter 3** cytokine and chemokine profiles in serum and exhaled breath condensate in BOS^{pos} and BOS^{neg} patients and healthy controls are determined to assess their usefulness as biomarkers for BOS. The samples were collected longitudinally after lung transplantation prior to the diagnosis of BOS.

In **chapter 4** the role of *MMP7*, a repair gene, in the development of BOS is discussed. MMP-7 is upregulated in response to injury of the airway epithelium to facilitate re-epithelialisation, cell migration and regulation of the inflammatory response, which are processes related to the development of BOS. The expression of MMP-7 is primarily regulated at the transcriptional level. Genetic polymorphisms in the *MMP7* gene might contribute to the development of BOS. In lung transplant recipients, with and without BOS, and in healthy controls seven SNPs in *MMP7* and MMP-7 serum levels have been investigated.

YKL-40 is a growth factor for fibroblasts and is involved in inflammation, remodeling and fibrosis. Besides, it might be a biomarker in sarcoidosis and asthma. MMP-9 facilitates the degradation and turnover of the extracellular matrix and migration of inflammatory cells. Several studies suggest that there is a link between MMP-9 and BOS. In **Chapter 5** YKL-40 and MMP-9 serum levels were assessed as biomarkers for BOS. Serial YKL-40 and MMP-9 serum levels are measured in BOS^{pos} and BOS^{neg} patients in the period after lung transplantation and prior to BOS.

Reduced expression of caveolin-1 (Cav-1) in different cells is linked to pulmonary fibrosis. Genetic polymorphisms in *CAV1* influence the function of Cav-1 in malignancies and associate with renal allograft fibrosis. In **Chapter 6** the distribution of four SNPs in *CAV1* are investigated in lung transplant recipients, with and without BOS, and in healthy controls. Furthermore, it is investigated whether Cav-1 serum levels are influenced by genotype and can be useful as biomarker for BOS.

Several risk factors contribute to the development of BOS. However, these risk factors alone do not explain the interindividual variability seen in the development of BOS. Genetic polymorphisms in innate immunity genes and cytokine genes have been frequently investigated, as in this thesis, as potential risk factors for BOS, and might contribute to an individual's susceptibility to BOS. **Chapter 7** gives a systematic review of the genetic polymorphisms that have so far been investigated in lung transplant recipients, and have been associated with the development of BOS.

In **Chapter 8** the results are summarized and concluding remarks are provided.

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CHAPTER 2

POLYMORPHISMS IN INNATE IMMUNITY GENES ASSOCIATED WITH DEVELOPMENT OF BRONCHIOLITIS OBLITERANS AFTER LUNG TRANSPLANTATION

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Abstract

Background

Activation of the immune system is suggested to prevent transplant tolerance and to promote the development of bronchiolitis obliterans syndrome (BOS). The innate immune system is activated by the interaction of pathogen-associated molecular patterns of microorganisms with Toll-like receptors (TLRs). Activation of innate immunity via TLRs was shown to be a barrier to the induction of transplantation tolerance after lung transplantation. We hypothesize that polymorphisms in 10 genes coding for *TLR1* to *TLR10* might contribute to an altered immune response and the subsequent development of BOS.

Methods

DNA was collected from 110 lung transplant recipients. Twenty patients developed BOS. The control group comprised 422 individuals. Sixty-four single nucleotide polymorphisms (SNPs) in 10 genes coding for *TLR1* to *TLR10* were genotyped.

Results

The genotype distribution of *TLR2* (*rs1898830* and *rs7656411*), *TLR4* (*rs1927911*) and *TLR9* (*rs352162* and *rs187084*) was significantly different between BOS^{pos} patients and BOS^{neg} patients and controls. The BOS^{pos} patients had significantly more patients with 3 or 4 of these risk alleles compared with BOS^{neg} patients and controls.

Conclusions

Polymorphisms in *TLR2*, *TLR4* and *TLR9* that recognize bacterial and viral pathogens are associated with BOS after lung transplantation.

Introduction

The 5-year survival rate after lung transplantation is 50%. This poor survival rate is mainly due to progressive and treatment-refractory airway remodeling that manifests clinically as bronchiolitis obliterans syndrome (BOS).[1] Lung transplant recipients are continuously exposed to stimuli of the innate immune system through commensal and pathogenic bacteria via inspiration of airborne particles and aspiration.[2-4]

Toll-like receptors (TLRs) recognize a spectrum of pathogen-associated molecular patterns of microorganisms that lead to enhanced presentation of alloantigen and increased alloresponses by upregulation of cytokines to initiate the adaptive immunity.[2, 5-8] Activation of innate immunity via TLRs was shown to be a barrier to the induction of transplantation tolerance after lung transplantation.[8, 9]

Variations in innate immune response can partly be explained by polymorphisms in innate immune response genes.[10] It is known that genetic polymorphisms of the recipient might correlate with the fate of the transplanted lung.[3, 11-13] We hypothesize that polymorphisms in the genes coding for TLRs might contribute to an altered immune response and finally to BOS.

We analyzed single nucleotide polymorphisms (SNPs) in 10 genes coding for *TLR1* to *TLR10* and sought to determine whether these polymorphisms are associated with BOS.

Materials and methods

Patients and clinical data

All lung transplant recipients who underwent a lung transplantation in the Heart Lung Centre of the University Medical Centre in Utrecht, the Netherlands, in the period from July 2001 to November 2008 were asked to donate DNA.

The diagnosis BOS was defined as a decline in forced expiratory volume in one second (FEV_1) of more than 20% from the baseline determined by an average of two measurements made at least three weeks apart in the absence of known causes for an acute declining FEV_1, such as acute rejection and infection.[14] All patients with the diagnosis BOS are patients with BOS grade 1 or higher.

Standard immunosuppressive therapy consisted of basiliximab (induction), tacrolimus, mycophenolate mofetil and prednisone for all patients. No surveillance bronchoscopies were performed.

After approval by the medical–ethical committee informed consent was obtained from each lung transplant recipient and healthy control and DNA was collected.

The control group comprised 422 healthy individuals (228 men and 194 women; age 48.2 ± 11.9 years, range 23 to 77 years).

Table 1. Distribution and features of 64 SNPs* in *TLR1* to *TLR10* in patients with BOS (BOS+), patients without BOS (BOS-) and controls.

					BOS+ (n=20)			BOS- (n = 90)			Controls (n = 422)			
Gene	**SNP**	**Major**	**Minor**		**AA**	**AB**	**BB**	**AA**	**AB**	**BB**	**AA**	**AB**	**BB**	**HWE**
TLR1	rs5743604	T	C	tag	15 (75)	4 (20)	1 (5)	57 (63)	31 (34)	2 (2)	238 (56)	158 (38)	26 (6)	yes
TLR1	rs5743595	T	C	tag	16 (80)	3 (15)	1 (5)	66 (73)	22 (25)	2 (2)	283 (67)	121 (29)	18 (4)	yes
TLR1	rs5743594	C	T	tag	12 (60)	8 (40)	0 (0)	61 (67)	26 (29)	3 (3)	273 (65)	127 (30)	22 (5)	yes
TLR1	rs4833113	C	A	regulatory	8 (40)	9 (45)	3 (15)	28 (31)	49 (54)	13 (15)	147 (35)	204 (48)	69 (16)	yes
TLR1	rs2134817	G	C	regulatory	8 (40)	9 (45)	3 (15)	28 (31)	49 (54)	13 (15)	146 (35)	209 (50)	67 (15)	yes
TLR1	rs4833115	A	G	regulatory	8 (40)	9 (45)	3 (15)	28 (31)	50 (56)	12 (13)	146 (35)	206 (49)	70 (16)	yes
TLR1	rs6810965	A	G	regulatory	8 (40)	9 (45)	3 (15)	28 (31)	50 (56)	12 (13)	147 (35)	206 (49)	69 (16)	yes
TLR1	rs6848109	C	T	regulatory	8 (40)	9 (45)	3 (15)	28 (31)	50 (56)	12 (13)	147 (35)	206 (49)	69 (16)	yes
TLR1	rs6848727	C	T	regulatory	8 (40)	9 (45)	3 (15)	28 (31)	50 (56)	12 (13)	147 (35)	206 (49)	69 (16)	yes
TLR2	rs1898830	A	G	tag	15 (75)	5 (25)	0 (0)	39 (43)	40 (45)	11 (12)	184 (44)	186 (44)	52 (12)	yes
TLR2	rs4696480	T	A	custom	1 (5)	10 (50)	9 (45)	21 (23)	42 (47)	27 (30)	105 (24)	205 (49)	112 (27)	yes
TLR2	rs3804099	T	C	tag	2 (10)	11 (55)	7 (35)	24 (27)	47 (52)	19 (21)	124 (29)	207 (49)	91 (22)	yes
TLR2	rs5743704	C	A	custom	19 (95)	1 (5)	0(0)	82 (92)	6 (7)	1 (1)	381 (90)	41 (10)	0 (0)	no
TLR2	rs5743708	G	A	custom	20 (100)	0 (0)	0 (0)	82 (91)	8 (9)	0 (0)	386 (91)	36 (9)	0 (0)	yes/n.a.
TLR2	rs7656411	T	G	tag	9 (45)	7 (35)	4 (20)	44 (49)	37 (41)	9 (10)	239 (57)	158(37)	25 (6)	yes
TLR3	rs7657186	G	A	tag	12 (60)	7 (35)	1 (5)	54 (60)	32 (36)	4 (4)	267 (63)	137 (33)	18 (4)	yes
TLR3	rs13126816	G	A	tag	12 (60)	7 (35)	1 (5)	49 (54)	34 (38)	7 (8)	243 (58)	160 (38)	19 (4)	yes
TLR3	rs10025405	A	G	tag	5 (25)	9 (45)	5 (25)	25 (28)	43 (48)	11 (12)	117 (28)	206 (49)	89 (21)	yes
TLR3	rs1473597	A	G	regulatory	4 (20)	13 (65)	3 (15)	28 (31)	47 (52)	13 (14)	133 (32)	215 (51)	72 (17)	yes
TLR4	rs1927914	T	C	tag	8 (40)	7 (35)	5 (25)	45 (50)	33 (37)	12 (13)	170 (40)	201 (48)	51 (12)	yes
TLR4	rs1927911	C	T	tag	9 (45)	6 (30)	5 (25)	53 (59)	31 (34)	6 (7)	216 (51)	175 (42)	31 (7)	yes
TLR4	rs5030728	G	A	tag	13 (65)	4 (20)	3 (15)	46 (51)	36 (40)	8 (9)	209 (49)	177 (42)	36 (9)	no
TLR4	rs4986790	A	G	custom	18 (90)	2 (10)	0 (0)	80 (89)	10 (11)	0 (0)	370 (87)	50 (12)	2 (0.5)	yes
TLR5	rs2788090	C	T	tag	12 (60)	8 (40)	0 (0)	58 (64)	27 (30)	5 (6)	264 (63)	137 (32)	21 (5)	yes
TLR5	rs4648445	A	G	tag	7 (35)	11 (55)	2 (10)	42 (47)	32 (36)	16 (18)	193 (46)	181 (43)	48 (11)	no
TLR5	rs12046158	T	C	tag	7 (35)	11 (55)	2 (10)	42 (47)	31 (34)	17 (19)	191 (45)	182 (43)	49 (12)	no
TLR5	rs5744174	T	C	custom	7 (35)	10 (50)	3 (15)	29 (32)	44 (49)	17 (19)	136 (32)	213 (51)	73 (17)	yes
TLR5	rs2072493	A	G	custom	14 (70)	6 (30)	0 (0)	67 (75)	21 (23)	2 (2)	298 (71)	118 (28)	6 (1)	yes
TLR5	rs5744168	C	T	custom	19 (95)	1 (5)	0 (0)	79 (88)	10 (11)	1 (1)	362 (85)	59 (14)	1 (0.5)	yes

					BOS+ (n=20)			BOS- (n = 90)			Controls (n = 422)			
Gene	**SNP**	**Major**	**Minor**		**AA**	**AB**	**BB**	**AA**	**AB**	**BB**	**AA**	**AB**	**BB**	**HWE**
TLR5	rs1773726	G	A	tag	7 (35)	10 (50)	3 (15)	49 (54)	25 (28)	16 (18)	136 (32)	213 (51)	73 (17)	no
TLR5	rs2241096	C	T	tag	15 (75)	4 (20)	1 (5)	66 (73)	21 (23)	3 (3)	342 (81)	77 (18)	3 (1)	yes
TLR5	rs1773767	T	C	tag	18 (90)	2 (10)	0 (0)	83 (92)	5 (6)	2 (2)	378 (90)	43 (10)	1 (0.5)	no
TLR5	rs5744105	G	A	tag	6 (30)	10 (50)	4 (20)	20 (22)	49 (54)	19 (21)	111 (26)	209 (50)	103 (24)	yes
TLR6	rs3775073	A	G	tag	11 (55)	7 (35)	2 (10)	37 (41)	45 (50)	8 (9)	186 (44)	196 (46)	40 (10)	yes
TLR6	rs1039559	T	C	tag	4 (20)	8 (40)	8 (40)	17 (19)	58 (64)	15 (17)	105 (25)	236 (56)	81 (19)	no
TLR6	rs5743794	G	A	tag	16 (80)	4 (20)	0 (0)	66 (73)	21 (23)	4 (4)	273 (65)	128 (30)	21 (5)	yes
TLR7	rs5935387	G	C	regulatory	13 (65)	4 (20)	3 (15)	57 (63)	17 (19)	13 (14)	246 (58)	87 (21)	89 (21)	no
TLR7	rs179008	A	T	functional	15 (75)	4 (20)	1 (5)	62 (69)	17 (19)	8 (9)	288 (68)	70 (17)	64 (15)	no
TLR7	rs5743780	G	A	tag	20 (100)	0 (0)	0 (0)	85 (95)	4 (4)	1 (1)	413 (98)	7 (2)	2 (0.5)	no
TLR7	rs3853839	C	G	tag	16 (80)	1 (5)	3 (15)	72 (80)	13 (14)	3 (3)	338 (80)	46 (11)	38 (9)	no
TLR7	rs5935438	G	C	tag	7 (35)	7 (35)	6 (30)	33 (37)	21 (23)	36 (40)	180 (43)	87 (20)	155 (37)	no
TLR8	rs2699999	G	A	regulatory	16 (80)	4 (20)	0 (0)	69 (77)	12 (13)	9 (10)	312 (74)	61 (14)	49 (12)	no
TLR8	rs3761623	G	A	tag	6 (30)	7 (35)	7 (35)	44 (49)	18 (20)	28 (31)	163 (39)	91 (21)	168 (40)	no
TLR8	rs5741886	T	A	tag	10 (50)	7 (35)	3 (15)	49 (55)	19 (21)	22 (24)	225 (53)	89 (21)	108 (26)	no
TLR9	rs352162	T	C	tag	9 (45)	7 (35)	4 (20)	17 (19)	37 (41)	35 (39)	77 (18)	222 (53)	123 (29)	yes
TLR9	rs352140	C	T	custom	10 (50)	6 (30)	4 (20)	17 (19)	36 (40)	37 (41)	79 (19)	219 (52)	124 (29)	no
TLR9	rs187084	T	C	tag	11 (55)	6 (30)	3 (15)	23 (26)	43 (48)	24 (26)	136 (32)	221 (52)	65 (16)	yes
TLR9	rs352143	A	G	tag	15 (75)	4 (20)	1 (5)	60 (67)	28 (31)	2 (2)	265 (63)	138 (33)	19 (4)	yes
TLR10	rs11096955	A	C	tag	13 (65)	6 (30)	1 (5)	37 (41)	47 (52)	6 (7)	175 (41)	198 (47)	49 (12)	yes
TLR10	rs4274855	G	A	tag	17 (85)	2 (10)	1 (5)	69 (77)	19 (21)	2 (2)	288 (68)	116 (28)	18 (4)	no
TLR10	rs7658893	G	A	tag	17 (85)	2 (10)	1 (5)	55 (61)	30 (33)	5 (6)	246 (58)	151 (36)	25 (6)	no
TLR10	rs9994896	T	C	regulatory	13 (65)	5 (25)	2 (10)	49 (55)	36 (40)	5 (5)	225 (53)	170 (40)	27 (7)	yes
TLR10	rs9999294	G	A	regulatory	4 (20)	9 (45)	7 (35)	16 (18)	48 (53)	26 (29)	106 (25)	220 (52)	96 (23)	yes

* Eleven SNPs were monomorphic: *TLR1* rs5743621 and rs5743559; *TLR3* rs5743316 and rs7674943; *TLR4* rs5030722; *TLR5* rs5744176; *TLR6* rs1310225; *TLR8* rs5744077; *TLR9* rs5743846; *TLR10* rs1146660 and rs17616831

AA refers to homozygotes major allele and BB refers to homozygotes minor allele; all data are shown as n (%).

Genotyping

Three haplotype tagging SNPs for the *TLR* genes were selected using the Tagger program for the genomic region of *TLR1* to *TLR10* ± 2500 bp on genome build 35. Preferential picking of SNPs was conducted under the pairwise tagging options, a minimum allele frequency setting of 25% and a high Illumina design score. The algorithm was set to select tags that would cover the Caucasian HapMap panel with an r^2 of 0.8 or more.[15] Furthermore, additional SNPs were selected on the basis of previously published data or presumed functionality. The SNPs for *TLR1* to *TLR10* which were genotyped are shown in Table 1.

DNA was extracted from whole blood samples and SNP typing was conducted using a custom Illumina goldengate bead SNP assay. The assay was performed in accordance with the manufacturer's recommendations (Illumina Inc., San Diego, CA, USA).

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) and tests for association were calculated using the programme available at: http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl. Statistical significance of the differences between the groups was determined with the χ^2 test and one-way ANOVA.

Correcting for type-1 errors using the false discovery rate method, results in a 5% probability of finding false positive significant SNPs, which translates into approximately two significant SNPs.

The linkage disequilibrium (LD) structure of the polymorphisms was determined using Haploview 4.1.[16, 17] Statistical analyses were performed with SPSS for Windows, version 15.0 (SPSS Inc, Chicago, IL). p-values ≤ 0.05 were considered statistically significant.

Results

In the period from July 2001 to November 2008, there were 139 lung transplant procedures performed in 138 recipients. In total, 110 lung transplant recipients gave written informed consent and donated DNA. Twenty patients developed BOS (BOS^{pos}) during their follow-up. The baseline characteristics of this study cohort are shown in Table 2.

Genotype distribution in the Toll-like receptors

The distribution of the 64 genotyped SNPs are shown in Table 1. Eleven SNPs were found to be monomorphic in the patient group as well as in the controls. Another 18 SNPs were not in Hardy-Weinberg equilibrium in one or more of the 3 groups. These 29 SNPs were excluded from further analysis.

The SNPs with a significantly different genotype distribution between BOS^{pos} patients and BOS^{neg} patients or controls were found in the genes of *TLR2* (*rs1898830* and *rs7656411*), *TLR4* (*rs1927911*) and *TLR9* (*rs352162* and *rs187084*) as shown in Table 3.

Table 2. Baseline characteristics.

Variable	BOS+	BOS-	controls
Total number	20	90	422
Gender, no. (%)			
male	9 (45%)	45 (50%)	228 (54%)
female	11 (55%)	45 (50%)	194 (46%)
Age, mean ± SD, years	53.4 ± 10.7	49.4 ± 12.7	48.2 ± 11.9
Diagnosis, no. (%)			NA
COPD	7 (35%)	29 (32%)	
CF	2 (10%)	24 (27%)	
IPF	4 (20%)	13 (14%)	
sarcoidosis	2 (10%)	3 (3%)	
alpha-1 antitrypsin deficiency	5 (25%)	7 (8%)	
others	0	14 (16%)	
Type of graft, no. (%)			NA
bilateral	16 (80%)	77 (86%)	
unilateral	4 (20%)	13 (14%)	
Time to BOS, mean ± SD, months	23.7 ± 15.2	NA	NA

COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; IPF, idiopathic pulmonary fibrosis; NA, not applicable; SD, standard deviation.

Table 3. Genotype distribution of SNPs in *TLR2*, *TLR4* and *TLR9* with a significantly different distribution in patients with BOS (BOS+) compared with the other groups.

				BOS+ (n = 20)			BOS- (n = 90)			controls (n = 422)		
				AA	AB	BB	AA	AB	BB	AA	AB	BB
TLR2	rs1898830	intron 1	A/G*	15 (75)	5 (25)	0 (0)	39 (43)	40 (45)	11 (12)	184 (44)	186 (44)	52 (12)
TLR2	rs7656411	3′ UTR	T/G**	9 (45)	7 (35)	4 (20)	44 (49)	37 (41)	9 (10)	239 (57)	158(37)	25 (6)
TLR4	rs1927911	intron 1	C/T***	9 (45)	6 (30)	5 (25)	53 (59)	31 (34)	6 (7)	216 (51)	175 (42)	31 (7)
TLR9	rs352162	3′ UTR	T/C****	9 (45)	7 (35)	4 (20)	17 (19)	37 (41)	35 (39)	77 (18)	222 (53)	123 (29)
TLR9	rs187084	promotor	T/C*****	11 (55)	6 (30)	3 (15)	23 (26)	43 (48)	24 (26)	136 (32)	221 (52)	65 (16)

AA refers to homozygotes major allele, BB refers to homozygotes minor allele; data are shown as n (%).
BOS+ vs controls: * p = 0.016, ** p = 0.044, *** p = 0.018, **** p = 0.013
BOS+ vs BOS-: * p = 0.026, *** p = 0.046, **** p = 0.039, ***** p = 0.036

Homozygotes for the major allele of *rs1898830*, *rs352162* and *rs187084* had an increased risk to develop BOS compared with the carriers of the minor allele with an odds ratio (OR) ranging from 2.57 to 3.88 (Table 4). Homozygotes for the minor allele of *rs7656411* and *rs1927911* had an increased risk to develop BOS compared with the carriers of the major allele with an OR ranging from 3.97 to 4.20 (Table 4).

Table 4. Odds ratio for homozygous carriers of the risk allele versus carriers of the protective allele.

SNP	rs1898830	rs7656411	rs1927911	rs352162	rs187084
Risk allele	A	G	T	T	T
OR	3.88	3.97	4.20	3.66	2.57
CI	1.39 - 10.87	1.23 - 12.82	1.43 - 12.35	1.47 - 9.17	1.04 - 6.33
p-value	0.006	0.013	0.005	0.003	0.035

For calculation of the odds ratio (OR) lung transplantation patients were compared with controls; CI, confidence interval.

Our data did not allow to construct haplotypes because the SNPs of *TLR2*, *TLR4* and *TLR9* are located on different chromosomes. As an alternative, we determined the number of the 5 aforementioned 'risk alleles' per patient in the different groups (Table 5). The BOS^{pos} group had significantly more patients with 3 or 4 'risk alleles' ($p < 0.006$) and significantly less patients with 0 'risk alleles' ($p = 0.015$) compared with BOS^{neg} patients and controls. There was no correlation between the number of 'risk alleles' per patient and the BOS-free period after lung transplantation in the BOS^{pos} patients (data not shown).

Table 5. Number of 'risk alleles' in patients of each of the groups.

number of risk alleles	**BOS+** (n = 20)	**BOS-** (n = 90)	**controls** (n = 422)
0	1 (5)	33 (37)	155 (37)
1	6 (30)	30 (33)	153 (36)
2	3 (15)	19 (21)	74 (18)
3	8 (40)	7 (8)	35 (8)
4	2 (10)	1 (1)	5 (1)
5	0 (0)	0 (0)	0 (0)

data are shown as n(%).

Discussion

The aim of this study was to assess whether polymorphisms in the genes of 10 TLRs associate with BOS. The results show an association between polymorphisms in *TLR2*, *TLR4* and *TLR9* and BOS. These findings support the hypothesis that genetic polymorphisms that alter the expression and/or function of innate immune receptors of the TLR family contribute to the interindividual differences in the onset and severity of lung transplant rejection.
In general, TLR2 and TLR4 are important in the signaling pathway for bacterial microorganisms. TLR9 was shown to be important in recognizing viral pathogens.[10]

One study showed that the homozygous *TLR2* Arg753Gln (*rs5743708*) polymorphism, a SNP not shown to be of risk in our study, is associated with allograft failure after liver transplantation.[18] TLR2 mediates cellular responses to various microbial danger signals

including lipoproteins, peptidoglycans from gram-positive bacteria and products from yeast and endogenous ligands.[10, 19, 20] The role of TLR2 in BOS is not known but polymorphisms in *TLR2* might be responsible for an impaired immune response to respective ligands by defective intracellular signaling and impaired cytokine secretion and an increased predisposition to microbial sepsis, as described for other polymorphisms in *TLR2*.[21-23]

TLR4 is highly expressed on alveolar macrophages and on airway epithelia.[3] It interacts with lipopolysaccharide to induce production of chemokines and cytokines, recruit and activate monocytes and macrophages and upregulate co-stimulatory molecules on antigen-presenting cells necessary for appropriate adaptive responses.[24]

Lung transplant recipients heterozygous for 1 of the 2 functional polymorphisms in the *TLR4*, Asp299Gly (*rs4986790*) and/or Thr399Ile (*rs4986791*), downregulated the response to endotoxin and had a lower incidence of acute rejection.[13] Acute rejection is an important risk factor for the development of BOS.[25, 26] This may explain the decreased rate of BOS in *TLR4* heterozygous patients. The mechanism underlying the reduced frequency of allograft rejection as observed in *TLR4* heterozygosity may be related to decreased circulating pro-inflammatory cytokines and chemokines and soluble adhesion molecules.[27]

Palmer et al. concluded that carriers of the G allele of Asp299Gly had a trend towards a reduced frequency of BOS.[3, 13] We genotyped the SNP coding for Asp299Gly and did not find an association with the development of BOS. We calculated the LD for Asp299Gly and the *TLR4* SNP (*rs1927911*) that was found to be significantly different between the BOS^{pos} patients and BOS^{neg} patients and controls. They segregate together with a low LD: $r^2 = 0.02$ and $D' = 1$. The G allele of Asp299Gly was thus completely linked to the C allele of *rs1927911* ($D' = 1$). We found that carriers of the C allele have a reduced risk to develop BOS, which is in agreement with the results of Palmer et al.

TLR9 is localized intracellularly and it is activated by unmethylated CpG motifs that are present in high frequency in DNA from various microbes, especially viruses.[10, 28, 29] TLR9 is critically required in the process of cytomegalovirus (CMV) sensing to assure rapid anti-viral responses.[30, 31] The TLR9 ligand CpG prevents graft acceptance both by interfering with natural regulatory T-cell (Treg) function and by promoting the differentiation of Th1 effector T cells in vivo.[8, 9] It is known that suppression of the Tregs is associated with the development of BOS.[32, 33]

This study was a single-centre retrospective investigation. Because of the relatively small number of patients it was difficult to determine whether these polymorphisms or a combination of risk alleles had a significant impact upon the severity and onset of BOS. Additional multi-centre studies with larger number of patients and a longer follow-up time would be useful to confirm our observations. In addition, we suggest that experimental studies be conducted to better understand the molecular mechanisms underlying our clinical observations.

In conclusion, our data demonstrate that polymorphisms in *TLR* genes, that recognize viral and bacterial pathogens, have a significantly different distribution in BOS^{pos} patients compared with BOS^{neg} patients and controls. The mechanism by which these polymorphisms contribute to the development of BOS might be an increased secretion of cytokines and chemokines and a suppression of the function of Tregs and, by this, a more severe and prolonged injury of the epithelium and subsequent the development of BOS. This implies that, in addition to multiple environmental causes like infection and aspiration, bacterial as well as viral pathogens may promote the development of BOS.

The functionality of these genetic polymorphisms and their association with rates of infection remains to be determined and studies with more patients and prolonged follow-up will be required to determine whether a combination of polymorphisms of *TLR2*, *TLR4*, and *TLR9* may lead to an earlier onset or more severe BOS.

References

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CHAPTER 3

SYSTEMIC AND EXHALED CYTOKINE AND CHEMOKINE PROFILES ARE ASSOCIATED WITH THE DEVELOPMENT OF BRONCHIOLITIS OBLITERANS SYNDROME

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Abstract

Background

The mechanisms that lead to the fibrotic obliteration in bronchiolitis obliterans syndrome (BOS) may involve the interactions between T-helper (Th)1 and Th2 cytokines. The aim of this study is to determine the Th1 and Th2 cytokine and chemokine profiles in serum and exhaled breath condensate (EBC) in lung transplant recipients and to assess their usefulness as biomarkers to predict the development of BOS.

Methods

Serum and EBC from 10 patients with BOS (BOS^{pos}) and 10 patients without BOS (BOS^{neg}), matched for clinical and demographic variables, were analyzed with a multiplex immunoassay to measure a panel of 27 cytokines and chemokines.

Results

The pro-inflammatory cytokines in serum were elevated in lung transplant recipients compared with controls. BOS^{pos} patients had significantly lower concentrations of interleukin (IL)-4, IL-13 and vascular endothelial growth factor (VEGF) compared with BOS^{neg} patients. The concentration of IL-5, however, was significantly higher in BOS^{pos} patients.

Levels of IL-4 and IL-5 were hardly detectable in EBC. IL-13 and VEGF, both decreased in serum in BOS^{pos} patients, were also decreased in EBC in BOS^{pos} patients compared with BOS^{neg} patients. Longitudinal analysis of cytokines and chemokines in serum and EBC from the time of lung transplantation onwards did not reveal significant trends in cytokines and chemokines that preceded the diagnosis of BOS.

Conclusions

Levels of pro-inflammatory cytokines were increased in lung transplant recipients compared with controls. From the moment of transplantation onwards, there is a different pattern of Th2 cytokines in serum in BOS^{pos} patients than in BOS^{neg} patients.

Introduction

Lung transplantation has emerged as an effective treatment for patients with end-stage lung disease. However, the 5-year survival after lung transplantation is only 50%.[1, 2] The main cause of the limited long-term survival is the development of chronic rejection, which is termed bronchiolitis obliterans syndrome (BOS).[3] BOS, the clinical definition of obliterative bronchiolitis, describes the deterioration of graft function after lung transplantation not due to acute rejection, infection or problems of the bronchial anastomosis. The process is characterized by cellular infiltration, fibrosis and occlusion of the small airways in the allograft.[4] The diagnosis BOS is made when a progressive sustained decline in forced expiratory volume in one second (FEV_1) of more than 20% occurs.[5]

The understanding of the immunologic processes that lead to BOS remains to be resolved. Intrinsic (i.e. human leucocyte antigen mismatch, acute rejection) as well as extrinsic factors (i.e. infection, gastroesophageal reflux disease) might lead to airway injury and might be involved in the development of BOS.[6-9]

Critical to airway repair is a balance between pro-inflammatory and anti-inflammatory cytokines and chemokines. The specific mechanisms that lead to the fibrotic obliteration of allograft airways during BOS may involve an imbalance between T-helper (Th)1 and Th2 cytokines.[10] Th1 pro-inflammatory cytokines are considered to induce cell-mediated immunity and allograft rejection. Several studies showed that a Th1-dominated immune response might be correlated with the development of BOS.[11, 12] Conversely, type 2 immune response can also promote rejection.[13] Increased levels of interleukin (IL)-13, a Th2 profibrotic cytokine, are associated with the development of BOS.[14, 15] Chronic rejection in renal transplantation was associated with high levels of IL-4.[16] Chemokines and their interaction with specific cell receptors are essential components of any immune responses through the recruitment of specific leukocyte sub-populations. Understanding of the immunologic processes that are involved in the development of BOS is an essential pre-requisite for ultimate improvement of the long-term survival after lung transplantation.

