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YKL-40 and matrix metalloproteinases as potential biomarkers of inflammation and fibrosis in the development of bronchiolitis obliterans syndrome

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Abstract. *Background and objective:* 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 for BOS. Furthermore, we investigate the relationship between these two potential biomarkers of BOS and MMP-7. *Design:* 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 concentrations 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 value 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.
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Key words: bronchiolitis oblyterans syndrome; lung transplantation; matrix metalloproteinases

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

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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 posttransplant 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 study 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).

The standard immunosuppressive therapy for all patients consisted of basiliximab (induction; day 0 to 4 20 mg), tacrolimus (targets levels 10-15 ng/ml and after 4 -6 months 5-10 ng/ml), mycophenolate mofetil (day 0 to 4: 1500 mg twice a day (bid), from day 4 to 2 months 1000 mg bid, from months 2-3 750 mg bid and after 1 year 500 mg bid) and prednisone (the first days high dosage of methylprednisolone and from day 4 to 8 30 mg prednisone, day 8 to week 3 25 mg, week 3 to 4 20 mg, week 4 to 7 months 15 mg, from months 7 onwards 10 mg). When infections were excluded as cause of FEV_1 decline, the patients were treated with corticosteroids and azithromycin (500 mg the first three days followed by 250 mg every second day). When no increase in lung functions was observed, the diagnosis BOS was made.

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, postoperative follow-up time (difference in post-operative follow-up time < 1 year), and unilateral or bilateral transplantation. Patients were matched on these 5 items with a median of 4.0 matching items (range, 2.0 –5.0 items). MMP-7 is 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 2 months before the diagnosis. According to this method, 5 samples were collected for the BOS^{pos} patients and 4 samples for the BOS^{neg} patients. However, for BOS^{pos} patients nr 3 and 4 respectively 4 and 3 samples were included due to a short BOS free survival which led to a smaller number of samples. For BOS^{neg} patient nr 4 only 2 samples were included. For BOS^{pos} patients nr 5, 7 and 9 and BOS^{neg} patient nr 2 one sample was missing.

The serum samples were stored at –80°C until analysis and were never thawed or only once before 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 chi square. 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 2 groups, a REML (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 x 2 table of the collected data.

Correlations between serum levels of the different markers were assessed with Spearman's rho. $p < 0.05$ was considered statistically significant.

Results

Patients

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.

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

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

BOS, bronchiolitis obliterans syndrome; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; IPF, interstitial pulmonary fibrosis; NA notapplicable; OB, obliterative bronchiolitis; SD, standard deviation; all data are shown as mean ± SD; * identical to survival; † less than 6 months after lung transplantation; ‡ more than 500 copies within 1 year after lung transplantation

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.

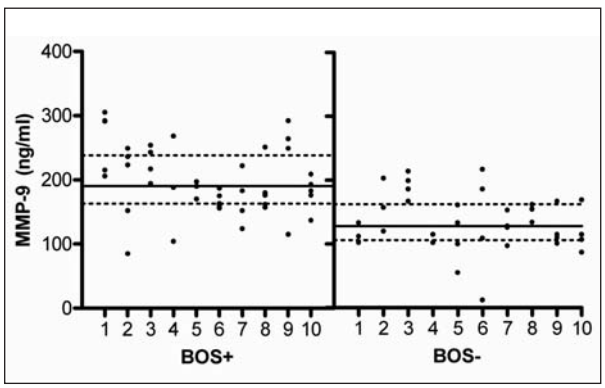

Fig. 1. Serum levels of MMP-9 in patients with bronchiolitis obliterans syndrome (BOS+) and patients without bronchiolitis obliterans syndrome (BOS-) ($p < 0.0001$). Matched pairs of BOS+ and BOS- patients have identical numbers. Horizontal lines represent group median (line) with interquartile range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

Table 2. Cut off points for MMP-9 serum levels for the diagnosis of bronchiolitis obliterans syndrome

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 concentrations with IQR of MMP-7 in 9 BOS^{pos} patients and in 9 matched BOS^{neg} patients were 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.

