

# Synergism of imatinib, vatalanib and everolimus in the prevention of chronic lung allograft rejection after lung transplantation (LTx) in rats

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**Summary.** Chronic lung allograft dysfunction (CLAD) still remains a major drawback in the outcome following lung transplantation (LTx). New therapeutic strategies are warranted. Growth factors and their receptors like platelet-derived growth factor-receptor (PDGFR) and vascular endothelial growth factor-receptor (VEGFR), may play a crucial role in the development of CLAD, especially bronchiolitis obliterans (BO) and vasculopathy. In this study, we used an orthotopic left lung transplantation model from Fischer (F344) to Wistar Kyoto (WKY) rats to investigate the effect of the receptor tyrosine kinase inhibitor (RTKI) vatalanib alone, the dual combination of the RTKIs vatalanib and imatinib and a triple therapy consisting of vatalanib, imatinib and the mammalian target of rapamycin inhibitor (mTORI) everolimus on the development of CLAD after LTx in rats. With this trial we demonstrated that monotherapy with vatalanib attenuated mild and severe chronic vascular rejection, whereas dual therapy (vatalanib and imatinib) after LTx also showed a significant reduction of chronic bronchiolar rejection and interstitial fibrosis. By adding everolimus, the effect of vatalanib and imatinib could additionally be increased. In conclusion, the combination of mTORI and RTKIs might be a possible strategy in the prevention of CLAD and BO.

**Key words:** Chronic lung allograft dysfunction, Bronchiolitis obliterans, Vatalanib, Imatinib, Everolimus

## Introduction

Lung transplantation has become an effective clinical treatment option for patients with end-stage lung diseases. In spite of improved surgical techniques, donor organ preservation solutions and immunosuppression regimes, CLAD still remains a severe complication after LTx and limits long term survival (Verleden et al., 2016). Currently, there is no generally accepted pharmacologic regimen following lung transplantation (Afshar, 2014). The most common immunosuppressive strategy consists of prednisolone, a calcineurin-inhibitor like tacrolimus or cyclosporine in combination with an antimetabolite like mycophenolate mofetil or azathioprine (Nakajima et al., 2011; Afshar, 2014; Yusen et al., 2016). The current therapy for CLAD mainly consists of augmentation or variation of immunosuppressive agents (Al-Githmi et al., 2006), which have little effect on the development of lung dysfunction (Tikkanen et al., 2006). Better therapeutic strategies are required in order to reduce proliferative and fibrotic processes in the long term follow up after lung transplantation. Nowadays a variety of chronic transplant rejection forms are known. The non-reversible type of CLAD comprises two forms, the non-reversible form of bronchiolitis obliterans syndrome (BOS) and/or restrictive allograft syndrome (RAS) (Verleden et al., 2015). RAS patients display more interstitial damage like peripheral lung fibrosis (Sato et al., 2011). Bronchiolitis obliterans (BO) describes the histological correlate of BOS and is characterized by the progressive luminal occlusion of small airways, often accompanied by intimal thickening and vascular sclerosis (Tikkanen et al., 2006; Stewart et al., 2007). A wide range of immune and nonimmune risk factors for

the development of BO have already been described (Nakajima et al., 2011).

It is commonly assumed that CLAD develops during two different phases: First tissue injury leads to inflammation, which is followed by an excessive fibroproliferative reaction that finally results in progressive scarring (Bonner, 2004). This aberrant tissue repair is presumably promoted by growth factors, such as platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (Bergmann et al., 1998; Knoop and Estenne, 2006). Several studies suggest a vital role of PDGF in the progression of CLAD and in lung fibrogenesis (Hertz et al., 1992; Ferrara et al., 2003; Abdollahi et al., 2005). It both works as a mitogen and survival factor of myofibroblasts and also plays an important role in collagen production (Bonner, 2004). Furthermore, VEGF has a mitogenic effect on endothelial cells and is crucial for vascular growth (Ferrara, 1999; Ferrara et al., 2003).

The effect of PDGF and VEGF is mediated via receptor tyrosine kinases (RTKs), which are therefore possible targets to prevent or at least attenuate the development of CLAD. In this context, co-application of imatinib with the mTORI everolimus has already shown promising results after experimental LTx (Suesskind-Schwendi et al., 2013b). In addition, the RTKIs imatinib and vatalanib successfully prevented obliterative airway disease in heterotopic tracheal transplantation (Tikkanen et al., 2006). However, this rodent model, commonly used to understand BO, has several drawbacks, including lack of physiological ventilation and perfusion (Sato et al., 2009; Suesskind-Schwendi et al., 2013b). Moreover, one cannot examine small bronchioles or peripheral lung tissue, which are primarily affected during the development of both forms of CLAD (Jungraithmayr et al., 2013). In contrast, in our model we can not only examine chronic vascular and bronchiolar rejection, but also interstitial and peripheral fibrosis (Suesskind-Schwendi et al., 2017).

Another important risk factor for the development of BO is acute allograft rejection (Hirt et al., 1999; Estenne and Hertz, 2002; Nakajima et al., 2011). Consequently, the treatment of acute rejection (AR) should prevent chronic rejection (CR) as well (Hirt et al., 1999). The mTORI everolimus reversed ongoing AR under cyclosporine maintenance therapy in a stringent rat lung allotransplant model (Hausen et al., 2000). In contrast, monotherapy of everolimus could not delay the progression of AR (Brunner et al., 2013). Everolimus suppresses growth-factor stimulated proliferation of haematopoietic cells (T cells and B cells), vascular smooth muscle cells and human lung fibroblasts in vitro (Schuler et al., 1997; Hausen et al., 2000; Azzola et al., 2004) and chronic lung allograft rejection (Suesskind-Schwendi et al., 2013a) in rats. By adding the RTKI imatinib, everolimus has been shown to reduce severe acute vascular and bronchiolar rejection (Suesskind-Schwendi et al., 2013b).

Therefore we hypothesize that co-application of

imatinib, vatalanib and everolimus shows a better long term outcome after experimental orthotopic lung transplantation compared to monotherapy or dual-combination of these anti-proliferative and anti-fibrotic substances.

## Material and methods

### Lung transplantation

Left lung allografts were transplanted orthotopically from pathogen-free inbred male F344 (RT1lv1) rats to MHC (RT1)-incompatible WKY (RT11) rats (Charles River, Sulzfeld, Germany; 250 to 300 g) as described in preliminary work (Matsumura et al., 1995; Hirt et al., 1999; Süßkind-Schwendi et al., 2012). The animals were kept according to the Principles of Laboratory Animal Care formulated by the European Union Guide for the Care and Use of Laboratory Animals. For assessment of rejection, recipients were killed on postoperative day (POD) 20 to evaluate AR and on POD 60 to assess CR.

### Experimental groups

The allograft recipients were divided into four experimental groups: group one received vatalanib (PTK/ZK, Novartis, Basel, Switzerland), group two a combination of imatinib (Glivec<sup>®</sup>, Novartis, Basel, Switzerland) and vatalanib (vata/ima), group three additionally received the mTORI everolimus (rapamycin-derivate-RAD, Certican<sup>®</sup>, Novartis, Basel) (vata/ima/RAD) and the control-group received no drugs after LTx. Within each group, some of the animals were killed on POD 20 and on POD 60 (Table 1). Drugs were dissolved in polyethylene glycol and administered daily via gavage. The animals received no additional immunosuppression. The dosage of imatinib: (20 mg/kg bw from POD -1) and everolimus (2.5 mg/kg/day intraperitoneal from POD 7) was based upon the experience of the research group (Suesskind-Schwendi et al., 2013b). Everolimus was administered later due to reduced wound healing and the clinical situation. The dosage of vatalanib (100 mg/kg from POD -1) was based on previous studies in rodent models (Tikkanen et al., 2006). To avoid massive weight loss as described earlier

**Table 1.** Experimental groups.

Experimental group	Postoperative day (POD)	Number of animals (n)
vata/ima/RAD- group	20	6
(triple combination group)	60	9
vata/ima-group	20	8
(dual combination group)	60	11
Vatalanib-group	20	6
	60	7
Control-group	20	6
	60	6

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(Suesskind-Schwendi et al., 2013a), we only used animals with an initial weight of over 250 g.

