THE PLEURAL MESOTHELIUM AND TGF-β_1 PATHWAYS IN RESTRICTIVE ALLOGRAFT SYNDROME – A PRE-CLINICAL INVESTIGATION

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ABSTRACT

Background: Chronic lung allograft dysfunction (CLAD) hampers long-term survival after lung transplantation. Common fibrosis-related mechanisms in idiopathic pulmonary fibrosis and CLAD, instigated the consideration of investigating the differential regulation of pleural mesothelium and TGF-β_1 in restrictive allograft syndrome (RAS).

Methods: TGF-β_1 was assessed in broncho-alveolar lavage (BAL) using ELISA and via immune staining of explant biopsies. To assess the role of the pleura, explanted BOS and RAS lungs were compared using CT scans, calretinin stainings, western blot and qRT-PCR. Lastly, a pleural mesothelial cell line was used to assess mesothelial-to-mesenchymal transition and its inhibition.

Results: TGF-β_1 was increased in BAL of RAS patients (p=0.035), and was present in the (sub)pleural area of biopsies. Explanted RAS lungs demonstrated an increased volume fraction of pleura (p=0.0004), a higher proportion of calretinin-positive stainings (p=0.0032), and decreased E-cadherin (p=0.019) and increased α-SMA (p=0.0089) mRNA expression and protein levels in isolated pleural tissue. Moreover, TGF-β_1 stimulation of pleural mesothelial cells led to a phenotypical switch to mesenchymal cells, accompanied with an increased migratory capacity. IL-1α was able to accentuate TGF-β_1-induced mesothelial-to-mesenchymal transition. None of the tested drugs could inhibit mesothelial-to-mesenchymal transition at the used concentrations.

Conclusion: Our results support an interplay between TGF-β_1 and the pleural mesothelium in the pathophysiology of restrictive allograft syndrome.

INTRODUCTION

Chronic lung allograft dysfunction (CLAD) continues to impair long-term survival after lung transplantation (LTx), which has the lowest survival rates of all solid organ transplants [1]. Nowadays, chronic rejection is considered a heterogeneous entity with at least two clinical phenotypes: bronchiolitis obliterans syndrome (BOS) and restrictive allograft syndrome (RAS). BOS is characterized by an obstructive pulmonary function defect, obliterative bronchiolitis (OB) on histopathology, and mostly air trapping on chest CT, while RAS is characterized by a restrictive pulmonary function defect accompanied by (sub)pleural thickening, persistent pleuroparenchymal infiltrates and fibrosis on chest CT [2]. Moreover, patients suffering from RAS have a worse prognosis (1-1.5 years) compared to patients with BOS (3-5 years) [3–5].

In RAS, fibrosis formation occurs in a pleuroparenchymal pattern [6] and although the exact nature of this fibrotic process has not been elucidated yet, parallels with idiopathic pulmonary fibrosis (IPF) have been suggested, evidenced by similar immune activation, increased production and deposition of extracellular matrix, and proliferation of fibroblasts, however there are not many comprehensive studies to compare the similarities and differences between IPF and RAS [7]. Recently, the pleural mesothelium was implicated as a potential source of (myo-)fibroblasts in IPF [8]. The pleura is a metabolically active membrane of fibrous tissue that lines the interior of the thoracic cavity and consists of two layers: the parietal layer lines the thoracic wall and the visceral layer is attached to the lung parenchyma [9]. Apart from facilitating movement of the lung, pleural mesothelial cells (PMCs) also respond to injury by proliferating and producing pro-inflammatory and extracellular matrix proteins [10,11]. During embryonic development, mesothelial cells migrate into lung parenchyma to form smooth muscle cells and (myo-) fibroblasts. Re-activation of this developmental pathway, during which PMCs differentiate into (myo-) fibroblasts after stimulation with TGF-β via a process called mesothelial-to-mesenchymal transition (MMT), is suggested to play a role in the pathogenesis of IPF and to actively contribute to fibrosis formation [8,12].

TGF-β is considered a key player in chronic fibrotic lung disorders and is responsible for recruitment of circulating fibrocytes and inflammatory cells, inducing a pro-fibrotic environment and activating

collagen [13]. Moreover, gene expression profiling of transbronchial biopsies implicated the TGF-β axis in CLAD [14].

In the present study, we investigated the role of TGF-β_1-induced transformation of PMCs into (myo-) fibroblasts in RAS.