The analysis of exhaled breath condensate (EBC) constituents as a way of monitoring inflammation of the lungs has gained interest in a number of common lung diseases.[17] Several constituents of EBC, including exhaled CO and NO, are being extensively investigated in chronic obstructive pulmonary disease and asthma.[18-24] Lung transplant recipients with chronic rejection have a lower EBC pH and, as a reflection of increased airway neutrophilia, an increased exhaled NO and CO level than stable lung transplant patients and healthy volunteers.[25-29] The value of other biomarkers, such as cytokines and chemokines, in the EBC of patients after transplantation has not been extensively studied.

At the moment the diagnosis BOS is made, the process of fibrosis, infiltration, and obliterations is already at an advanced and mostly irreversible stage.[1] This emphasizes the

need of biomarkers that could detect processes leading to BOS before a deterioration of FEV_1 occurs.

The aim of this study is to determine the Th1 and Th2 cytokine and chemokine profiles in serum and EBC in lung transplant recipients and to assess their usefulness as biomarkers to predict the development of BOS.

For this study, the levels of cytokines and chemokines in serum and EBC were longitudinally collected after lung transplantation and analyzed with a multiplex immunoassay to measure a panel of 27 cytokines and chemokines. To our knowledge, this is the first study to measure in such detail the cytokine and chemokine profiles in serum and EBC in lung transplant recipients.

Material and methods

Patients and clinical data

Between September 2003 and November 2008, all patients who underwent lung transplantation in the Heart Lung Centre in Utrecht, the Netherlands, were asked to participate in a study on biomarkers for development of BOS. After approval by the medical–ethical committee, informed consent was obtained and blood samples were taken every month in the first year after transplantation and once every 3 months in the following years. EBC was usually collected at the time of blood sampling.

The BOS diagnosis was made when a decline in FEV_1 of greater than 20% from the baseline occurred at two separate measurements with an interval of at least three weeks and in the absence of infection or other etiology.[5]

Standard immunosuppressive therapy for all patients consisted of basiliximab (induction; day 0 to 4, 20 mg), tacrolimus (targets levels, 10-15 ng/ml; after 4 - 6 months, 5 - 10 ng/ml), mycophenolate mofetil (day 0 to 4, 1500 mg twice daily; day 4 to month 2, 1000 mg twice daily; from month 2 to 3, 750 mg twice daily; after 1 year, 500 mg twice daily), and prednisone (the first days, high dosage of methylprednisolone; from day 4 to 8, 30 mg prednisone; day 8 to week 3, 25 mg; week 3 to 4, 20 mg; week 4 to month 7, 15 mg; month 7 onward, 10 mg).

No surveillance bronchoscopies were performed. When infections were excluded as cause of FEV_1 decline, the patients were treated with corticosteroids and azithromycin (500 mg the first 3 days, followed by 250 mg every second day). BOS was diagnosed when no increase in lung functions was observed.

Cytomegalovirus (CMV)-positive recipients and CMV-negative recipients with a CVM-positive donor were treated with valganciclovir for 6 months, according to protocol.

To exclude clinical and demographic variables influencing the results, each patient with BOS (BOS^{pos}) was paired with the closest matched patient who did not develop BOS (BOS^{neg}). The clinical and demographic variables used to match BOS^{pos} and BOS^{neg} patients included

age (difference in age < 3 years), sex, primary lung pathology, post-operative follow-up time (difference in post-operative follow-up time < 1 year), and type of transplantation (unilateral or bilateral). Patients were matched on 5 items, with a median of 4.0 matching items (range, 2.0 – 5.0 items).

The control group comprised 40 healthy adult volunteers who were not receiving any medical treatment at the time of analysis. The characteristics of this group in terms of blood and EBC cytokines and chemokines are described elsewhere (unpublished data by Nagtegaal et al.).

EBC and serum

For the BOS^{pos} patients in this study, serum and EBC were collected every 2 to 3 months after transplantation until BOS was diagnosed. For the matched BOS^{neg} patients, serum and EBC samples with similar intervals after lung transplantation from which the samples for their BOS^{pos} counterparts were obtained were analyzed. Each patient provided 3 to 15 serum samples and 3 to 16 EBC samples (Table 1).

The serum samples were stored at –80 °C, and multiplex immunoassay analysis was performed en bloc. EBC was collected using an Ecoscreen breath condenser (Jaeger Inc, Hoechberg, Germany). Participants were breathing tidal for 15 minutes as recommended, and the collected EBC (approximately, 0.5-2 ml) was stored at -80 °C for subsequent multiplex immunoassay analysis.[30] Contamination of EBC with saliva was avoided by a saliva trap incorporated in the mouthpiece of the Ecoscreen breath condenser.

Multiplex immunoassay analysis of cytokines and chemokines

A 27 multiplex immunoassay (Bio-Rad Laboratories Inc., Hercules, CA, USA) was used to analyse serum and EBC. Cytokines and chemokines were measured using a xMAP technology (Luminex Corporation, Austin, TX, USA) on a Bioplex 100 instrument. Data analysis was performed with Bioplex Manager 4.1 software (Bio-Rad Laboratories). An 8-point standard curve in duplicate was included on every 96-well plate. Measurements that were not on the linear part of standard curves (low concentrations) were considered unreliable and marked as below detection limit. The detection limit (pg/ml) for every variable and the percentage of the samples above the detection limit are reported in Table 2. Results with more than 50% of the samples above the detection limit were used for further analysis. All cytokine and chemokine concentrations are expressed in pg/ml.

Table 1. Baseline characteristics.

Variable	BOS+	BOS-	controls
Total Number	10	10	40
Gender, no.			
male	3	4	24
female	7	6	16
Mean age, mean ± SD, years	45.2 ± 15.0	45.7 ± 13.1	22.4 ± 5.5
Diagnosis, no.			NA
COPD	3	3	
CF	4	5	
IPF	1	0	
sarcoidosis	1	0	
alpha-1 antitrypsin deficiency	1	1	
others	0	1	
Type of graft, no.			NA
bilateral	10	10	
unilateral	0	0	
Survival, mean ± SD, months	33.6 ± 20.0	46.4 ± 9.5	NA
BOS-free survival, mean ± SD, months	19.3 ± 12.5	46.4 ± 9.5*	NA
BOS grade at diagnosis, no.		NA	NA
1	7		
2	3		
3	0		
Histology, no.		NA	NA
biopsy - histological BOS	4		
biopsy - no histological BOS	2		
no biopsy	4		
Acute rejection, no.**	1	0	NA
CMV infection, no.***	2	1	NA
Samples (serum/EBC), no. ****			NA
pair 1	12/15	15/16	
pair 2	6/4	4/6	
pair 3	8/6	10/10	
pair 4	3/6	3/3	
pair 5	5/7	7/7	
pair 6	11/13	13/15	
pair 7	6/4	6/8	
pair 8	10/12	6/5	
pair 9	8/5	9/8	
pair 10	5/5	4/5	
PGD (at T0/T24/T48/T72)*****			NA
grade 0	6/7/7/7	8/7/6/5	
grade 1	1/3/2/2	0/0/0/2	
grade 2	3/0/0/1	2/3/3/1	
grade 3	0/0/1/0	0/0/1/2	

COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; IPF, idiopathic pulmonary fibrosis; CMV, cytomegalovirus; PGD, primary graft disfunction; NA, not applicable; SD, standard deviation.
* identical to survival; ** less than 6 months after lung transplantation; *** more than 500 copies within 1 year after lung transplantation; **** number of available samples for serum/EBC during follow-up until BOS; ***** number of patients with PGD at 0 (T0), 24 (T24), 48 (T48), and 72 (T72) hours after transplantation (T0/T24/T48/T72).

Table 2. Characteristics of the 27 cytokines and chemokines in serum and exhaled breath condensate (EBC) in lung transplant recipients and controls.

Factor	Detection limit (pg/ml)	BOS+ serum	BOS - serum	BOS + EBC	BOS - EBC	Detection limit (pg/ml)	controls serum	controls EBC
Pro-inflammatory								
IL-1b	0.09	100	99	35	100	0.01	98	39
IL-6	0.25	100	100	81	92	0.01	100	46
IL-17	4.66	100	99	ND	4	2.10	100	5
IP-10	2.60	100	99	62	96	1.16	100	90
MCP-1	0.18	100	100	99	100	0.30	98	15
Anti-inflammatory								
IL-1ra	8.84	100	99	6	3	5.52	100	18
Th1 cytokines								
IL-2	0.47	73	72	ND	47	0.50	80	13
IFN-gamma	3.17	100	99	4	3	1.67	100	3
TNF-alpha	2.04	86	100	100	100	1.76	95	56
Th2 cytokines								
IL-4	0.03	100	99	1	ND	0.02	100	26
IL-5	0.10	98	77	3	ND	0.02	98	59
IL-9	0.79	100	99	3	4	0.56	100	31
IL-13	0.10	100	99	14	85	0.10	100	ND
Regulatory cytokines								
IL-10	0.22	100	100	70	93	0.05	100	89
IL-12	0.76	100	99	ND	30	0.20	98	18
Chemokines								
Eotaxin	3.52	100	99	1	7	0.91	100	31
IL-8	0.17	100	99	82	100	0.07	100	49
MIP-1alpha	2.20	90	65	13	19	0.42	100	21
MIP-1beta	2.03	100	100	ND	1	0.62	98	ND
RANTES	0.79	100	85	3	93	0.50	100	ND
Growth factors								
IL-7	0.08	100	99	10	ND	3.73	98	ND
IL-15	0.89	100	100	100	100	0.45	15	82
FGF-bb	6.37	29	53	51	100	7.53	50	44
G-CSF	2.39	100	99	ND	ND	0.71	100	5
GMCSF	1.60	97	99	57	95	1.61	50	100
PDGF	1.00	100	100	10	58	0.60	100	5
VEGF	0.90	100	99	5	46	0.10	100	54

FGF-bb, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IFN, interferon; IP, induced protein; MCP, monocyte chemotactic protein; MIP, macrophage-inflammatory protein; ND, not detectable; PDGF, platelet-derived growth factor; RANTES, regulated upon activation normal T-cell expressed and secreted; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.
Columns 3 to 6 and 8 to 9 show the percentage of the samples above the detection limit.

Statistical analysis

Statistical analyses were performed with SPSS for Windows, version 17.0 (SPSS Inc, Chicago, IL, USA). Statistical significance of the characteristics of the groups was calculated using the paired samples t-test and χ^2 test.

A linear mixed model was used to compare the levels of cytokines and chemokines of BOS^{pos} and BOS^{neg} patients over time.[31] Non-normal data were log-transformed, by which normality was achieved.

The data follow a non-parametric distribution; therefore medians are used instead of means. The median concentration used is the median of all the samples of one group. The Mann-Whitney U-test was used to compare the results of the lung transplant recipients with the results of the healthy controls. For statistical analysis, cytokine and chemokine-concentrations with undetectable values were assigned a value of half of the respective lower limit of detection.

The longitudinal analysis of cytokine and chemokine concentrations used all the available samples of serum and EBC from the first 6 months after lung transplantation and the last 6 months before the diagnosis BOS was made (Figures 3 and 4). When the BOS-free survival was less than 12 months, this period was divided in 2 equal periods. The first period was used for the first 6 months and the last period for the last 6 months; for example; 8 months to BOS, then the samples of the first 4 months were used for the six months after lung transplantation, and samples of the last 4 months were used for the 6 months before transplantation. Values of $p \leq 0.05$ were considered statistically significant.

Results

In the period from September 2003 to November 2008, 105 patients received lung transplantation in the Heart Lung Centre of the University Medical Centre Utrecht. In this group 13 (12%) patients developed BOS. Written informed consent was provided and serum and EBC samples were available for longitudinal analysis for 87 patients, of whom 10 patients were diagnosed with BOS. Each BOS^{pos} patient was matched as closely as possible with a transplanted BOS^{neg} patient (Figure 1). The baseline characteristics are shown in Table 1.[32]

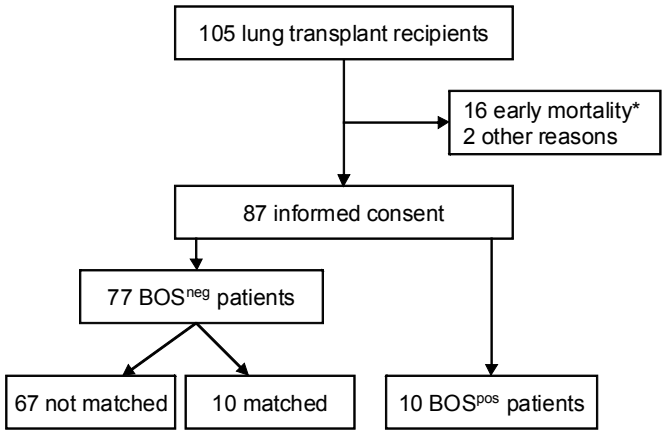

Figure 1. Flow diagram of matched patients with bronchiolitis obliterans syndrome (BOS^{pos}) and without (BOS^{neg}). * Including primary graft dysfunction.

Lung transplant recipients compared with healthy controls

Most of the cytokines and chemokines measured by the 27 multiplex immunoassay in serum, except IL-4, IL-5 and IL-6, showed a significant difference between lung transplant recipients (BOS^{pos} and BOS^{neg}) and controls. The most remarkable finding was that despite all the immunosuppressive medication, the pro-inflammatory cytokines in serum revealed a marked elevation in lung transplant recipients: IL-1b and tumor necrosis factor (TNF)-α were increased compared with the healthy participants. IL-1ra, antagonist of IL-1b and inhibitor of inflammatory processes, was decreased compared with healthy individuals. Increases in lung transplant recipients were found for the pro-inflammatory chemokine MCP-1, the Th1 cytokine interferon (IFN)-γ and IFN-γ-induced protein (IP)-10. The immunomodulatory cytokine IL-10 was increased 20-fold in lung transplant recipients, as well as the chemokine eotaxin (Figure 2).

In the EBC of the lung transplant recipients, more than 50% of the results in 9 cytokines and chemokines were above the detection limit, compared with 7 cytokines and chemokines in the controls. In lung transplant recipients IL-6, IL-8, IL-10, IL-15, granulocyte-macrophage colony-stimulating factor (GM-CSF), MCP-1 and TNF-α were significantly increased compared with healthy controls. Lung transplant recipients thus had increased concentrations of pro-inflammatory cytokines and the regulatory cytokine IL-10 in blood as well as in EBC.

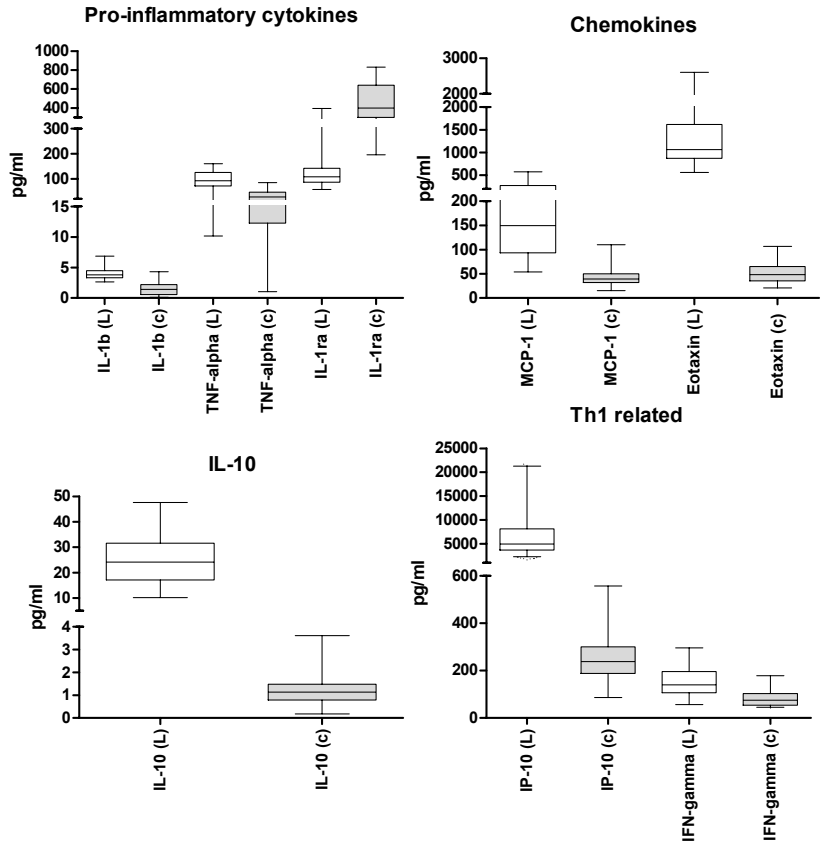

Figure 2. Distribution of pro-inflammatory cytokines, chemokines, interleukin (IL)-10 and T-helper (Th)1 related cytokines and chemokines in lung transplant recipients (L) and controls (c; grey boxes). The horizontal line in the middle of each box indicates the median: the top and bottom borders of the box mark the 75th and 25th percentiles, and the whiskers mark the 5th and 95th percentiles. IFN, interferon; IP-10, induced-protein; MCP-1, monocyte chemotactic protein-1; TNF, tumor necrosis factor.

BOS^{pos} patients compared with BOS^{neg} patients

The median concentrations with interquartile range of the 27 cytokines and chemokines in serum of the first available sample of the first 3 months after lung transplantation are reported in Table 3.

The BOS^{pos} patients had a significantly lower concentrations of the Th2 cytokines IL-4 ($p = 0.003$) and IL-13 ($p < 0.0001$) as well as vascular endothelial growth factor (VEGF; $p = 0.002$) than their BOS^{neg} counterparts. The concentration of IL-5, however, was significantly higher in the BOS^{pos} patients than in the BOS^{neg} patients ($p = 0.006$).

Longitudinal analysis of serum cytokines and chemokines from the time of lung transplantation onwards did not reveal significant changes or trends in cytokines and chemokines profiles that preceded the clinical signs of BOS (Figure 3).

Table 3. Median concentration of cytokines and chemokines of serum of the first available sample of the first 3 months after lung transplantation.

Factor	BOS + Median (IQR) pg/ml	BOS - Median (IQR) pg/ml	Controls Median (IQR) pg/ml
Pro-inflammatory			
IL-1b	3.67 (3.33 - 4.55)	3.77 (3.43 - 4.07)	1.39 (0.56 - 2.07)
IL-6	8.15 (6.32 - 22.81)	11.49 (8.89 - 15.87)	7.21 (5.28 - 10.48)
IL-17	111.17 (98.97 - 148.17)	130.82 (122.69 - 149.60)	27.30 (17.11 - 31.12)
IP-10	5480.26 (3687.33 - 6063.65)	4601.97 (3582.77 - 5118.53)	238.40 (192.74 - 298.36)
MCP-1	162.49 (92.40 - 219.43)	259.36 (164.97 - 445.97)	39.33 (32.90 - 49.71)
Anti-inflammatory			
IL-1ra	116.07 (88.50 - 164.50)	112.76 (88.72 - 171.02)	406.71 (311.35 - 673.02)
Th1 cytokines			
IL-2	2.89 (0.32 - 5.58)	4.85 (0.79 - 15.26)	6.74 (2.31 - 12.51)
IFN-gamma	122.77 (107.29 - 189.09)	192.86 (123.96 - 263.37)	74.95 (53.54 - 100.76)
TNF-alpha	130.25 (91.66 - 146.72)	86.63 (74.11 - 128.21)	27.54 (13.72 - 44.33)
Th2 cytokines			
IL-4	2.65 (2.17 - 3.09)*	3.09 (2.76 - 3.69)	2.69 (2.31 - 3.22)
IL-5	1.09 (0.93 - 1.62)**	0.19 (0.05 - 0.80)	0.70 (0.48 - 1.27)
IL-9	51.57 (35.07 - 87.74)	44.42 (37.93 - 50.04)	20.29 (14.15 - 27.16)
IL-13	6.93 (4.87 - 10.50)***	12.92 (11.04 - 14.50)	2.34 (1.56 - 3.18)
Regulatory cytokines			
IL-10	21.89 (18.45 - 32.14)	25.53 (22.63 - 29.93)	1.14 (0.80 - 1.45)
IL-12	44.81 (37.84 - 68.72)	56.33 (50.97 - 64.11)	6.56 (5.09 - 11.22)
Chemokines			
Eotaxin	1142.33 (1011.70 - 1658.71)	1378.57 (711.28 - 1885.28)	48.44 (36.88 - 64.90)
IL-8	51.41 (26.01 - 126.36)	37.58 (21.42 - 111.62)	15.79 (12.44 - 22.02)
MIP-1alpha	12.79 (9.45 - 17.50)	9.04 (2.43 - 19.13)	5.01 (3.92 - 7.05)
MIP-1beta	217.53 (164.93 - 332.88)	217.53 (175.65 - 268.31)	64.49 (50.45 - 81.05)
RANTES	all > 88.74	all > 891.49	all > 565.00
Growth factors			
IL-7	9.31 (7.89 - 13.39)	9.33 (5.45 - 12.92)	15.99 (12.49 - 21.15)
IL-15	20.92 (19.44 - 26.33)	26.03 (22.13 - 34.64)	0.23 (0.23 - 0.23)
FGF-bb	6.90 (3.19 - 12.23)	15.30 (3.19 - 33.30)	8.43 (3.77 - 77.11)
G-CSF	22.91 (19.89 - 29.93)	23.28 (12,72 - 36.47)	39.37 (33.92 - 51.76)
GMCSF	33.20 (22.24 - 47.88)	39.91 (27.20 - 59.04)	2.38 (0.81 - 13.90)
PDGF	all > 5183.83	all > 5205.43	5265.18 (4328.17 - 6483.71)
VEGF	174.29 (131.02 - 285.64)****	346.19 (315.51 - 460.66)	41.83 (29.98 - 81.36)

FGF-bb, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IFN, interferon; IP, induced protein; IQR, interquartile range; MCP, monocyte chemotactic protein; MIP, macrophage-inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated upon activation normal T-cell expressed and secreted; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Lung transplant recipients compared with controls: IL-4, IL-5, IL-6 were not significantly different, the remaining cytokines/chemokines were significantly different ($p < 0.0001$).

BOS+ vs BOS-: * $p = 0.003$, ** $p = 0.006$, *** $p < 0.0001$, **** $p = 0.002$

Compared with the serum levels of IL-4 and IL-5, both cytokines were hardly detectable in EBC. IL-13 and VEGF, both decreased in serum in BOS^{pos} patients, were also decreased in EBC in BOS^{pos} patients compared with BOS^{neg} patients. The BOS^{pos} patients had a significant lower concentration of IL-8 ($p < 0.001$) and IL-10 ($p = 0.04$) in EBC compared with their BOS^{neg} counterparts. The concentration of IL-15 was significantly increased in BOS^{pos} patients than in the BOS^{neg} patients ($p = 0.013$). The following chemokines were significantly decreased in the BOS^{pos} patients compared to their counterparts: GM-CSF ($p < 0.0001$), basic fibroblast growth factor ($p = 0.033$), IP-10 ($p < 0.0001$) and TNF-α ($p = 0.029$).

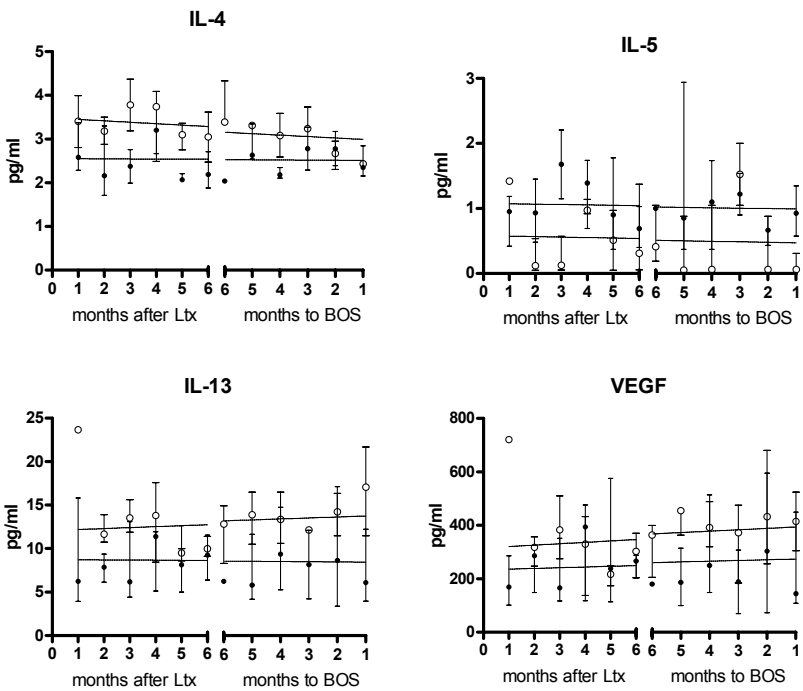

Figure 3. Comparison of interleukin (IL)-4, IL-5, IL-13 and vascular endothelial growth factor (VEGF) in serum from patients with BOS (BOS^{pos}; solid points) and without BOS (BOS^{neg}; open points) 6 months after lung transplantation (Ltx) and 6 months before the diagnosis BOS was made. The median at each month is shown with interquartile range.

As with serum, longitudinal analysis of cytokines and chemokines in EBC from the time of lung transplantation onwards did not reveal significant changes or trends in cytokines and chemokines profiles that preceded the clinical signs of BOS (Figure 4). From the moment of transplantation onwards, the differences between BOS^{pos} and BOS^{neg} patients were already detectable and remained throughout the period of analysis.

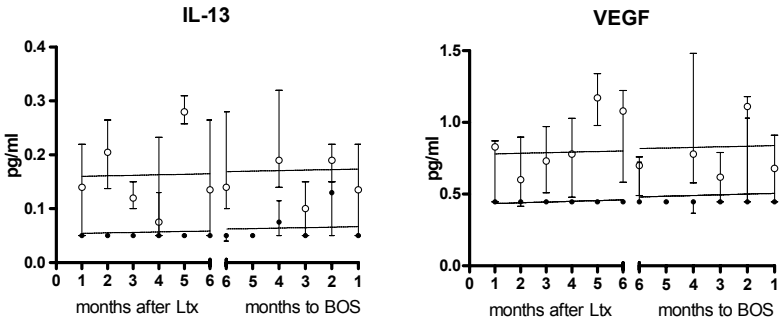

Figure 4. Comparison of interleukin (IL)-13 and vascular endothelial growth factor (VEGF) in exhaled breath condensate (EBC) in patients with BOS (BOS^{pos}; solid points) and without BOS (BOS^{neg}; open points) 6 months after lung transplantation (Ltx) and 6 months before the diagnosis BOS was made. The median at each month is shown with interquartile range.

Discussion

In lung transplant recipients immunologic processes are considered to be involved in the development of BOS.[10-12, 33] The results of this study showed that the immune status of lung transplant recipients, such as reflected in the local cytokine and chemokine profile in the lung as well as in the systemic circulation, is significantly different compared with healthy individuals. A remarkable finding was that despite all the immunosuppressive medication used by lung transplant recipients, levels of the pro-inflammatory cytokines were increased compared with healthy individuals. Contributing to the inflammatory process were the low concentrations of IL-1ra (Figure 2).

The BOS^{pos} patients and the BOS^{neg} patients revealed significant differences in the Th2 cytokines IL-4, IL-5, and IL-13 in serum (Table 3). Levels of IL-4, IL-13 were decreased and levels of IL-5 were increased in the BOS^{pos} patients. In addition, growth factor VEGF was significantly lower in BOS^{pos} than in BOS^{neg} patients.

In EBC, 9 of the 27 cytokines and chemokines had more than 50% of the samples above the detection limit. Most levels of these cytokines and chemokines were elevated in the lung transplant recipients compared with healthy controls. Different pro-inflammatory markers, IL-8, IL-10, IP-10 and TNF-α, were decreased in the BOS^{pos} patients compared with the BOS^{neg} patients.

Results of a study that investigated serum levels of 25 cytokines in BOS^{pos} and BOS^{neg} transplant recipients and healthy controls were recently published.[12] The study found an early post-transplant elevation of basal serum levels of pro-inflammatory chemokines IP-10 and MCP-1, and Th1-cytokines IL-1β, IL-2, Il-12 and IL-15 in BOS^{pos} patients compared with BOS^{neg} patients and controls was found. In addition, a 3-fold decline in IL-10 levels was found

during BOS development. We also found increased levels of pro-inflammatory cytokines and chemokines in the early stage after lung transplantation, but the pattern of cytokines and chemokines was different and the levels did not change during follow-up.

Unfortunately, it is difficult to compare the results of Bharat et al. with our data for several reasons.[12] First, they used a different standard immunotherapy protocol. Second, we used a different assay kit for determination of the cytokines and chemokines. Our respective panels partly overlapped but were not totally identical. Third, they applied a different statistical method. They used a quadrant based sampling method, whereas we included all data points during the first 6 months after lung transplantation and 6 months before the onset of BOS.

We found Th2 cytokines IL-4, IL-5 and IL-13 were specifically different in BOS^{pos} compared with BOS^{neg} patients. The potential role of Th2 cytokines in the development of BOS has not been investigated extensively. Mamessier et al. found an increased IL-4 concentration in serum in evolving BOS and no difference in IL-4 in stable BOS patients compared with healthy controls.[34] This is apparently in contrast with our data, but we did not discriminate between stable and evolving BOS. An investigation of the role of IL-5 in liver transplantation found IL-5 was increased in patients with liver allograft rejection.[35, 36] Although primary graft dysfunction (PGD) after lung transplantation is a different clinical entity than BOS, Hoffman et al., showed that IL-13 is decreased at the moment PGD is diagnosed.[37] In animal models, IL-13 prolongs graft rejection in hearts allografts.[38] The findings of reduced concentrations of IL-4 and IL-13 in BOS^{pos} patients compared to BOS^{neg} are in concert with the results of Paantjens et al. They found that median serum Th2 chemoattractant thymus and activation regulated chemokine levels were significantly lower in BOS^{pos} patients compared with BOS^{neg} patients.[39]

Studies of the role of VEGF in the development of BOS are scarce and therefore the role of VEGF in BOS remains controversial. One study of VEGF in serum in lung transplant recipients revealed that the level of VEGF is not significantly different between BOS^{pos}, BOS^{neg}, and healthy controls.[40] VEGF measured in bronchoalveolar lavage (BAL) is decreased in BOS^{pos} patients compared with stable lung transplant recipients.[40] In the Meyer et al. study $VEGF_{165}$, which is the major secreted form of VEGF, was determined, but a fair amount of other isoforms of VEGF remains detectable.

Except for IL-13, which is decreased in serum as well as in EBC in BOS^{pos} patients compared with BOS^{neg} patients, our study revealed that the spectrum of cytokines and chemokines in the systemic circulation of BOS^{pos} and BOS^{neg} patients significantly differs from that in the lung compartment (EBC).