### Histology

Transplanted left lungs and non-transplanted right lungs were removed at the time points indicated above. Thereafter, they were fixed in 5% paraformaldehyde (Merck, Darmstadt, Germany), embedded in paraffin, and cut into 5  $\mu$ m sections. For histopathological analysis, sections were stained with haematoxylin-eosin (HE) to assess acute rejection and Sirius-red and Masson Goldner Trichrome (MGT) to assess CR. AR was graded as described in the revised working formulation of The International Society for Heart and Lung Transplantation (Stewart et al., 2007). In summary, acute vascular rejection was graded from A0 to A4 according to the intensity and distribution of perivascular mononuclear cells whereas acute airway inflammation/lymphocytic bronchiolitis was graded from B0 to B2R depending on infiltration of the submucosa by mononuclear cells and epithelial damage. Chronic vascular and airway rejection was classified according to the modified scale by Suesskind et al. 2012 (Süßkind-Schwendi et al., 2012). Briefly, chronic vascular rejection was ranked from D0 to D2R by assessing the obstruction of the lumen and perivascular fibrosis and chronic airway rejection was graded from C0 to C2R according to intraluminal granulation tissue and peribronchiolar fibrosis (Fig. 1). The exact classification has been described previously (Süßkind-Schwendi et al., 2012).

In addition, interstitial fibrosis was also graded from 0 (normal lung) to 8 (complete interstitial fibrosis) according to the modified Ashcroft scale by Hübner et al. (Table 2) (Hübner et al., 2008).

### Immunohistochemistry

The expression of PDGF-A, PDGFR- $\alpha$ , VEGF-A and VEGFR-2 was analysed by immunohistochemistry using rabbit anti-rat as primary antibodies (PDGF-A: Santa Cruz sc7958, diluted 1:300; PDGFR- $\alpha$ : Santa Cruz sc338, diluted 1:1000; VEGF-A: BioLogo, diluted 1:80; VEGFR-2: Dianova, diluted 1:100) in 5  $\mu$ m sections. After deparaffinization, tissue sections were

heated for 20 minutes in 1 x Target Retrieval Solution (Dako, Denmark) and treated with hydrogen peroxide to quench non-specific peroxidases. Thereafter, tissue sections were incubated in 10% normal goat serum (Sigma, Munich, Germany) to block unspecific binding-sites. Then, the sections were incubated with the primary antibody (Table 3) overnight at 4°C. After rinsing, sections were incubated with secondary goat-anti-rabbit antibody (Vector Laboratories, Burlingame, USA; diluted 1:300) and then with the tertiary antibody, an avidin-biotin-complex (Vector Laboratories, Burlingame, USA, diluted 1:100). HistoGreen (Linaris, Dossenheim, Germany) was used as a specific substrate chromogen. Counterstaining was performed with haematoxylin (Merck, Germany). The number of positive cells was counted in a blinded fashion by two independent operators using five randomly selected microscopic power fields (200x, diameter 1.1 mm). To detect the positive staining according to cell type, each sample was searched for the specific cells listed in Tables 4 and 5 and was semi-quantified by colour intensity.

### Statistical analysis

Histological and immunohistochemical scoring was performed by two independent investigators in a blinded fashion. Data were expressed as means  $\pm$  standard deviation (SD). The Kruskal-Wallis test was used to compare the non-treated control group with the study groups (vata, vata/ima, vata/ima/RAD). As non-parametric statistical hypothesis test, Wilcoxin-Mann-Whitney-U-test was used to decide whether one experimental group improved after therapy as compared to another group.  $P \leq 0.05$  was considered statistically significant.

## Results

### Survival and general health

All successfully transplanted rats showed good general conditions (normal social and grooming behaviour, acceptable feed consumption, inconspicuous defecation). Drugs were well tolerated.

As shown in Fig. 2, independent of drug treatment,

**Table 2.** Grading of interstitial fibrosis according to Hübner et al., 2008.

Grade of fibrosis	Significance
0	Normal lung tissue
1	Rare interlobular septal thickening
2	Increased interlobular septal thickening
3	Confluent fibrotic walls
4	Isolated fibrotic masses (<10%)
5	Confluent fibrotic masses (<50%)
6	Confluent fibrotic masses (>50%)
7	Nearly complete fibrosis
8	Complete fibrosis

**Table 3.** Primary antibodies.

Primary antibody	Dilution	Origin
Anti-PDGF-A (rabbit-anti-rat)	1:300	Santa Cruz Biotechnology, USA
Anti-PDGFR- $\alpha$ (rabbit-anti-rat)	1:1000	Santa Cruz Biotechnology, USA
Anti-VEGF-A (rabbit-anti-rat)	1:80	BioLogo, Susteren, Netherlands
Anti-VEGFR-2 (rabbit-anti-rat)	1:100	Dianova, Hamburg, Germany

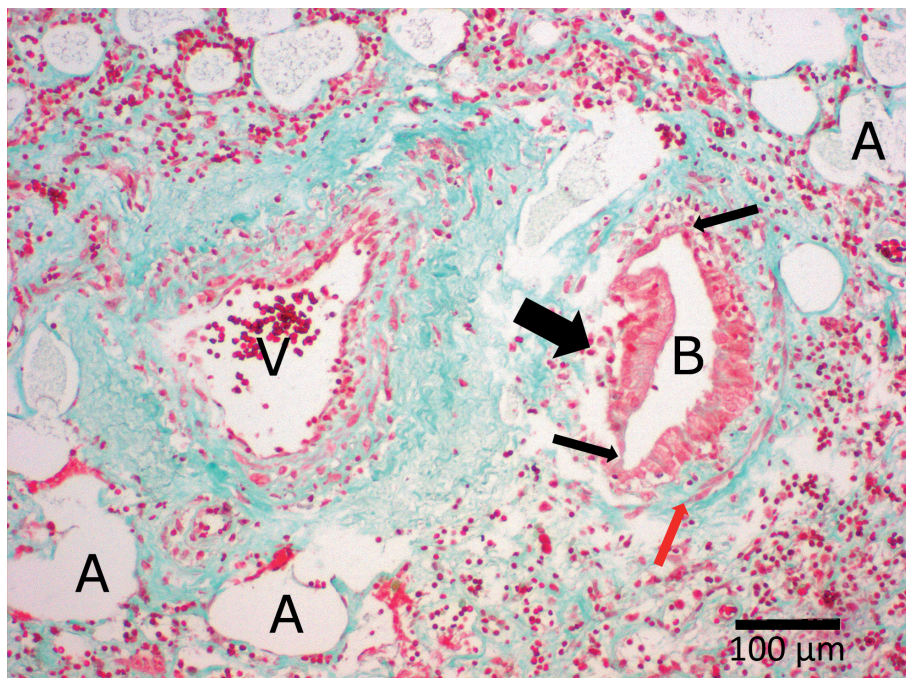
early after LTx feed consumption was restricted (data not shown) and resulted in a significant decrease in mean body weight. Rats from the control group, vatalanib group and vata/ima group regained their initial body weight within 15, 14, and 16 days, respectively. Afterwards, body weight increased significantly over time ( $p \leq 0.05$  each) in these groups. In contrast, additional application of everolimus on POD 7 to the vata/ima treated animals caused a secondary weight loss up to POD 30. Animals in this group never regained their initial body weight. Compared to the other groups animals were significantly lighter in weight at the end of the trial (on POD 60 compared to vata/ima:  $p < 0.0001$ ; compared to vata  $p = 0.001$ ; compared to control:  $p = 0.004$ ). Apart from that, the animals' general health was unaffected and they showed no altered feeding

behaviour, urine and faeces production was normal. Two animals showed crusty skin lesions in the area of the elbow which vanished after a singular treatment with Braunol® (Braun, Melsungen, Germany). Nevertheless, all animals had pale mucous membranes at the time of organ harvesting.