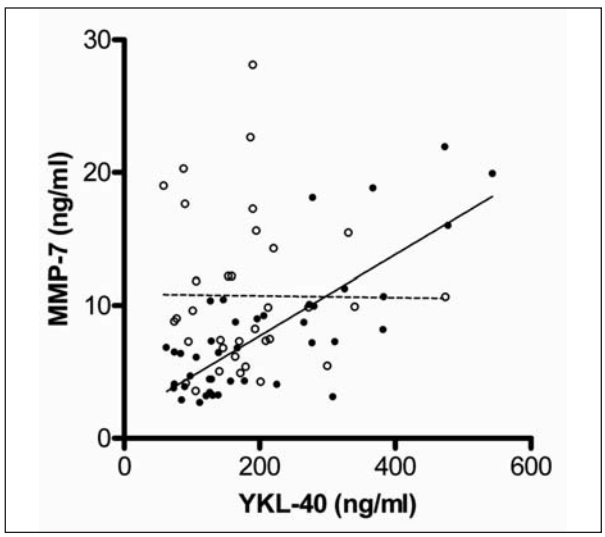

Fig. 2. Correlation between YKL-40 and MMP-7. Presence of correlation in serum of patients with bronchiolitis obliterans syndrome (solid points, solid line, Spearman rho 0.65, $p < 0.0001$) and absence of correlation in patients without bronchiolitis obliterans syndrome (open points, dotted line, spearman rho 0.06, $p = 0.76$). Lines represents linear regression

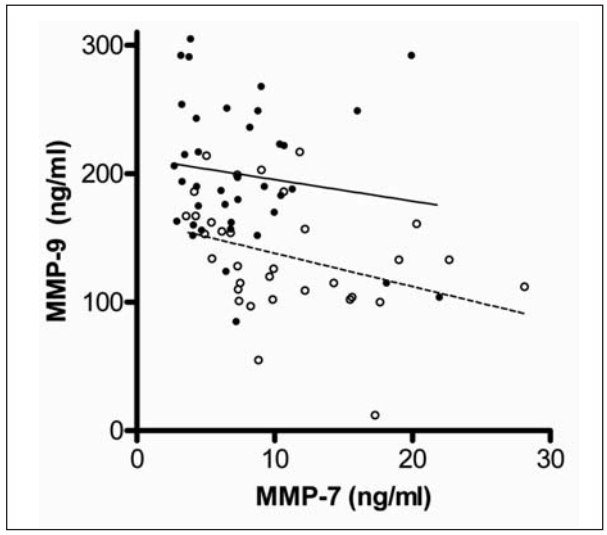

Fig. 3. Correlation between MMP-7 and MMP-9. Presence of correlation in serum of patients without bronchiolitis obliterans syndrome (open points, dotted line, Spearman rho 0.42, $p = 0.015$) and absence of correlation in patients with bronchiolitis obliterans syndrome (solid points, solid line, Spearman 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 intraindivid-

ual 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 immunosoppressive 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 serum levels of MMP-7 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, i.e. 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 multicenter studies with larger number of patients and a longer follow-up are required to substantiate our conclusions. Besides, in future studies the functional activity of MMPs can be determined by zymography to extend the knowledge about the role of MMPs in the development of BOS. Another interesting aspect for future studies is the ratio between MMP-9 and its natural tissue inhibitors (TIMPs). It has been found that TIMP-1 is decreased and the MMP-9/TIMP-1 ratio is increased in BOS^{pos} patients (26).

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 (TGF)-beta and, thereby, decreases the MMP-9 concentration (41). 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 of lung function. Furthermore, azithromycin prevents the upregulation of the MMP9 gene. Interestingly, in our study the patients that were treated with azithromycin, the BOS^{pos} patients, revealed an increased MMP-9 serum level despite the treatment with azithromycin. This suggests that the increased MMP-9 serum levels contribute to the development of BOS (44).

In summary, the development of BOS is a multi-factorial 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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