IL-13 is the most closely related cytokine to IL-4 and shares many functional similarities.[41] The role of Th2 cytokines IL-4 and IL-13 in the process of allograft rejection remains controversial and dependent on their cellular targets. On one hand, Th2 cytokines are thought to blunt the severity of allograft rejection by inhibiting Th1-mediated cytotoxic T lymphocyte and delayed-type hypersensitivity responses.[42] In clinical situations they play a role in chronic inflammation

and fibrosis.[15, 16] On the other hand, studies have demonstrated a protective effect of Th2 cytokines on the allograft. Th2 cytokines, especially IL-4, are necessary for induction of the allograft tolerance.[43, 44] Macrophages activated by IL-4 and IL-13 reduce allograft rejection.[38, 45] Both cytokines increase the cytoprotective molecules and downregulate responses of dendritic cells to lipopolysaccharides and IFN-α and thereby activation of the immune system.[46-49] In our BOS^{pos} patients, IL-4 and IL-13 were decreased compared with BOS^{neg} patients. This might lead to reduced induction of tolerance of the allograft and, finally, to BOS.

IL-5 is a T-cell-derived cytokine that acts as a specific eosinophil differentiation factor through enhancement of eosinophil survival, cytotoxic activity and degranulation.[50] Several investigators have observed that IL-5 and eosinophils may be present during rejection of tissue.[35, 51, 52] There is also evidence that IL-5-dependent eosinophil infiltrate in chronic skin allograft rejection is associated with interstitial graft fibrosis.[53, 54] An increased IL-5 level in our BOS^{pos} patients may lead to an increased number of eosinophils and to a process of rejection and fibrosis. Several studies demonstrated that besides IL-5, eotaxin is also needed for the recruitment of eosinophils.[55-57]

VEGF is a pro-angiogenic and pro-inflammatory growth factor that is highly expressed in pulmonary epithelial cells and is involved in lung injury and wound healing.[40, 58, 59] Krebs et al. provided an explanation for the reduced VEGF levels in serum in BOS^{pos} patients compared with BOS^{neg} patients.[60] They suggest that epithelial cells may secrete VEGF to BAL fluid in stable allografts, and epithelial injury, as part of the development of BOS, leads to a reduction in this secretion. Th2 cytokines and VEGF are both involved in the airway remodeling and inflammation that occurs in asthma.[61-63] Several studies have shown that IL-4 and IL-13 especially stimulate the production of VEGF.[64, 65] Therefore, a decreased level of IL-4 and IL-13 might be an explanation for the low levels of VEGF.

This study was a single-centre retrospective investigation. In our cohort from whom serum was available, clinical signs of BOS developed in 10 patients after a BOS-free survival period of 19 months. This is comparable with most reported series, which show a median time to diagnosis BOS of 16 to 20 months.[66] The number of patients and samples may be too low to detect more subtle differences in cytokines with great intraindividual variation. Additional multicentre studies with larger number of patients and a longer follow-up period would be required to substantiate our conclusions.

For EBC, many of the cytokines and chemokines had levels that were more than 50% below the detection limit. Therefore, our negative results should be interpreted with caution. Rosias et al. measured 8 cytokines in EBC of healthy adults. The overall level of detection was 46% to 97%.[67] In our study, the overall level of detection for these 8 cytokines ranged from 1% to 100%.

The increased concentrations of EBC in the lung transplant recipients are logically due to the local process of inflammation and fibrosis in the lungs. However, the cellular source of the cytokines and chemokines of EBC is unclear. Whether the EBC is derived from the airways

or the alveolar space, or both, is unknown. Furthermore, it is not known which proportion of EBC consists of epithelial lining fluid and which of exhaled water. The value of the decreased pro-inflammatory cytokines and chemokines in BOS^{pos} patients is not clear and needs further investigation. Nagtegaal et al. (unpublished data) concluded that concentration in EBC are not comparable with BAL fluid, except for IL-10 and TNF-α. This might be an explanation for the difference in the levels of IL-8 in EBC and BAL in BOS^{pos} patients. The level of IL-8 in the BAL of BOS^{pos} patients is significantly increased compared with BOS^{neg} patients.[68, 69]

In conclusion, this study shows that the concentration of cytokines and chemokines in lung transplant recipients are completely different than in healthy controls. Despite all the immunosuppressive medications, the pro-inflammatory cytokines in serum were increased in lung transplant recipients. Already shortly after transplantation, there is a different pattern of IL-4, IL-5, IL-13 and VEGF in the serum of BOS^{pos} patients compared with BOS^{neg} patients. These cytokines and chemokines might be useful as a prognostic factor to predict the development of BOS in the period early after lung transplantation and also allow the initiation of early intervention strategies. Because there is no significantly difference in concentration in time between the BOS^{pos} and BOS^{neg} patients, these cytokines and chemokines cannot be used to predict the moment of onset of BOS.

This study evaluated 27 cytokines and chemokines in EBC in lung transplant recipients. Most detectable cytokines and chemokines were significantly increased in the EBC of lung transplant recipients compared with controls. This might be explained by the local process of stress and inflammation in the transplanted lungs. The diagnostic value of cytokines and chemokines in EBC in lung transplant recipients needs further investigation. For the future, a prediction rule in which a combination of certain cytokines and chemokines of serum or EBC, or both, are included could make it possible to determine the risk of developing BOS early after transplantation. Our data indicate that such a panel of cytokines should include IL-4, IL-5, IL-13 and VEGF in serum, and IL-8, IL-10 and IL-15 in EBC.

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CHAPTER 4

GENETIC POLYMORPHISMS IN *MMP7* AND REDUCED SERUM LEVELS ASSOCIATE WITH THE DEVELOPMENT OF BRONCHIOLITIS OBLITERANS SYNDROME AFTER LUNG TRANSPLANTATION

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Abstract

Background

Pulmonary epithelium is the primary target of injury in the development of bronchiolitis obliterans syndrome (BOS) after lung transplantation. Matrix metalloproteinases (MMP)-8 and -9 already have been implicated in the pathogenesis of BOS. MMP-7, which is involved in the repair of the lung epithelium, has not been studied in this respect. We hypothesized that genetic polymorphisms in *MMP7* influence its expression and correlate with serum MMP-7 levels and the development of BOS.

Methods

DNA was collected from 110 lung transplant recipients, including 21 patients with BOS. We genotyped seven single nucleotide polymorphisms in *MMP7* and measured serum MMP-7 levels. The control group comprised 422 healthy individuals.

Results

BOS^{pos} patients had lower levels of MMP-7 than BOS^{neg} patients (7.87 vs 10.18 ng/ml). Significant differences in genotype and haplotype distribution between the BOS^{pos} and BOS^{neg} patients and controls were found. An increased risk for BOS development was found in patients homozygous for the major alleles of *rs17098318*, *rs11568818* and *rs12285347*, and for the minor allele of *rs10502001* (odds ratio, 3.88 - 5.30). Haplotypes constructed with 3 or 4 risk alleles correlated with lower MMP-7 levels.

Conclusions

Genetic polymorphisms of *MMP7* predispose to the development of BOS. Patients carrying these risk alleles express lower levels of MMP-7, which may contribute to aberrant tissue repair and culminate in the development of BOS.

Introduction

The major complication after lung transplantation is the manifestation of bronchiolitis obliterans syndrome (BOS), which affects 50% of the lung transplant recipients after 5 years and is characterized by inflammation and fibrosis.[1, 2] The pathogenesis of BOS remains to be resolved.[3] Recent evidence implicates that the epithelium is the primary target of injury and is prominently involved in the pathogenesis and development of BOS.[4-6]

The production of matrix metalloproteinases (MMPs) is induced by injury. MMPs are essential for lung repair and re-epithelialization, especially MMP-7 is important in the repair of lung epithelium.[7-11] MMPs are key enzymes in regulation of turnover and degradation of connective tissue proteins as well as cell-cell and cell-matrix signaling.[7, 8] Previous work has shown that MMP-8 and MMP-9 are involved in the development of BOS.[12-14] MMP-7 thus far has not been studied in the context of BOS, although its function has been established in fibrotic lung diseases such as idiopathic pulmonary fibrosis and, in particular, the development of various cancers has been established.[15-21]

MMP-7 has a broad substrate specificity, being able to degrade elastin, proteoglycans, type IV collagen, and other components found in the lung matrix.[9, 22] It is a protein constitutively produced by the epithelium of several non-injured, non-inflamed tissues, such as lung, liver, and breast. It is also produced in bone marrow-derived macrophage-like cells, newly deposited tissue macrophages, blood monocytes, and monocyte-derived macrophages.[23, 24] In response to injury of the airway epithelium MMP-7 is upregulated and released basally towards the underlying matrix to facilitate re-epithelialization and cell migration and to regulate the inflammatory response.[7-10, 25] Re-epithelialization, cell migration, and inflammation are all processes related to the development of BOS.

The expression of MMP-7 is primarily regulated at the transcriptional level.[26] We hypothesized that genetic polymorphisms in the *MMP7* gene influence the expression or function of MMP-7 and contribute to the development of BOS. The twofold aims of this study were (1) to establish whether polymorphisms of *MMP7* are associated with the development of BOS and (2) to investigate if these polymorphisms influence the MMP-7 serum levels and thus contribute to disturbances in tissue repair that occur in BOS.

Materials and Methods

Patients and clinical data

All lung transplant recipients who underwent lung transplantation in the Heart Lung Centre in Utrecht, the Netherlands, in the period from July 2001 to November 2008 were asked to donate DNA and serum.

The diagnosis BOS was defined as a decline in forced expiratory volume in one second (FEV_1) of greater than 20% from the baseline determined by average of two measurements made at least three weeks apart in the absence of known acute causes of declining FEV_1, such as acute rejection and infection.[1]

Standard immunosuppressive therapy consisted of basiliximab (induction), tacrolimus, mycophenolate mofetil, and prednisone for all patients. No surveillance bronchoscopies were performed. When infections were excluded as cause of FEV_1 decline, the patients were treated with corticosteroids and azithromycin. When no increase in lung functions was observed, the diagnosis BOS was made.

After study approval by the medical–ethical committee, 110 patients gave written informed consent and DNA and serum was collected. Twenty-one patients had developed BOS (BOS^{pos}). The control group comprised 422 healthy individuals (228 men and 194 women) who were 48.2 ± 11.9 years old (range, 23-77 years).

To exclude that the native lung disease was associated with the differences in *MMP7* genotypes, the diagnoses of the BOS^{pos} patients were matched to a group of 84 patients with similar distributions of diagnoses. These 84 patients were extracted from the transplanted patients without BOS (BOS^{neg}) and the patients on the waiting list (WL). Of these 84 patients, 24 patients were diagnosed with chronic obstructive pulmonary disease (6 BOS^{neg} +18 WL), 23 patients with α-1 antitrypsin deficiency (8 BOS^{neg} + 15 WL), 9 patients with sarcoidosis (3 BOS^{neg} + 6 WL), 14 patients with idiopathic pulmonary fibrosis (5 BOS^{neg} + 9 WL) and 14 patients with cystic fibrosis (5 BOS^{neg} + 9 WL).

Single nucleotide polymorphism selection and genotyping

Three haplotype tagging single nucleotide polymorphisms (SNPs) for the *MMP7* gene were selected using the Tagger program (Broad Institute, Cambridge, MA) for the genomic region of *MMP7* ± 2500 bp on genome build 35. Preferential picking of SNPs was conducted under the pairwise tagging options, a minimum allele frequency setting of 25% and a high Illumina (Illumina Inc; San Diego, CA, USA) design score. The algorithm was set to select tags that would cover the Caucasian HapMap panel with an $r^2 < 0.8$.[27] Furthermore, additional SNPs were selected on the basis of previously published literature or presumed functionality. The following single nucleotide polymorphisms were genotyped; *rs1996352*, *rs12285347*, *rs17884789*, *rs10502001*, *rs11568818*, *rs11568819*, *rs17098318*.

DNA was extracted from whole blood samples and SNP typing was conducted using a custom Illumina Goldengate bead SNP assay according to the manufacturer's manual (Illumina Inc).

Serum MMP-7 levels

Serum MMP-7 levels were measured in 78 healthy controls selected by genotype. Sequential serum samples were analyzed in 18 lung transplantation recipients, 9 BOS^{pos} patients and 9 BOS^{neg} patients matched for several clinical and demographic variables. The baseline characteristics of this cohort are summarized in Table 1. Serum levels of MMP-7 were determined at a single moment for another group of 35 unmatched lung allograft recipients without BOS.

Table 1. Baseline characteristics of the matched cohort.

Variable	BOS+	BOS-
Total number	9	9
Gender, no.		
male	3	3
female	6	6
Age, mean ± SD, years	46.0 ± 15.4	46.9 ± 13.3
Diagnoses, no.		
COPD	3	3
CF	3	4
IPF	1	0
sarcoidosis	1	0
alpha-1 antitrypsin deficiency	1	1
others	0	1
Type of graft, no.		
bilateral	9	9
unilateral	0	0
Time to BOS, mean ± SD, months	19.1 ± 13.3	NA
BOS grade at diagnosis, no (%)		NA
1	6 (67)	
2	3 (33)	
3	0 (0)	

COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; IPF, idiopathic pulmonar fibrosis; NA, not applicable; SD, standard deviation.

To compare serum MMP-7 levels between BOS^{pos} and BOS^{neg} patients at similar time points, a quadrant-based sampling model was used.[28] In BOS^{pos} patients, the follow-up period after lung transplantation until the development of BOS was divided in four equal quadrants, and one sample at the midpoint of each interval was analyzed. The samples from the BOS^{neg} patients were obtained from a chronologically similar visit from which the samples for their BOS^{pos} counterparts were analyzed. In the BOS^{pos} patients one extra sample was analyzed that was obtained within two months before the diagnosis.

An enzyme-linked immunosorbent assay (ELISA) MMP-7 kit (Quantikine, R&D systems Inc, Minneapolis, USA) was used to determine the human active and pro-MMP-7 levels in serum according to the manufacturer's manual.

Statistical analysis

To assess whether the genotype distribution of the polymorphisms was in Hardy-Weinberg equilibrium (HWE), the tests for deviation from Hardy-Weinberg equilibrium and tests for association programme were used (http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl). Statistical significance of the characteristics between the groups were compared with the χ^2 test and the one-way analysis of variance (ANOVA).

The significance threshold was set after accounting for multiple comparisons using a Bonferroni correction for the effective number of independent SNPs proposed by Li and Ji.[29] Owing to linkage disequilibrium, the effective number of SNPs was 4.000 for *MMP7* (6 genotyped SNPs), resulting in an adjusted significance threshold of 0.05 / 4.000 = 0.0127. Therefore, p-values were multiplied by four to adjust for multiple comparisons. Thus obtained p-values ≤ 0.05 were considered statistically significant. To study differences between MMP-7 serum levels of BOS^{pos} and BOS^{neg} patients over time, a linear mixed model was used. Non-normal distributed data were log-transformed. An unpaired t-test was used to compare serum levels between the different groups.

Haplotypes were reconstructed with PHASE program and linkage disequilibrium (LD) structure of the polymorphisms was determined using Haploview 4.1.[30, 31]

Statistical analyses were performed with SPSS 15.0 software (SPSS Inc, Chicago, IL, USA). All continuous data are shown as mean ± standard deviation.

Results

Of the 138 patients who received a lung allograft, BOS developed in 23 (17%). DNA samples were obtained from 110 patients who gave written informed consent, including 21 BOS^{pos} patients. The baseline characteristics are shown in Table 2.

Genotype distribution of *MMP7*

Genotype distribution of the 6 polymorphic SNPs in BOS^{pos} and BOS^{neg} patients and controls are reported in Table 3. One SNP (*rs17884789*) was not polymorphic in our study population (data not shown). The other 6 SNPs were in Hardy-Weinberg equilibrium.

There was a significant difference in the genotype distribution of polymorphisms of the *MMP7* gene between the BOS^{pos} patients and the healthy controls in *rs17098318* (p = 0.016), *rs11568818* (p = 0.016), *rs10502001* (p = 0.040) and *rs12285347* (p = 0.024; Table 3).

Table 2. Baseline characteristics of the 110 lung transplantation recipients and the healthy controls.

Variable	BOS+	BOS-	Controls
Total number	21	89	422
Gender, no. (%)			
male	9 (43%)	45 (51%)	228 (54%)
female	12 (57%)	44 (49%)	194 (46%)
Age, mean ± SD, years	52.4 ± 11.2	49.4 ± 12.7	48.2 ± 11.9
Diagnoses, no. (%)			NA
COPD	7 (33%)	29 (32%)	
CF	3 (14%)	23 (26%)	
IPF	4 (19%)	13 (15%)	
sarcoidosis	2 (10%)	3 (3%)	
alpha-1 antitrypsin deficiency	5 (24%)	7 (8%)	
others	0	14 (16%)	
Type of graft, no. (%)			NA
bilateral	17 (81%)	76 (85%)	
unilateral	4 (19%)	13 (15%)	
Time to BOS, months	23.6 ± 14.8	NA	NA

COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; IPF, idiopathic pulmonary fibrosis; NA, not applicable; SD, standard deviation.

Table 3. Genotype frequency distribution in *MMP7* single nucleotide polymorphisms in patients with BOS (BOS+), patients without BOS (BOS-) and controls.

		SNP	**rs12285347**	**rs10502001**	**rs17098318**	**rs11568818**	**rs1996352**	**rs11568819**
		Gene region	intron 3	exon 2	promoter	promoter	intron 3	promoter
		Major/minor	T/C^	C/T^^	G/A^^^	A/G^^^^	T/C	C/T
Group	**no.**	genotype*	no. (%)	no. (%)	no. (%)	no. (%)	no. (%)	no. (%)
BOS+	21	AA	12 (57)	11 (52)	16 (76)	13 (62)	11 (52)	20 (95)
		AB	6 (29)	6 (29)	4 (19)	6 (28)	8 (38)	1 (5)
		BB	3 (14)	4 (19)	1 (5)	2 (10)	2 (10)	0 (0)
BOS-	89	AA	28 (32)	53 (60)	41 (46)	28 (31)	55 (61)	79 (89)
		AB	40 (44)	32 (36)	34 (38)	40 (45)	28 (32)	10 (11)
		BB	21 (24)	4 (4)	14 (16)	21 (24)	6 (7)	0 (0)
Controls	422	AA	108 (26)	257 (61)	169 (40)	109 (26)	274 (65)	367 (87)
		AB	200 (47)	147 (35)	181 (43)	199 (47)	131 (31)	54 (12,8)
		BB	114 (27)	18 (4)	72 (17)	113 (27)	17 (4)	1 (0,2)

* The A refers to major allele; B to minor allele.
Genotype distritubion in BOS+ vs controls: ^ p = 0.024, ^^ p = 0.040, ^^^ p = 0.016, ^^^^ p = 0.016
Genotype distribution between BOS+ vs BOS- patients and controls and BOS- patiënts is not significantly different.

No significant difference was found in the genotype distribution of these SNPs between all the allograft recipients and the controls, the BOS^{pos} and BOS^{neg} patients and between the BOS^{neg} patients and the controls.

The BOS^{pos} patients had a significantly higher G allele frequency in *rs17098318* compared with the controls (0.86 vs 0.62, p<0.05). The risk that BOS will develop is increased in the following groups:

- homozygotes with GG (odds ratio (OR) 4.78; 95% confidence interval (CI) 1.72-13.33; p = 0.00105) compared with carriers of the A allele;
- homozygotes for the major allele of *rs12285347* (OR 3.88; 95% CI 1.59-9.43; p = 0.00150) compared with carriers of the minor allele;
- homozygotes for the minor allele of *rs10502001* (OR 5.30; 95% CI 1.61-17.24; p = 0.00234) compared with carriers of the major allele; and
- homozygotes for the major allele of *rs11568818* (OR 4.62; 95% CI 1.88 –11.49; p = 0.00031) compared with carriers of the minor allele.

There was no significant difference in the genotype distribution of the SNPs between the BOS^{pos} group and the 84 patients with similar underlying lung disease (data not shown).

MMP-7 serum levels in controls and transplantation recipients

Compared with controls, BOS^{pos} and BOS^{neg} patients have significantly increased serum levels of MMP-7 (Figure 1). The mean serum levels of MMP-7 were 7.87 ng/ml ± 4.92 in the 9 BOS^{pos} patients and 10.18 ng/ml ± 5.89 in the 44 BOS^{neg} patients. In the controls, the mean serum level of MMP-7 was 3.45 ng/ml ± 1.16. In the matched cohort, the BOS^{pos} patients had a significantly lower mean level of MMP-7 than BOS^{neg} patients (p = 0.013). However, longitudinal analysis of subsequent serum levels of MMP-7 during follow-up after lung transplantation in this cohort did not reveal a significantly different course in the BOS^{pos} patients compared with BOS^{neg} patients (data not shown).

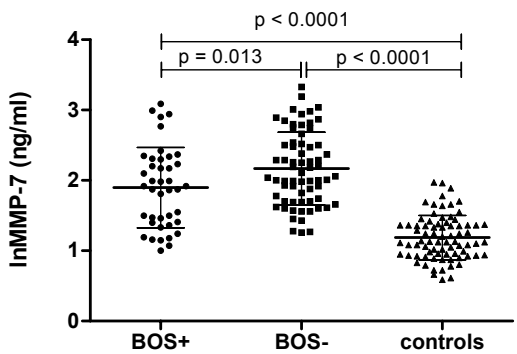

Figure 1. Serum levels of MMP-7 are shown in patients with BOS (BOS+) and patients without BOS (BOS-) and in healthy controls. Horizontal bar represents mean with standard deviation.

Genotypes and haplotypes of *MMP7* and correlation with serum MMP-7

Serum MMP-7 levels in controls did not correlate with the genotypes of the 4 relevant SNPs. In lung transplant recipients, we did observe significant correlations between the *MMP7* genotype and MMP-7 serum levels: homozygotes for the major allele of *rs12285347* and *rs11568818* had a significant lower concentration of MMP-7 than did homozygotes for the minor allele.

The LD structure revealed one haplotype block (Figure 2); therefore, haplotypes of the *MMP7* polymorphisms were constructed and analyzed. Haplotypes with a frequency exceeding 5% were used in this cohort, and 4 haplotypes in the best reconstruction were found (Table 4). The distribution of haplotypes between the BOS^{neg} patients and controls was not significantly different. The BOS^{pos} patients had significantly more homozygotes for haplotype 2 and fewer carriers of haplotype 4 compared with the BOS^{neg} patients and controls.

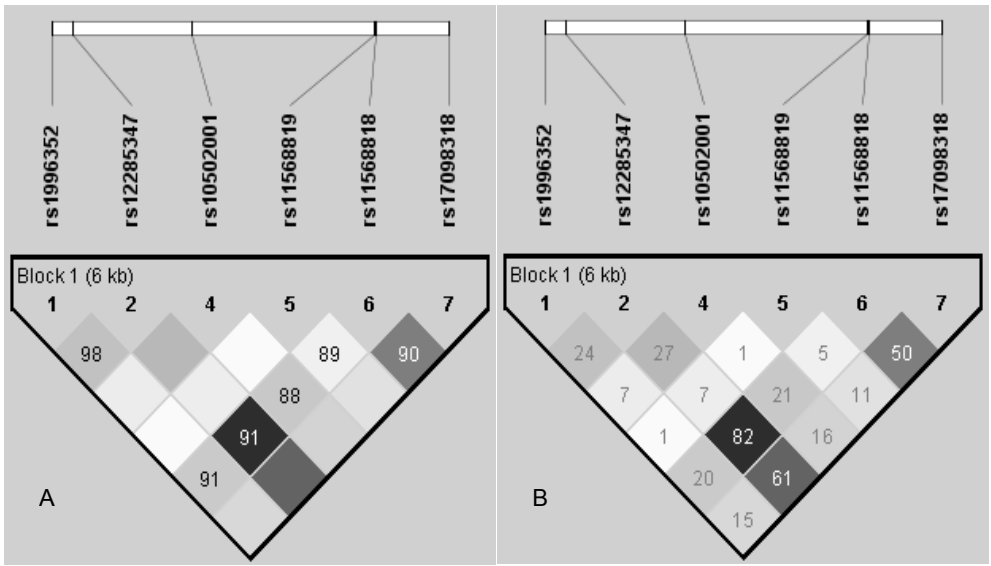

Figure 2. LD structure of single nucleotide polymorphisms in the *MMP7* gene in our cohort. A: values shown are D', blank cells indicate that D'=1. B: values shown are r^2 values.

To analyze the effect of haplotype on serum MMP-7 levels in allograft recipients, we used the serum of the 9 BOS^{pos} patients and the 44 BOS^{neg} patients. Serum levels of MMP-7 were not haplotype-dependent for the heterozygotes and carriers of the 4 haplotypes. Patients homozygous for haplotype 2 had a significantly lower serum level of MMP-7 compared with the patients homozygous for haplotype 3 ($p = 0.004$). Patients with the risk haplotype (haplotype 1 + 2) had significantly lower levels of MMP-7 than did patients with the non-risk haplotype (haplotype 3 + 4; Figure 3).

Table 4. Distribution of haplotypes in patients with BOS (BOS+) and patients without BOS (BOS-) and controls.

Haplotypes	**homozygotes** no. (n%)	**heterozygotes** no. (n%)	**carriers** no. (n%)
1:**TCGACC**			
BOS+	2 (10)	8 (38)	10 (48)
BOS-	6 (7)	27 (30)	33 (37)
controls	17 (4)	123 (29)	140 (33)
2:**TTGATC***			
BOS+	4 (19)	6 (29)	10 (48)
BOS-	4 (4)	33 (37)	37 (41)
controls	18 (4)	133 (32)	151 (36)
3:**CCGGTC**			
BOS+	1 (5)	2 (10)	3 (14)
BOS-	2 (2)	16 (18)	18 (20)
controls	8 (2)	79 (19)	87 (21)
4:**CCAGTC****			
BOS+	1 (5)	3 (14)	4 (19)
BOS-	11 (12)	30 (34)	41 (46)
controls	51 (12)	153 (36)	203 (48)

Haplotype distributions between controls and BOS- patients was not significantly different.
BOS+ vs controls: * p = 0.02, ** p = 0.01; BOS+ vs BOS-: * p = 0.04, ** p = 0.03.
locus 1 rs12285347; locus 2 rs10502001; locus 3 rs17098318; locus 4 rs11568818; locus 5 rs1996352; locus 6 rs11568819.

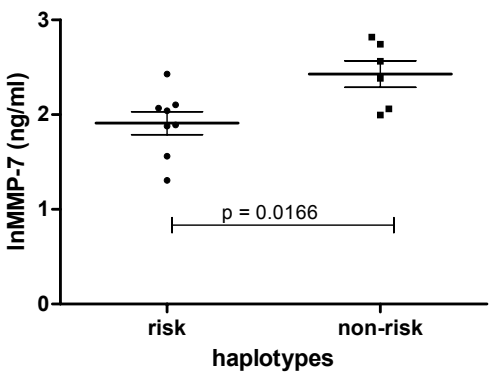

Figure 3. Distribution is shown of serum levels of MMP-7 in lung transplant recipients between haplotype risk alleles (1+2) and haplotype non-risk alleles (3+4). Haplotype risk alleles predispose to the development of bronchiolitis obliterans syndrome.

Discussion

Genetic polymorphisms in the *MMP7* gene predispose to the development of BOS. The risk that BOS would develop was increased in homozygotes for the major alleles of *rs17098318*, *rs11568818*, and *rs12285347*, and homozygotes for the minor allele of *rs10502001* compared with homozygotes of the other alleles. Significantly lower serum levels of MMP-7 were found in patients with a combination of these 'risk alleles' than in those without the 'risk alleles'.

Distribution of *MMP7* genotypes of patients in the total group of lung allograft recipients were not significantly different than in controls. However, BOS^{pos} patients had a significantly different genotype distribution in 4 SNPs compared with controls and to BOS^{neg} patients. Thus as a group, lung transplant recipients are not different from the normal population, but BOS^{pos} patients are a specific subgroup within the total group of recipients.

As also reported by others, serum levels of MMP-7 were low in controls and were not genotype- or haplotype-dependent.[21, 32] We found that serum levels of MMP-7 were increased in the allograft recipients and that this increase was genotype- and haplotype-dependent. These data indicate that *MMP7* genotype has an effect on protein levels but only under conditions of cellular stress, such as after lung transplantation, and not during steady-state conditions, such as in healthy individuals. Therefore, disrepair processes in controls with certain *MMP7* genotypes are not perceptible.

In this study, we observed no relation between the genotype distribution and the BOS grade or the severity of FEV_1 decline. A study with more patients is needed to determine whether there is a relationship between the genotypes of *MMP7* and these clinical parameters.

A final reason that needs to be explored in explaining the difference in MMP-7 levels between controls and patients may be related to azithromycin, which is known to influence the gene expression of some MMPs in human airway epithelia, other than MMP-7.[33]

Two functional SNPs have been identified in the promoter of *MMP7*, *rs11568818* (-181A/G) and *rs11568819* (-153C/T), which have been shown to modulate transcription by influencing the binding of nuclear proteins.[34] The combination of the minor alleles of these SNPs, *rs11568818*G and *rs11568819*T, conferred a twofold to threefold higher level of protein expression.[35] However, the minor allele of *rs11568819* is very rare in the normal population. In this study, the *rs11568819*T allele is only present in 1 out of 422 controls. This individual with *rs11568819*TT also had the GG genotype of *rs11568818* and had a serum MMP-7 level of 7.10 ng/ml. This was the highest level of MMP-7 measured in the controls and was twice the mean of this group.

MMP-7 is induced in response to injury.[7, 10] The airway epithelium is the primary target for injury after lung transplantation.[5, 6] In this study, we showed that lung transplantation recipients had a higher serum level of MMP-7 than controls. MMP-7 levels were significantly higher in BOS^{neg} patients than in BOS^{pos} patients, even before the development of BOS. In

BOS^{pos} patients, the insufficient upregulation of MMP-7 as part of the repair of the lung might be an explanation for the development of BOS.

MMP-7 also has a function in defence against microorganisms and is induced by bacterial exposure. Patients with a decreased level of MMP-7 might be more vulnerable for bacterial infections, and therefore, BOS is more prone to develop.

Animal models of *MMP7* knockout (KO) mice showed that MMP-7 is important in the re-epithelialization of the lung. Wounds in the trachea of *MMP7*-KO mice showed no epithelial migration, and the size of the wound opening did not change significantly.[9] Another study showed that damage to the respiratory epithelium after tracheal transplant in *MMP7*-KO recipient mice induced changes consistent with chronic allograft rejection.[6] Apparently, aberrant airway re-epithelialization is sufficient for the progression of fibrosis during allograft rejection.[5]

In summary, besides the alloimmune-dependent and -independent risk factors already known for the development of BOS, a specific genetic profile for *MMP7* predisposes to the development of BOS. As part of the repair process and potentially supportive to optimize immunosuppressive treatment after lung transplantation, MMP-7 might contribute to the risk of infection and aberrant repair, culminating in the development of BOS.

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CHAPTER 5

YKL-40 AND MATRIX METALLOPROTEINASES AS POTENTIAL BIOMARKERS OF INFLAMMATION AND FIBROSIS IN THE DEVELOPMENT OF BRONCHIOLITIS OBLITERANS SYNDROME AFTER LUNG TRANSPLANTATION

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Submitted

Abstract

Background

The development of bronchiolitis obliterans syndrome (BOS) after lung transplantation is characterized by inflammation, remodeling and fibrosis. Both YKL-40 and matrix metalloproteinase (MMP)-9 have shown to be involved in these processes.