MATERIALS & METHODS (Additional information in online supplement)

Study design & population

In this cross-sectional study, patient inclusion was based on retrospective diagnosis of BOS and RAS via a combination of spirometry, CT and availability of samples (supplementary method 1). When patients changed CLAD phenotype during their disease course [4], the last phenotype prior to redo-transplantation or death was used for analysis. The Leuven University Hospital Ethics Committee (S57742) and the local biobank board (S51577) approved the study and all patients provided written informed consent that collected material could be used for research.

Specimen collection & processing

Depending on availability, different specimens were obtained from different patients (figure S1).

Broncho-alveolar lavage & TGF-β_1 measurement

Broncho-alveolar lavage (BAL) was performed as described previously, using 2x50 cc of saline [15]. TGF-β_1 was measured in BAL supernatant of BOS (n=23) and RAS (n=26) patients at CLAD diagnosis using sandwich ELISA (R&D Systems, Abingdon, UK) according to manufacturer's instructions. As a control group, BAL of stable (never CLAD) lung transplant patients (n=20) were used.

Patient blood samples and mesothelin measurement

Mesothelin protein levels were measured in serum of BOS (n=26) and RAS (n=27) patients using sandwich ELISA (R&D Systems) according to manufacturer's instructions. As a control group, serum samples of stable (never CLAD) lung transplant patients (n=22) were used.

Explant lungs & CT analysis

Explanted lung specimens were collected from BOS (n=8) and RAS (n=8) patients undergoing redo-transplantation and processed as described previously [16]. Unused donor lungs (n=7) served as controls and were obtained under existing Belgian law. CT images were processed using Mimics® software (Materialise NV, Leuven, Belgium) and resulting segmentations enabled calculation of the volume fraction of pleura and attached infiltrates relative to the total lung volume.

Lung biopsies & immunohistochemistry for calretinin and TGF-β_1

Lung biopsies were obtained during redo-transplantation, VATS-biopsy or autopsy of patients with BOS (n=19) and RAS (n=19). Non-transplant control biopsies (n=14) were obtained during autopsy of patients without underlying lung disease (n=11) or during resection of non-diseased lung tissue from lung carcinoma patients with normal spirometry (n=3). All biopsies were retrieved at random by the Hospitals Pathology Department for routine clinical purposes and not specifically for this study. Paraffin sections were prepared from each biopsy and stained with calretinin (Dako, Agilent, Heverlee, Belgium) and/or TGF-β_1 (Atlas, Bromma, Sweden) antibody (supplementary method 2 & 3). Control stainings for TGF-β_1 were performed on liver and tonsil biopsies.

Multiplex immunofluorescent staining for immune subsets

After deparaffinization and heat-induced antigen retrieval, quadruple staining with CD4, CD8, CD20 and CD68 (table S2, supplementary method 4) was performed via automated multiplex immunofluorescence using the Ventana Benchmark Discovery (Ventana Medical Systems Inc., AZ, USA).

Protein analysis & quantitative real-time PCR analysis of MMT markers in pleural tissue

Pleural tissue was isolated from frozen explanted lungs of BOS (n=5), RAS (n=6) and unused donors (n=6) and used for protein and total RNA extraction (supplementary method 5 & 6). Subsequently, western blot (supplementary method 9) and qRT-PCR of MMT markers was performed (table S3). To quantify the band intensity of the western blots, 1D (Totallab, Newcastle upon Tyne, UK) was used including background correction. For the qRT-PCR, a 260/280 ratio of at least 1.8 was ensured for all

RNA samples (table S4). Data analysis was carried out with Eco™ Real-Time PCR System (Illumina, USA) via the comparative cycle threshold (Ct) method.

Pleural mesothelial cell culture

Human pleural mesothelial cells (MeT-5A cells, ATCC, Molsheim, France) between passages two and twelve were used. Cells were either (i) stimulated with TGF-β_1, IL-6, or IL-1α (10 µg/mL, Bioké, Cell Signaling Technology, Leiden, The Netherlands), (ii) treated with pirfenidone (5 mM; GenenTech, Michigan, USA), azithromycin (0.01 µM, Sigma-Aldrich, Bornem, Belgium), montelukast (1 µM, Cayman, Michigan, USA), or dexamethasone (5 µM, Sigma-Aldrich), or (iii) exposed to a combination of these agents for 48 hours prior to western blot analysis. Negative control cells were stimulated with an equivalent amount of vehicle. Migration was assessed using Ibidi® chamber migration assays (Culture-Insert 2 well µ-dishes, REF81176, Ibidi GmbH, Germany). Additional information can be found in supplementary methods 7-10.