### Histology

Effect of vatalanib, imatinib and everolimus on acute allograft rejection

Figure 3a-d show representative micrographs from allografts of all study groups on POD 20. In the control group, alloimmune activation peaked at that time. Severe acute vascular (ISHLT-A3-4) and airway rejection



**Fig. 1.** Representative image of beginning BO in our rat model (vatalanib group, POD 20, MGT). A, alveole; B, bronchiole; V, vessel. In this sample, we can see eccentric fibrosis of the submucosa (thick black arrow, C1). Moreover, the bronchiolar epithelium is damaged at various points (thin black arrows) as is the lamina muscularis (red arrow), which is often associated with BO. This example can therefore be classified as a beginning form of BO.

**Table 4.** Immunohistology of allografts on POD 20.

Study group	PDGF-A				PDGFR- $\alpha$				VEGFR-2			
	C	V	D	T	C	V	D	T	C	V	D	T
Bronchiolar epithelium	++	+	++	+++	+	++	-	++	+	+	+	+++
Type I cells	+	-	-	-	-	-	-	+	-	-	-	-
Type II cells	-	-	-	++	-	-	-	++	-	-	-	+
Fibroblasts	+++	+	-	+	++	++	+	+	+	-	+	+
Vascular endothelium	++	+	-	+	-	-	-	+	+	-	-	+
Alveolar macrophages	+++	+	++	+	+++	++	+++	++	+++	++	+++	+
Other mononuclear cells	+++	+++	+++	+	+++	+++	+++	+	+++	+++	++	++
Metaplastic epithelial cells	+	+++	+	+	-	-	-	-	-	-	-	+

Intensity [-] absent, [+], moderate and [+++] strong) of specific positive stained cells (C, control group; V, vatalanib group; D, vata/ima group; T, vata/ima/RAD group).

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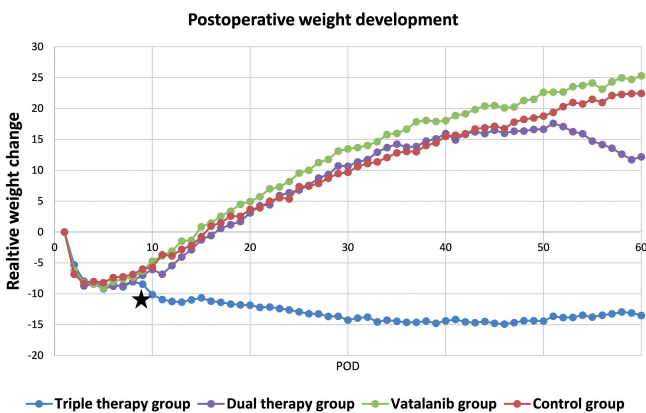
(ISHLT-B1R-2R) dominated the tissue sections of all study groups (Fig. 3a-d). Prominent alveolar pneumocyte damage with varying degrees of organization in combination with diffuse perivascular, interstitial, and airway infiltration of mononuclear cells and endothelialitis, dominated the sections on POD 20. Bronchiolar epithelial hyperplasia, epithelial destruction and vascular obstruction by mononuclear cells were common (Fig. 3a).

In addition to the acute inflammatory response, the first signs of chronic rejection could be detected in all groups on POD 20. Especially in the control group, there was evidence of chronic vascular rejection. A multitude of small and medium sized vessels were obstructed by leucocytes. Incipient subendothelial and perivascular fibrosis of the vessels were diagnosed as first signs of severe chronic vascular rejection (D2R). Chronic vascular rejection could be reduced by increasing the

number of drug applications. Within the vatalanib group, a multitude of small vessels was obstructed by mononuclear cells adhered to the endothelium (D1:  $60 \pm 19.24\%$ , Fig. 3b). However, compared to the control group, there were no severe grade rejected vessels to be found ( $p=0,015$ , Fig. 5). In the vata/ima group, half of the vessels were completely free of chronic alterations (D0:  $51.25 \pm 28.35\%$ , Fig. 3c), the effect being increased by adding everolimus (D0:  $61.12 \pm 33.71\%$ , Fig. 3d). All in all, dual application (vata/ima) increased the percentage of unaffected vessels, lowered the amount of mild CR and significantly reduced severe CR already on POD 20 ( $p=0,029$ ) whereas triple application (vata/ima/RAD) significantly reduced both mild and severe chronic vascular rejection ( $p(D1)=0.002$  and  $p(D2)=0.015$ , Fig. 5). The latter was also significantly superior to the vatalanib group regarding mild vascular rejection ( $p=0.009$ , Fig. 5).

Investigation of terminal bronchioles on POD 20 showed that on the bronchiolar level, mild rejection with intraluminal signs of granulation tissue, fibroblasts and fibrin filaments dominated the sections of the control group (C1:  $78,5 \pm 40,17\%$ ). There was no evidence of severe chronic bronchiolar rejection on POD 20. In comparison to the control group, vatalanib reduced chronic bronchiolar rejection, although no significant improvement could be observed (C0:  $52.17 \pm 45.78\%$  as compared to  $4.83 \pm 11.84\%$ , Fig. 5). In contrast, the percentage of unaltered bronchioles was significantly higher in the vata/ima group than in the control group ( $p=0,008$ , Figs. 3c, 5). This effect was further increased by everolimus. Triple application significantly reduced the amount of bronchioles affected by mild CR as compared to the control group already on POD 20 ( $p(C1)=0.041$ )  $p(D1)= p=0.002$  and  $p(D2)= p=0.015$ , Fig. 5). Moreover, no severe chronic bronchiolar rejection was found in any lung and the percentage of non-altered bronchioles was significantly increased in comparison to the control group ( $p=0.015$ , Fig. 5), analogous to the vata/ima group.

On POD 20, all allografts presented beginning interstitial fibrosis comparable to the control group with



**Fig. 2.** Postoperative weight development of the different study groups until POD 60. The preoperative weight is used as zero-point. Whereas the control, the vatalanib, and the vata/ima group significantly increased in weight after an initial weight loss ( $p \leq 0.05$ ), the vata/ima/RAD group significantly lost weight after the application of RAD on POD 9 ( $p < 0.0005$ , star) and never reached their preoperative weight.

**Table 5.** Immunohistology of allografts on POD 60.

Study group	PDGF-A				PDGFR-α				VEGFR-2			
	C	V	D	T	C	V	D	T	C	V	D	T
Bronchiolar epithelium	++	-	++	+++	+	+	+	++	+	+	+	+++
Type I cells	-	-	-	-	-	-	-	-	-	-	-	-
Type II cells	-	-	-	+	-	-	-	++	-	-	-	+
Fibroblasts	+++	-	-	-	+	-	+	-	-	-	-	-
Vascular endothelium	++	-	-	+	+	+	-	+	++	-	+	+
Alveolar macrophages	+++	+++	+++	+	+++	++	+++	+	++	+	+	++
Other mononuclear cells	+++	+++	+++	+	+++	+++	+++	++	++	++	+	+
Metaplastic epithelial cells	-	-	+	-	-	+	-	++	+	-	-	++