We measured serial YKL-40 and MMP-9 serum levels in lung transplant recipients and assessed their usefulness as biomarker to predict the development of BOS. Furthermore, we investigate the relationship between these two potential biomarkers of BOS and MMP-7.

Methods

Ten patients with BOS (BOS^{pos}) and 10 matched patients without BOS (BOS^{neg}) were included. Serial serum samples were collected after lung transplantation and prior to BOS. YKL-40, MMP-9 and MMP-7 serum levels were determined by ELISA.

Results

The median concentration of YKL-40 did not differ between BOS^{pos} and BOS^{neg} patients ($p > 0.05$).

The median concentration of MMP-9 in BOS^{pos} patients was significantly higher than in BOS^{neg} patients ($p < 0.0001$). For MMP-9 as possible risk factor for BOS, a cut off point of 145 ng/ml has a sensitivity of 90% and a negative predictive value of 83%.

Longitudinal analysis of YKL-40 and MMP-9 serum levels from the early post-transplant period onwards did not reveal a significant trend in time in both serum levels preceding BOS. In BOS^{neg} patients MMP-9 showed an inverse relationship with MMP-7, that was absent in BOS^{pos} patients.

Conclusions

From the moment of transplantation onwards, patients who eventually developed BOS had significantly increased MMP-9 serum levels in comparison with patients who did not develop BOS. Therefore, increased MMP-9 serum levels might be useful as risk factor for BOS.

Introduction

Lung transplantation is the final therapeutic option for patients with end-stage lung disease. Long-term survival after lung transplantation is limited due to the development of chronic rejection, called bronchiolitis obliterans syndrome (BOS).[1] The development of BOS is characterized by injury of the airway epithelium that is caused by events, such as infection, acute rejection or gastroesophageal reflux. After injury, inflammation and remodeling of the airway epithelium take place and might lead to excessive fibroblastic repair and BOS.[2-5] The histopathologic findings in BOS show a broad spectrum of cellular infiltrates, active fibroplasia and inactive fibrosis.[6]

Besides spirometry, no biomarker is available that can confirm the diagnosis BOS or predict putative BOS^{pos} patients. Because established BOS is a process that responds poorly to augmented immunosuppression, biomarkers that detect processes leading to BOS before the deterioration in lung function occurs are needed.[5] YKL-40 and matrix metalloproteinases (MMPs) have shown to be involved in inflammation, remodeling and fibrosis and, therefore, are candidate biomarkers for the development of BOS after lung transplantation.[7-13]

YKL-40 is a chitinase-like protein secreted by several cells, including alveolar macrophages and neutrophils.[14, 15] It is a growth factor for fibroblasts and vascular endothelial cells.[14, 16] The biological properties of YKL-40 suggest that it plays a role in inflammation, remodeling and fibrosis.[11, 17] In asthma, serum YKL-40 may be suitable as biomarker through its increase in severe asthma suggestive for a contribution of YKL-40 to airway remodeling.[10, 18, 19] In pulmonary sarcoidosis, YKL-40 may be a biomarker of disease activity and ongoing fibrogenesis.[11, 12] Furthermore, in patients with idiopathic pulmonary fibrosis (IPF) increased expression of YKL-40 may be associated with fibrosis.[15, 20]

The role of YKL-40 in lung transplantation is not known, however, in heart transplant recipients post-transplant YKL-40 serum levels were associated with rejection and fibrosis,[21] and in liver transplantation elevated post-transplant YKL-40 serum levels were found to accurately predict rapid progression of fibrosis.[22] These studies indicate that YKL-40 can be used as marker for remodeling as well as for fibrosis.

MMPs are a family of enzymes responsible for the turnover and degradation of the extracellular matrix (ECM) through their capacity to cleave structural proteins, as collagens and elastin.[7] MMP-9 is present in low quantities in the healthy adult lung, but much more abundant in the lungs of patients suffering from asthma, chronic obstructive pulmonary disease (COPD) and IPF.[23-25] In addition to the turnover and degradation of the ECM, MMP-9 contributes to the migration of inflammatory cells, as lymphocytes and neutrophils, through the ECM, the basement membrane and the endothelial layer.[7]

Several studies suggest that MMP-9 is correlated with the development of BOS.[2, 26-29] Increased concentrations of MMP-9 in bronchoalveolar lavage (BAL) fluid have been shown

to be indicative for the development of BOS, but were also considered non-specific and attributed to lung transplantation itself.[2, 26-29] Concerning MMP-9 in serum, only one study has been conducted, which revealed a difference in BAL MMP-9 levels between patients with BOS (BOS^{pos}) and without BOS (BOS^{neg}), but no difference in MMP-9 serum levels.[27]

MMP-7 is involved in the repair of the lung by facilitating cell migration and re-epithelialisation and regulation of the inflammatory response.[8, 9] Lung transplant recipients carrying risk alleles leading to lower levels of MMP-7 were shown to be predisposed to BOS.[30]

We determined whether YKL-40 and MMP-9 serum levels are potentially useful biomarkers for BOS. Furthermore, we investigated the relationship between these two potential biomarkers and MMP-7 in an attempt to further elaborate the pathogenesis of BOS.

Material and Methods

Patients

Between September 2003 and November 2008, all patients who underwent lung transplantation in the Division of Heart & Lungs of the University Medical Centre Utrecht, the Netherlands, were asked to participate in a study on biomarkers for development of BOS. After approval by the medical–ethical committee, informed consent was obtained. From the participating lung transplant recipients blood samples were taken every month in the first year post-transplantation and once every three months in the following years.

The diagnosis BOS was made when a decline in forced expired volume in one second (FEV_1) of greater than 20% from the baseline occurred which was determined by average of two measurements made at least three weeks apart in the absence of known acute causes of declining FEV_1, as acute rejection and infection.[6]

Standard immunosuppressive therapy consisted of basiliximab (induction), tacrolimus, mycophenolate mofetil and prednisone for all patients.

To exclude the influence of clinical and demographic variables, each BOS^{pos} patient was paired with the closest matched BOS^{neg} patient (Table 1). The variables used to match BOS^{pos} and BOS^{neg} patients included age (difference in age < 3 years), gender, primary lung pathology, post-operative follow-up time (difference in post-operative follow-up time < 1 year), and unilateral or bilateral transplantation. Patients were matched on these five items with a median of 4.0 matching items (range, 2.0 –5.0 items). MMP-7 in serum was measured in the same patient groups.[30]

MMP-9,YKL-40 and MMP-7 serum levels

To compare serum levels between BOS^{pos} and BOS^{neg} patients at similar time points, a quadrant based sampling model was used.[31] In BOS^{pos} patients the follow-up period after lung

transplantation until the development of BOS was divided in four equal quadrants and one sample at the midpoint of each interval was analyzed. The samples from the BOS^{neg} patients were obtained from chronologically similar visits from which the samples for their BOS^{pos} counterparts were analyzed. In the BOS^{pos} patients one extra sample was analyzed that was obtained within two months before the diagnosis. According to this method, five samples were collected for the BOS^{pos} patients and four samples for the BOS^{neg} patients. However, for BOS^{pos} patients no. 3 and 4 respectively four and three samples were included due to a short BOS-free survival which led to a smaller number of samples. For BOS^{neg} patient no. 4 only two samples were included. For BOS^{pos} patients no. 5,7 and 9 and BOS^{neg} patient no. 2 one sample was missing.

The serum samples were stored at –80 °C until analysis. MMP-9 serum levels were determined with a MMP-9 enzyme-linked immunosorbent assay (ELISA, Human Biotrak Elisa System, GE Healthcare, Buckinghamshire, UK). YKL-40 serum levels were determined with a YKL-40 ELISA (Quidel Corporation, San Diego, CA, USA). MMP-7 serum levels were determined with a MMP-7 ELISA (Quantikine, R&D systems Inc, Minneapolis, USA) as described previously.[30] All performed in accordance with the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed with SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA). Differences between groups were determined using the paired samples t-test and χ^2 test. Serum levels were not normally distributed and are expressed as median with interquartile range (IQR). Mann-Whitney U-test was used to calculate the differences in serum levels between the groups.

To determine whether there is a trend in the serial serum levels over time in a single subject, and to compare this trend between the two groups, a restricted maximum likelihood linear mixed model was used.[32] Time was treated as a random factor (effect) and the fit of the model was assessed via the -2 restricted log likelihood (lowest value indicated best fit). The unstructed covariance matrix led to the lowest -2 restricted log likelihood.

The diagnostic accuracy of MMP-9 in serum was evaluated using receiver operating characteristic (ROC) curve analysis, which correlates true- and false-positive rates (sensitivity and (1-specificity)). An area under the ROC curve (AUC) with 95% confidence intervals (CI) was calculated for MMP-9 serum levels. The best cut off point was determined by using the intersection of the sensitivity with the specificity. Sensitivity, specificity, positive predictive value and negative predictive value were calculated using a 2×2 table of the collected data.

Correlations between serum levels of the different markers were assessed with Spearman's rho. p-values ≤ 0.05 were considered statistically significant.

Table 1. Baseline characteristics of the patients with BOS (BOS+) and the patients without BOS (BOS-) patients.

Variable	BOS+	BOS-
Total number, no.	10	10
Gender		
male	3	4
female	7	6
Mean age, mean ± SD, years	45.2 ± 15.0	45.7 ± 13.1
Diagnosis, no.		
COPD	3	3
CF	4	5
IPF	1	0
sarcoidosis	1	0
alpha-1 antitrypsin deficiency	1	1
others	0	1
Type of graft, no.		
bilateral	10	10
unilateral	0	0
Survival, mean ± SD, months	33.6 ± 20.0	46.4 ± 9.5
BOS-free survival, mean ± SD, months	19.3 ± 12.5	46.4 ± 9.5*
BOS grade at diagnosis, no.		NA
1	7	
2	3	
3	0	
Histology, no.		NA
biopsy - histological OB	4	
biopsy - no histological OB	2	
no biopsy	4	
Acute rejection,** no.	1	0
CMV infection*,** no.	2	1

COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; IPF, interstitial pulmonary fibrosis; CMV, cytomegalovirus; all data are shown as mean ± SD; NA, not applicable; * identical to survival; ** less than 6 months after lung transplantation; *** more than 500 copies within 1 year after lung transplantation.

Results

In the study period 105 patients received a lung transplantation in our centre of whom 13 (12%) patients developed BOS. Eighty-seven patients, including 10 patients with BOS, gave a written informed consent and serum samples were available for longitudinal analysis. The baseline characteristics of the BOS^{pos} and BOS^{neg} patients are shown in Table 1.

YKL-40 and MMP-9 serum levels

For YKL-40, the median concentration (IQR) of all serial samples in the BOS^{pos} patients was 160 (126 – 279) ng/ml and in the BOS^{neg} patients 164 (95 – 209) ng/ml ($p > 0.05$).

For MMP-9, the median concentration of all serial samples was significantly different between BOS^{pos} and BOS^{neg} patients: 190 (163 – 238) ng/ml versus 128 (106 – 162) ng/ml ($p < 0.0001$). For every matched pair the median MMP-9 serum level was higher in the BOS^{pos} patient than in the BOS^{neg} counterpart (Figure 1).

Longitudinal analysis of YKL-40 and MMP-9 serum levels from the time of transplantation onwards did not reveal a significant decrease or increase in serum levels in the period preceding BOS.

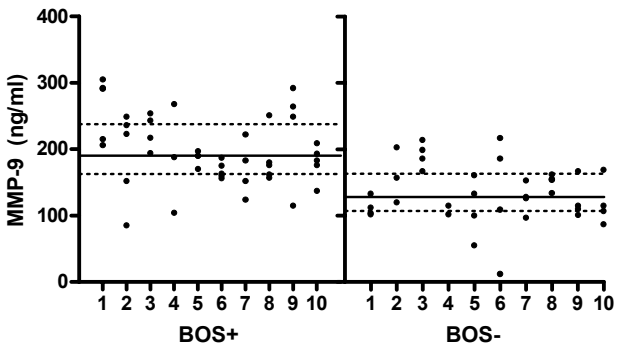

Figure 1. Serum levels of MMP-9 in patients with BOS (BOS+) and patients without BOS (BOS-) ($p < 0.0001$). Matched pairs of BOS+ and BOS- patients have identical numbers. Horizontal lines represent group median (line) with interquartile range (dotted line).

MMP-9 serum level as biomarker for BOS

Because MMP-9 serum levels were significantly different between the BOS^{pos} and BOS^{neg} patients a ROC curve analysis was performed with the samples of the first quadrant after lung transplantation. The AUC and 95% confidence interval of MMP-9 were 0.79 and 0.58 – 0.98, respectively. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of several cut off points for MMP-9 are presented in Table 2. The best cut off point was 162 ng/ml with a sensitivity, specificity, PPV and NPV of 60%, 70%, 67% and 63%, respectively. A lower cut off point of 145 ng/ml with a sensitivity of 90% and a NPV of 83% might be more useful in clinical practice for identifying putative BOS patients.

Table 2. Cut off points for MMP-9 serum levels for the diagnosis of BOS.

Cut off point	Sensitivity	Specificity	PPV	NPV
145 ng/ml	90%	50%	64%	83%
155 ng/ml	70%	60%	63%	66%
162 ng/ml	60%	70%	67%	63%

PPV = positive predictive value; NPV = negative predictive value.

Correlation between YKL-40, MMP-9 and MMP-7 serum levels

The median concentration with IQR of MMP-7 in 9 BOS^{pos} patients and in 9 matched BOS^{neg} patients was 7 (4 – 10) ng/ml and 9 (7 – 15) ng/ml, respectively (p = 0.010).

YKL-40 serum levels in BOS^{pos} patients correlated with MMP-7 serum levels (Spearman rho 0.65, p < 0.0001, Figure 2). There was no correlation between YKL-40 and MMP-7 in BOS^{neg} patients (Spearman rho 0.06, p = 0.76). YKL-40 did not correlate with MMP-9, neither in the BOS^{pos} nor in the BOS^{neg} patients (Spearman rho 0.02, p = 0.88 and 0.03, p = 0.87, respectively). In BOS^{neg} patients, MMP-9 showed an inverse relationship with MMP-7 (Spearman rho - 0.42, p = 0.015, Figure 3), but in BOS^{pos} patients no correlation between MMP-7 and MMP-9 was found.

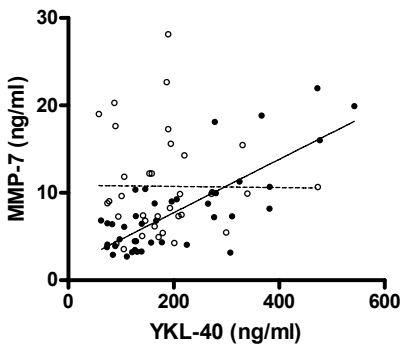

Figure 2. Correlation between YKL-40 and MMP-7 in serum of BOS^{pos} patients (solid points, solid line, Spearman rho 0.65, p < 0.0001) and the absence of correlation in BOS^{neg} patients (open points, dotted line, Spearman rho 0.06, p = 0.76). Lines represents linear regression.

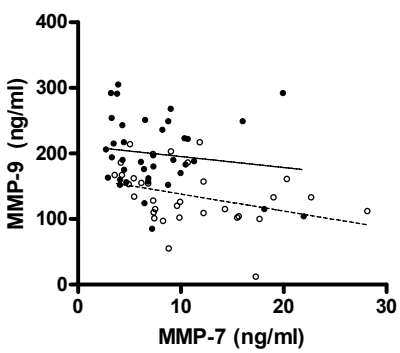

Figure 3. Correlation between MMP-7 and MMP-9 in serum of BOS^{neg} patients (open points, dotted line, Spearman rho -0.42, p = 0.015) and the absence of correlation in BOS^{pos} patients (solid points, solid line, Spearman rho -0.14, p = 0.40). Lines represents linear regression.

Discussion

In this study we measured YKL-40 and MMP-9 serum levels in lung transplant recipients with and without BOS. We found significantly increased MMP-9 serum levels in BOS^{pos} patients compared with BOS^{neg} patients in the period after lung transplantation and before the diagnosis BOS was made. This difference in MMP-9 serum levels can be detected from the moment of lung transplantation onwards. Therefore, MMP-9 serum levels after lung transplantation might be useful for risk stratification of putative BOS^{pos} patients.

A few recent studies have suggested a role for MMP-9 in the development of BOS, but their results were not conclusive.[2, 26-29] In one study it was found that MMP-9 levels in BAL fluid were increased in all lung transplant recipients.[2] Other studies showed that MMP-9 levels in BAL fluid and the activity of MMP-9 were increased in BOS^{pos} patients compared with BOS^{neg} patients.[26-29]

Taghavi et al. performed the only study that measured MMP-9 in serum at one time point after lung transplantation, but these authors did not find a difference in serum levels between BOS^{pos} and BOS^{neg} patients.[27] However, because of the intraindividual variation of MMP-9 serum levels, as shown in Figure 1, a single sample might not be representative of a patient's average level. We found variable, but consistently increased MMP-9 serum levels in BOS^{pos} patients, which strongly suggest that an increased MMP-9 serum level after lung transplantation is a time-independent risk factor for the development of BOS. Consequently, no change in MMP-9 serum levels in time was determined between the two groups. Therefore, it cannot be used as a marker to predict the BOS-free survival period.

To identify putative BOS^{pos} patients the cut off point of 145 ng/ml MMP-9 in serum might be useful. This cut off point has a NPV of 83% which means that 83% of the lung transplant recipients with a MMP-9 serum level below 145 ng/ml are correctly diagnosed not to be at risk to develop BOS. Furthermore, 90% of the lung transplant recipients that are diagnosed with BOS have a MMP-9 serum level above 145 ng/ml. Clinically, MMP-9 serum levels above 145 ng/ml might be indicative of a high risk of developing BOS. These lung transplant recipients might benefit from intensive follow-up and augmented immunosuppressive treatment in order to prevent or slow down the development of BOS.

In BOS^{neg} patients an inverse relationship between MMP-9 and MMP-7 was found. This is consistent with the function of MMP-9 and -7 in wound healing. In the situation of epithelial repair, MMP-9 serum levels decrease and MMP-7 serum levels increase resulting in less inflammation, remodeling and degradation of the ECM and more repair.[7, 9] In BOS^{pos} patients this relationship was not found and insufficient release of MMP-7 and excessive increase of MMP-9 result in fibrosis.

The other potential biomarker measured in this study was YKL-40. We did not find significant differences in YKL-40 serum levels between the BOS^{pos} and BOS^{neg} patients. The median YKL-40 concentration in healthy controls (9 men and 21 women, age (years) 45 ± 14.1) as recently

described by Korthagen et al.[33], was 38 (29 – 47) ng/ml, which was significantly lower than in the lung transplant recipients (164 (106 – 265) ng/ml, $p < 0.0001$).

YKL-40 is produced by neutrophils and macrophages in tissues that are characterized by chronic inflammation.[13, 17] The increased YKL-40 serum levels in both BOS^{pos} and BOS^{neg} patients might be caused by the continuously exposure of transplanted lungs to stimuli of the immune system, for example via inhalation and inspiration or via alloimmune-dependent factors, that lead to injury of the airway epithelium and to chronic inflammation with attraction of neutrophils and macrophages.[34, 35]

We found that MMP-7 positively correlates with YKL-40 in BOS^{pos} patients and previously demonstrated that the increase of MMP-7 is insufficient in BOS^{pos} patients.[30] The correlation might be explained by the activation of neutrophils and the involvement of fibroblasts in the development of BOS. In the situation of epithelial injury and repair, epithelial cells release MMP-7 that lead to an influx and activation of neutrophils, which are an important source of YKL-40.[14, 36] Furthermore, fibroblasts are involved in the development of BOS and YKL-40 is known to be a growth factor for fibroblasts.[37, 38]

This study was a single-centre retrospective investigation. The number of patients included in this study is small, however, they are matched for several variables and, therefore, the influence of confounding factors will be limited. Additional multicentre studies with larger number of patients and a longer follow-up are required to substantiate our conclusions.

Earlier research suggests that the overwhelming activity of MMP-9 in patients with BOS is not sufficiently inhibited.[26] This might be an interesting goal for the limited treatment options for BOS. MMP-9 inhibitors as anticancer agents have already created interest.[7, 39] In animal models, mice treated with doxycyclin, a nonspecific MMP inhibitor, did not develop obliterative airway disease.[40] In an experimental model, simvastatin attenuates transforming growth factor beta and, thereby, decreases the MMP-9 concentration.[41] Finally, azithromycin reduces airway neutrophilia and increases the survival after lung transplantation.[42, 43] Neutrophils are suggested to be a major source of MMP-9 in BAL fluid.[2, 7] Reduced airway neutrophilia and subsequent decreased levels of MMP-9 might thus be one of the mechanisms by which azithromycin may reverse or halt the decline in lung function.

In summary, the development of BOS is a multifactorial process in which several cytokines, chemokines, and other growth factors are involved. In this study we investigated YKL-40 and MMPs as potential biomarkers for the development of BOS because they have shown to be involved in inflammation, remodeling, repair and fibrosis. While YKL-40 cannot be used as risk factor for BOS, increased MMP-9 serum levels after lung transplantation appear to be a risk factor for BOS. A cut off point of 145 ng/ml MMP-9 in serum might aid the diagnosis of putative BOS^{pos} patients, but this requires further confirmation. A promising role for MMP-9 inhibitors in the treatment of BOS needs to be further prioritized.

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CHAPTER 6

A GENETIC POLYMORPHISM IN THE *CAV1* GENE ASSOCIATES WITH THE DEVELOPMENT OF BRONCHIOLITIS OBLITERANS SYNDROME AFTER LUNG TRANSPLANTATION

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Abstract

Background

Caveolin-1 (Cav-1) is the primary structural component of cell membrane invaginations called caveolae. Expression of Cav-1 is implicated in the pathogenesis of pulmonary fibrosis. Genetic polymorphisms in the *CAV1* gene influence the function of Cav-1 in malignancies and associate with renal allograft fibrosis. Chronic allograft rejection after lung transplantation, called bronchiolitis obliterans syndrome (BOS), is also characterized by the development of fibrosis. In this study, we investigated whether *CAV1* genotypes associate with BOS and whether Cav-1 serum levels are influenced by the *CAV1* genotype and can be used as a biomarker to predict the development of BOS.

Methods

Twenty lung transplant recipients with BOS (BOS^{pos}), 90 without BOS (BOS^{neg}) and 422 healthy individuals donated DNA samples. Four single nucleotide polymorphisms (SNPs) in *CAV1* were genotyped. Serial Cav-1 serum levels were measured in a matched cohort of 10 BOS^{pos} and 10 BOS^{neg} patients. Furthermore, single-time point Cav-1 serum levels were measured in 33 unmatched BOS^{neg} patients and 60 healthy controls.

Results

Homozygosity of the minor allele of *rs3807989* was associated with an increased risk for BOS (odds ratio 6.13; p = 0.0013). The median Cav-1 serum level was significantly higher in the BOS^{pos} patients than in the matched BOS^{neg} patients (p = 0.026). Longitudinal analysis did not show changes in Cav-1 serum levels over time in both groups. The median Cav-1 serum level in the group of 43 BOS^{neg} patients was lower than that in the healthy control group (p = 0.046). In lung transplant recipients, homozygosity of the minor allele of *rs3807989* and *rs3807994* was associated with increased Cav-1 serum levels.

Conclusion

In lung transplant recipients, the *CAV1* SNP *rs3807989* was associated with the development of BOS and Cav-1 serum levels were influenced by the *CAV1* genotype.

Introduction

Caveolae are 50 to 100 nm flask-shaped cell membrane invaginations in which the primary structural component is caveolin-1 (Cav-1).[1] Cav-1 has been found in many cell types, but is abundantly expressed in endothelial cells, type 1 pneumocytes, epithelial cells, smooth muscle cells and fibroblasts.[2-5] It has many cellular functions, including vesicular transport, signal transduction and cholesterol homeostasis.[1, 4, 6]

Kasper et al. were the first investigators to link Cav-1 to a fibrotic phenotype in the lungs of rats.[5] Subsequently, studies of the role of Cav-1 in pulmonary fibrosis in humans were conducted. In patients with idiopathic pulmonary fibrosis (IPF), Cav-1 mRNA expression was found to be reduced in epithelial cells and fibroblasts.[7] In patients with systemic sclerosis, Cav-1 expression was markedly decreased in tissue of affected lungs and skin.[8] Knock-down of Cav-1 resulted in a fivefold increase of collagen gene expression by normal human lung fibroblasts, whereas increased Cav-1 expression caused a reduction in collagen.[9] *CAV1*$^{-/-}$ mice developed pulmonary and skin fibrosis.[8] On the other hand, during fibrogenesis, increased expression of Cav-1 was observed in endothelial cells.[5, 10] Taken together, the results of these studies support a pivotal role for Cav-1 in the fibrogenesis of the lungs.[8, 11]

The *CAV1* gene is localized on chromosome 7, a highly conserved region that includes a known fragile site which is deleted or associated with loss of heterozygosity in a variety of human cancers.[12] Studies that have addressed whether genetic variations of *CAV1* increases propensity towards fibrosis are scarce. Among kidney transplant donors, a single nucleotide polymorphism (SNP) in *CAV1* was significantly associated with renal allograft fibrosis in two independent cohorts.[13]

After lung transplantation the major limitation on long-term survival is the development of chronic rejection in the form of obliterative bronchiolitis (OB) or its clinical surrogate marker, the bronchiolitis obliterans syndrome (BOS).[14] OB is characterized by inflammation and remodeling of the pulmonary epithelium of the small airways.[15] This process results in recruitment and proliferation of fibroblasts, which ultimately leads to fibrosis. Advanced OB can include a spectrum ranging from partial to complete acellular fibrotic obliteration whereby only scar tissue remains of the airway lumen.[16, 17] When BOS is diagnosed on the basis of a decline in lung function, the process of inflammation and fibrosis is usually at an advanced and irreversible stage and treatment options are limited.[15] This emphasizes the need for biomarkers that predict the development of BOS before a decline in lung function has occurred.

The primary objective of this study was to determine whether SNPs in the *CAV1* gene are associated with the development of BOS after lung transplantation. In addition, Cav-1 serum levels in controls and lung transplant recipients were measured to evaluate whether Cav-1 serum levels are influenced by genotype and can be useful as biomarker to predict the development of BOS. To establish whether Cav-1 expression is indeed present in OB lesions, lung tissue

sections from BOS^{pos} patients and controls were studied using immunohistochemical staining.

The role of Cav-1 in lung transplant recipients and BOS has never been investigated. However, the role of Cav-1 in another pulmonary fibrotic disease, such as IPF, has been described previously.[7] To improve the understanding of Cav-1, we also measured Cav-1 serum levels in patients with IPF.

Materials and methods

Patients and clinical data

All lung transplant recipients who underwent transplantation in the Heart Lung Centre of the University Medical Centre in Utrecht, The Netherlands, in the period from July 2001 to November 2008 were asked to donate DNA and serum.

The diagnosis BOS was defined as a decline in forced expiratory volume in one second (FEV_1) of greater than 20% from the baseline determined by average of two measurements made at least three weeks apart in the absence of known causes for an acute declining FEV_1, such as acute rejection and infection.[16] Standard immunosuppressive therapy consisted of basiliximab (induction), tacrolimus, mycophenolate mofetil and prednisone for all patients.
After approval by the medical–ethical committee, informed consent was obtained from each lung transplant recipient and healthy control, and DNA and serum were collected.

Genotyping

Three haplotype-tagging SNPs for the *CAV1* gene were selected using the Tagger program (Broad Institute, Cambridge, MA, USA) for the genomic region of *CAV1* ± 2500 bp on genome build 35. Preferential picking of SNPs was conducted under the pairwise tagging options, a minimum allele frequency setting of 25% and a high Illumina design score (Illumina, San Diego, CA, USA). The algorithm was set to select tags that would cover the Caucasian HapMap panel with an r^2 of 0.8 or more.[18] Furthermore, additional SNPs were selected on the basis of previously published data or presumed functionality. The following SNPs were genotyped: *rs12154695*, *rs10256914*, *rs3807989* and *rs3807994*.

DNA was extracted from whole-blood samples, and SNP typing was conducted using a custom-made Illumina goldengate bead SNP assay in accordance with the manufacturer's recommendations (Illumina, San Diego, CA, USA). The characteristics of the lung transplant recipients and controls from whom DNA was taken are shown in Table 1. In three BOS^{neg} patients, the genotyping of the *CAV1* SNPs failed. The control group comprised 422 healthy individuals who were not receiving any medical treatment at the time of analysis.

Table 1. Baseline characteristics of BOS^{pos} patients (BOS+) and BOS^{neg} patients (BOS-) and healthy controls.

Variable	BOS+	BOS-	controls
number of patients, no.	20	90	422
Gender, no. (%)			
male	9 (45%)	46 (51%)	228 (54%)
female	11 (55%)	44 (49%)	194 (46%)
Age, mean ± SD, years	53.4 ± 10.7	49.4 ± 12.7	48.2 ± 11.9
Diagnoses, no. (%)			NA
COPD	7 (35%)	29 (32%)	
CF	2 (10%)	24 (27%)	
IPF	4 (20%)	13 (14%)	
sarcoidosis	2 (10%)	3 (3%)	
alpha-1 antitrypsin deficiency	5 (25%)	7 (8%)	
others	0	14 (16%)	
Type of graft, no. (%)			NA
unilateral	4 (20%)	13 (14%)	
bilateral	16 (80%)	77 (86%)	
Time to BOS, mean ± SD, months	23.7 ± 15.2	NA	NA

COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; IPF, idiopathic pulmonary fibrosis; NA, not applicable; SD, standard deviation.

Cav-1 serum levels in patient groups and healthy controls

Cav-1 serum levels were measured in different groups of lung transplant recipients to perform longitudinal analysis and to investigate whether Cav-1 can be used as biomarker for BOS.

For longitudinal analysis of Cav-1 serum levels, 10 BOS^{pos} patients were matched with 10 BOS^{neg} patients to reduce the influence of confounding factors. In this matched cohort of 10 BOS^{pos} patients and 10 BOS^{neg} patients, sequential serum samples (samples per patient range from two to five samples) were analyzed. These patients were matched for several clinicodemographic variables to reduce the influence of confounding factors, including age (difference in age < 3 years), gender, primary lung pathology, postoperative follow-up time (difference in post-operative follow-up time < 1 year) and unilateral or bilateral transplantation (Table 4). Patients were matched on these 5 items with a median of 4.0 matching items (range, 2.0 –5.0 items).

A quadrant-based sampling model was used to compare serum Cav-1 levels between the matched BOS^{pos} and BOS^{neg} patients at similar time points after lung transplantation and prior to BOS as described previously.[19, 20] The time period from lung transplantation until the diagnosis BOS was made varied in the cohort of BOS^{pos} patients with a mean of 19 months (Table 4).

To investigate whether Cav-1 serum levels are useful as biomarker for BOS, Cav-1 serum levels were measured at one moment in 33 BOS^{neg} patients, who were not matched with a BOS^{pos} patient. The baseline characteristics of these three groups of lung transplant recipients are summarized in Table 4.