Statistical analysis

All results are presented as mean ± standard error of mean or as median (interquartile range). Discrete data were compared with contingency tables. Comparing two continuous variables was done using Mann–Whitney U test; for multiple groups, one-way ANOVA with Tukey's multiple comparisons test, Kruskal-Wallis test with Dunn's post hoc test or the two-way ANOVA with Sidak's multiple comparisons test was used. Survival analysis was performed using Log-rank test. GraphPad prism 6.0 software (San Diego, CA, USA) was used for statistical analysis. $P<0.05$ was considered significant.

RESULTS

Patient characteristics and distribution of sample types among the cohort are shown in table S1 and figure S1.

Importance of TGF-β_1 in RAS patients

At diagnosis, RAS patients had higher TGF-β_1 levels in BAL compared to stable patients (p=0.02, figure 1A). Based on the concentrations of TGF-β_1 in RAS, the median level (14.5 pg/mL) was defined as a threshold to stratify RAS patients. RAS patients with high TGF-β_1 levels demonstrated a worse post-diagnosis graft survival compared to RAS patients with low TGF-β_1 levels (p=0.033, HR 2.77, 95% CI 1.1-7.1, figure 1B).

Immunohistochemical stainings of TGF-β_1 on lung biopsies from RAS patients demonstrated three different patterns: (i) relatively low TGF-β_1 expression, with primarily subpleural staining and TGF-β_1 positivity in stromal cells and macrophages (21%; figure 2A-C); (ii) intermediate TGF-β_1 expression with subpleural and intra-alveolar staining (47%; figure 2D); and (iii) diffuse staining of the extracellular matrix, particularly in biopsies with advanced fibrosis (32%; figure 2E).

Lymphocyte subpopulations in the three TGF-β_1 patterns were investigated by multiplex immunofluorescence for CD4 (T helper cells), CD8 (cytotoxic T cells), CD20 (B cells) and CD68 (macrophages). In the low TGF-β_1 group (figure 3A), there was a low degree of inflammation, with little and dispersed CD4, CD20 and CD68 positive staining. In the intermediate TGF-β_1 group (figure 3B), there was a low number of CD4, CD8, CD20 and CD68 positive cells. In the high TGF-β_1 group (figure 3C and S2), there was abundant CD4, CD8, CD20 and CD68 positive cells.

Pleural volume is increased in RAS

Analysis of CT scans of inflated, explanted lungs showed an increased volume percentage of pleura and attached infiltrates in RAS lungs (p=0.0004, figure 4) compared to non-transplant controls (p=0.025) and BOS (p=0.0003). No differences were observed between non-transplant controls and BOS (p>0.05).

Increased pleural reactivity in RAS

Given this increase in pleural volume, we sought to investigate the reactivity of the pleural mesothelial cells. First, the presence of PMCs within the lung was assessed using calretinin, a marker for reactive mesothelial cells. A bigger proportion of RAS biopsies showed positivity for calretinin

(53%, p=0.0001, figure 5A), compared to BOS (5%) and controls (0%). Calretinin-positive cells were found deeper within the parenchyma in RAS biopsies (figure 5B). To associate the previous TGF-β_1 staining patterns with calretinin positivity, biopsies of low and intermediate TGF-β_1 expression were grouped together and compared to biopsies with high TGF-β_1 expression. In the low or intermediate TGF-β_1 biopsies, only 5 out of 13 (38%) were calretinin-positive, while in the high TGF-β_1 biopsies 5 out of 6 (83%) were calretinin-positive (figure 5C, p=0.069).

Secondly, mesothelin protein levels were measured in serum, as mesothelin has been reported to be expressed in reactive mesothelial cells [17]. Mesothelin levels were increased in RAS (p=0.0022, figure 6) and tended to be higher in BOS (p=0.055) compared to stable patients.

Pleural tissue of RAS lungs shows evidence of MMT

To further confirm reactivity of the pleura, pleural tissue was extracted from explanted lungs and MMT markers were assessed. At mRNA level, RAS pleura showed decreased expression of E-cadherin compared to controls (p=0.019, figure 7A) and a trend for lower expression compared to BOS (p=0.056). On the other hand, expression of α-SMA was increased in RAS compared to controls (p=0.0089, figure 7C), but not compared to BOS (p=0.15). There were no differences in fibronectin (p=0.88, figure 7E) or Wilms' tumor 1 (Wt1) (p=0.19, figure 7G) expression between groups.