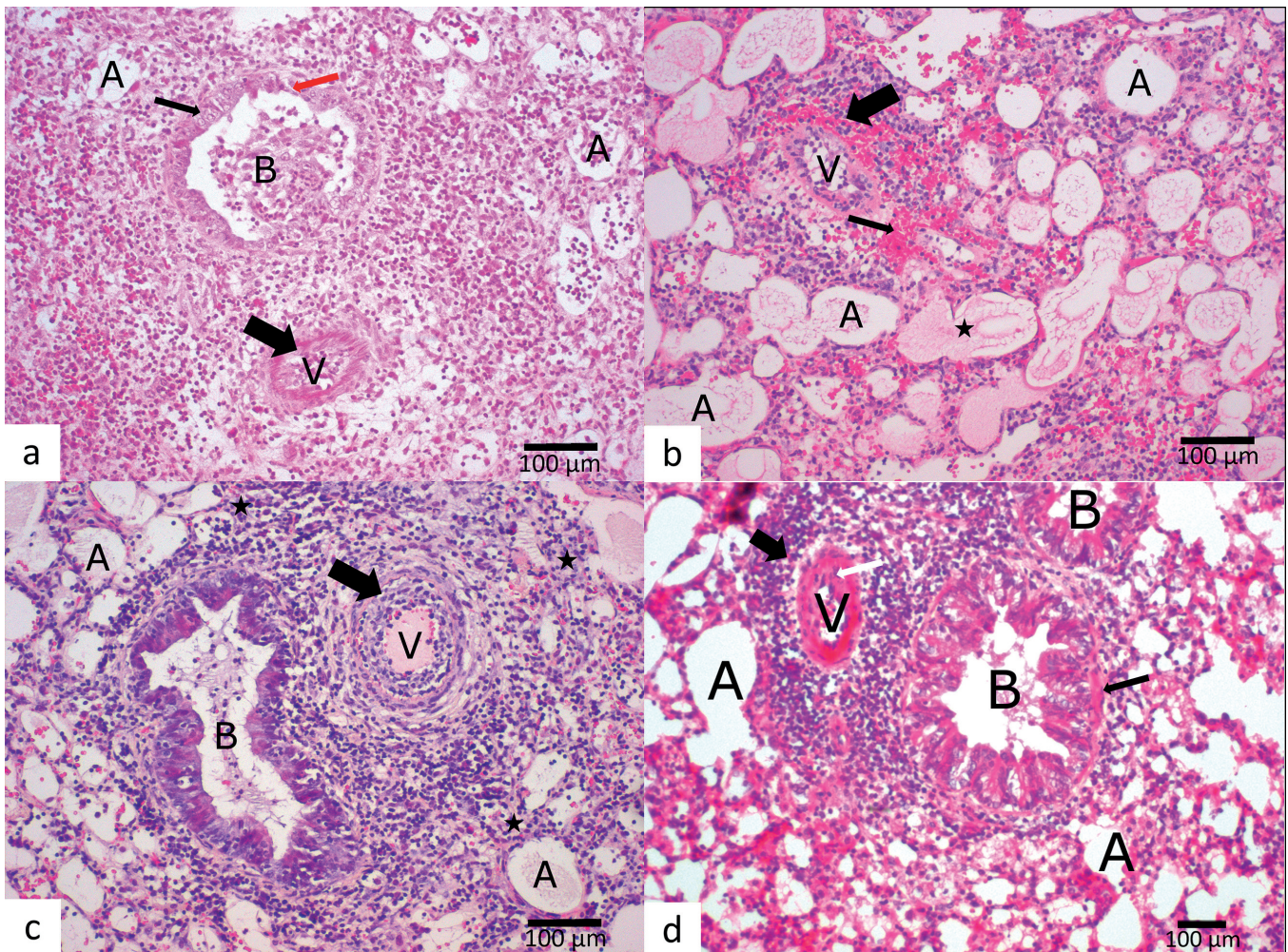
Intensity [-] absent, [+ ] weak, [++ ] moderate and [+++ ] strong) of specific positive stained cells (C, control group; V, vatalanib group; D, vata/ima group; T, vata/ima/RAD group).

thickening of alveolar septa and single fibrotic masses (grade of interstitial fibrosis:  $4 \pm 0.63$ ). Aside from this, exudate and bleeding into alveolar spaces was commonly seen. Despite this, unaffected lung parenchyma was more commonly seen in the vata/ima group than in the control and vatalanib group and two allografts showed no augmentation of connective tissue (mean grade of interstitial fibrosis:  $2.5 \pm 1.69$ ). By adding everolimus, interstitial fibrosis could significantly be decreased already on POD 20 in comparison to the vatalanib and

control group ( $p=0.004$  and  $p=0.015$ , Fig. 6).

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On POD 60, chronic alterations dominated whereas inflammatory infiltrations had receded. In the control group, mononuclear cell infiltrates had vanished. Instead, perivascular fibrosis, intimal thickening, and destruction of the epithelium were commonly seen (Fig.