Cav-1 serum levels were measured in 60 healthy controls (Table 4). The minor allele frequencies of the four SNPs in this cohort were 37% (*rs12154695)*, 30% (*rs10256914)*, 50% (*rs3807989)* and 28% (*rs3807994)*. To improve our understanding of the role of Cav-1 in pulmonary fibrosis Cav-1 serum levels were measured in 25 patients with IPF at one time point (Table 4). These patients were diagnosed according to the current American Thoracic Society/ European Respiratory Society guidelines.[21]

Protocol for serum Cav-1 assay

All serum samples were stored at –80 ºC until analysis. Serum Cav-1 was measured using the enzyme-linked immunosorbent assay (ELISA) developed by Tahir et al.[22] with some minor modifications. Briefly, Nunc maxisorb microplate wells were coated overnight at 4 ˚C with 100 µl 0.25 µg/well polyclonal anti-Cav-1 antibody (BD Transduction Laboratories, San Diego, CA, USA) and blocked with Tris-buffered saline containing 1.5% bovine serum albumin and 0.05% vol/vol Tween 20 (blocking buffer). All incubations were done at room temperature. To 50 µl blocking buffer, 50 µl of serum samples, calibrators and controls were added. We used full-length Cav-1 recombinant protein as a calibrator (Abnova, Taipei City, Taiwan). After shaking, the plate was incubated for two hours. After washing, 100 µl 0.1 µg/well monoclonal Cav-1 antibody (BD Transduction Laboratories) were incubated for 90 minutes, followed by 60 minutes of incubation with 100 µl 0.13 µg/well polyclonal rabbit antimouse horseradish peroxidase (Dako, Glostrup, Denmark). After washing, 90 µl 3,3',5,5'-tetramethylbenzidine substrate solution (Biolegend, San Diego, CA, USA) were added and the blue colour was allowed to develop for 20 minutes in the dark. The reaction was stopped by adding 50 µl of 2 N H_2SO_4, and the absorbance was read at 450 nm using a microplate reader (BioTek, Winooski, VT, USA).

Immunohistochemistry of human lung tissue sections

Paraffin embedded tissue was available from six BOS^{pos} patients (lung biopsy, autopsy or lung explant). Excess tissue of a lung donor and an area of normal lung tissue from a lobectomy specimen were used as healthy controls.

Serial cross-sections (4µm) were deparaffinized and rehydrated, boiled in sodium citrate and blocked in 10% normal goat serum. The sections were incubated for one hour at room temperature with 1.25 ug/ml polyclonal rabbit antihuman Cav-1 antibody (BD Transduction Laboratories) as determined by titration and analyzed using the Novocastra PowerVision Poly-HRP Anti-Rabbit IHC Detection System (Leica Microsystems, Buffalo Grove, IL, USA). Staining was developed with 3,3'-diaminobenzidine substrate and counterstained with haematoxylin. Negative controls were obtained avoiding the primary antibody.

Statistical analysis

The statistical significance of the differences between the groups was determined with the χ^2 test and one-way analysis of variance.

The Hardy-Weinberg equilibrium (HWE) and tests for association were calculated using the online programme available at: http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl. The HWE cut off for significant disequilibrium and subsequent exclusion from analysis was set at $p = 0.05$.

The significance threshold was set after accounting for multiple comparisons using a Bonferroni correction for the effective number of independent SNPs proposed by Li and Ji.[23] Owing to linkage disequilibrium (LD), the effective number of SNPs was three for *CAV1*, resulting in an adjusted significance threshold of $0.05/3 = 0.017$. Therefore, p-values were multiplied by three to adjust for multiple comparisons. Thus, obtained p-values ≤ 0.05 were considered statistically significant.

LD structure of the polymorphisms was determined using Haploview 4.2 software and haplotypes were reconstructed using the PHASE programme software.[24, 25]

Cav-1 serum levels were not normally distributed and are expressed as medians with interquartile ranges (IQR). To determine whether there is a trend in the serial serum levels over time in a single subject, and to compare this trend between the two groups, a restricted maximum likelihood linear mixed model was used.[26] The Mann-Whitney U-test was applied to comparisons between genotype and serum levels. Statistical analyses were performed with SPSS for Windows version 17.0 software (SPSS Inc., Chicago, IL).

Results

Genotype and haplotype distribution of *CAV1* in patients and controls

During the study period, 139 lung transplant procedures were performed in 138 patients. One hundred ten recipients gave their written informed consent and donated DNA, of whom twenty patients developed BOS during follow-up (Table 1). The genotype distribution of the four SNPs in *CAV1* in the different groups are reported in Table 2.

Table 2. Genotype distribution of BOS^{pos} patients (BOS+) and BOS^{neg} patients (BOS-) and healthy controls.

			BOS+ (n = 20)			BOS- (n = 87)**			controls (n = 422)		
SNP	**gene region**	**major/ minor**	AA	AB	BB	AA	AB	BB	AA	AB	BB
rs12154695	unknown	C/A	12 (60)	6 (30)	2 (10)	34 (39)	47 (54)	6 (7)	181 (43)	189 (45)	52 (12)
rs10256914	intron	T/C	12 (60)	6 (30)	2 (10)	50 (57)	29 (33)	8 (9)	228 (54)	158 (37)	36 (9)
rs3807989*	intron	C/T	4 (20)	9 (45)	7 (35)	33 (38)	47 (54)	7 (8)	144 (34)	206 (49)	72 (17)
rs3807994	intron	C/T	10 (50)	7 (35)	3 (15)	57 (66)	28 (32)	2 (2)	240 (57)	156 (37)	26 (6)

data are shown as n (%). A refers to major, B to minor.
* genotype distribution in BOS+ vs BOS-: p = 0.015; allele frequency in BOS+ and BOS- patients: p = 0.027
** CAV1 genotyping failed in three BOS- patients.

Table 3. Haplotype distribution in BOS^{pos} patients (BOS+, n = 20), BOS^{neg} patient (BOS-, n = 87) and healthy controls (n = 422).

		homozygotes	**heterozygotes**	**carriers**
	Haplotype	No. (%)	No. (%)	No. (%)
1	CTCC			
	BOS+	1 (5)	9 (45)	10 (50)
	BOS-	12 (14)	49 (56)	61 (70)
	controls	61 (14)	218 (52)	279 (66)
2	CTTC			
	BOS+	1 (5)	4 (20)	5 (25)
	BOS-	0 (0)	12 (14)	12 (14)
	controls	2 (0.5)	52 (12)	54 (13)
3	CCTT*			
	BOS+	2 (10)	3 (15)	5 (25)
	BOS-	0 (0)	15 (17)	15 (17)
	controls	4 (1)	75 (18)	79 (19)
4	ATCC			
	BOS+	0 (0)	4 (20)	4 (20)
	BOS-	0 (0)	20 (23)	20 (23)
	controls	10 (2)	60 (14)	70 (16)
5	ATTC			
	BOS+	0 (0)	4 (20)	4 (20)
	BOS-	0 (0)	17 (20)	17 (20)
	controls	4 (1)	76 (18)	80 (19)
6	ACTT			
	BOS+	0 (0)	1 (5)	1 (5)
	BOS-	0 (0)	10 (11)	10 (11)
	controls	1 (0.5)	70 (17)	71 (17)

BOS+ vs controls: * p = 0.03
BOS+ vs BOS-: * p = 0.03
Sequence of SNPs in haplotype: rs12154695, rs10256914, rs3807989, rs3807994.

All SNPs were found to be polymorphic and in Hardy-Weinberg equilibrium. The genotype distribution of *rs3807989* was significantly different between BOS^{pos} and BOS^{neg} patients (p = 0.015), which is related to a significantly higher minor allele frequency of *rs3807989* in BOS^{pos} patients than in BOS^{neg} patients (minor allele frequency 0.58 vs 0.35; p = 0.027). Homozygotes of this minor allele had an increased risk of developing BOS compared with carriers of the major allele (odds ration 6.13; p = 0.0013; 95% confidence interval 1.85 – 20.41). For the other SNPs, no significant differences were found in the genotype distribution and allele frequency between the patient groups and healthy controls.

The LD structure revealed one haplotype block between *rs3807989* and *rs3807994* with D' = 1 and r^2 = 0.45; therefore, haplotypes of the *CAV1* polymorphisms were constructed and analyzed. Fourteen haplotypes were constructed, and the six most frequent haplotypes with a frequency exceeding 5% were used for further analysis (Table 3). BOS^{pos} patients had significantly more homozygotes of haplotype 3 than BOS^{neg} patients and controls (p = 0.03).

Table 4. Baseline characteristics of BOS^{pos} patients (BOS+), BOS^{neg} patients (BOS-, matched and unmatched) and healthy controls and patients with IPF.

Variable	BOS+	BOS- (matched)	BOS- (unmatched)	Controls	IPF
Total number, no.	10	10	33	60	25
Gender, no.					
male	3	4	18	30	18
female	7	6	15	30	7
Mean age, mean ± SD, years	45.2 ± 15.0	45.7 ± 13.1	48.2 ± 13.9	46.7 ± 11.3	64.7 ± 11.3
Diagnosis, no.				NA	NA
COPD	3	3	8		
CF	4	5	10		
IPF	1	0	5		
sarcoidosis	1	0	2		
alpha-1 antitrypsin deficiency	1	1	3		
others	0	1	5		
Type of graft, no.				NA	NA
bilateral	10	10	27		
unilateral	0	0	6		
Survival, mean ± SD, months	33.6 ± 20.0	46.4 ± 9.5	51.6 ± 21.3	NA	NA
BOS-free survival, mean ± SD, months	19.3 ± 12.5	46.4 ± 9.5*	51.6 ± 21.3*	NA	NA
BOS grade at diagnosis, no.		NA	NA	NA	NA
1	7				
2	3				
3	0				
Histology		NA	NA	NA	NA
biopsy - histological OB	4				
biopsy - no histological OB	2				
no biopsy	4				

* identical to survival; COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; IPF, idiopathic pulmonary fibrosis; OB, obliterative bronchiolitis; NA, not applicable; SD, standard deviation.

Cav-1 serum levels in patients and controls

In our cohort of lung transplant recipients, serum samples were collected from 10 BOS^{pos} patients and 43 BOS^{neg} patients (Table 4). Initially, we matched 10 BOS^{pos} patients with 10 BOS^{neg} patients to reduce the influence of confounding factors and performed longitudinal analysis of Cav-1 serum levels. These patients were matched for several clinico-demographic variables, including age, gender and primary lung pathology. Serial serum samples were used to perform this longitudinal analysis, and two to five serum samples were collected for every matched BOS^{pos} patient and BOS^{neg} patient.

The median (IQR) Cav-1 serum level of all samples in the 10 BOS^{pos} was significantly higher than that of the 10 matched BOS^{neg} patients: 555 ng/ml (447 to 747) and 468 ng/ml (418 to 558), respectively (p = 0.026, Figure 1). The median Cav-1 serum level of healthy controls (n = 60, one sample per individual) was 609 ng/ml (531 to 678) and differed significantly from that of all samples from the 20 matched lung transplant recipients (10 BOS^{pos} and 10 BOS^{neg} patients): 492 ng/ml (426 to 629) (p = 0.0003, Figure 1).

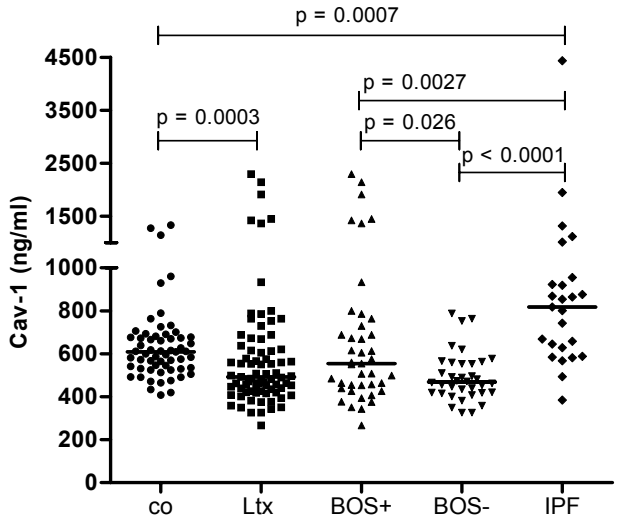

Figure 1. Cav-1 serum levels in patient groups and healthy controls.
Groups: controls (co, n = 60), the cohort of matched lung transplant recipients (Ltx; n = 10 BOS^{pos} patients and n = 10 matched BOS^{neg} patients, the samples per patient range from two to five samples), BOS^{pos} patients (BOS+, n = 10), matched BOS^{neg} patients (BOS-, n = 10), patients with idiopathic pulmonary fibrosis (IPF, n = 25). Horizontal bars represent medians.

We analyzed Cav-1 serum levels in the matched cohort from the time of lung transplantation until the BOS diagnosis was made. Samples were selected using a quadrant-based sampling model. In this model, the follow-up period after lung transplantation until the development of BOS was divided into four equal quadrants, and one sample at the midpoint of each interval was analyzed. The samples from the BOS^{neg} patients were obtained from chronologically similar visits at which the samples for their BOS^{pos} counterparts were analyzed. In the BOS^{pos} patients, one extra sample was obtained within two months before the BOS diagnosis was made. The mean time period between lung transplantation and the onset of BOS was 19 months with a variation ranging from 8 to 49 months (Table 4). The serial Cav-1 serum levels in both groups did not reveal a significant increase or decrease at similar time points after lung transplantation and prior to BOS. Because Cav-1 serum levels did not change in time, the samples of the first quadrant of this matched cohort were used for further analysis.

To investigate whether the difference in Cav-1 serum levels between the matched BOS^{pos} and BOS^{neg} patients would hold in an unmatched BOS^{neg} group, 33 extra unmatched BOS^{neg} patients were added to the cohort of 10 BOS^{neg} patients. In these 33 BOS^{neg} patients, Cav-1 serum levels were measured at one moment after lung transplantation. We found that the median Cav-1 serum levels were not significantly different between the 43 BOS^{neg} patients and 10 BOS^{pos} patients, respectively: 550 ng/ml (433 to 736) and 565 ng/ml (421 to 738) ($p = 0.89$). The 43 BOS^{neg} patients had significantly lower Cav-1 serum levels than the controls ($p = 0.046$).

To improve the understanding of the role of Cav-1 in pulmonary fibrosis, Cav-1 serum levels were also measured in patients with IPF (n = 25; one sample per individual). The median Cav-1 serum concentration in this group was 818 ng/ml (609 to 940), which was significantly higher than that in 10 BOS^{pos} patients ($p = 0.0027$), 10 BOS^{neg} patients ($p < 0.0001$) and controls ($p = 0.0007$, Figure 1).

Correlation of Cav-1 serum levels with genotype and haplotype

In all lung transplant recipients (10 BOS^{pos} and 43 BOS^{neg} patients), homozygotes of the minor allele of the following SNPs had significantly increased serum levels compared with the carriers of the major alleles: *rs3807989* (689 vs 520 ng/ml; $p = 0.03$) and *rs3807994* (731 vs 520 ng/ml; $p = 0.02$, Figure 2).

Heterozygotes of haplotype 2 (CTTC) had lower Cav-1 serum levels than heterozygotes of haplotype 3 (CCTT; 448 vs 689 ng/ml; $p = 0.04$). Also, carriers of haplotype 2 had lower Cav-1 serum levels than carriers of haplotype 3 (448 vs 672 ng/ml; $p = 0.02$, Figure 3).

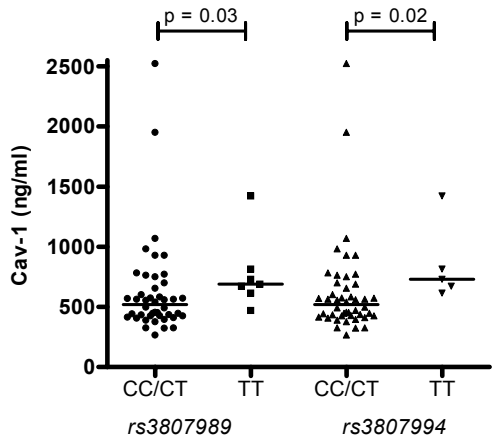

Figure 2. Correlation of Cav-1 serum levels with genotype in lung transplant recipients.
Cav-1 serum levels in lung transplant recipients (10 BOS^{pos} and 43 BOS^{neg} patients) are increased in homozygotes of the minor alleles compared with carriers of the major allele of *rs3807989* (p = 0.03) and *rs3807994* (p = 0.02). Horizontal bars represent medians.

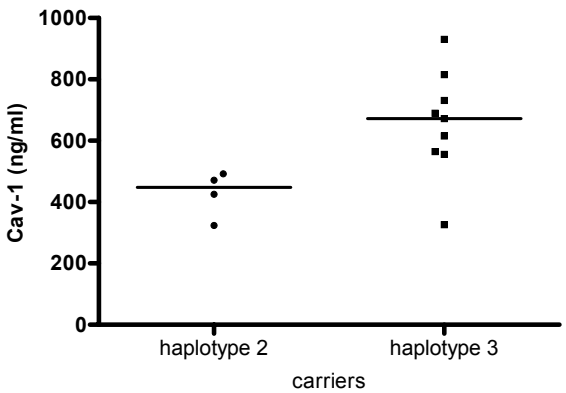

Figure 3. Cav-1 serum levels in carriers of haplotype 2 and haplotype 3 in lung transplant recipients. Carriers of haplotype 2 (CTTC, n = 4) had lower Cav-1 serum levels compared with carriers of haplotype 3 (CCTT, n = 9) (p = 0.02). The number of individuals is smaller than reported in Table 3, because serum samples were not available from all BOS^{pos} and BOS^{neg} patients. Horizontal bars represent medians.

Localization of Cav-1 in obliterative bronchiolitis

Qualitative immunohistochemical staining of Cav-1 was studied in lung tissue from six lung transplant recipients and two controls. In normal lung tissue, the Cav-1 staining was intense in the cell membranes of endothelial cells and the alveolar epithelium. The Cav-1 staining in the bronchiolar epithelial cells was less intense (Figure 4, left panel).

In OB lesions, the intensity of the Cav-1 staining in endothelial cells and alveolar epithelium was similar to that in normal lung tissue. The staining of Cav-1 in the bronchiolar epithelium of

lung transplant recipients with OB was slightly accentuated compared with normal bronchiolar epithelium. In the OB lesions, some staining of the cellular infiltrate, that is, partially obliterating the bronchiolus, was observed. (Figure 4, right panel).

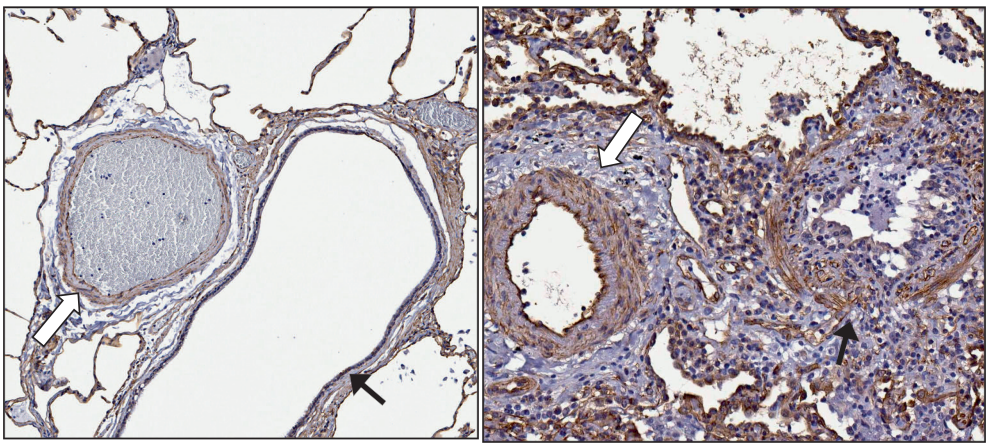

Figure 4. Immunohistochemical staining for Cav-1 in lung tissue sections.
Left panel: control tissue from a lobectomy specimen (left lower lobe) with positive staining of alveolar epithelium, endothelium, smooth muscle cells and minimal staining of the bronchiolar epithelium.
Right panel: one representative sample of an obliterative bronchiolitis (OB) lesion from the right lower lobe, with some staining of the cellular infiltrate partially obliterating the bronchiole. In this sample, the staining of bronchiolar epithelium seemed slightly increased. The staining of alveolar epithelium and endothelium are similar to normal lung tissue.
Open arrows indicate the arterial branch. Closed arrows indicate the bronchiole (original magnification, x100).

Discussion

We found an association between a genetic polymorphism in the *CAV1* gene and the development of BOS. Homozygosity of the minor allele of *rs3807989* is associated with a sixfold increased risk of developing BOS. Cav-1 serum levels were genotype dependent. In lung transplant recipients, increased Cav-1 serum levels were observed in homozygotes of the minor alleles of two SNPs, including *rs3807989*, which genotype was associated with an increased risk of BOS.

Cav-1 is an integral protein of caveolae and has been identified in a wide variety of cells.[1] Although this protein was originally identified as membrane protein, Cav-1 has also been reported to be present in the secretory cellular components of the pancreas and salivary glands, in differentiating osteoblasts and in cancer cells.[27-30] This might explain the detectable serum levels of Cav-1 in healthy controls. Cav-1 serum levels were never measured before in lung transplant recipients or in patients with IPF. However, Cav-1 serum levels in patients with

prostate cancer have been shown to be a potential biomarker in this disease.[22, 28, 31, 32] We found that Cav-1 in serum was lower in lung transplant recipients than in healthy controls. Within the matched cohort of 10 BOS^{pos} and 10 BOS^{neg} patients, the BOS^{pos} patients had higher Cav-1 serum levels than the BOS^{neg} patients. The six highest Cav-1 serum levels in the BOS^{pos} cohort, illustrated in Figure 1, were measured in two patients who are both homozygous for the risk allele of SNP *rs3807989*. Serial Cav-1 serum levels of the matched cohort did not reveal a trend in Cav-1 concentration with time after lung transplantation and prior to BOS. The total group of 43 BOS^{neg} patients had lower Cav-1 serum levels than the healthy controls, but in contrast to the matched cases there was no difference in Cav-1 serum concentration between the 10 BOS^{pos} patients and the total group of 43 BOS^{neg} patients. For these reasons, Cav-1 serum levels cannot be used as a biomarker to predict the development of BOS.

We also measured Cav-1 serum levels in 25 patients with IPF. Although the pathogenesis of BOS and IPF remains to be determined, there may be some similarities. The general hypothesis is that BOS and IPF are caused by injury of the lung followed by an aberrant repair response and ultimately fibrosis.[15, 33] During fibrogenesis in IPF and BOS, the epithelial to mesenchymal transition is a critical cellular mechanism,[33, 34] and neutrophils play an important role in both diseases.[35, 36] Differences between these two entities are the localization of the lesions in the lung and the stimuli that cause the initial injury. BOS is localized within the respiratory bronchioli and IPF is localized within the alveoli.[17, 37] Several immune-dependent and -independent mechanisms are known to be risk factors for the development of BOS.[15] However, the stimuli that cause the injury in IPF are still unknown.[33]

Cav-1 serum levels are significantly increased in patients with explicit pulmonary fibrosis, as illustrated by the increased Cav-1 serum levels in patients with IPF in this study. On the other hand, the expression of Cav-1 in lung tissue and fibroblasts of patients with IPF was previously described to be decreased and associated with enhanced transforming growth factor (TGF)-β1 signaling and increased collagen deposition.[2, 3, 7] The relationship between Cav-1 expression in lung tissue and pulmonary fibroblasts and the Cav-1 serum concentration is unknown. One can only speculate about the discrepancy between the decreased Cav-1 expression in IPF described in literature and the increased concentration of its soluble form in patients with IPF. Cav-1 is present in caveolae of the cell membrane, and it might also be part of secretory pathways, that is, of the pancreas or salivary glands, which might influence Cav-1 serum levels.[27]

Increased Cav-1 serum levels in BOS^{pos} patients compared with BOS^{neg} patients were expected because of the process of fibrogenesis in BOS. In the matched cohort, we were able to detect this difference in Cav-1 concentration. The hypothesis that Cav-1 is increased in BOS^{pos} patients is supported by the correlation of *rs3807989* minor T allele with both an increased risk of BOS and increased Cav-1 serum levels. For future studies, the presence of extremely high Cav-1 serum levels might be specific to BOS^{pos} patients.

The increased Cav-1 serum levels in BOS^{pos} patients compared with the matched BOS^{neg} patients may be explained by TGF-β1. Researchers in several studies have shown that TGF-β1 is involved in the development of BOS,[38-41] although others could not confirm this.[34, 42]

During pulmonary fibrosis, it has been shown that Cav-1 expression is decreased in epithelial cells and fibroblasts compared with controls, but is increased in endothelial cells.[5, 7, 10, 43] We found that Cav-1 expression in normal lung tissue was similar to the findings of Odajima et al.[44], who localized Cav-1 in normal lung tissue and in lung tissue of patients with interstitial pneumonias. A description of Cav-1 expression in OB after lung transplantation is not available. We found that in OB lesions, Cav-1 expression in the bronchiolar epithelial cells seemed to be slightly increased compared with normal lung tissue. In the OB lesions, cellular infiltrates were observed that showed some degree of Cav-1 expression and were partially obliterating the lumen of the bronchiole. These lesions may represent an early phase in the development of BOS and might explain the tendency towards an overall increase in Cav-1 serum levels in BOS^{pos} patients. In the development of BOS, fibrointimal changes involving pulmonary arteries and veins are seen, but they have been overshadowed by the airway lesions.[45]

Cav-1 expression in BOS seems increased but Wang et al.[7] found a decreased epithelial expression of Cav-1 in IPF. The limited number of tissue sections from BOS^{pos} patients and the absence of multiple comparisons based on image analysis clearly limit the conclusions we can draw from this part of our study. Pulmonary fibrosis in patients with IPF and systemic sclerosis is different from that in patients with BOS and is localized in other parts of the lung. Different cell types and molecular pathways may be involved in the pathogenesis of these diseases. Furthermore, there is evidence that different types of caveolae exist and that there is more than one regulatory mechanism of Cav-1 expression.[43] Two Cav-1 isoforms, α and β, are known, both of which were detected by the antibodies in our study.[43, 46] The α isoform is mainly expressed by endothelial cells and the alveolar cells predominantly express the β isoform.[46] This underlines the complexity of Cav-1 in pulmonary pathology.

Regarding the source of the serum Cav-1 in BOS^{pos} patients, we hypothesize that the Cav-1 expression in OB lesions could have a relationship with increased serum levels we observed. However, further research based on quantitative analysis is needed.

The mechanism by which Cav-1 contributes to fibrosis might be found in the signaling pathway. Cav-1 functions as a part of the TGF-β pathway through its participation in TGF-β receptor internalization. TGF-β is involved in the development of fibrosis by stimulation of extracellular matrix production and accumulation of collagens and other matrix proteins.[2, 47] In addition, Cav-1 serves as a scaffolding protein for other signaling molecules, such as members of the mitogen-activated protein kinase family, G-proteins and other growth factors receptors.[48] These signaling molecules are involved in the regulation of α-smooth muscle actin-positive fibroblasts and collagen.[48, 49]

Some limitations of our study have to be acknowledged. First, the study has a retrospective design and the number of patients may be too low to detect more subtle differences in Cav-1 serum levels within the lung transplant recipients. However, the procedure that we used to match the BOS^{pos} and BOS^{neg} patients might have reduced the influence of confounding factors. Additional studies with more lung transplant recipients and a longer follow-up period are required to replicate our association between the *CAV1* genotype and BOS. In addition, experimental studies and quantitative analysis using immunohistochemistry need to be conducted to better understand the molecular mechanisms of Cav-1 underlying our observations.

Application of the genetic variability of lung transplant recipients in the management and treatment of these patients could be a promising approach in the future. Genetic risk profiling might provide a tool for individualized risk stratification and for personalized immunosuppressive treatment after lung transplantation. Personalized immunosuppressive treatment might lead to better graft survival and less drug toxicity. The *CAV1* genotype of SNP *rs3807989* is associated with the development of BOS and therefore could be included in such a genetic risk profile.

Our data demonstrate that the *CAV1* SNP *rs3807989* is associated with the development of BOS after lung transplantation and that Cav-1 serum levels are influenced by the composition of the coding gene. The risk allele associates with increased Cav-1 serum levels, and OB lesions might show increased Cav-1 expression. The mechanism through which increased Cav-1 expression contributes to the development of BOS needs to be explored further.

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CHAPTER 7

GENETIC POLYMORPHISMS AND BRONCHIOLITIS OBLITERANS SYNDROME AFTER LUNG TRANSPLANTATION: PROMISING RESULTS AND RECOMMENDATIONS FOR THE FUTURE

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Summary

Survival rates after lung transplantation are the lowest among solid organ transplantations. Long-term survival is limited by the development of chronic rejection, known as bronchiolitis obliterans syndrome (BOS).

Risk factors, such as acute rejection and cytomegalovirus infection, contribute to the development of BOS. However, these risk factors alone do not explain the interindividual variability seen in the development of BOS. There is growing evidence that genetic variations might contribute to an individual's susceptibility to rejection.

In this systematic review, based on a literature search through Medline and Embase, an overview is given of the genetic polymorphisms that have been investigated in lung transplant recipients in relation to the development of BOS.

Functional genetic polymorphisms in the genes of *IFNG* (+874 A/T), *TGFB1* (+915 G/C) and *IL6* (-174 G/C) have been found to be associated with the development of BOS and allograft fibrosis after lung transplantation. However, confirmation was not consistent across all studied cohorts.

Genetic polymorphisms in the genes of several Toll-like receptors, mannose-binding lectin, *CD14*, killer immunoglobulin-like receptors, and matrix metalloproteinase-7 were also found to be associated with the development of BOS, but these studies need to be replicated in independent cohorts.

This review shows that there may be involvement of genetic polymorphisms in the development of BOS. Genetic risk profiling of lung transplant recipients could be a promising approach for the future, enabling individualized risk stratification and personalized immunosuppressive treatment after transplantation. Further studies are needed to define risk alleles.

Bronchiolitis obliterans syndrome after lung transplantation

Lung transplantation is a therapeutic option for patients with end-stage pulmonary diseases. However, the survival rates after lung transplantation are the lowest among solid organ transplantations.[1] The long-term survival is mainly limited by the development of chronic rejection, known as bronchiolitis obliterans syndrome (BOS).[2, 3]

The initial step in the development of BOS is damage to the pulmonary epithelium, caused by several risk factors, such as acute rejection, lymphocytic bronchiolitis, cytomegalovirus infection, and gastroesophageal reflux.[2-4] This injury is followed by an inflammatory response and upregulation of cytokines and chemokines.[2] The inflammatory cascade is believed to cause repetitive damage and subsequent remodeling of the bronchioli leading to fibrosis and obliteration of the airway lumen.[3, 5]

However, these risk factors alone do not explain the interindividual variability seen in the development of BOS. There is growing evidence that genetic factors could play a role in the interindividual variation in susceptibility to complications after transplantation, to differences in time of onset of the clinical symptoms of BOS in particular, and to differences in the way a recipient responds to immunosuppressive therapy.[6, 7] Various genetic polymorphisms in innate immunity genes and cytokine genes have already been investigated as potential independent risk factors for the development of BOS.[8-11] Cytokine gene polymorphisms have been found to alter the secretion or function of cytokines, which might influence the activation of the immune system.[12-14] Moreover, variations in the innate immune response were found to be influenced by genetic polymorphisms in innate immunity genes.[15]

Application of the genetic variability of lung transplant recipients in the management and treatment of these patients might be a promising approach for the future. Genetic risk profiling could help clinicians to better stratify the risk of developing BOS on an individual basis and to start with targeted immunosuppressive therapy accordingly.