At protein level, RAS pleura showed decreased levels of E-cadherin compared to BOS (p=0.017) and a trend for lower levels compared to controls (p=0.054, figure 7B). On the other hand, α-SMA levels were increased in RAS compared to controls (p=0.015), but not compared to BOS (p=0.13, figure 7D). There were no differences in fibronectin (p=0.36, figure 7F).

Exploration of possible triggers and treatments in a cell culture model

To investigate the TGF-β_1-pleural axis in more depth, an *in vitro* MMT model was used. MeT-5A cells stimulated with TGF-β_1 (figure S3) showed (i) morphological changes (figure S5B), (ii) decreased E-cadherin (p<0.0001) and increased fibronectin levels (p<0.0001, figure S4), and (iii) increased migratory capacity (p=0.046, figure S5); evident of a mesothelial to mesenchymal phenotype change.

The ability of inflammation to influence the TGF-β_1-pleural axis was assessed by exposing cells to IL-6 or IL-1α, two proteins that are upregulated in RAS [18]. Stimulation with IL-6 or IL-1α alone (figure S3 and S6A-B) led to decreased E-cadherin levels (p=0.0011 and p=0.0022 respectively), but no change in fibronectin (p=0.24 for both IL-6 and IL-1α) compared to control cells. Cells exposed to TGF-β_1 and IL-6 simultaneously (figure S6C) showed no differences in E-cadherin or fibronectin compared to TGF-β_1-stimulated cells (p=0.31 and p=0.95 respectively). Cells exposed to TGF-β_1 and IL-1α simultaneously (figure S6D) showed decreased E-cadherin levels (p=0.041) and a trend towards increased fibronectin levels (p=0.064) compared to TGF-β_1-stimulated cells, indicating that IL-1α-related inflammation could aggravate MMT.

Lastly, we investigated if known therapeutic agents could inhibit MMT. However, there was no difference in E-cadherin or fibronectin levels in TGF-β_1-stimulated cells treated with either pirfenidone, montelukast, azithromycin or dexamethasone at the used doses compared to TGF-β_1-stimulated cells without treatment (all agents p>0.05, figure S7).

DISCUSSION

In the present study, we provide evidence for a role of the TGF-β_1-pleural axis in RAS. We demonstrated that TGF-β_1 was increased in RAS in both BAL and lung biopsies. Abundant TGF-β_1 expression was accompanied with inflammation. Moreover, the degree of pleural fibrosis and adjacent fibrotic parenchymal infiltrates and thereby also the proportion of affected lung tissue was quantified, showing that this was increased in RAS. This was also accompanied by a higher proportion of calretinin-positive staining in the parenchyma, increased mesothelin in serum, and decreased E-cadherin and increased α-SMA mRNA and protein levels in isolated pleural tissue of RAS. TGF-β_1 stimulation of a pleural mesothelial cell line demonstrated a transition into a mesenchymal cell phenotype and increased migratory capacity, which is aggravated by IL-1α. Finally, we investigated the effect of drugs (azithromycin, montelukast, pirfenidone and dexamethasone) in ameliorating the change in E-cadherin

and fibronectin expression in cells. However, we were unable to demonstrate any convincing signal for benefit.

TGF-β_1 is an important pro-fibrotic mediator, playing key roles in chronic respiratory diseases [19]. Within LTx, TGF-β_1 is shown to induce epithelial-to-mesenchymal transition (EMT) and the production and secretion of matrix metalloproteinases in bronchial epithelial cells, while smaller studies directly implicated TGF-β_1 with chronic rejection [20,21]. Our results support the hypothesis of an important role for TGF-β_1, especially in RAS via MMT.