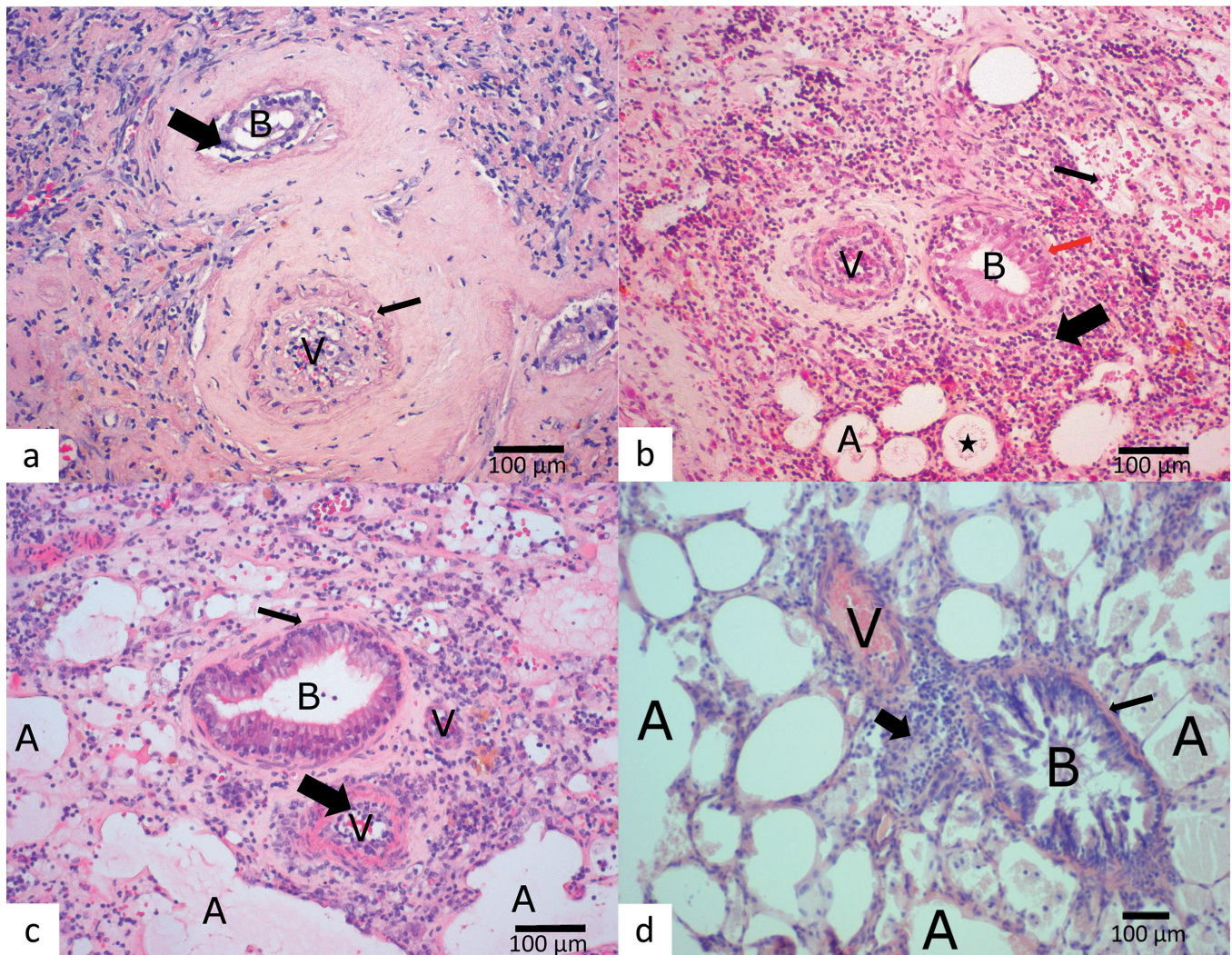


**Fig. 3.** Representative histopathology for all experimental groups on POD 20 (HE). A, alveole; B, bronchiole; V, vessel. **a.** Control group. **b.** Vatalanib group. **c.** Vata/ima group. **d.** Vata/ima/RAD group. **a.** This sample shows a terminal bronchiole (high grade bronchiolar rejection, B2R) and a small vessel (severe acute vascular rejection, A4). Damaged bronchiolar epithelium (red arrow) and in addition epithelial hyperplasia (thin black arrow) is present. Both bronchioles and vessel are surrounded by mononuclear cells. The lumen of the vessel is obstructed by leucocytes (thick black arrow). **b.** Diffuse mononuclear cell infiltrates (thick black arrow) dominate the view and also spread into adjacent alveolar septa (severe acute vascular rejection, A4). On top of that, inflammatory cells obstruct the vessel by adhering to the endothelium (low grade chronic vascular rejection, D1R). Contemporaneously, alveolar exudate (star) and bleeding can be seen (thin black arrow). **c.** No increased connective tissue can be seen in this staining. Inflammatory cells surround the vessel and spread into the adjacent alveolar septa (severe acute vascular rejection, A4, stars). The lumen of the vessel is obstructed by leukocytes (low grade chronic rejection, D1R, thick black arrow). In contrast, the bronchiole with its continuous lamina muscularis is free from chronic rejection. **d.** The small vessel on the left side is surrounded by multiple layers of mononuclear cells, which also infiltrate the adjacent alveolar septa (severe acute vascular rejection, A4, thick black arrow). Moreover, leucocytes attached to the endothelium obstruct the lumen (low grade chronic vascular rejection, D1R, white arrow). The bronchiole is marked by beginning epithelial damage (severe acute bronchiolar rejection, B2R). Its lamina muscularis is still intact (no chronic airway rejection, C0, thin black arrow).

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4a). All vessels that were investigated in the section were affected by chronic fibrotic disorders (vasculopathy  $100\pm 0\%$ ). However, as opposed to the control group, the inflammatory process persisted in all drug-applied groups on POD 60 (Fig. 4b-d). 76 $\pm$ 36.83% of the vessels of the vatalanib group developed distinct perivascular fibrosis and/or fibrointimal thickening. One allograft even exhibited complete fibrosis and vasculopathy. Despite this, vasculopathy was significantly less

pronounced within the vatalanib-group as compared to the control group on POD 60 ( $p(D1R)=0.035$ ,  $p(D2R)=0.035$ , Figs. 4b, 5), whereas the combination of vatalanib and imatinib achieved significantly better results concerning all three grades of rejection. Finally, the vata/ima/RAD group significantly exceeded dual therapy by lowering severe vascular rejection ( $p<0.0001$ ) as well as in preventing vascular rejection altogether ( $p(D0)<0.0001$ , Fig. 5).



**Fig. 4.** Representative histopathology for all experimental groups on POD 60 (HE). A, alveole; B, bronchiole; V, vessel. **a.** Control group. **b.** Vatalanib group. **c.** Vata/ima group. **d.** Vata/ima/RAD group. **a.** In this staining, the epithelium of the central bronchioles is completely destroyed and fragmented (thick black arrow). The vessel can only be recognized by its elastica (thin black arrow). The whole area is almost completely fibrotic. **b.** Mononuclear cell infiltrations are still present (thick black arrow). The lumen of the small vessel is obstructed by leucocytes adhered to the endothelium and first signs of fibrointimal thickening can be distinguished (low to high grade chronic vascular rejection, D1R-D2R). The bronchiole's lamina muscularis is intact (red arrow) and shows no peribronchiolar fibrosis (no chronic bronchiolar rejection, C0). Bleeding into alveolar spaces (thin black arrow) and exudate (star) can be found. **c.** The bronchiole is characterized by its continuous lamina muscularis (thin black arrow). No signs of fibrosis can be seen (no chronic bronchiolar rejection, C0). The lumen of the small vessel is partly filled with leucocytes (thick black arrow, low grade chronic vascular rejection, D1R). The surrounding tissue rather resembles an acute than a chronic rejection. **d.** The central vessel and bronchiole are not affected by chronic rejection (no chronic bronchiolar or vascular rejection, C0 and D0). The peribronchiolar and perivascular tissue is infiltrated by mononuclear cells (thick black arrow), hence inflammation is still present (severe acute vascular rejection, A4). The layer of smooth muscle cells is not destroyed (thin black arrow).

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Regarding chronic bronchiolar rejection, all untreated animals exhibited C2R alterations. Peribronchiolar fibrosis, destruction of the lamina muscularis and lamina epithelialis associated with luminal obstruction due to granulation tissue were commonly seen. In contrast, vatalanib lowered the number of bronchioles affected by chronic alterations. Though three allografts had completely developed BO, a small amount of bronchioles remained free of chronic rejection on POD 60 (C0: 6±12.37%, Fig. 5). But only the combination with imatinib significantly reduced chronic bronchiolar rejection. This effect was even exceeded in the triple combination group: It showed significantly superior results in the category C0 and C2R as compared to dual therapy and vatalanib alone (p(C0)=0.01 and p(C2R)=0.008 and p(C0)<0.0001 and p(C2R)=0.02, respectively; Fig. 5).

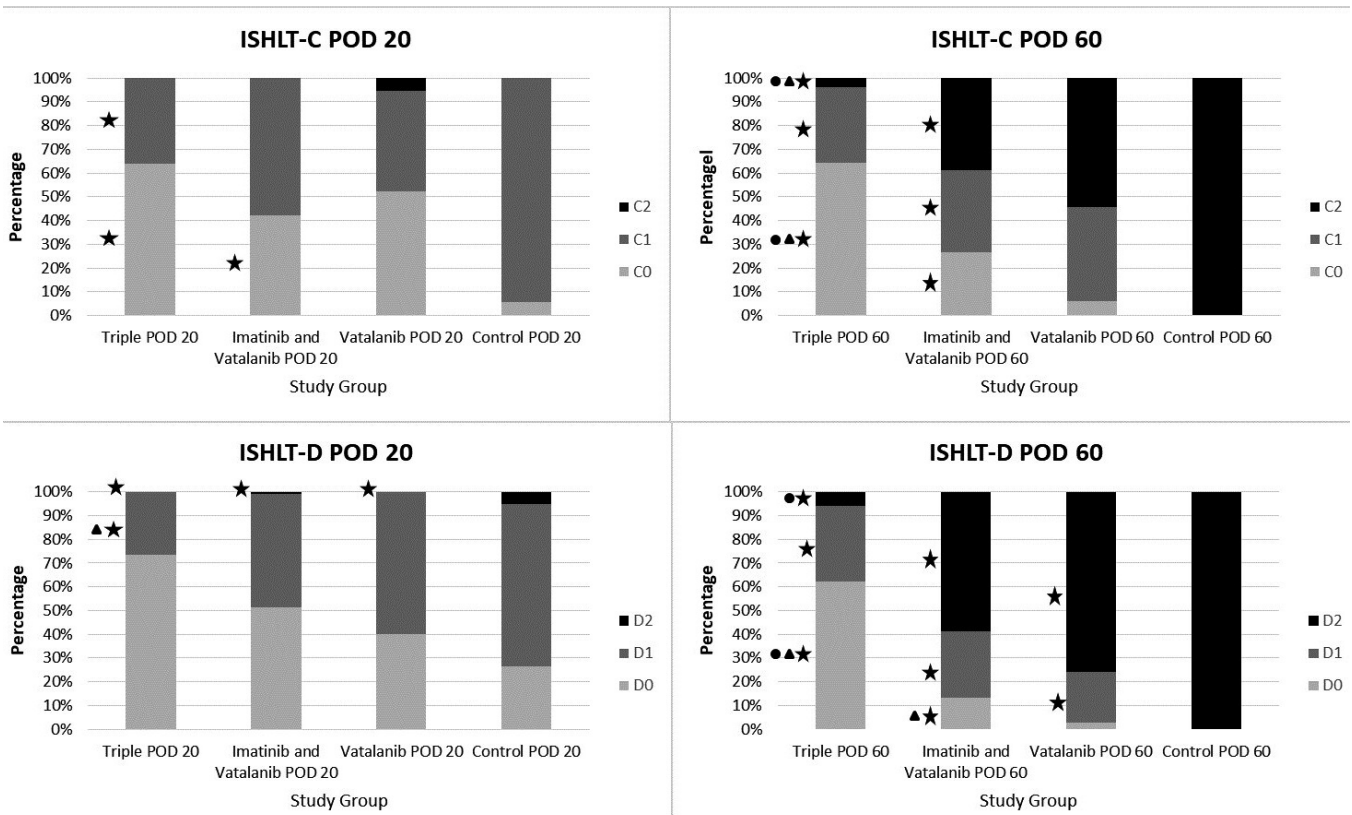
On POD 60, interstitial fibrosis could be significantly reduced in all intervention groups (p=0.035 (vatalanib alone), p=0.001 (vata/ima) and p=0.001 (vata/ima/RAD))

in comparison to the control group (Fig. 6).