In this article, we describe the results of a systematic review based on a literature search through Medline and Embase from 1948 until March 2011. The genetic variations that have been investigated in lung transplant recipients and that have shown to be associated with the susceptibility to develop BOS after lung transplantation are summarized.

Search strategy and selecting criteria

A search through Ovid Medline (1948 to March 2011) and Embase (1988 to March 2011) was performed for all medical literature published in English-language journals.

For Medline, the following search strategy was used: ((bronchiolitis obliterans.mp. or Bronchiolitis Obliterans/ OR allograft fibrosis.mp. OR chronic rejection.mp.) AND (exp Polymorphism, Genetic/ OR haplotype$.mp. or Haplotypes/ OR genetic predisposition.mp. or Genetic Predisposition to Disease/)) OR Bronchiolitis Obliterans/ge OR ((lung transplantation. mp. or Lung Transplantation/) AND *Graft Rejection/ge)) OR (*lung transplantation/ AND Graft

Rejection/ge). For Embase, the same keywords were used, but the search strategy was adapted to Embase-specific indexation.

In addition, we reviewed the reference lists from all relevant articles to identify additional studies. Studies that met the following criteria were included in the study: 1) lung transplant recipients were included; 2) the development of BOS or allograft fibrosis was reported as outcome; and 3) any genetic polymorphism was determined.

Data extraction

Numbers of controls and lung transplant recipients with (BOS^{pos}) and without (BOS^{neg}) BOS or allograft fibrosis and allele and genotype frequencies were extracted from the included articles and summarized in a consistent manner to aid comparison. If a study reported results in percentage instead of absolute data, absolute data were calculated when possible.

Statistical analysis

The effect of genetic variation in cytokine, innate immunity, and repair genes on the development of BOS was estimated by testing the significance of differences in distribution of alleles or genotypes between BOS^{pos} and BOS^{neg} patients. At first, differences between the allele frequencies were calculated with the Pearson's chi-square test together with the corresponding odds ratio (OR) and 95% confidence interval (CI). Second, when $p < 0.15$ for the allelic distribution, we determined the risk associated with carriership or homozygosity of the risk allele with Pearson's chi-square, together with the OR and 95% CI. To determine the additive effect of the risk allele, we performed the Armitage's trend test; however, this could only be performed for studies that supplied counts of the three different genotypes in both BOS^{pos} and BOS^{neg} patient groups. Computations were performed online at: http://faculty.vassar.edu/lowry/VassarStats.html and http://ihg2.helmholtz-muenchen.de/ihg/snps.html. p-values ≤ 0.05 were considered statistically significant.

Results of literature search

Database searching identified 269 potential citations. After removing the duplicates and initial screening of titles and abstracts, 16 studies were assessed for possible inclusion in the review, and 13 studies met the inclusion criteria (Figure 1). In these 13 articles, 40 different genetic polymorphisms in eight cytokine genes, seven innate immunity genes and one repair gene were investigated in relation to BOS or allograft fibrosis.

In Table 1, the numbers of cases and controls in each study are reported. In Table 2, an overview is given of the investigated genetic polymorphisms in lung transplant recipients with the respective risk calculations for the development of BOS or allograft fibrosis.

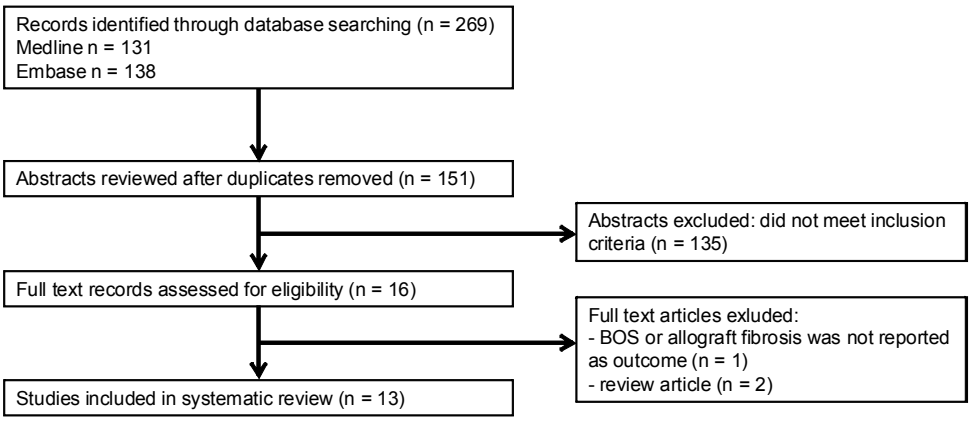

Figure 1. Flowchart of review.

Cytokine genes and BOS or allograft fibrosis

A number of studies have investigated the association between genetic polymorphisms in tumor necrosis factor alpha (*TNFA*), interferon gamma (*IFNG*), transforming growth factor beta-1 (*TGFB1*), interleukin (*IL*)*6*, and *IL10*, and the development of BOS or allograft fibrosis after lung transplantation.[10, 11, 16, 17] These genetic polymorphisms were chosen on account of the proven inflammatory, profibrotic, or anti-inflammatory properties of their gene products.

In four independent studies, no association was detected between genetic polymorphisms in *TNFA* and *IL10* and the development of BOS or allograft fibrosis.[10, 11, 16, 17]

A significant association was detected between homozygosity for the major T allele of *IFNG* at position +874 A/T and the development and earlier onset of BOS.[11] Two other studies did not confirm this association.[10, 16] but a fourth study showed that allele #2 of the CA repeat in *IFNG* was most commonly observed in the group with allograft fibrosis compared with the group without allograft fibrosis,[18] but this association was not replicated.

Homozygosity for the major allele of codon 25 of *TGFB1* was associated with allograft fibrosis diagnosed by histology in two studies.[19, 20] One of these studies showed that a second genetic polymorphism (cytosine deletion at position +72) was also associated with allograft fibrosis and that the G allele at position -800 was associated with lung transplant recipients who developed fibrosis compared with healthy controls, although the frequency was not significantly different between recipients with and without allograft fibrosis.[20] Other studies, that used either the BOS criteria according to the International Society of Heart and Lung Transplantation (ISHLT)[10, 11] or the term chronic rejection,[16, 17] did not confirm this association.

Homozygosity for allele #1 of the 86 bp repeat of the IL-1 receptor antagonist (*IL1RN*) gene was associated with chronic rejection in a cohort of thoracic transplant recipients and an almost twofold increased risk for the major allele at position 8061 C/T in *IL1RN* was found.[17, 21] These associations were not replicated in another independent cohort.

In *IL6*, carriership of the G allele of the *IL6* gene (-174 G/C) was associated with the development and an earlier onset of BOS in two studies,[10, 11] but could not be validated in three other cohorts.[10, 16, 17]

Innate immunity genes and BOS

Five studies have investigated the associations between genetic polymorphisms in innate immunity genes and BOS, but none of the following positive associations have been replicated in another independent cohort.

Lung transplant recipients carrying the minor allele for either one of the functional single nucleotide polymorphisms (SNPs) in Toll-like receptor 4 (*TLR4)*, Asp299Gly (*rs4986790*) or Thr399Ile (*rs4986791*), showed a trend toward reduced onset of BOS grade 2 or 3.[9] Other genetic polymorphisms in *TLR2* (*rs1898830*), *TLR4* (*rs1927911*) and *TLR9* (*rs352162* and *rs187084*) were associated with an increased risk to develop BOS.[22] In this study, the BOS^{pos} patients had significantly more risk alleles in *TLR2*, *TLR4*, and *TLR9* together compared with the BOS^{neg} patients and controls.[22]

Homozygotes for the minor allele (T) of *CD14* at position -159 C/T had a higher overall incidence and an earlier onset of BOS than patients with other genotypes.[8]

Patients who received a graft from a donor homozygous for the Y allele of the mannose-binding lectin (*MBL*) gene had a worse BOS-free survival compared with patients who received a graft from a donor with a X/X or X/Y genotype. Furthermore, a negative effect of the donor HYPA haplotype on the development of BOS was observed. However, these negative effects disappeared after introduction of a new immunosuppressive regimen because of a dramatic increase in the 1-year BOS-free survival. Recipient *MBL* genotype was not associated with transplant outcome.[23]

Furthermore, the presence of the inhibitory haplotype A of the killer immunoglobulin-like receptors (*KIRs*) and the absence of *KIR2DS5* were reported to be associated with BOS.[24]

Repair gene and BOS

Only one study investigated the association between genetic polymorphisms in repair genes and BOS. Lung transplant recipients homozygous for the major alleles of *rs17098318*, *rs11569919*, and *rs12285347*, and for the minor allele of *rs10502001* of the matrix metalloproteinase (*MMP*)*7* gene had an increased risk to develop BOS. Haplotypes constructed with three or four of these risk alleles correlated with lower serum levels of MMP-7 and were more often present in the BOS^{pos} patients.[25]

Table 1. Numbers of cases, controls and patients with BOS or allograft fibrosis in each study.

Author	Ref. number	Genes	No. of cases	No. of controls	No. of patients with BOS/allograft fibrosis
Awad et al. (1998)	20	*TGFB1*	95 recipients	107 controls	66-68 patients with allograft fibrosis
Awad et al. (1999)	18	*IFNG*	82 recipients	164 controls	66 patients with allograft fibrosis
El-Gamel et al. (1999)	19	*TGFB1*	91 recipients	96 controls	39 patients with allograft fibrosis
Jackson et al. (2001)	16	*TNFA, IFNG, TGFB1, IL6, IL10*	119 recipients		No. n.a.; chronic rejection as diagnosis
Kastelijn et al. (2010)	22	*TLR2, TLR4, TLR9*	110 recipients	422 controls	20 patients with BOS
Kastelijn et al. (2010)	25	*MMP7*	110 recipients	422 controls	21 patients with BOS
Kwakkel et al. (2008)	24	*KIR*	48 recipients		7 patients with BOS
Lu et al. (2002)	11	*TNFA, IFNG, TGFB1, IL6, IL10*	93 recipients	29 controls	38 patients with BOS
Munster et al. (2008)	23	*MBL*	189 donors 200 recipients		No. n.a.; BOS as diagnosis
Palmer et al. (2005)	9	*TLR4*	170 recipients		45 patients with BOS
Palmer et al. (2007)	8	*CD14*	226 recipients		No. n.a.; BOS as diagnosis
Snyder et al. (2006)	10	*TNFA, IFNG, TGFB1, IL6, IL10*	(1) 78 recipients (2) 198 recipients (validation)		55 patients with BOS 71 patients with BOS
Vamvakopoulos et al. (2002)	17	*TNFA,TGFB, IL1B1, IL1R1, IL1RN, IL6, IL10, FCGRIIA*	15 out of 96 thoracic transplant recipients received a lung transplant	83 controls	15 patients BOS

n.a., not available, no absolute data available to determine or calculate the number of patients with BOS or allograft fibrosis
TNFA, tumor necrosis factor alpha; IFNG, interferon gamma; IL, interleukin; IL1R1, IL1 receptor; IL1RN, IL1 receptor antagonist; TGFB, transforming growth factor beta; TLR, Toll-like receptor; FCGRIIA, Fc gamma receptor IIa; MBL, mannose binding lectin; KIR, killer immunoglobin-like receptor; MMP, matrix metalloproteinase.

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34 R35 R36 R37 R38 R39

Comments on the published genetic associations

The results of this review show that significant associations have been reported between functional genetic polymorphisms in several cytokine genes and the development of BOS or allograft fibrosis after lung transplantation. In addition, significant associations in innate immunity genes and a repair gene were found in relation to BOS.

In the majority of the cytokine gene association studies, the same subset of cytokine genes was analyzed. The association between the genetic polymorphism in the *IL6* gene and BOS was reported by Lu et al. and Snyder et al.[10, 11] Snyder et al. were unable to confirm the association of *IL6* and *IFNG* with BOS.[10] However, they could conclude that SNPs in the *IL6* and *IFNG* genes were associated with an earlier onset of BOS and suggested that the conflicting results might be attributed to small sample size and differences in ethnic backgrounds, immunosuppressive regimens and follow-up time.[10] The existence of an association between *IFNG* and BOS is supported by genetic linkage of the T allele at position +874 and allele #2 of the CA-repeat.[26]

IL10 and *TNFA* have never been associated with BOS or allograft fibrosis, and therefore, in our opinion these two cytokines can be excluded from future gene association studies.

The associations of both *IL1* and *TGFB1* with BOS or allograft fibrosis need to be interpreted with caution. The study that reported the association between *IL1* and chronic rejection used a cohort of different types of thoracic transplant recipients of which the number of lung transplant recipients was too small to analyze separately.[17] In the studies of El Gamel et al.[19] and Awad et al.[20] the cohorts of lung transplant recipients were largely overlapping. Therefore, the association between codon 25 in the *TGFB1* gene and allograft fibrosis is not positively replicated in another independent cohort. In addition, these studies found an association between a genetic polymorphism in the *TGFB1* gene and allograft fibrosis. Allograft fibrosis and BOS may not be equivalent entities as the presence of fibrotic changes on transbronchial biopsies does not necessarily identify patients with changes of obliterative bronchiolitis. The difference between allograft fibrosis and BOS is recently illustrated by a new concept describing BOS no longer as the only form of chronic lung allograft dysfunction (CLAD). Another form of CLAD, called restrictive allograft syndrome (RAS), exhibits restrictive functional changes with fibrotic processes in peripheral lung tissue, rather than the classical finding of small airway obliteration seen in BOS.[27]

Furthermore, in the past years, two different phenotypes of BOS are distinguished based on the response to the treatment with azithromycin.[28, 29] The first phenotype is called neutrophilic reversible allograft dysfunction (NRAD) and showed increased bronchoalveolar lavage (BAL) levels of neutrophils and different proteins, inflammatory active lesions on histology, and is responding to azithromycin. The second phenotype includes the fibroproliferative BOS that showed no neutrophils and another protein pattern in BAL, pure fibrosis on histology, and no response to azithromycin.[28, 30] RAS and the two different phenotypes of BOS were described recently and have therefore not been included in the definitions of BOS or allograft fibrosis

in the gene association studies in this review. Nevertheless, part of the patients that were diagnosed with BOS or allograft fibrosis in these studies might meet the criteria of these new subtypes, which might influence the present conclusions. For example, as azithromycin seems to reduce inflammation by inhibiting components of the innate immune response,[29, 31] treatment of lung transplant recipients with azithromycin might influence the associations found between innate immunity genes and BOS. Before RAS and NRAD can be used in future association studies, they need to be evaluated and confirmed.

Early after lung transplantation, the transplanted lungs exist of donor cells. Nevertheless, chimerism between donor and recipient cells is reported to occur in the lungs of lung transplant recipients.[32] Epithelial structures displaying signs of chronic injury, as present in the development of BOS, showed a higher degree of chimerism.[32] From this point of view, Palmer et al. concluded that *TLR4* recipient genotype could influence the epithelial response to innate pathogens.[9] Besides chimerism, shown to be present in transplanted lung, the genetic profile of the donor will also be involved in the development of BOS. Munster et al. showed that the genetic profile of the donor, and not of the recipient, is associated with the development of BOS.[23]

Functionality of the genetic polymorphisms

The functionality of the genetic polymorphisms in the cytokine genes has been previously investigated.

The T allele (+874 A/T) and the CA repeat allele #2 of *IFNG* are in linkage disequilibrium with each other and are associated with an increased production of IFN-γ.[33] Furthermore, the -174 G allele of *IL6* is also associated with an increased production of its gene product.[14]

Homozygosity for the major allele of codon 25 of the *TGFB1* gene, which is in linkage disequilibrium with a cytosine deletion at position +72, is also associated with a higher TGF-β1 production than the other genotypes.[20]

The mechanisms by which these genetic variations contribute to the development of BOS are currently not exactly known. It is, however, likely that they influence the immune response toward inflammation and fibrosis. IL-6 and IFN-γ are involved in acute inflammatory responses in general, but both are also known for their profibrotic properties.[34, 35] TGF-β plays a pivotal role in the development of fibrosis.[35] This suggests that genetically determined variability in cytokine production capacity could play a role in interindividual differences in the intensity of the inflammatory process and in the subsequent fibrogenesis leading to BOS.

Table 2. Overview of polymorphisms in cytokine, innate immunity and repair genes and its association with the development of BOS.

Gene	Polymorphism	Association[1]	Odds ratio[2]	95% CI	p-value	Odds ratio[3]	95% CI	p-value	Risk allele[4]	Odds ratio[5]	p-value	Ref.
TNFA	- 308 G/A - rs1800629	no	n.a.[6]	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	10, 16
	- 308 G/A	no	n.a.	n.a.	n.a.	1.85	0.79 - 4.33	0.15	carrier, A allele	n.a.	n.a.	11
	- 308 G/A	no	1.05	0.60 - 1.84	0.86	n.s.[10]	n.s.	n.s.		n.a.	n.a.	17
IFNG	+ 874 A/T - rs62559044	no	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	10, 16
	+ 874 A/T	yes	2.82	1.54 - 5.18	0.0006	3.83	1.42 - 10.34	0.006	major, homozygous	3.05	0.0007	11
	CA repeat	yes	n.a.	n.a.	n.a.	5.60	1.70 - 18.38	0.005	allele #2, carrier	n.a.	n.a.	18
TGFB1	+ 869 T/C - rs1800470	no	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	10, 11, 16
	+ 869 T/C	no	1.02	0.54 - 1.96	1	n.s.	n.s.	n.s.		1.38	0.15	19
	+ 869 T/C	no	1.90	0.85 - 4.24	0.11	1.77	0.58 - 5.41	0.31		2.18	0.10	20
	+ 915 G/C - rs1800471	no	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	10, 11, 16
	+ 915 G/C	yes	19.68	5.74 - 67.45	< 0.0001	72	16.71 - 310.31	< 0.001	major, homozygous	33.60	0.000003	19
	+ 915 G/C	yes	6.11	1.53 - 24.25	0.01	7.3	1.68 - 31.38	0.01	major, homozygous	3.85	0.003	20
	+ 915 G/C	no	1.32	0.58 - 3.03	0.51	n.s.	n.s.	n.s.		n.a.	n.a.	17
	- 800 G/A - rs1800468	yes	n.a.†	n.a.	n.a.	n.a.†	n.a.	n.a.		1.26	0.27	20
	- 509 C/T - rs1800469	no	1.24	0.52 - 24.25	0.63	n.s.	n.s.	n.s.		1.42	0.63	20
	+ 72 C insertion/deletion	yes	6.11	1.53 - 24.25	0.01	7.3	1.68 - 31.38	0.01	cytosine insertion	3.85	0.003	20
IL1B	-31 C/T	no	1.17	0.74 - 1.83	0.50	n.s.	n.s.	n.s.		n.a.	n.a.	17
	+ 3953 C/T	no	1.17	0.70 - 1.93	0.55	n.s.	n.s.	n.s.		n.a.	n.a.	17
IL1R1	131 C/T	no	1.09	0.70 - 1.70	0.70	n.s.	n.s.	n.s.		n.a.	n.a.	17
IL1RN	8061 C/T	yes	1.70	1.08 - 2.70	0.02	1.73	0.96 - 3.13	0.06	major, homozygous	n.a.	n.a.	17
	86 bp repeat	yes	2.02	1.30 - 3.20	0.002	2.14	1.17 - 3.90	0.01	allele #1, homozygous	n.a.	n.a.	17
IL6	- 174 G/C - rs1800795	no[7]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	10, 16
	- 174 G/C	yes	n.a.	n.a.	n.a.	3.13	1.22 - 8.03	0.016	major, carrier	n.a.	n.a.	11
	- 174 G/C	no	1.25	0.82 - 2.02	0.18	n.s.	n.s.	n.s.		n.a.	n.a.	17
IL10	- 1082 A/G - rs1800896	no	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	10, 11, 16
	- 1082 A/G	no	1.33	0.88 - 2.02	0.18	n.s.	n.s.	n.s.		n.a.	n.a.	17
	- 819 C/T - rs3021097	no	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	10, 11, 16
	- 819 C/T	no	1.04	0.62 - 1.74	0.86	n.s.	n.s.	n.s.		n.a.	n.a.	17
	- 592 C/A - rs1800872	no	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	10, 11, 16
FCGRIIA	507 A/G - rs1801274	no	1.05	0.69 - 1.59	0.82	n.s.	n.s.	n.s.		n.a.	n.a.	17
TLR2	rs1898830	yes	3.68	1.37 - 9.86	0.006	3.92	1.31- 11.72	0.01	major, homozygous	8.73	0.007	22
	rs7656411	no	1.36	0.67 - 2.79	0.45	n.s.	n.s.	n.s.		1.40	0.41	22

Gene	Polymorphism	Association[1]	Odds ratio[2]	95% CI	p-value	Odds ratio[3]	95% CI	p-value	Risk allele[4]	Odds ratio[5]	p-value	Ref.
TLR4	Asp299Gly - rs4986790	no[8]	n.a.	n.a.	n.a.	0.32	0.07 - 1.44	0.16		n.a.	n.a.	9
	Thr399Ile - rs4986791	no[8]	n.a.	n.a.	n.a.	0.32	0.07 - 1.44	0.16		n.a.	n.a.	9
	rs1927911	yes	2.12	1.03 - 4.36	0.04	4.67	1.26 - 17.26	0.03	minor, homozygous	2.07	0.05	22
TLR9	rs352162	yes	2.51	1.24 - 5.10	0.009	3.51	1.26 - 9.81	0.009	major, homozygous	2.17	0.02	22
	rs187084	yes	3.10	1.46 - 6.48	0.002	3.56	1.31 - 9.68	0.009	major, homozygous	2.04	0.03	22
CD14	-159 C/T - rs2569190	yes	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	8
MBL	-619 C/G - rs11003125	no	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	23
	-290 G/C - rs7096206	no/yes[9]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	23
	-66 C/T - rs7095891	no	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	23
	+154 C/T - rs5030737	no	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	23
	+161 G/A - rs1800450	no	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	23
	+170 G/A - rs1800451	no	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.		n.a.	n.a.	23
KIR	haplotype A (homozygous)	yes	3.67	0.95 - 14.12	0.05	4.82	0.83 - 28.10	0.10	haplotype A, homozygous	2.35	0.09	24
	KIR2DS5 (absence)	yes	n.a.	n.a.	n.a.	0.00†	n.a.	n.a.	KIR2DS5 (absence)	n.a.	n.a.	24
MMP7	rs12285347	yes	2.14	1.03 - 4.44	0.04	2.91	1.11 - 7.69	0.03	major, homozygous	1.81	0.05	25
	rs10502001	yes	1.73	0.83 - 3.59	0.14	5.00	1.14 - 21.98	0.04	minor, homozygous	1.90	0.15	25
	rs17098318	yes	3.21	1.28 - 8.03	0.01	3.75	1.30 - 11.11	0.01	major, homozygous	2.50	0.018	25
	rs11568818	yes	2.73	1.27 - 5.90	0.009	3.54	1.31 - 9.51	0.009	major, homozygous	2.30	0.014	25
	rs1996352	no	1.38	0.65 - 2.94	0.40	n.s.	n.s.	n.s.		1.32	0.42	25
	rs11568819	no	2.44	0.30 - 19.61	0.47	n.s.	n.s.	n.s.		1.05	0.37	25

TNFA, tumor necrosis factor alpha; IFNG, interferon gamma; IL, interleukin; IL1R1, IL1 receptor; IL1RN, IL1 receptor antagonist; TGFB, transforming growth factor beta; TLR, Toll-like receptor; FCGRIIA, Fc gamma receptor IIa; MBL, mannose binding lectin; KIR, killer immunoglobin-like receptor; MMP, matrix metalloproteinase; OR, Odds ratio; CI, confidence interval; n.a., not available.

1. Association reported in original article.
2. Odds ratio for allele frequency distribution between BOS+ and BOS- patients.
3. Odds ratio for carriership or homozygosity of the risk allele.
4. Risk allele represent allele that gives increased OR for the development of BOS.
5. Odds ratio determined by Armitage's trend test.
6. No absolute data available to determine allele frequency or genotype distribution/frequency and to construct a 2 x 2 table, accounts for all n.a.
7. Ref 10. in first cohort association with an earlier onset of BOS, a second cohort did not validate this association.
8. Carriers of minor allele showed a trend towards reduced onset of BOS grade 2 and 3.
9. Recipients who received a graft from a donor with an Y/X or X/X genotype compared to Y/Y had a better BOS-free survival.
10. Allele frequencies is not significantly different, therefore no OR for genotype distribution is calculated, accounts for all n.s.

† due to the absence (n = 0) of an allele or genotype it is not possible to calculate an OR with a CI.

Data highlighted in bold denote significant differences between BOS+ and BOS- patients, italicised values denote trend ($0.05 \leq p \leq 0.15$).

R39 R38 R37 R36 R35 R34 R33 R32 R31 R30 R29 R28 R27 R26 R25 R24 R23 R22 R21 R20 R19 R18 R17 R16 R15 R14 R13 R12 R11 R10 R9 R8 R7 R6 R5 R4 R3 R2 R1

Significant associations between genetic polymorphisms in the innate immunity genes and BOS were also found. Especially the association of two genetic variants in *TLR4* (Asp299Gly and Thr399Ile) is of great interest because there is evidence that carriers of the minor allele have a reduced production of pro-inflammatory cytokines and chemokines on stimulation, which might have a protective effect upon the pulmonary epithelium.[36] The functionality of the other SNPs in the *TLR2*, *TLR4* and *TLR9* genes has not been investigated;[22] however, the risk alleles of these SNPs might contribute to the development of BOS by an increased secretion of cytokines and chemokines that is followed by injury of the pulmonary epithelium. The functionality of the genetic polymorphisms in the *CD14*, *MBL* and *KIR* genes is known as well. First, CD14 binds to lipopolysaccharide and promotes signaling through TLR4.[37] Homozygotes for the risk allele of *CD14* had higher levels of CD14, TNF-α, and IFN-γ in their peripheral blood implying a heightened state of innate immune activation.[8] Second, the Y allele of *MBL* was found to be associated with high production of the gene product that may result in more inflammation and tissue damage and an increased antigen presentation.[38] Third, natural killer (NK) cells are important components of the innate immunity and their activation is influenced by KIRs.[39] *KIR* haplotypes are associated with the number of functional inhibitory and activating *KIR* genes. Haplotype A contains six inhibitory and one activating *KIR* gene, and this haplotype is associated with functional down-regulation of the NK-cell activity. Haplotype B contains a mixture of functional activating and inhibiting KIRs.[40, 41] The association between haplotype A and BOS is against the expectation, because the presence of haplotype A on NK cells is associated with less reactivity against donor cells recognized on lung allografts and thus the absence of BOS.[24]

Finally, a genetic association was found between BOS and *MMP7*, a repair gene. MMP-7 is involved in the repair of the pulmonary epithelium, and its expression is primarily regulated at the transcriptional level.[42] The genetic polymorphisms in the *MMP7* gene may contribute to aberrant tissue repair and fibrosis through insufficient levels of MMP-7.[25]

The foregoing evidence supports that genetic polymorphisms in innate immunity genes and in a repair gene might contribute to the development of BOS by influencing the inflammatory response and the process of fibrogenesis. However, the association of genetic polymorphisms in the innate immunity genes and in *MMP7* with BOS has never been replicated; therefore, validation in an independent cohort is required.

Application of genetic risk profiling to clinical practice

In the future, genetic risk profiling may become a tool for the clinician to stratify the risk of developing BOS after lung transplantation and to adjust the treatment. Palmer et al. already suggested that *TLR4* genotyping before transplant permits assessment of the risk for acute rejection.[43] In addition, genetic risk profiling may allow individualization of the immunosuppressive treatment. For example, if a lung transplant recipient has a genetic profile

conferring a greater risk of BOS after lung transplantation, it is not unlikely that he/she may benefit from adaptation of the standard immunosuppressive treatment regime. Furthermore, knowledge of the genetic polymorphisms that contribute to BOS might lead to alternative therapies to prevent or treat BOS, such as prevention of the activation of innate immunity through TLRs or inhibition of IL-6, IFN-γ, and TGF-β, that is, by blocking their receptors, to slow down the inflammation and fibrosis. Lung transplant recipients receive multiple anti-inflammatory medications to prevent acute and chronic rejection. Nowadays, the treatment of BOS consists of augmenting or changing the type of immunosuppressive drug.[3] Recently, there is evidence that treatment of lung transplant recipients with azithromycin has promising results. A randomized controlled trial showed that azithromycin prophylaxis after lung transplantation attenuates the inflammatory response, improves the FEV_1 and reduced the occurrence of BOS.[44] Furthermore, treatment of BOS^{pos} patients with azithromycin led to an increase in FEV_1 and to a better survival.[45, 46] Azithromycin modulates, in particular, the innate immune response by decreasing the response of several cytokines, as IL-4, IL-8, and TNF-α, inhibiting the chemotaxis of neutrophils, inducing the apoptosis of neutrophils and lymphocytes, and disturbing the interaction between host and pathogen.[31]

With the knowledge that BOS is also a fibrotic disease, the question arises whether the treatment of BOS might profit from antifibrotic agents, next to the anti-inflammatory agents.

Although risk stratification of lung transplant recipients with genetic profiling seems to be a promising approach for the future, which absolutely warrants further research, the results of the present studies discussed in this review are not yet sufficient to implement the use of a genetic profile into clinical practice.

Recommendations for the future

While comparing and summarizing the literature, several limitations were encountered in the studies on genetic polymorphisms and the development of BOS.

First, in most studies only a few genetic polymorphisms or the same subset of genes were studied, which makes the list of candidate gene studies far from exhaustive. There is evidence that a combination of risk alleles is present in BOS^{pos} patients. For example, in a study on genetic polymorphisms in several *TLR* genes, BOS^{pos} patients had more risk alleles compared with BOS^{neg} patients and controls.[22] Furthermore, concomitant presence of high-expression SNPs in both the *IL6* and the *IFNG* gene was higher in BOS^{pos} patients than in BOS^{neg} patients.[11] In the light of genetic profiling, future association studies should investigate a combination of multiple genes. For example, in addition to MMP-7, other MMPs have shown to be involved in the development of BOS by their role in remodeling and degradation of the extracellular matrix and, therefore, might be interesting candidate genes.[47-49] For the future, aiming at identifying genes relevant in BOS candidate genes can also be selected on the basis of their assumed involvement in pathways leading to BOS. Genetic polymorphisms in *IFNG* and its gene

product are both associated with the development of BOS,[50-52] therefore, receptors of IFN-γ and pathways that are activated by IFN-γ might be promising as well. An alternative way of identifying pathways involved in the development of BOS might benefit from whole genome association studies or SNP chips for specific pathway analysis. However, these approaches require a large group of patients to correct for type 1 errors.

Second, the sizes of most study populations were small which might influence the results through insufficient statistical power. In addition, other risk factors for BOS, such as human leukocyte antigen mismatches, autoimmune responses, cytomegalovirus infection, and type of transplantation, are difficult to control in a statistical analysis because of the small sample size. In larger cohorts, these different risk factors should be included in a multivariate analysis together with the genetic profile, thus enabling a more accurate prediction of the risk of developing BOS.