Mesothelial-to-mesenchymal transition (MMT) is very reminiscent of EMT, which has been implicated in the pathophysiology of OB [22]. Several biomarkers can be used to demonstrate this mesenchymal transitioning of cells [23]. We showed a decrease of E-cadherin in protein and mRNA in RAS pleura, which represents the onset of MMT. Afterwards, contact with the basal membrane and therefore also polarity of the cells is forfeited. This step during transition is often – as in our results – characterized by increased expression of α-SMA, a marker for active (myo-)fibroblasts [24]. Another crucial factor for the progression of mesenchymal transitioning is remodeling of the ECM, with an important role for fibronectin [25]. Although our *in vitro* data confirmed the increase in fibronectin in experimental conditions, our protein and mRNA results from pleura could not corroborate this. This might be explained by the fact that only pleural tissue - not parenchymal tissue - was sampled. Cells within the pleura are still undergoing transition and may therefore be unable to secrete fibronectin. Expression of Wt1 mRNA remained unchanged between groups. However, similar as for fibronectin, this is a late marker of MMT [26], explaining the lack of difference. The lack of a significant difference in some markers, although a clear trend is observed, could be due to the low number of analyzed samples as tissue availability remains scarce.

As (alveolar) inflammation is considered a key player in CLAD [27], we wanted to assess the effect of inflammation on MMT by stimulating the pleural mesothelial cell line with interleukin (IL)-6 and IL-1α, as these proteins were upregulated in BAL of RAS patients and were of prognostic importance [18]. Cells exposed to both TGF-β_1 and IL-1α showed a decrease in E-cadherin and increase in fibronectin expression. This suggests that IL-1α enhances TGF-β_1-induced MMT, consistent with the hypothesis

that this alarmin is of considerable importance in the pathophysiology of CLAD [28], which could be an important area for further research.

Given the current lack of treatment and the bad prognosis for RAS patients, there is a substantial need for new therapeutic approaches. TGF-β_1 presents as an intuitive therapeutic target when attempting to prevent this process of MMT and consequently the progression of fibrosis, especially given its association with inflammation (figure 3). However, general antagonism of TGF-β_1 would be unfavorable because of its beneficial roles [13]. Other potential drugs may already be part of clinical practice and therefore, we examined a number of drugs that are currently in use to treat LTx patients such as azithromycin, montelukast, pirfenidone and steroids [29]. Unfortunately, there were no differences in E-cadherin or fibronectin expression between the different agents and TGF-β_1-stimulated cells. However, as we did not assess any other changes – such as morphology, migration, etc. – we cannot state with certainty that these drugs do not affect or inhibit the MMT process.

Admittedly, our study has several limitations. First, only 10 out of 19 RAS biopsies stained positive for calretinin, a marker of reactive mesothelial cells [12,26,30]. The relatively low number of calretinin positive stainings is most likely due to the location where the biopsy is taken and the heterogeneity within the lungs as well as the possibility that even RAS itself is not homogeneous [31]. Nevertheless, we were also able to show an increase of mesothelin, another marker of reactive mesothelial cells [17], in serum of RAS patients. A second limitation is the use of a cell line for our experiments. Ideally, primary PMCs obtained from explanted lungs would have been used. However, this was challenging due to; (i) scarcity of organs available for isolation, (ii) concurrent infection, and (iii) fibroblast overgrowth. Although cell lines might not truly represent the patient, this allowed us to establish a controllable and reproducible *in vitro* MMT model. Thirdly, there is a great variation in n-values per experiment; however, this was dependent on availability of the required samples. Ideally, one patient cohort with each patient providing all three sample types would have been used. However, gathering these samples remains difficult due to rarity of the disease and the low incidence of (re-)transplantations/autopsies. Another limitation is the doses of TGF-β_1 and drugs used to stimulate and treat the cells. A dose of 10ng/mL TGF-β_1 was used in our experiments, which is higher than the concentrations measured in

BAL. However, concentrations of proteins are known to be diluted in BAL and we believe that this dose might be physiologically relevant given the high abundance of TGF-β_1 found in peripheral lung tissue on immunostainings. For the treatment experiments, we used concentrations based on literature and toxicity testing since the exact amount of drug that reaches the pleura is not known [32–34].

Despite these limitations, we were able to implicate the pleura in the pathophysiology of restrictive allograft syndrome, via TGF-β_1-induced mesothelial-to-mesenchymal transition. This is worth further investigation, especially to assess and develop possible pleural/TGF-β_1-based treatment strategies. Moreover, given that mesothelial-to-mesenchymal transition is implicated in IPF, this suggests there may be at least a partial overlap in pathophysiological mechanisms.