Immunohistochemistry

Receptor and ligand expression in non-treated allografts

PDGF-A, PDGFR- $\alpha$ , and VEGFR-2 were all expressed in the lungs of the control group. Immunohistochemical analysis showed strong PDGF-A expressions in fibroblasts, alveolar macrophages, and other mononuclear cells and moderate expression in bronchiolar epithelium and vascular endothelium on POD 20 and POD 60. Strong expression of VEGFR-2 and PDGFR- $\alpha$  was also detected in alveolar macrophages and other mononuclear cells on POD 20, but VEGFR-2 expression decreased on POD 60 (Tables 4, 5). In contrast, there was only weak expression of PDGF-A in type I cells on POD 20, and apart from that no expression of ligands or their receptors in type I or II



**Fig. 5.** Effect of triple and dual combination therapy and of vatalanib alone on the development of chronic bronchiolar and vascular rejection. POD 20 (n1) and POD 60 (n2): Percentage of vessels with no (pale bar), mild (intermediate bar) and severe (black bar) chronic rejection of each group (vata/ima/RAD group, n1=6 and n2=9; vata/ima group, n1=8 and n2=11; vatalanib group, n1=6 and n2=7; control group, n1=6 and n2=6). Significance compared to the control group is shown by stars, compared to the vatalanib group by triangles and compared to the vata/ima group by circles. On POD 60, severe and mild chronic vascular rejection could significantly be reduced by the treatment with vatalanib. Both chronic vascular and bronchiolar rejection was significantly reduced regarding all three grades in the vata/ima group as compared to the control group. This effect could further be improved by adding everolimus.



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cells on POD 20 or 60. Also, only weak to no expression was observed in metaplastic epithelial cells.

Receptor and ligand expression in the vatalanib-treated allografts

In the vatalanib group, PDGF-A, VEGF-A, and VEGFR-2 were all strongly expressed in mononuclear cells other than alveolar macrophages on POD 20, whereas there was no expression of ligands or their receptors in type I or II cells. Except for PDGF-A expression in metaplastic epithelial cells, there were only slight differences between POD 20 and 60. No significant difference between the vatalanib group and

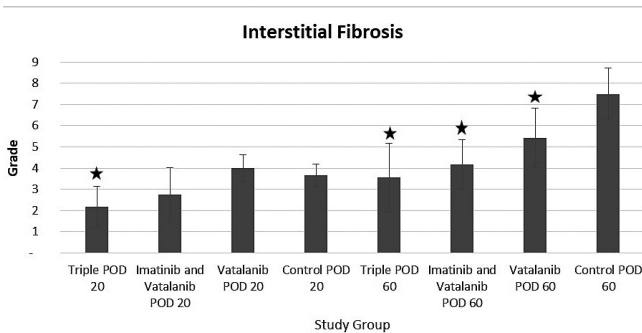
the control group was to be found.

Receptor and ligand expression in the vatalanib/imatinib-treated allografts

Generally, there was lower expression of growth factors and their receptors in the vata/ima group as compared to the control or vatalanib group. For example, as opposed to the control group, fibroblast and endothelial cells were PDGF-A negative on POD 20 and POD 60 (Tables 4, 5). Expression of VEGFR-2 was significantly decreased on POD 20 as compared to the vatalanib group (Fig. 7,  $p=0.003$ ).

Receptor and ligand expression in the vatalanib/imatinib/RAD-treated allografts

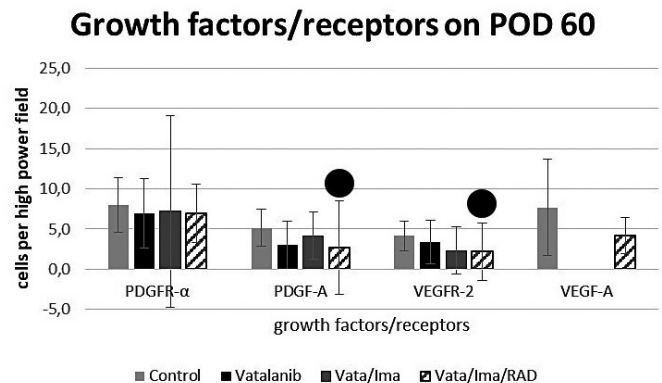
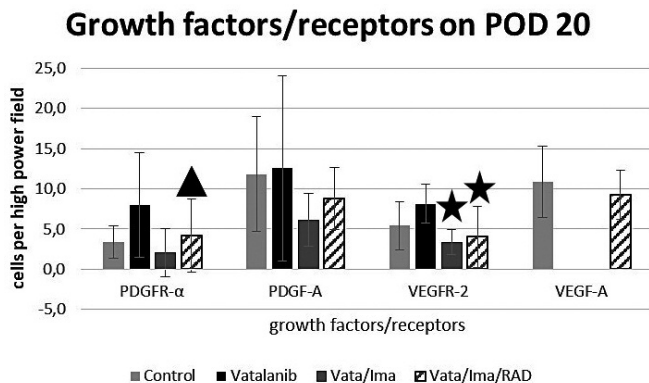
Similar to the vata/ima-group, there was generally lower expression of PDGF-A, PDGFR- $\alpha$ , and VEGFR-2 than in the vatalanib and control group. Strong expression of PDGF-A and VEGFR-2 was found in the bronchiolar epithelium and only moderate to weak expression of all investigated growth factors and receptors in macrophages and other mononuclear cells on POD 20 and POD 60. On POD 20, VEGFR-2 was significantly decreased as compared to the vatalanib group ( $p=0.004$ , Fig. 7). In contrast, PDGFR- $\alpha$  was significantly higher in the vata/ima/RAD group than in the vata/ima group ( $p=0.02$ , Fig. 7). On POD 60, there was a significantly lower expression of PDGF-A and VEGFR-2 as compared to the control group ( $p=0.037$  and  $p=0.038$ , Fig. 7).



**Fig. 6.** Effect of triple and dual combination therapy and of vatalanib alone on the development of interstitial fibrosis. Each bar represents the mean grade of interstitial fibrosis of each group on POD 20 and 60. The degree of interstitial fibrosis was significantly decreased by vatalanib ( $p=0.035$ ), the dual combination of vatalanib and imatinib ( $p=0.001$ ), and the triple therapy ( $p=0.001$ ) compared to the control group on POD 60. Furthermore, the triple combination group showed significantly less interstitial fibrosis on POD 20 as compared to the vatalanib ( $p=0.004$ ) and the control group ( $p=0.015$ ) and showed significantly superior results to the vatalanib group on POD 60 ( $p=0.035$ ).