Third, the follow-up period between the studies is different. The development of BOS is a time-dependent diagnosis, therefore, studies with a relatively short follow-up do not allow BOS to develop and this may lead to false conclusions.

Fourth, the definition BOS or allograft fibrosis is different between studies. Some studies use the BOS criteria according to the ISHLT guidelines, while others use histological criteria to grade fibrosis, and in some studies, the definition of allograft fibrosis or BOS is lacking. In addition, RAS and two different phenotypes of BOS are identified as described earlier.[27-29] The existence of these subtypes needs to be taken into consideration in future studies.

Fifth, in the majority of studies, the ethnic composition is not described, which influences the results because ethnicity influences the distribution of genetic polymorphisms, as reported in cytokine genes.[53, 54]

Finally, differences in immunosuppressive treatment might lead to discrepancies in the results of the various groups, because immunosuppressive medication might mask a possible effect of the genetic polymorphisms. To promote the implementation of genetic profiling, we underline the proposal of Holweg et al.[7] of starting a database, in which allele and genotype frequencies of both donor and recipient, standardized definitions for complications after transplantation, and characteristics of transplant recipients are collected to improve gene association studies on BOS in the future.

Conclusions

The results of this review show that genetic polymorphisms in cytokine, innate immunity, and repair genes have been linked to the susceptibility to develop BOS after lung transplantation. However, exact causality of many of the associations, for example, by regulating the inflammatory response, cytokine and chemokine production, and facilitation of repair, still

needs to be proven. Combining of the relevant genetic associations into a SNP chip for the stratification of the risk to develop BOS might be a promising approach. Genetic profiling could help clinicians to set out individualized treatment regimens for the prevention and treatment of BOS. Further studies are, however, needed to prove this concept.

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CHAPTER 8

SUMMARY
GENERAL DISCUSSION
AND FUTURE PERSPECTIVES

Summary

Lung transplantation is the treatment of choice for patients with end-stage lung disease. However, long-term survival is limited by the development of bronchiolitis obliterans syndrome (BOS) in the donor lung of the transplant recipient.

BOS is diagnosed after lung transplantation when a decline in forced expired volume in one second (FEV_1) occurs, which is not due to rejection, infection or problems of the bronchial anastomosis. The development of BOS is characterized by injury of the airway epithelium by alloimmune-dependent and -independent mechanisms. This process leads to cellular infiltration, inflammation and remodeling, which is followed by aberrant repair, and finally fibrosis and occlusion of the small airways of the allograft.

When BOS is diagnosed, the process is already at an advanced and mostly irreversible stage and treatment options are limited. The current status of diagnosis of BOS clearly indicates the need for markers, in serum, exhaled breath condensate (EBC) or DNA, that may detect processes leading to BOS before the decline in FEV_1 occurs. These unmet needs are the basis of this thesis.

We hypothesize that biomarker levels in serum or EBC or the genotype distribution were significantly different between BOS^{pos} patients and BOS^{neg} patients. Each of these biomarkers was selected for its role in one or more pathways that lead to the development of BOS.

Toll-like receptors (TLRs) are involved in the activation of innate immunity by recognition of a spectrum of micro-organisms. Activation of the innate immunity via TLRs was shown to be a barrier for induction of transplant tolerance after lung transplantation. Variations in the innate immune response can partly be explained by genetic polymorphisms in innate immunity genes. In **chapter 2**, we analyzed 64 single nucleotide polymorphisms (SNPs) in 10 genes coding for *TLR1* to *TLR10* to determine whether these genetic polymorphisms were associated with the development of BOS. Twenty BOS^{pos} patients, 90 BOS^{neg} patients and 422 controls were included.

In the genes coding for *TLR2* (2 SNPs), *TLR4* (1 SNP) and *TLR9* (2 SNPs) a significantly different genotype distribution was found between BOS^{pos} patients and BOS^{neg} patients and controls. The BOS^{pos} patients carried significantly more risk alleles (alleles with an increased risk of developing BOS) compared with the BOS^{neg} patients and the controls.

TLR2 and TLR4 are important in the signaling pathway for bacterial micro-organisms. TLR9 is involved in the recognition of viral pathogens. These results imply that bacterial as well as viral pathogens may promote the development of BOS. Genetic polymorphisms in *TLR2*, *TLR4* and *TLR9* may contribute to the development of BOS by increased secretion of cytokines and chemokines and suppression of regulatory T-cells which may lead to more severe injury of the airway epithelium, and finally to the development of BOS.

The specific mechanisms that lead to inflammation and remodeling of the airway epithelium and finally to fibrosis may involve an imbalance between T-helper (Th)1 and Th2 cytokines. In **chapter 3**, we determined the cytokine and chemokine profiles in serum and EBC of lung transplant recipients with and without BOS and assessed their usefulness as biomarkers for BOS.

Samples of serum and EBC were longitudinally collected in the period after lung transplantation until the diagnosis BOS was made and analyzed with a 27 multiplex immunoassay. Ten BOS^{pos} patients, 10 matched BOS^{neg} patients and 40 healthy controls were included.

Despite immunosuppressive medication, pro-inflammatory cytokines in serum revealed a marked elevation in lung transplant recipients compared with controls. Comparison of serum levels of the Th2 cytokines in BOS^{pos} and BOS^{neg} patients showed lower interleukin (IL)-4, IL-13 and vascular endothelial growth factor (VEGF) levels and higher IL-5 levels in BOS^{pos} patients. In EBC, IL-4 and IL-5 were hardly detectable. IL-13 and VEGF, both decreased in serum, were also decreased in EBC of BOS^{pos} patients compared with BOS^{neg} patients. Longitudinal analysis of cytokines and chemokines in serum and EBC from the time of lung transplantation onwards did not reveal an increase or decrease prior to BOS.

This study shows that after lung transplantation BOS^{pos} patients and BOS^{neg} patients exhibit a different pattern of Th2 cytokines in serum. It needs to be further explored whether these patterns, or parts of it, can be used for risk stratification of BOS after lung transplantation.

BOS is characterized by an aberrant repair response that results in fibrosis obliterating the airway lumen. Matrix metalloproteinases (MMPs) are a family of enzymes involved in the turnover and degradation of the extracellular matrix (ECM), in cell-cell and cell-matrix signaling, and in the process of re-epithelialization and repair. Especially MMP-7 is important in repair of the airway epithelium. In **chapter 4**, we investigated whether genetic polymorphisms in *MMP7* are associated with the development of BOS and whether they are related to MMP-7 serum levels. Twenty-one BOS^{pos} patients, 89 BOS^{neg} patients and 422 healthy controls were genotyped. MMP-7 serum levels were measured in 9 BOS^{pos} and 9 matched BOS^{neg} patients, in 35 unmatched BOS^{neg} patients and in 78 healthy controls.

Compared with controls, lung transplant recipients had significantly increased MMP-7 serum levels, but BOS^{pos} patients had significantly lower MMP-7 serum levels than BOS^{neg} patients.

The genotype and haplotype distribution of *MMP7* was significantly different between BOS^{pos} and BOS^{neg} patients and controls. Specific alleles were associated with an increased risk to develop BOS and shown to be risk alleles. Haplotypes constructed with three or four risk alleles correlated with lower MMP-7 serum levels. Low MMP-7 serum levels might contribute to aberrant repair of the airway epithelium. Thus, lung transplant recipients carrying risk alleles of *MMP7* express lower serum levels of MMP-7 and might be more prone to the development of BOS due to ineffective epithelial repair.

YKL-40 acts as a growth factor for fibroblasts and is involved in inflammation, remodeling and fibrosis. In asthma and sarcoidosis YKL-40 may be a biomarker for disease activity and fibrosis formation. MMP-9 is involved in the degradation and turnover of the ECM and in the migration of inflammatory cells. Several studies suggest that there is a relation between MMP-9 and BOS, though, the results are controversial. In **chapter 5**, we assessed whether YKL-40 and MMP-9 serum levels can be useful as biomarker for BOS.

We measured serial YKL-40 and MMP-9 serum levels in 10 BOS^{pos} and 10 matched BOS^{neg} patients.

There was no significant difference in YKL-40 serum levels between the two patient groups. The MMP-9 serum levels were significantly higher in BOS^{pos} patients than in BOS^{neg} patients. Longitudinal analysis after lung transplantation did not reveal a significant increase or decrease in YKL-40 and MMP-9 serum levels prior to the diagnosis of BOS.

These results suggest that measuring the post-transplant MMP-9 serum levels might be useful for risk stratification of BOS. Increased MMP-9 serum levels in BOS^{pos} patients might contribute to the degradation of the ECM and to migration of inflammatory cells which might lead to the development of BOS. Post-transplant YKL-40 serum levels cannot be used as biomarker for BOS.

A second aim of this study was to investigate the relationship between YKL-40, MMP-9 and MMP-7. In the BOS^{neg} patient group MMP-9 showed an inverse relationship with MMP-7. Apparently, normal epithelial repair, as represented by increased MMP-7 serum levels, correlates with less degradation, as shown by lower MMP-9 serum levels. MMP-7 and MMP-9 serum levels in the BOS^{pos} patient group did not show this relationship and only demonstrated low MMP-7 serum levels and high MMP-9 serum levels, indicative of little repair and high degradation.

Reduced expression of Caveolin-1 (Cav-1) is found in animals and humans with pulmonary fibrosis. Genetic polymorphisms in *CAV1* influence the function of its gene product in malignancies and are associated with renal allograft fibrosis. In **chapter 6**, genetic polymorphisms in the *CAV1* gene and Cav-1 serum levels have been investigated in BOS.

Twenty BOS^{pos} patients, 90 BOS^{neg} patients and 422 healthy controls were genotyped. Cav-1 serum levels were measured in 10 BOS^{pos} patients, in 10 matched BOS^{neg} patients, in 33 unmatched BOS^{neg} patients and in 60 healthy controls.

Cav-1 serum concentrations were lower in all lung transplant recipients than in healthy controls. Within the matched cohort, the BOS^{neg} patients had lower Cav-1 serum levels than the BOS^{pos} patients. Serial Cav-1 serum levels did not reveal an increase or decrease in Cav-1 concentration in time after lung transplantation and prior to BOS. Cav-1 serum levels in the 43 (10 matched + 33 unmatched) BOS^{neg} patients were lower than in healthy controls, but not different compared with the BOS^{pos} patients.

The *CAV1* genotype distribution was significantly different between BOS^{pos} and BOS^{neg} patients. Homozygosity of the minor allele of *rs3807989* was associated with a sixfold increased risk to develop BOS. Lung transplant recipients homozygous for the minor allele of *rs3807989* and *rs3807994* had increased Cav-1 serum levels compared with other genotypes.
Concluding, *CAV1* SNP *rs3807989* was associated with an increased risk to develop BOS and Cav-1 serum levels correlated with genetic polymorphisms in *CAV1*. The mechanisms through which these genetic polymorphisms contribute to increased Cav-1 serum levels and to the development of BOS need to be further explored.

An individual's susceptibility to BOS is inevitably determined by genetic variations in cytokine and innate immunity genes. **Chapter 7** gives an overview of the genetic polymorphisms that have been investigated in lung transplant recipients and that have been associated with the development of BOS or allograft fibrosis. This systematic review is based on a literature search through Medline and Embase.
Functional genetic polymorphisms in several cytokine genes were associated with the development of BOS and allograft fibrosis after lung transplantation. However, these associations have not been confirmed in independent studies. Genetic polymorphisms in several innate immunity genes and in a repair gene were also found to be associated with the development of BOS, but these studies also need to be replicated in other cohorts.
Genetic risk profiling of lung transplant recipients could be a promising approach for the future. It might support individualized risk stratification and personalized immunosuppressive treatment after lung transplantation.

Conclusions

The development of BOS is initiated by alloimmune-dependent and -independent triggers that lead to injury and inflammation of the airway epithelium, which end in fibrosis and obliteration of the airway lumen (Figure 1).

TLRs are critical molecules for activation of the innate immune system by recognition of pathogens, and they can prevent the induction of allograft tolerance.[1] Genetic polymorphisms in *TLR2*, *TLR4* and *TLR9* might contribute to patients' susceptibility for BOS. These genetic polymorphisms could predispose to increased secretion of pro-inflammatory cytokines, causing injury and inflammation of the airway epithelium (Chapter 2).

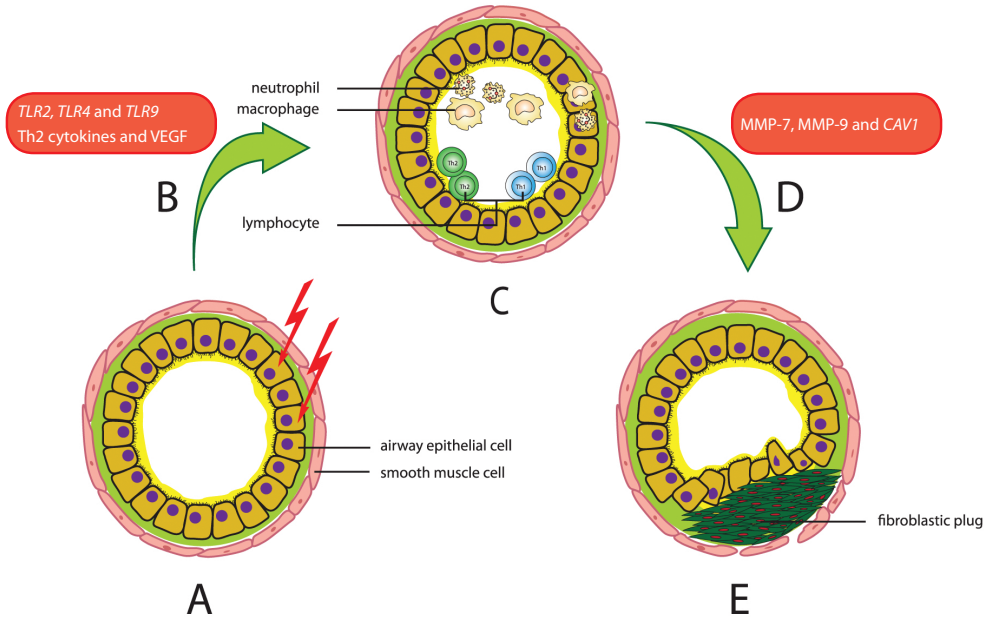

Figure 1. Primary damage to airway epithelium by alloimmune-dependent and -independent mechanisms (A) leads to activation of the innate immune system i.e. via **Toll-like receptors** which is followed by release of Th1 and **Th2 cytokines** (B). The injury and inflammatory response of the airway epithelium (C) is followed by an aberrant repair response with fibroblast proliferation and extracellular matrix deposition mediated by **metalloproteinases** and several growth factors (D). The last step in this process is fibrosis of the airway epithelium with obliteration of the lumen (E).

The exact role of different types of cytokines in rejection or tolerance of the allograft is under debate.[2] On one hand, Th1 cytokines often lead to allograft rejection, while Th2 cytokines promote tolerance of the allograft. On the other hand, Th2 cytokines may not be necessary for the induction of tolerance and Th1 cytokines may be beneficial in promoting allograft acceptance.[2] We showed that the Th1 cytokines were similar between BOS^{pos} and BOS^{neg} patients, but the Th2 cytokines revealed a different pattern between these two groups (Chapter 3). These results suggest that Th2 cytokines are involved in the process of chronic rejection, possibly due to the inhibition of transplant tolerance, the absence of inhibition of the Th1 response and the influence on proliferation of regulatory T-cells.[3-6]

In relation to excessive injury and chronic inflammation, the process of fibrogenesis is considered to be of central importance to the development of BOS.[7] Normally, after injury of the airway epithelium an adequate repair mechanism is required to prevent fibrogenesis. Lung transplant recipients who have developed BOS, however, seem to have an impaired repair mechanism and a profibrotic airway milieu. BOS^{pos} patients had a different genotype distribution of *MMP7* and lower levels of MMP-7 than BOS^{neg} patients that might contribute to an impaired repair mechanism of the airway epithelium (Chapter 4). Besides, there

is more degradation and turnover of the ECM in BOS^{pos} patients than in BOS^{neg} patients as shown by increased levels of MMP-9 (Chapter 5). Although, the functionality of the genetic polymorphisms in the *CAV1* gene is not yet known, it might contribute to fibrogenesis as well through the transforming growth factor (TGF)-β signaling pathway (Chapter 6).

In conclusion, genetic polymorphisms in Toll-like receptors, *MMP7* and *CAV1* and biomarkers in serum, such as Th2 cytokines, MMP-7 and MMP-9, are related to the development of BOS after lung transplantation, and might be potential biomarkers for clinical decision making.

General discussion

The results described in this thesis suggest that several markers in serum and DNA can be used for risk stratification of BOS after lung transplantation. In this paragraph both the promises and limitations of using biomarkers for risk stratification of BOS are described. One of the promises in the field of biomarker studies on BOS is the use of a set of biomarkers and the auspicious role of MMPs in the development of BOS. The differences in the genetic profile of both the donor and recipient and the use of several immunosuppressive drugs worldwide are possible limitations and might have consequences for the applicability of biomarkers.

The main limitation of our studies concerns the relatively small sample size. We recognize that the small number of especially the BOS^{pos} patients limits the power of our studies. For the genetic association studies we calculated the effect size that could be measured with a power of 0.80 for the different minor allele frequencies and the limited number of 20 BOS^{pos} patients. In a dominant gene model minor allele frequencies (MAF) of 0.05 result in a detectable odds ratio (OR) of five, MAF of 0.10 to 0.25 result in an OR of four and MAF of 0.30 to 0.50 result in an OR of five to fifteen. With a similar MAF range the additive gene model results in an OR of 2.50 to 4.50. This shows that genetic association studies with a small number of patients will provide significant results when the effect size, as given by the OR, is large.
In addition, the matching procedure between the BOS^{pos} and BOS^{neg} patients reduced the influence of confounding factors. Furthermore, the diagnosis of BOS is made accurately according to the guidelines of the International Society of Heart and Lung Transplantation (ISHLT), which makes it a consistent group of BOS^{pos} patients. Therefore, the findings of this thesis are clinically relevant and warrant further research.

Use of biomarkers in clinical practice

Early detection of allograft dysfunction will allow timely treatment and possibly prevention of the progressive decline in lung function that occurs after the onset of BOS. One of the focuses in the field of lung transplantation is the search for biomarkers, which will enable the prediction or early detection of BOS before a decline in lung function has occurred.

We did not find a correlation between lung function and a biomarker in the longitudinal analysis (adjusted for sex and age). This might be explained by the fact that when biomarker levels decrease or increase, the process of inflammation and fibrosis is already present, though, not advanced it will have caused a decline in lung function. To detect the development of BOS before lung function decline, the biomarker level needs to change earlier than the onset of decline in lung function. Ideally, the aim is NOT to find a marker that correlates with lung function decline, but to find a marker that identifies the process of BOS before lung function declines, and that contributes in clinical decision making to improve BOS outcome.

Given the multifactorial nature of BOS, more than one biomarker is needed to discriminate between lung transplant recipients who will develop BOS and those who will not. A set of biomarkers will have more power to accurately identify those lung transplant recipients that are at increased risk to develop BOS. To enclose the several pathways of BOS, the following serum markers and genetic polymorphisms are proposed to be included in the set of biomarkers for risk stratification of BOS after lung transplantation: serum IL-4, IL-5, IL-13, VEGF, MMP-7 and MMP-9 and genetic polymorphisms in the genes of *TLR2*, *TLR4*, *TLR9*, *MMP7* and *CAV1*.

In clinical practice, the genetic profile must be determined before transplantation and the serum markers must be measured early after lung transplantation, for example at 6 months, at a stable condition without infection or acute rejection. This risk profile will give an individualized risk stratification of a lung transplant recipient to develop BOS and might lead to personalized immunosuppressive treatment after lung transplantation.

Challenges of using multiple parameters in a clinical setting are increased analysis time and data complexity, which have to be taken into account when using sets of biomarkers as a diagnostic entity into clinical practice.

Differences in the genetic profile of donor and recipient

Chimerism, the presence of two genetically distinct types of cells in one individual, is present in the lungs of lung transplant recipients.[9]

The question is whether determination of the 'systemic' genetic profile of a lung transplant recipient by extracting DNA from blood cells, does represent the 'local' or pulmonary genetic profile of the lung where the processes of inflammation, remodeling and repair occur.

To investigate the effect of chimerism on the outcome after lung transplantation, it is most important which cell types express specific genes. For example, TLRs are mainly present on hematopoietic cells, but can also be found on the airway epithelium.[10] Hematopoietic cells are recipient-derived and, by that, receiving transplanted lungs will not influence the genetics of these innate immunity genes. As chimerism is present in the airway epithelium, Palmer et al. concluded that the *TLR4* genotype of the recipient could influence the epithelial response to innate pathogens.[11] Consequently, determination of the 'systemic' genetic profile of TLRs will be a good reflection of the genetic profile of the recipient.

MMP-7 and Cav-1 are expressed on the airway epithelium, although Cav-1 can also be found on other cells, such as endothelial cells and fibroblasts. For these enzymes the 'local' genetic profile might be different from the 'systemic' genetic profile. This difference will attenuate in time after lung transplantation because chimerism will be more obvious in case of high cell turnover as present in chronic rejection.[9] This makes it plausible that, especially in BOS^{pos} patients, the genetic profile of the airway epithelium will become more recipient-derived and less donor-derived.

In the fields of genetic research and transplantation medicine, identifying genetic markers that may improve clinical decision making is very challenging. For this purpose, the genetic background of both the recipient and the donor need to be taken into consideration.

The influence of immunosuppressive agents on biomarkers in serum and on the outcome after transplantation

Two aspects of the use of different immunosuppressive agents worldwide will be discussed below.

First, does a specific pattern of cytokines as measured in our study under specific immunosuppressive medication, appear in another cohort with patients under different immunosuppressive medication? For example, in renal transplant recipients, a tacrolimus-based immunosuppression regimen was associated with an increased response of IL-2 and IL-4 compared with a cyclosporine-based treatment. Another example is mycophenolate mofetil (MMF) which leads to a reduced response of IL-10 and a reduced expression of IL-6 compared with azathioprine.[12]

In renal transplants, cyclosporine contributes to the development of renal fibrosis and to the molecular mechanisms underlying epithelial-mesenchymal transition (EMT).[13] EMT, in which epithelial cells differentiate into fibroblasts, has been shown to contribute to the development of BOS.[14] Accordingly, another group showed that in airway epithelial cells the immunosuppressive drugs cyclosporine, MMF and sirolimus can trigger an EMT response mediated through TGF-β1 and lead to an increased production of the extracellular matrix proteins. Tacrolimus and prednisone did not trigger this response.[15] These results suggest that immunosuppressive agents may influence cytokine levels and the process of remodeling and fibrosis, which may influence the applicability of biomarkers.

Secondly, do different regimens of immunosuppressive agents influence the outcome, for example the development of BOS, after lung transplantation? In our transplant centre tacrolimus and MMF are used as standard maintenance immunosuppressive drugs. In the latest report of the ISHLT, a combination of tacrolimus and MMF showed the lowest rejection rates compared with other combinations, including cyclosporine and azathioprine.[16] These lower rejection rates will decrease the incidence of BOS and, by that, the number of lung transplant recipients with BOS. Therefore, to detect significant associations between a biomarker, c.q. pattern of cytokines, and the development of BOS a longer follow-up and more patients are needed.

The influence of genetic polymorphisms on biomarkers in serum

In the scope of factors that might influence biomarker levels in serum the question arises whether genetic polymorphisms might influence serum biomarker levels. Healthy controls with the *TLR4* Asp299Gly allele had lower levels of certain pro-inflammatory cytokines,

such as IL-6, than healthy controls with the other genotype.[17] We investigated whether the risk alleles in *TLR2*, *TLR4* and *TLR9* (chapter 2) correlate with cytokine levels (chapter 3). We found that the BOS^{pos} patients carrying the risk allele of *TLR9* (*rs187084*) and *TLR4* (*rs1927911*) had increased serum levels of respectively IL-12 and interferon (IFN)-γ compared with BOS^{pos} patients carrying the non-risk allele. The sample size is small, but these results might suggest that genetic polymorphisms in the *TLR* genes have consequences for cytokine levels.

Biomarkers integrated

In this thesis several potential biomarkers for the development of BOS are investigated separately. We here describe potential connections between these biomarkers in serum and the development of BOS.

To integrate all our measured biomarkers, we used the principal component analysis (PCA). PCA involves a mathematical procedure to reduce the dimensionality of a data set consisting of a large number of interrelated variables, while retaining as much as possible of the variation present in the data set. This is achieved by transforming the existing data set to a new set of variables, the principal components, which do not correlate, and which are ordered so that the first few retain most of the variation present in all of the original variables. In the scope of a biomarker study, one component can be seen as a possible pathophysiological pathway.

For this analysis all markers in the 27 multiplex assay were included, except RANTES and platelet derived growth factor because these two variables could not be determined. Furthermore, MMP-7, MMP-9, YKL-40 and Cav-1 were included. Variables with a coefficient of ≥ 0.75 in one component were included. This analysis revealed a different pattern of biomarkers between BOS^{pos} and BOS^{neg} patients. In the BOS^{pos} patients, Cav-1, MMP-7, IL-1b (pro-inflammatory), IL-1ra (anti-inflammatory), IL-2 (Th1), IL-9 (Th2), IL-12 (regulatory, stimulating Th1 response) and IL-15 (growth factor) are responsible for the first component. IFN-γ had a coefficient of 0.72 and, therefore, was not included in the first component. In the BOS^{neg} patients the first component was driven by IL-4 (Th2), IL-7 (growth factor), IFN-γ (Th1) and macrophage inflammatory protein (MIP)-1α (chemokine). Remarkably, none of the markers of the first component overlap between BOS^{pos} and BOS^{neg} patients. Further, in both groups Th1 cytokines as well as Th2 cytokines are involved, but none of these cytokines are identical. Another remarkable finding in the cytokines between the BOS^{pos} and BOS^{neg} patients is the presence of IL-1b and IL-1ra in the BOS^{pos} group. This suggest the importance of a pro-inflammatory milieu in the development of BOS. Besides, in the BOS^{pos} patients, also Cav-1 and MMP-7 are involved in the first component, which suggest that fibrogenesis is also an important process in the development of BOS.

Future perspectives

Matrix metalloproteinases and BOS

In the development of BOS it is important to know to which extent a lung transplant recipient is able to react to the noxious insults towards the airway epithelium by facilitating adequate repair mechanisms, and to prevent or promote fibrogenesis as final common pathway of BOS. MMPs have shown to be involved in matrix remodeling, repair and fibrogenesis.[18] MMPs are promising markers for BOS as MMP levels are not influenced by immunosuppressive agents, as opposed to Th2 cytokines.

Besides the inclusion of MMP-7 and MMP-9 in a risk profile, also other MMPs might be interesting because of their involvement in processes leading to the development of BOS. For example, MMP-2, which plays a role in the degradation of type IV collagen, has been investigated in the development of BOS in both humans and animals.[19-21] MMP-8 and MMP-14 might also be involved in degradation and remodeling of the allograft airways after lung transplantation.[20, 22, 23]

In future, exploring the role of other members of the MMP family, combined with cell counts in BAL fluid, will improve our understanding of the development of BOS and the consequences of treatment.

New treatment options for BOS

In search for new treatment options for BOS, results of other fibrotic diseases are of great interest. The pathogenic mechanism leading to idiopathic pulmonary fibrosis (IPF) is a yet unidentified insult to the airway epithelium, initiating injury and chronic inflammation that lead to aberrant wound healing and fibrosis.[24] Traditionally, treatment is based on regimens containing corticosteroids, cytotoxic or immunosuppressive drugs.[25] However, it is now clear that these treatment options are not effective. Based on the concept that IPF is a fibrotic condition treatment options shift towards antifibrotic drugs. For example, pirfenidone, a novel antifibrotic oral agent, appears to improve progression-free survival and pulmonary function in patients with IPF.[26]

Lung transplant recipients receive multiple immunosuppressive agents to prevent rejection. With the knowledge that BOS is a fibrotic disease and antifibrotic therapies in IPF may be effective, the question arises whether the treatment of BOS needs to be expanded with antifibrotic agents. This hypothesis has been investigated in several animal models with positive findings. All studies showed that pirfenidone given orally inhibited development of obliterative bronchiolitis-like lesions in the murine tracheal transplant model of chronic airway rejection, especially when given early after transplantation.[27-30] These findings suggest that pirfenidone might be a candidate drug for prevention of airway fibrosis in lung transplant recipients.

Treatment of lung transplant recipients with MMP-9 inhibitors can also be of interest. MMP-9 inhibitors are already used as anticancer agent.[31] In mice, MMP-9 inhibitors, including doxycyclin, were effective against airway obliteration if treatment was introduced early after transplantation.[32, 33] Besides, treatment with azithromycin prevented upregulation of several MMP genes, including *MMP9*.[34] In addition to the effect of MMP inhibitors on the MMP activity, it was shown that inhibition by a nonspecific MMP inhibitor also acts as a potent immunomodulatory agent able to alter the immunobiology of the rejection response.[35]

Recommendations for future studies

The concept of risk profiling using several candidate gene polymorphisms and serum biomarkers might be a promising approach for the future, because it is helpful for the clinician in clinical decision making after transplantation, e.g. regarding the type and level of immunosuppressive treatments. This might lead to a better outcome regarding the development of BOS and less drugs toxicity. However, improvements are required before gene and biomarker studies will have clinical implications:

Sample size - due to the small number of patients, additional studies with a larger cohort of lung transplant recipients are needed to confirm the results. The relatively small number of patients in our studies is inherent to the number of lung transplantation in our centre per year (ca 20-25 procedures), and to the low incidence of BOS in our centre. A possibility to enlarge the cohort of patients with BOS is to set up a multicentre BOS database as will be discussed later. Another possibility is to include patients who developed BOS after allogeneic bone marrow transplantation, because in both groups the clinical and radiographic presentation, pathology, and immunologic pathogenesis show similarities.[36-38]

Follow-up - as every lung transplant recipient will develop BOS sooner or later after lung transplantation, studies with limited follow-up do not allow time for BOS to develop. The median follow-up time needs to be at least 2 years, because the median time to the onset of BOS after lung transplantation varies between 16 to 20 months.[39]

All lung transplant recipient are prone to develop BOS, therefore, gene and biomarker associations, that have been found to be a risk factor for BOS, might be able to discriminate early onset BOS from late onset BOS instead of predicting the development of BOS itself.

Standard therapy - as discussed earlier, uniformity in the immunosuppressive treatment is needed to exclude discrepancies in the results which are caused by the influence of immunosuppressive agents.

Definition of BOS - in literature, the diagnosis BOS has been made by different diagnostic tools, such as histological investigations or according to the guidelines of the ISHLT. To create uniformity in the outcome of the studies the use of a well-defined, standardized definition of BOS is needed.

Genotyping - in genetic profiling, future studies need to focus on combinations of genetic polymorphisms in which the genetic profile of both the recipient and donor is included.

We support the proposal of Holweg et al.[40] to set up a database, in which patient characteristics, genetic information of recipient and donor, complications after transplantation and serial serum biomarker values are collected. This will enhance the development of risk profiling using genetic polymorphisms and serum markers and contribute to early detection of the development of BOS. A close cooperation between the three transplantation centers in the Netherlands and initiation of this database would be a significant step forward to more conclusive research and to improve understanding of the development of BOS.