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FIGURES

Figure 1 – TGF-β_1 protein levels in broncho-alveolar lavage fluid of stable, BOS and RAS patients

(A) TGF-β_1 protein levels of stable (n=20), BOS (n=23) and RAS (n=26) patients, measured in BAL at CLAD diagnosis, presented as median ± IQR, showing increased levels in RAS compared to stable patients (p=0.024). (B) Kaplan-Meier survival curve showing a significant graft survival benefit for RAS patients with TGF-β_1 levels lower than the median 14.5 pg/mL (black curve, p=0.033, HR 2.77, 95% CI 1.1-7.1). Median survival was 0.49 years in group > median and 2.45 years in group < median. $^{*}p<0.05$

Figure 2 – Immunohistochemical patterns of TGF-β_1 on RAS biopsies

Representative RAS biopsies demonstrating three different TGF-β_1 staining patterns: (A-C) represents pattern (i) with relatively low TGF-β_1 expression, primarily subpleural staining and TGF-β_1 positivity in stromal cells and macrophages (4/19 or 21% of biopsies); (D) represents pattern (ii) with intermediate TGF-β_1 expression and mainly subpleural and alveolar staining (9/19 or 47% of biopsies); (E) represents pattern (iii) with diffuse staining of the extracellular matrix (6/19 or 32% of biopsies). Liver (F) and tonsil (G) biopsies were used as positive controls to validate the TGF-β_1 staining. TGF-β_1 positivity was found in Kupffer cells and histiocytes respectively.

Figure 3 - Lymphocyte subpopulations in TGF-β_1 expressing RAS biopsies

Representative images of multiplex immunofluorescent stainings of RAS biopsies with low (A), intermediate (B) and high (C) TGF-β_1 expression. Staining was performed for CD4 (red, T helper cells), CD8 (blue, cytotoxic T cells), CD20 (green, B cells) and CD68 (white, macrophages). The low TGF-β_1 group showed low degree of inflammation, with little and dispersed CD4, CD20 and CD68 positive staining. The intermediate TGF-β_1 group showed low number of CD4, CD8, CD20 and CD68 positive cells. The high TGF-β_1 group showed abundant CD4, CD8, CD20 and CD68 positive cells.

Figure 4 – Relative fraction of pleura and attached infiltrates on CT in control, BOS and RAS lungs

(A) shows the results of computed tomography (CT) scans of unused donor lungs, BOS and RAS lungs. Results are presented as mean ± SEM. A significant increase of volume percentage of pleura and

attached infiltrates in RAS compared to BOS (p=0.0003) and non-transplant control (p=0.025) lungs was observed (p=0.0004). (B) shows a representative CT scan of a slice from a RAS lung, while (C) also shows how pleura and attached infiltrates are highlighted (blue segmentation) to obtain results. N=8 lungs in each group; * p<0.05; *** p<0.001

Figure 5 – Calretinin staining of control, BOS and RAS explanted human lung biopsies

(A) the proportion of biopsies with calretinin-positive stainings was significantly higher in RAS lungs (p=0.0001, n=19) compared to BOS (n=19) and non-transplant control (n=14) lungs. (B) shows representative calretinin stainings of BOS, non-transplant control, and RAS biopsies. Calretinin-positive cells were visible in normal pleura (arrowhead) as well as deeper within the subpleural fibrosis of the parenchyma (arrows) in RAS biopsies. Scale bars = 200 μm. (C) TGF-β_1 staining severity showed a trend towards association with calretinin positivity. Biopsies with high TGF-β_1 staining (i.e., diffuse staining of the extracellular matrix) were almost exclusively calretinin-positive (5/6 or 83% of biopsies, p=0.69), while in the low to intermediate TGF-β_1 biopsies (i.e., subpleural and alveolar staining with TGF-β_1 positivity in stromal cells and macrophages) only few were calretinin-positive (5/13 or 38% of biopsies).

Figure 6 - Mesothelin protein levels in serum samples of stable, BOS and RAS patients

Mesothelin protein levels measured in serum samples of stable (n=22), BOS (n=26) and RAS (n=28) patients. Results are presented as median ± IQR, showing increased levels in RAS compared to stable patients (p=0.0032).

Figure 7 –Mesothelial-to-mesenchymal transition markers in pleural tissue

MMT markers were assessed at mRNA and protein level in pleural tissue of non-transplant control, BOS and RAS lungs. E-cadherin (A-B), α-SMA (C-D), fibronectin (E-F), and Wt1 (G) were used as markers. Every dot represents the relative mean mRNA or protein amount of a single sample and horizontal lines represent median ± IQR per group. # 2 samples in the RAS group proved inadequate for protein extraction. * p<0.05; ** p<0.01