## Discussion

The present study describes the efficiency of the co-application of the two RTKIs, vatalanib and imatinib,



**Fig. 7.** Immunohistologic evaluation of the expression of growth factors PDGF-A and VEGF-A and their receptors PDGFR- $\alpha$  and VEGFR-2 on POD 20 (left) and POD 60 (right). Each bar represents the mean  $\pm$  SD of positive stained cells in each group. Number of positive stained cells were counted per power field (200x). On POD 20, PDGFR- $\alpha$  was significantly higher in the vata/ima/RAD group than in the vata/ima group ( $p=0.02$ ). VEGFR-2 was significantly decreased in the vata/ima/RAD group and in the vata/ima group as compared to the vatalanib group ( $p=0.004$  and  $p=0.003$ ). On POD 60, PDGF-A ( $p=0.037$ ) and VEGFR-2 ( $p=0.038$ ) were significantly higher in the allografts of the control group than in the vata/ima/RAD group. (Star, as compared to vatalanib group; circle, as compared to control group; triangle, as compared to vata/ima group).

alone and in combination with the mTORI everolimus in the development of CLAD after left lung allo-transplantation in a rat model. We demonstrated that the use of vatalanib alone significantly attenuated chronic vascular rejection which often accompanies BO (Stewart et al., 2007). Furthermore, the combination with imatinib almost completely prevented obliterative bronchiolitis, i.e. the development of BO in our rats. By adding everolimus, vascular and bronchiolar rejection could be reduced even further. In contrast, acute allograft rejection could not be reduced by either of the treatment groups.

The term CLAD predominantly consists of three entities: bronchiolitis obliterans (BO), restrictive allograft rejection syndrome (RAS) and neutrophilic reversible allograft dysfunction (NRAD) (Gauthier et al., 2016). While the first two are irreversible, NRAD can be attenuated by azithromycin and will not be discussed in this study.

RAS is hallmarked by peripheral lung fibrosis as well as diffuse alveolar damage and approximately accounts for 30% of CLAD (Sato et al., 2011; Verleden et al., 2015). To further understand its development and possible treatment options, an animal model is needed, which shows both BO and RAS. As shown previously, the left lung LTx rat model from F344 to WKY rats exhibits histological features of BO and RAS (Suesskind-Schwendi et al., 2017). To the present day, no general classification system for the pathological changes in RAS exists. Therefore, we described interstitial and peripheral fibrosis using the modified Ashcroft scale (Hübner et al., 2008), which reflects histological features of RAS. Within this scoring system, we were able to demonstrate that interstitial and peripheral fibrosis could be significantly reduced by the treatment with vatalanib on POD 60. This effect could further be enhanced by adding imatinib alone and both imatinib and everolimus. Consequently, the RTKs and the mTORI used not only prevent the development of BO in our rats but also of RAS, targeting both aspects of CLAD.

To date, there is no efficient established therapy for CLAD. The immunosuppressive agents commonly used have several drawbacks, including higher risk of infection, nephrotoxicity especially when using calcineurin-inhibitors and malignancy (Ivulich et al., 2018). New therapeutic targets are warranted which both help eliminate these side effects and also provide successful treatment options.

Several studies have demonstrated that growth factors signalling cascades targeted by imatinib and vatalanib are involved in the pathogenesis of CLAD after LTx. Al-Dossari et al. demonstrated that PDGF and fibroblast growth factor-2 (FGF-2) induce fibroproliferation, occluding the tracheal lumen in tracheal isografts in mice (al-Dossari et al., 1995). Significantly increased expression of FGF-1, FGF-2, and PDGF-B but not PDGF-A were found during BO 4 weeks after transplantation in a mouse model of

heterotopic tracheal transplantation (Aris et al., 2002). Tikkanen et al. already showed the beneficial effect of imatinib and vatalanib on the development of obliterative airway disease using a heterotopic tracheal model. Blocking VEGF receptor tyrosine kinase activity with vatalanib (PTK/ZK) in combination with inhibiting PDGF receptor tyrosine kinase activity with imatinib significantly attenuated the development of tracheal occlusion in an early or prophylactic treatment regimen (Tikkanen et al., 2006). On top of that, VEGFR-1-inhibition also reduced vasculopathy after heart transplantation in mice (Chatur et al., 2016), indicating a potentially beneficial effect on chronic vascular rejection after transplantation. In a large animal model of BO, Alho et al. showed that the expression of PDGF-A, PDGF receptors, and transforming growth factor  $\beta$  were up-regulated in allografts that were rejected (Alho et al., 2007). By targeting PDGF receptors, imatinib treatment reduced the rate of obliterative processes in this model. Our own study group indicated the role of elevated growth factors and receptors in the pathogenesis of CLAD (Suesskind-Schwendi et al., 2017). In a previous study, our group also showed in an orthotopic lung transplantation model in the rat that early application of imatinib decreased the number of animals with BO (Suesskind-Schwendi et al., 2013b) whereas monotherapy with vatalanib significantly reduced vasculopathy in the present study. These findings suggest a synergistic effect of imatinib and vatalanib with imatinib mainly affecting the bronchioles and vatalanib the vessels. Similarly, the additional application of a mTORI (everolimus) to imatinib further reduced the number of animals with vasculopathy (Suesskind-Schwendi et al., 2013b). Moreover, imatinib reduced collagen deposit after lung transplantation (Suesskind-Schwendi et al., 2013b), indicating an anti-fibrotic effect of imatinib.

The effect of imatinib and vatalanib, inhibitors of PDGFR and VEGFR, on the development of CLAD could lead to the hypothesis that by inhibiting tyrosine kinase activity after ligand binding, they would also influence their expression. As demonstrated previously, the treatment with nintedanib significantly lowered the expression of PDGF-A, VEGF-A and their receptors (Suesskind-Schwendi et al., 2017). Nintedanib is also a tyrosine kinase inhibitor, inhibiting VEGFR-1, -2 and -3, PDGFR- $\alpha$  and - $\beta$  and fibroblast growth factor receptor-1, -2 and -3 (FGFR-1, -2 and -3) (Hilberg et al., 2008). Therefore we hypothesized that imatinib and vatalanib have a similar effect on the expression of growth factors and their receptors. Contrary to our assumption, immunohistological analysis showed no conclusive difference in the expression of PDGF-A, PDGFR- $\alpha$ , and VEGFR-2 between allografts in the treatment groups and the control group. Although there was generally lower expression of receptors and ligands in the treated allografts, a significant reduction as compared to the control group was only found in the vata/ima/RAD group on POD 60. These findings correlate with the

## Prevention of chronic lung allograft dysfunction

results of Tikkanen et al. and Nykänen et al., who observed no effect of vatalanib and imatinib on the expression of VEGF or PDGF-A. Unfortunately, they do not refer to the expression of their receptors (Nykanen et al., 2005; Tikkanen et al., 2006). In addition, it is possible that receptor and ligand expression declines in the control group due to progressive fibroproliferation. As a consequence, fewer cells can produce proteins such as growth factors, whereas the allografts of the treatment groups exhibited nearly unaffected lung tissue with living cells. As we described previously, blocking only growth factors with nintedanib did not have any effect on the expression of CLAD and resulted in the same histologic features as detected in the control group (Suesskind-Schwendi et al., 2017). This may be the reason for the different findings concerning the significance between treatment group and control group in the present study.

Unfortunately, immunohistology did not show results conclusive enough to understand the mechanism of action of vatalanib and imatinib in our model. In addition, a RT-PCR on POD 20 and 60 showed no significant differences in the expression of PDGFA, VEGF-A, and their receptors between allografts, non-transplanted and naïve lungs (data not shown). How can we explain this lack of decisive outcomes?

First of all, we have to distinguish between the active and non-active form of the receptors concerned. As imatinib and vatalanib are RTKIs, they prevent the autophosphorylation of their targets PDGFR and VEGFR and therefore their activation (Heldin et al., 2002). We have to take into account that while we cannot discern convincing results regarding the non-active forms of PDGFR and VEGFR in immunohistochemistry, possible differences between the study groups may occur when investigating the activated forms. To examine these phosphorylated forms, further studies are warranted. In contrast, many studies suggest that even by testing specific phosphorylated proteins may not lead to additional benefit (Mandell, 2008; Brumbaugh et al., 2011).