BOS was first described in 1984. Nowadays, almost 30 years later, the mechanisms underlying the multifactorial process that lead to the development of BOS after lung transplantation are partly resolved. However, there are still many questions that need to be answered in order to understand this complex disease and, more importantly, to improve the diagnostic pathway and treatment of BOS.

To improve further understanding of the development of BOS after lung transplantation, the establishment of a multicentre database with information of recipient and donor, and creation of a risk profile of serum markers and genetic polymorphisms for risk stratification of BOS early after lung transplantation, need to be accomplished.

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NEDERLANDSE SAMENVATTING

Inleiding

Longtransplantatie is de laatste behandeling die mogelijk is voor patiënten met eindstadium longlijden. Echter, de overleving na een longtransplantatie is beperkt: slechts 50 tot 60% van de patiënten is vijf jaar na de transplantatie nog in leven. De belangrijkste oorzaak van dit hoge sterftecijfer is de ontwikkeling van chronische afstoting, genaamd bronchiolitis obliterans syndroom (BOS). BOS ontstaat door schade aan het *epitheel* van de kleine luchtwegen. Verschillende factoren veroorzaken deze schade, zoals de vorming van antilichamen door de ontvanger tegen de donorlong, een infectie, reflux van maaginhoud of inademen van *micro-organismen*. De schade leidt tot activatie van het immuunsysteem met aantrekking van verschillende cellen en vervolgens tot het ontstaan van chronische *inflammatie*. Dit proces leidt uiteindelijk tot de vorming van *fibrose* waardoor vernauwing of afsluiting van de luchtwegen van de getransplanteerde long(en) ontstaat. Fibrose is verlittekening van de wand van de kleine luchtwegen door schade ten gevolge van chronische inflammatie en een continu proces van *remodeling*.

Het ontstaan van BOS kan sluipend en aspecifiek verlopen zonder dat de patiënt daar klachten van ervaart, maar de ziekte kan ook klachten geven, zoals benauwdheid, hoesten en opgeven van slijm. BOS ontwikkelt zich meestal 16-20 maanden na longtransplantatie en de gemiddelde overleving na het stellen van de diagnose is drie tot vier jaar.

De diagnose BOS wordt gesteld door middel van het meten van de longfunctie, waarbij er sprake is van een afname van de longfunctie van meer dan 20% ten opzichte van de basiswaarden na longtransplantatie. De basiswaarde wordt gedefinieerd als het gemiddelde van de twee hoogste waarden die op enig moment na transplantatie zijn geblazen. Deze achteruitgang in longfunctie mag niet het gevolg zijn van een acute afstoting van de getransplanteerde long, een infectie of een probleem met de transplantatienaden. Als de diagnose BOS is gesteld worden de medicijnen die het immuunsysteem onderdrukken opgehoogd met als doel de afname van de longfunctie te stoppen dan wel af te remmen.

Op het moment dat de diagnose BOS wordt gesteld is het proces van chronische afstoting vaak al in een vergevorderd en onomkeerbaar stadium. Dit benadrukt het belang van andere diagnostische mogelijkheden die het proces van afstoting vaststellen voordat de achteruitgang in longfunctie plaatsvindt.

Doel van het proefschrift

Het doel van dit promotie onderzoek is om te analyseren of bepaalde *biomarkers* in *serum* of uitademingslucht, zoals *cytokinen*, *chemokinen*, groeifactoren en *enzymen*, geschikt zijn om de ontwikkeling van BOS te detecteren voordat de achteruitgang in longfunctie wordt vastgesteld. Naast deze biomarkers in serum en uitademingslucht wordt ook de aanwezigheid van genetische variaties in bepaalde *genen* onderzocht en gekeken of die geassocieerd zijn met het ontwikkelen van BOS.

De biomarkers beschreven in dit proefschrift zijn geselecteerd op basis van hun betrokkenheid in processen die leiden tot de ontwikkeling van BOS, zoals inflammatie, herstel en fibrose. Wij verwachten dat de onderzochte biomarkers in serum of gecondenseerde uitademingslucht verhoogd of verlaagd zijn in patiënten die BOS ontwikkelen (BOS^{pos}) in vergelijking met patiënten die geen BOS ontwikkelen (BOS^{neg}) en dat de genetische variatie tussen deze twee groepen anders is.

Hoofdstuk 2: Polymorfismen in de genen van het aangeboren immuunsysteem zijn geassocieerd met het ontstaan van het bronchiolitis obliterans syndroom na longtransplantatie

Longtransplantatie patiënten worden continu blootgesteld aan stimulatoren van het *aangeboren immuunsysteem*. Toll-like receptoren (TLR's) maken onderdeel uit van het aangeboren immuunsysteem. Ze vormen een groep van eiwitten op de celmembraan die belangrijk zijn voor het herkennen van micro-organismen en activeren, na herkenning van deze micro-organismen, het immuunsysteem. Dit gaat gepaard met secretie van cytokinen en chemokinen die op hun beurt weer andere cellen activeren. Uit onderzoek is gebleken dat activatie van het aangeboren immuunsysteem via TLR's verhindert dat het lichaam de getransplanteerde long kan accepteren. In de mens zijn vele verschillende TLR's bekend met elk een eigen functie. Bepaalde *single nucleotide polymorfismen* (SNP's) in de genen van de *TLR*'s zouden kunnen bijdragen aan een veranderde reactie van het immuunsysteem.

In dit hoofdstuk wordt de verdeling van verschillende SNP's in de tien genen van *TLR1* tot en met *TLR10* geanalyseerd en wordt vervolgens gekeken of deze SNP's geassocieerd zijn met de ontwikkeling van BOS. Er zijn 422 gezonde controle personen en 110 longtransplantatie patiënten onderzocht, waarvan 20 patiënten BOS hebben ontwikkeld na longtransplantatie.

In *TLR2 (*2 SNP's), *TLR4 (*1 SNP) en *TLR9 (*2 SNP's) is de genetische verdeling significant verschillend tussen BOS^{pos} patiënten, BOS^{neg} patiënten en gezonde controle personen. Het blijkt dat de BOS^{pos} groep meer patiënten heeft die meerdere risico *allelen* (allel met een verhoogd risico op BOS) bezitten dan de BOS^{neg} groep.

TLR2 en TLR4 zijn van belang bij de herkenning van bacteriën en TLR9 speelt een rol in de immuunreactie gericht tegen virussen wat suggereert dat bacteriële en virale *pathogenen* betrokken zijn in de ontwikkeling van BOS. Een verhoogde secretie van cytokinen en chemokinen en een onderdrukking van regulatoire cellen kan het mechanisme zijn waardoor deze genetische polymorfismen bijdragen aan de ontwikkeling van BOS. Hierdoor raakt het epitheel van de longen ernstiger en langduriger beschadigd en ontstaat uiteindelijk BOS.

Hoofdstuk 3: Systemische en uitgeademde cytokine en chemokine profielen zijn geassocieerd met de ontwikkeling van het bronchiolitis obliterans syndroom

Een disbalans tussen *T-helper* (Th)1 en *Th2* cytokinen kan mogelijk leiden tot inflammatie en remodeling van het epitheel van de long en uiteindelijk tot fibrose. Uit de literatuur blijkt dat zowel Th1 als Th2 cytokinen kunnen bijdragen aan de acceptatie of afstoting van het transplantaat.

In dit hoofdstuk wordt de concentratie van 27 verschillende cytokinen en chemokinen in serum en gecondenseerde uitademingslucht van 10 BOS^{pos} patiënten en 10 BOS^{neg} patiënten gemeten en wordt gekeken of ze nuttig zijn als biomarker voor het ontstaan van BOS. Voor dit onderzoek zijn 10 BOS^{pos} en 10 BOS^{neg} patiënten gematcht op verschillende items zoals geslacht, leeftijd, onderliggende longziekte, type transplantatie (enkel- of dubbelzijdig) en follow-up tijd na transplantatie.

Het patroon van de Th2 cytokinen in serum is verschillend in BOS^{pos} patiënten vergeleken met BOS^{neg} patiënten: de concentratie van *interleukine* (IL)-4, IL-13 en vascular endothelial groei factor (VEGF) is lager en IL-5 hoger in de BOS^{pos} patiënten vergeleken met hun BOS^{neg} tegenhangers. In longtransplantatie patiënten is de concentratie IL-4 en IL-5 in gecondenseerde uitademingslucht niet detecteerbaar. De concentraties van IL-13 en VEGF zijn in BOS^{pos} patiënten verlaagd ten op zichte van de BOS^{neg} groep. De concentraties van de 27 cytokinen en chemokinen in serum en gecondenseerde uitademingslucht veranderden niet over de tijd na transplantatie en voorafgaand aan het ontstaan van BOS.

Samengevat is er, tussen de patiënten met en zonder BOS, direct na transplantatie een verschil in concentratie van Th2 cytokinen en chemokinen in serum. Deze cytokinen en chemokinen zouden nuttig kunnen zijn voor het inschatten van het risico op BOS na longtransplantatie.

Hoofdstuk 4: Genetische polymorfismen in *MMP7* en verlaagde concentraties in serum zijn geassocieerd met de ontwikkeling van het bronchiolitis obliterans syndroom na longtransplantatie

Matrix metalloproteinases (MMP's) zijn een groep van enzymen die van belang zijn in het herstel en de afbraak van het epitheel. MMP-7, een lid van deze groep, is met name van belang bij het herstel van het epitheel en in reactie op epitheelschade is de uitscheiding van dit enzym door het epitheel verhoogd.

In dit hoofdstuk wordt de verdeling van verschillende SNP's in het gen voor *MMP7* en de invloed van deze genetische polymorfismen op de concentratie MMP-7 in het bloed beschreven. De genetische variatie in het *MMP7* gen is onderzocht in 422 gezonde controle personen en in 110 longtransplantatie patiënten, waarvan 21 patiënten BOS hebben ontwikkeld. MMP-7 in serum is gemeten in 9 BOS^{pos} patiënten, in 9 gematchte BOS^{neg} patiënten, in 35 niet gematchte BOS^{neg} patiënten en in 78 gezonde controle personen.

Longtransplantatie patiënten hebben een hogere concentratie MMP-7 in hun bloed vergeleken met controle personen. Binnen de groep van longtransplantatie patiënten hebben BOS^{pos} patiënten een lagere concentratie van MMP-7 in hun bloed dan BOS^{neg} patiënten.
De *genotype* verdeling van SNP's in *MMP7* tussen de BOS^{pos} patiënten en de controle personen is verschillend. Enkele van deze allelen zijn gecorreleerd met een lagere concentratie van MMP-7. Er zijn *haplotypes* gemaakt van de verschillende allelen in het *MMP7* gen. De haplotypes die in de BOS^{pos} patiënten veel voorkomen zijn gecorreleerd met een lagere concentratie van MMP-7. Samenvattend, genetische variaties in het *MMP7* gen hangen samen met de ontwikkeling van BOS. Patiënten die deze risico SNP's dragen, hebben een lagere concentratie van MMP-7 in het bloed, wat een relatie kan hebben met onvoldoende herstel van het epitheel waardoor uiteindelijk BOS ontstaat.

Hoofdstuk 5: YKL-40 en matrix metalloproteinases als potentiële biomarkers van inflammatie en fibrosis in de ontwikkeling van bronchiolitis obliterans syndroom na longtransplantatie

YKL-40 is een eiwit dat wordt geproduceerd door verschillende cellen in de long, waaronder de *macrofagen* en *neutrofielen*. Het is een groeifactor voor onder andere *fibroblasten* en speelt een rol in inflammatie, remodeling en fibrose. In astma is YKL-40 een goede marker gebleken voor de ernst van de ziekte. Ook in hart- en levertransplantaties blijkt de hoogte van de concentratie van YKL-40 gerelateerd te zijn met fibrose en afstoting.
MMP-9 speelt een rol in de afbraak en turnover van de *extracellulaire matrix* en in de migratie van ontstekingscellen. MMP-9 is bij gezonde mensen in een lage concentratie in de longen aanwezig, maar bij patiënten met bepaalde longziekten is die concentratie veel hoger. De rol van MMP-9 bij longtransplantatie is al eerder onderzocht. Een verhoogde concentratie van MMP-9 in de *bronchoalveolaire lavage* vloeistof kan samengaan met BOS, maar wordt aan de andere kant ook geduid als niet-specifiek en geassocieerd met de longtransplantatie zelf. De concentratie MMP-9 in serum, eenmaal beschreven in een artikel, liet geen verschil zien tussen BOS^{pos} en BOS^{neg} patiënten.
In hoofdstuk 5 is de concentratie van YKL-40 en MMP-9 op verschillende momenten na longtransplantatie gemeten in 10 BOS^{pos} patiënten en in 10 gematchte BOS^{neg} patiënten. Er is geen verschil in de YKL-40 concentratie tussen de BOS^{pos} en BOS^{neg} patiënten. De concentratie MMP-9 in BOS^{pos} patiënten is veel hoger vergeleken met BOS^{neg} patiënten, hetgeen suggereert dat MMP-9 een rol speelt in het ontstaan van BOS en als risicofactor voor BOS gebruikt kan worden.
Een tweede doel van deze studie is om te beoordelen wat de relatie is tussen YKL-40, MMP-7 en MMP-9 als potentiële biomarkers voor BOS. In BOS^{pos} patiënten is YKL-40 gecorreleerd met MMP-7, dat wil zeggen als de YKL-40 concentratie toeneemt dan neemt ook de MMP-7 concentratie toe. In BOS^{neg} patiënten liet MMP-9 een omgekeerd verband zien met MMP-7,

dat wil zeggen dat wanneer MMP-7 stijgt de MMP-9 concentratie daalt. Dit komt overeen met de functie van MMP-7 en MMP-9. In een situatie van herstel van het epitheel, weerspiegelt in een verhoogde concentratie van MMP-7, is er minder afbraak van het epitheel met daardoor een lagere concentratie van MMP-9. In BOS^{pos} patiënten lijkt dit verband verstoord door onvoldoende toename van MMP-7 en/of een extreem verhoogd MMP-9.

Hoofdstuk 6: Een genetisch polymorfisme in *CAV1* is geassocieerd met de ontwikkeling van het bronchiolitis obliterans syndroom na longtransplantatie

Caveolin-1 (Cav-1) is een eiwit dat zich bevindt in een instulping van de celmembraan en is betrokken bij de ontwikkeling van pulmonale fibrose. In dieren en mensen met pulmonale fibrose is er sprake van een verlaagde celexpressie van Cav-1. Genetische variaties in het *CAV1* gen beïnvloeden de functie van Cav-1 in patiënten met maligniteiten en zijn geassocieerd met het optreden van fibrose in getransplanteerde nieren. In hoofdstuk 6 wordt de verdeling van verschillende SNP's in het gen voor *CAV1* en de invloed van deze genetische polymorfismen op de Cav-1 serum levels beschreven.

De genetische variatie in het *CAV1* gen is onderzocht in 110 longtransplantatie patiënten, waarvan 20 BOS^{pos} patiënten en in 422 gezonde controle personen. Het Cav-1 in serum is gemeten in 10 BOS^{pos} patiënten, in 10 gematchte BOS^{neg} patiënten, in 33 niet gematchte BOS^{neg} patiënten en in 60 gezonde controle personen.

De Cav-1 serum concentratie is lager in de longtransplantatie patiënten vergeleken met gezonde personen. In het gematchte cohort hebben de 10 BOS^{neg} patiënten lagere Cav-1 serum concentraties dan de 10 BOS^{pos} patiënten. De totale groep van 43 BOS^{neg} patiënten heeft een lagere Cav-1 serum concentratie dan controle personen, maar het verschil in Cav-1 concentratie tussen de 10 BOS^{pos} en 43 BOS^{neg} patiënten is niet meer aantoonbaar.

Homozygoten van het minst frequente allel van SNP *rs3807989* hebben een zesmaal verhoogd risico op het ontwikkelen van BOS. Longtransplantatie patiënten die homozygoot zijn voor het minst frequente allel van SNP *rs3807989* en *rs3807994* hebben hogere Cav-1 serum concentraties vergeleken met de andere genetische variaties.

Samengevat, de *CAV1* SNP *rs3807989* is geassocieerd met de ontwikkeling van BOS en de Cav-1 serum concentratie is genotype afhankelijk. Cav-1 in serum is echter niet geschikt als risicofactor voor de ontwikkeling van BOS, omdat er geen verschil werd gevonden tussen de Cav-1 concentratie van BOS^{pos} patiënten en BOS^{neg} patiënten. Het mechanisme waardoor de genetische polymorfismen in het *CAV1* gen bijdragen aan de verhoogde Cav-1 serum concentratie en aan de ontwikkeling van BOS dient nog verder onderzocht te worden.

Hoofdstuk 7: Genetische polymorfismen en het ontstaan van het bronchiolitis obliterans syndroom na longtransplantatie: veelbelovende resultaten en aanbevelingen voor de toekomst

Er is steeds meer bewijs dat, naast de reeds bekende risicofactoren voor BOS zoals acute afstoting en infecties, ook genetische variaties bijdragen aan de gevoeligheid van een longtransplantatie patiënt voor het ontwikkelen van BOS. In hoofdstuk 7 wordt een overzicht gegeven van verschillende genetische polymorfismen die onderzocht zijn in longtransplantatie patiënten en mogelijk geassocieerd zijn met de ontwikkeling van BOS.

Genetische variaties in de genen van interferon-gamma, interleukine-6 en transforming growth factor-beta zijn geassocieerd met de ontwikkeling van BOS en met fibrose van de *allograft*. Deze bevindingen zijn echter niet bevestigd in andere studies.

Genetische variaties in de genen van *TLR´s*, mannose binding lectin, *CD14*, killer-immunoglobulin-like receptoren en *MMP7* zijn ook geassocieerd met de ontwikkeling van BOS. Deze bevindingen zijn eveneens niet gerepliceerd in een onafhankelijk cohort.

Het mechanisme waardoor deze genetische variaties bijdragen aan de ontwikkeling van BOS is niet precies bekend, maar zou mogelijk verklaard kunnen worden door het sturen van de immuunreactie in de richting van inflammatie en fibrose.

Dit review laat zien dat het genetisch profiel van betekenis is bij de ontwikkeling van BOS. Het bepalen van het genetische profiel van een longtransplantatie patiënt kan een veelbelovende benadering voor de toekomst zijn, omdat het individuele risico-inschatting mogelijk maakt en de behandeling met immuunsuppressiva aangepast kan worden aan de patiënt.

Conclusie

In dit proefschrift zijn op moleculair niveau verschillende mechanismen onderzocht die mogelijk van belang kunnen zijn bij de ontwikkeling van BOS: initiële schade aan het epitheel van de long gevolgd door inflammatie, remodeling, een gestoord herstel en uiteindelijk fibrose (zie Figuur 1 in hoofdstuk 8).

TLR´s zijn van belang bij inflammatie en kunnen invloed hebben op het proces van acceptatie van de getransplanteerde long. Bepaalde SNP's in de *TLR* genen maken een longtransplantatie patiënt mogelijk vatbaarder voor de schadelijke invloeden van bacteriën en virussen door verhoogde secretie van cytokinen en chemokinen en daardoor meer kans op beschadiging van het epitheel.

De exacte functie van de verschillende cytokinen en chemokinen in het proces van afstoting en acceptatie van de allograft is niet geheel duidelijk. Enerzijds zijn er aanwijzigen dat Th1 cytokinen leiden tot afstoting van de allograft en Th2 cytokinen tot acceptatie ervan. Anderzijds zouden Th2 cytokinen niet nodig zijn voor de acceptatie en zijn Th1 cytokinen nuttig voor het bevorderen van de acceptatie van de getransplanteerde long. Uit ons onderzoek blijkt dat de BOS^{pos} en BOS^{neg} patiënten een vergelijkbaar patroon van Th1 cytokinen hebben, maar dat de BOS^{pos} patiënten een ander patroon van Th2 cytokinen hebben dan de BOS^{neg}

patiënten. In tegenstelling tot de Th1 cytokinen lijken Th2 cytokinen dus wel van belang te zijn in het proces van afstoting.

Na beschadiging van het epitheel is een adequaat herstelmechanisme nodig om de ontwikkeling van fibrose en BOS te voorkomen. Longtransplantatie patiënten die BOS ontwikkelen lijken een gestoord herstelmechanisme te hebben vergeleken met BOS^{neg} patiënten vanwege een ander genetisch profiel van *MMP7* en lagere concentraties van MMP-7. Daarnaast is er in BOS^{pos} patiënten sprake van een toegenomen afbraak van het epitheel van de long, wat blijkt uit een verhoogd MMP-9. In verschillende vormen van pulmonale fibrose is Cav-1 betrokken bij het ontstaan van de fibrose. Ondanks dat de functionaliteit van het genetisch polymorfisme in *CAV1* onbekend is, zou dit ook bij kunnen dragen aan het ontstaan van BOS.

Combinatie van biomarkers

De resultaten van dit proefschrift tonen aan dat verschillende biomarkers in serum en DNA gebruikt kunnen worden voor het inschatten van het risico op BOS. Echter mede gezien het multifactoriële ontstaansmechanisme van BOS zal een set van biomarkers nauwkeuriger zijn om potentiële BOS patiënten te identificeren. De volgende biomarkers in serum en DNA kunnen in een dergelijk set geïncludeerd worden om het risico op BOS na longtransplantatie in te schatten: serum IL-4, IL-5, IL-13, VEGF, MMP-7 en MMP-9 en de genetische polymorfismen in *TLR2*, *TLR4*, *TLR9*, *MMP7* en *CAV1*. Het genetisch profiel kan bepaald worden voor longtransplantatie. De markers in serum dienen vroeg na transplantatie, in een stabiele situatie zonder infectie, gemeten te worden.

Toekomstperspectieven

Matrix metalloproteinases en BOS

In de ontwikkeling van BOS is het van belang hoe een longtransplantatie patient reageert op beschadiging van het epitheel van de kleine luchtwegen. Daarbij is een adequaat herstelmechanisme van belang om de ontwikkeling van fibrose tegen te gaan. Onderzoek aan MMP's heeft laten zien dat deze enzymen betrokken zijn in het proces van herstel, remodeling en fibrose. MMP's lijken, in tegenstelling tot cytokines, niet gevoelig te zijn voor de invloed van het gebruik van immuunsuppressieve medicatie.

Naast de inclusie van MMP-7 en MMP-9 in een risicoprofiel voor BOS zijn ook andere MMP's, bijvoorbeeld MMP-2, -8 en -14, interessant om nader te onderzoeken, gezien hun betrokkenheid in verschillende processen die bijdragen aan de ontwikkeling van BOS, zoals afbraak en remodeling. In de toekomst zal kennis over de functie van de MMP's kunnen bijdragen aan het begrip van de ontwikkeling van BOS en mogelijk gevolgen hebben voor de behandeling ervan.

Aanbevelingen voor toekomstig onderzoek

Dit proefschrift geeft aanleiding om in de toekomst een aantal aspecten van longtransplantatie onderzoek in ogenschouw te nemen en te verbeteren.

Aantal patiënten - door het kleine aantal patiënten dat gebruikt is in onze studies, maar ook in andere studies, is onderzoek met een groter cohort met een langere follow-up noodzakelijk. Patiënten die een stamceltransplantatie hebben gehad kunnen ook BOS ontwikkelen en inclusie van deze groep patiënten zou kunnen bijdragen aan een groter cohort.

Follow-up - omdat er wordt aangenomen dat elke longtransplantatie patiënt vroeg of laat BOS zal ontwikkelen, zal in een studie met een beperkte follow-up tijd een patiënt niet de kans krijgen om BOS te ontwikkelen. Een minimale follow-up periode van twee jaar lijkt daarom noodzakelijk.

Behandeling - zowel in Nederland als wereldwijd zijn de medicamenten die longtransplantatie patiënten krijgen verschillend. Uniformiteit in de medicatie is nodig om de invloeden van medicatie op de concentraties van cytokinen en chemokinen en ook op het effect van de genetische variaties te kunnen uitsluiten als mogelijke oorzaak van een verschil in resultaat.

Definitie van BOS - in de studies die wereldwijd zijn gedaan worden verschillende methoden gebruikt om de diagnose BOS te stellen, zoals door middel van histologie of aan de hand van de richtlijnen van de International Society of Heart and Lung Transplantation. Om te zorgen dat de uitkomsten in de studies vergelijkbaar zijn, moeten gestandaardiseerde definities gebruikt gaan worden.

Genetisch onderzoek - in de meeste genetische studies met BOS wordt een enkele of een klein aantal genetische polymorfismen onderzocht. In het kader van de toepassing van een genetisch profiel in de klinische praktijk zouden toekomstige studies een combinatie van verschillende genetische polymorfismen moeten onderzoeken.

Het opstarten van een database waarin genetische informatie, maar ook patiënten karakteristieken en gestandaardiseerde definities voor complicaties worden verzameld, zou een goed initiatief zijn om het longtransplantatie onderzoek te verbeteren. In het kader hiervan zou een meer intensieve samenwerking tussen de longtransplantatiecentra in Nederland een goede stap vooruit zijn om het longtransplantatie onderzoek op een hoger niveau te brengen.

Verklarende woordenlijst

- *Allel*: onderdeel van het DNA dat staat voor een bepaalde eigenschap.
- *Allograft:* het weefsel dat van mens op mens is getransplanteerd.
- *Biomarker*: een biochemisch kenmerk dat gebruikt wordt om progressie van ziekte of effect van een behandeling te meten.
- *Bronchoalveolaire lavage*: wassing van de kleinste luchtwegen en de longblaasjes waarbij een monster wordt genomen met materiaal (cellen, vloeistoffen en ander materiaal).
- *Chemokine*: een familie van structureel verwante cytokinen. Zij induceren een gerichte migratie van witte bloedcellen naar plaatsen van ontsteking in het lichaam, en spelen daardoor een cruciale rol in het immuunsysteem.
- *Cytokine*: een eiwit dat een rol speelt in de afweer. Er bestaan verschillende soorten, die uitgescheiden worden door verschillende soorten lichaamscellen. Sommige soorten worden alleen uitgescheiden door geactiveerde cellen tijdens een reactie van het immuunsysteem, andere worden continu geproduceerd. Sommige hoeveelheden uitgescheiden cytokines werken alleen lokaal, andere door het hele lichaam.
- *Enzym*: een actief eiwit dat niet-werkzame stoffen in werkzame stoffen kan omzetten.
- *Epitheel*: een laag cellen aan de buitenkant van het lichaam of in organen grenzend aan het extern milieu.
- *Extracellulaire matrix*: structuur die deel uitmaakt van biologische weefsels, zich buiten de cellen bevindt en zorgt voor stevigheid en structuur aan weefsels.
- *Fibroblast*: de belangrijkste cel van het bindweefsel. Het is verantwoordelijk voor de synthese van alle elementen van de extracellulaire matrix.
- *Fibrose*: overmatige toename van de hoeveelheid bindweefsel in een orgaan, verlittekening.
- *Gen*: een stukje van het DNA dat de code bevat voor de productie van een bepaald eiwit en de informatie voor alle erfelijke eigenschappen van dat eiwit.
- *Genotype*: het geheel van erfelijk materiaal dat een individu in zich draagt, maar wat niet noodzakelijk tot uiting komt.
- *Haplotype:* een vaste combinatie van een aantal genetische variaties verspreid over een bepaald stuk DNA.
- *Homozygoot*: individu dat gelijke allelen heeft voor een bepaald gen.
- *Immuunsysteem:* verdedigingssysteem van het lichaam, met als doel indringers of veranderde eigen cellen te bestrijden. Het immuunsysteem kan worden onderverdeeld in een aspecifiek (aangeboren, zoals huid, slijmvliezen, witte bloedlichamen) en een adaptief (verworven, o.a. het vormen van antilichamen) deel.
- *Inflammatie*: reactie op een schadelijke prikkel (chemische aard, trauma, micro-organismen). Het ontstekingsproces dat daardoor ontstaat, bestaat voornamelijk uit celbeschadiging, vaatverwijding, vochtophoping, afzetting van witte bloedcellen.

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- *Interleukine*: een cytokine die geproduceerd wordt door geactiveerde macrofagen en witte bloedcellen gedurende een immuunrespons met als doel om met andere witte bloedcellen te communiceren.
- *Macrofaag*: grote eenkernige cel die in staat is resten van dode of beschadigde lichaamseigen cellen, veranderd intercellullair materiaal en lichaamsvreemde cellen (bijvoorbeeld micro-organismen) in zich op te nemen.
- *Matrix metalloproteinases* (MMP's): een groep van enzymen die van belang zijn in zowel het herstel als afbraak van het bindweefsel tussen de cellen.
- *Micro-organisme*: een organisme dat te klein is om met het blote oog te zien, bijvoorbeeld een virus of bacterie.
- *Neutrofiel*: een kort levende witte bloedcel die zowel in het bloed als in ons weefsel voorkomt. Deze cel zorgt voor de primaire immuunrespons die maakt dat infecties snel gecontroleerd worden, doordat het bacteriën en schimmels in zich opneemt en zorgt dat deze vernietigd worden.
- *Pathogeen:* stof die ziekte veroorzaakt.
- *Remodeling*: de aanpassing van structuur van weefsel in pathologische condities.
- *Serum:* de vloeistof die overblijft als men bloed laat stollen en het stolsel afcentrifugeert.
- *Single nucleotide polymorfisme (SNP)*: een kleine 1-punts variatie in het DNA die bij meer dan 1% van de bevolking voorkomt en kan leiden tot veranderingen in het coderende eiwit waardoor de functie of expressie van dat eiwit veranderd.
- *T-helper cellen*: een groep van witte bloedcellen die een belangrijke regulerende rol heeft in het immuunsysteem door het uitstoten van verschillende soorten cytokinen die verschillende processen reguleren. Twee typen: type 1 (Th1) cellen zijn belang in de afweer tegen intracellulaire infecties, en type 2 Th (Th2) cellen zijn van belang in de afweer tegen extracellulaire infecties.

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CURRICULUM VITAE

Elisabeth Anna (Lisanne) Kastelijn was born on August 10th, 1983 in Rotterdam, the Netherlands. After finishing secondary education at Ichthus College, Veenendaal, she started with her study medicine at the University of Utrecht in 2001. In the last year of her study she performed research at the department of pulmonology at the University Medical Centre of Utrecht (head: Prof. dr. J-W. J. Lammers). This was her first research on lung transplantation.

In 2007 she obtained her medical degree. From October 2007 she worked as a resident at the department of cardiology of the Diakonessenhuis, Utrecht (head: Dr. J.J.J. Bucx), but eventually it turned out that she had a preference for pulmonology. From October 2008 she was employed for one year as a research fellow at the department of pulmonology, St Antonius Hospital, Nieuwegein (head: Prof. dr. J.M.M. van den Bosch, succeeded by Prof. dr. J.C. Grutters). During this period, her research was centered on the role of biomarkers in the development of bronchiolitis obliterans syndrome. In October 2009 she started her specialist training in respiratory medicine in the same hospital (head: Dr. F.M.N.H. Schramel). As part of this training she worked as a resident at the department of internal medicine (head: Dr. A.B.M. Geers). The term (October 2009 – October 2011) at the department of internal medicine has been interrupted for six months to accomplish this thesis. Currently, Lisanne is working as a specialist registrar at the department of pulmonology.

Lisanne is married to Michel Tacken (May 2011).