Furthermore, we did not perform synergetic lung transplantations in the present study. As a consequence, we do not know the expression of the receptors in genetically identical transplanted lungs. Does the transplantation itself lead to an up-regulation of PDGFR and VEGFR in the lung? Similarly, Biallas et al. found an up-regulation of the  $\alpha 9$  subunit of the nicotinic acetylcholine receptor up to POD 4 following isogenic left lung transplantation in a Lewis rat strain combination (Biallas et al., 2007). However, this could be due to general activation of inflammatory mediators (Biallas et al., 2007) at an early time point and may already be overcome on POD 20 or 60. Indeed, the previous work of Hirt et al. showed no sign of rejection in syngrafts of WKY until POD 100 (Hirt et al., 1999).

The results may therefore also lead to the assumption that imatinib and vatalanib reduce CLAD via other mechanisms.

Supporting this theory, we had suggested that

nintedanib would reduce the development of chronic bronchiolar rejection and interstitial fibrosis due to its inhibition of growth factors (Suesskind-Schwendi et al., 2017). The lack of efficacy in the prevention of CLAD suggests that the antifibrotic effect of imatinib and vatalanib is mediated through other pathways than blocking VEGFR and PDGFR.

One can also conclude that PDGF and VEGF may be expressed already in an earlier phase of BO. For instance, Tikkanen found an elevated level of VEGF on POD 10 (Tikkanen et al., 2006).

In addition, PDGF and VEGF could only play a minor role, if any, in this experimental model of BO. Other factors may in contrast be responsible for the fibroproliferative process and the effect of imatinib and vatalanib could thus also be due to alternative ways. For instance, imatinib also blocks c-Kit, Abl, Bcr-Abl and c-Fms in addition to the PDGF-receptors (Buchdunger et al., 2002; Dewar et al., 2005) while vatalanib likewise inhibits c-Kit and c-Fms (Wood et al., 2000). Supporting this theory, c-Kit-positive mast cells have already been found to play a possible role in BO (Fuehrer et al., 2009). Ding et al. found elevated levels of its ligand, stem cell factor (SCF), in bleomycin-induced pulmonary fibrosis in mice (Ding et al., 2013), indicating a potential role in the pathogenesis of fibrosis. Furthermore, TGF- $\beta$  is known to mediate fibrotic processes through stimulating collagen deposit (Bonner, 2004) and was also shown to promote fibroblast proliferation via activation of c-Abl (Daniels et al., 2004; Wang et al., 2005). C-Abl itself was shown to promote collagen production that could be inhibited by imatinib (Wang et al., 2005; Pannu et al., 2008). Watanabe et al. found that by inhibiting c-Abl, imatinib reduced the number of fibrocytes and suppressed the differentiation of CD14<sup>+</sup> monocytes and the fibrocyte migration in a murine heterotopic tracheal model of BO (Watanabe et al., 2017). All these findings suggest that other pathways than VEGFR and PDGFR activation may play a crucial role in the pathogenesis of BO.

As described previously, the co-application of everolimus and imatinib significantly reduces acute vascular and bronchiolar rejection on POD 20 in our left orthotopic rat LTx model (Suesskind-Schwendi et al., 2013b). In contrast, the present study did not show reduced acute rejection after treatment with two RTKIs and everolimus. We speculated that the different time of application – on POD 7 in the present study versus POD 14 – may have contributed to the divergent results. Several studies indicate pulmonary toxicity with the administration of mTORIs, like everolimus, including interstitial pneumonitis and BO (Parada et al., 2011; Solazzo et al., 2016; Fine and Kushwaha, 2016). Despite the unaffected clinical appearance of our rats, we can assume that the early application of everolimus may influence the histological presentation and lead to enhanced inflammatory infiltration. On the other hand, one can also assume that maybe a more sensitive classification system would show more distinct differences. For instance, counting CD4<sup>+</sup> and CD8<sup>+</sup> cells

could help to improve accuracy in grading acute rejection (Tikkanen et al., 2006). However, the additional application of everolimus showed significantly superior results in the prevention of BO in our rats as compared to the combined treatment with vatalanib and imatinib, indicating a synergic antifibrotic and antiproliferative effect of the mTORI and the RTKIs as already described earlier (Suesskind-Schwendi et al., 2013b).

In order to assess possible side effects, we also compared the postoperative weight development in all study groups. Interestingly, in spite of significantly reducing BO and vasculopathy, the vata/ima-treated animals suffered from secondary weight loss at the end of the tested time period. Moreover, the additional administration of everolimus even prevented the animals from regaining their preoperative weight altogether. One could assume that the application of a VEGFR-inhibitor would disturb angiogenesis. Nevertheless, research indicates the influence of VEGF on vascular remodelling, but not on neo-vascularization (Belperio et al., 2005; Tikkanen et al., 2006). In spite of generally being well-tolerated drugs (Wood et al., 2000; Manley et al., 2002; Scott et al., 2007), vatalanib and imatinib are both known to cause diarrhoea and nausea (Guilhot, 2004; Joensuu et al., 2011). Hence, maybe the combination of both drugs leads to secondary weight loss after long term application. The decrease in body weight by treatment with everolimus has already been described earlier (Suesskind-Schwendi et al., 2013a). On top of that, animals also exhibited pale mucous membranes. Furthermore, wound healing disorders (van der Vliet et al., 2006) and pulmonary toxicity (Expósito et al., 2008; Otton et al., 2009; Almeida et al., 2018) have been described during therapy with everolimus. However, in the present study, we did not detect any wound healing disorders when harvesting the lungs nor did the animals show abnormal behaviour following lung transplantation. Nevertheless, we cannot exclude pulmonary toxicity or wound healing impairment in our rats. In future studies, a dose reduction of everolimus should be taken into consideration, presumably reducing negative side effects on wound healing and postoperative weight development (Fine and Kushwaha, 2016).

The transferability to humans is limited due to the persistence of inflammation (Suesskind-Schwendi et al., 2013b). Nevertheless, BO features like eccentric scarring, protruding granulation tissue into the bronchiolar lumen, peribronchiolar scarring and the loss of bronchiolar epithelium, fragmentation of the smooth muscle layer surrounding the small airways and chronic vascular rejection which often accompanies BO are commonly to be found (Matsumura et al., 1995; Hirt et al., 1999; Süßkind-Schwendi et al., 2012). Moreover, despite being discussed controversially in the literature (Lama et al., 2017), the left orthotopic lung transplantation from F344 to WKY has been shown as a relevant model to study BO (Süßkind-Schwendi et al., 2012; Jungraithmayr et al., 2013).

Furthermore, the possibly different roles of growth

factors in rats has to be taken into account. For instance, PDGFR- $\alpha$  underlies other regulation mechanism in humans (Bonner, 2004). Future studies are imperative to determine the role of proteins other than PDGF-A and VEGF, such as c-Abl, c-KIT, c-Fms and TGF- $\beta$ , in the pathogenesis of CLAD and in our animal model of LTx. However, our model allowed studying interstitial lung fibrosis, the development of BO after LTx and possible treatment options for CLAD (Hirt et al., 1999; Süßkind-Schwendi et al., 2012, 2013b). Regarding safety concerns, both vatalanib and imatinib are generally well tolerated in humans (Joensuu et al., 2011; Iqbal and Iqbal, 2014). Moreover, everolimus has already been successfully used in clinical trials as a maintenance therapy after lung transplantation in combination with cyclosporine (Snell et al., 2006; Glanville et al., 2015), whereas clinical data for cyclosporine-free regimen are scarce (Ivulich et al., 2018). Our study provides a novel approach for a possible combination therapy consisting of everolimus and RTKIs and thus offers new possibilities in the treatment of CLAD and in avoiding current side effects by other immunosuppressive strategies.

In conclusion, the dual therapy with the RTKIs imatinib and vatalanib significantly reduces not only chronic vascular and bronchiolar rejection but also interstitial fibrosis after rat LTx. This effect is synergistically increased by adding the mTORI everolimus. Therefore, the combination of mTORI and RTKIs might be a possible strategy in the prevention of BO and chronic lung allograft rejection. Finally, our rat LTx model may contribute to further understand and find treatment options for RAS as a novel form of CLAD.

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