Table 1 Demographic data of study subjects.

Characteristics	n	%	Mean ± SD (minimum–maximum)
Number	46		
Age (years)			50.5 ± 14.9 (16–85)
Gender			
Male	29	63	
Female	17	37	
Clinical symptoms			
None	12	26	
Dyspnea on effort	25	54	
Cough	18	39	
Others	3	7	
Arterial blood gas analysis			
PaO_2 (Torr)	46		69.3 ± 13.8 (40.6–99.7)
$PaCO_2$ (Torr)	46		38.4 ± 4.3 (28.6–47.5)
$AaDO_2$ (Torr) [a]	46		33.1 ± 14.7 (2.6–60.2)
Pulmonary function test			
%VC	38		90.4 ± 14.9 (55.6–116.9)
$\%FEV_{1.0}$	35		83.3 ± 10.3 (67.8–126.5)
$\%DL_{CO}$	30		61.2 ± 23.4 (8.4–99.7)
Disease severity score	46		2.5 ± 1.1 (1–5)
Serum markers			
KL-6 (U/ml)	45		4431 ± 4707 (147–23,000)
SP-D (ng/ml)	45		184.4 ± 129.4 (17.3–498)

Definition of abbreviations: $AaDO_2$ = alveolar–arterial oxygen difference; GM-CSF = granulocyte/macrophage-colony stimulating factor; NS = not significant.

P_B; barometric pressure measured by local observatories, P_{H2O}; partial pressure of water vapor in inspired air (assumed to be 47 Torr), F_iO_2; fractional concentration of oxygen in dry gas (assumed to be 0.21), $PaCO_2$; partial pressure of arterial CO_2 measured in arterial blood, R; respiratory quotient (assumed to be 0.8), PaO_2; partial pressure of arterial oxygen measured in arterial blood.

The serum concentration of GM-CSF autoantibody with λ isotype (Igλ-GMAb) was measured by GMAb ELISA(2) using recombinant GM-CSF (Leukine™, Immunex Co., WA) for antigen capture and peroxidase labeled anti-lambda antibody (Bethyl Laboratories Inc., Montgomery, TX) for detecting antibody. The serum concentration of whole GM-CSF autoantibody was measured using peroxidase labeled anti-whole immunoglobulin antibody as the detecting antibody. For the standard, monoclonal human GM-CSF antibody (kindly provided from Dr. Kenzo Takada, Evec Co. Ltd., Sapporo, Japan) with lambda isotype was used. Kappa/lambda ratio was calculated by the following equation.

kappa/lambda ratio = (whole immunoglobulin concentration − Igλ-GMAb) / Igλ-GMAb.

[a] Calculated using the following equation: $AaDO_2 = (P_B - P_{H2O}) \times F_iO_2 - R + \{PaCO_2 \times F_iO_2 \times (1 - R) / R\} - PaO_2$.

of carbon monoxide ($\%DL_{CO}$) demonstrated moderate correlations with the degree of hypoxemia.

3.3. Correlation between κ/λ ratios of GMAb and disease severity

Another important property of GMAb that might affect the blocking capacity of GM-CSF bioactivity is the binding of paratopes, consisting of some parts of variable regions in both light and heavy chains. As the light chain κ/λ ratio is known to change in some autoimmune diseases, we focused on the ratio of GMAb in aPAP patients as compared to IVIG. The mean κ/λ ratio in total IgG was similar between aPAP patients and IVIG with values of 1.23 ± 0.60 and 1.41 ± 0.27, respectively (Fig. 2A), which were comparable to the data in normal subjects reported previously [5,6]. On the other hand, the mean κ/λ ratio of GMAb was higher in IVIG (3.60 ± 0.71) than in aPAP patients (2.12 ± 1.37) (P b 0.01, Fig. 2B). The κ/λ ratio of GMAb was strikingly higher in both aPAP patients and IVIG than the κ/λ ratio of the corresponding total IgG (Fig. 2C, P b 0.001 and P b 0.05, respectively). The κ/λ ratio of GMAb in patients was highly variable, ranging from 0.002 to 5.75 as compared with total IgG (Fig. 2C), suggesting that GMAb-producing B cells or plasma cells are polyclonally activated. The κ/λ ratio of GMAb was significantly higher than the ratio of the total IgG (P b 0.001).

When we evaluated the correlation between the κ/λ ratio and the disease severity parameters in aPAP patients, we found that the κ/λ ratio was moderately correlated with PaO_2 (Fig. 3A, $\rho = 0.411$, P b 0.01) and inversely and moderately correlated with both $AaDO_2$ (Fig. 3B, $\rho = -0.484$, P b 0.001) and DSS (Fig. 3C, $\rho = -0.378$, P b 0.01). When the κ-type or λ-type GMAbs were compared between patients with mild and severe aPAP (PaO_2 higher and lower than 67.5 mm Hg, respectively), the κ/λ ratio of GMAb was significantly higher in the former group than the latter (Fig. 4A, P b 0.05). Interestingly, the proportion of λ-type GMAb per whole λ-type IgG was significantly higher in the latter group than the former, whereas the proportion of κ-type GMAb per total κ-type IgG was equal between the two groups (Figs. 4B (P b 0.05) and 4C). This result suggested that λ-type GMAb predominantly increased in the severe aPAP.

3.4. Correlation between κ/λ ratio of GMAb and other parameters

As both serum markers and pulmonary functions are widely accepted as disease severity parameters, we evaluated whether serum κ/λ ratio correlated with these parameters. The κ/λ ratio weakly to moderately correlated with KL-6 ($\rho = -0.297$, P b 0.05) and SP-D ($\rho = -0.360$, P b 0.05), but not with %VC and $\%DL_{CO}$ (Supplemental Table 2). Neither the concentrations nor binding avidities of GMAb correlated with the disease severity or the κ/λ ratio of GMAb (Supplemental Table 2). Then, the patients were classified into two groups; group 1: patients with high binding avidity less than 0.24 nM (the mean + 2SD for IVIG) and group 2: patients with low binding avidity over 0.24 nM. In group 1, the serum κ/λ ratio was similar between severe (b 67.5 mm Hg in PaO_2) and mild (≧67.5 mm Hg in PaO_2) disease, whereas, in group 2, the κ/λ ratio was higher in mild patients (2.85 + 1.60) than severe (1.43 + 1.08) patients (P b 0.05, Fig. 4D). Thus, the association of serum κ/λ ratio with disease severity was obvious in patients with low avidity GMAb but not in patients with high avidity GMAb.

4. Discussion

In this study, we demonstrated that the light-chain ratio (κ/λ ratio) of GMAb in patients with aPAP and IVIG was higher than total IgG, and the κ/λ ratio was significantly correlated with

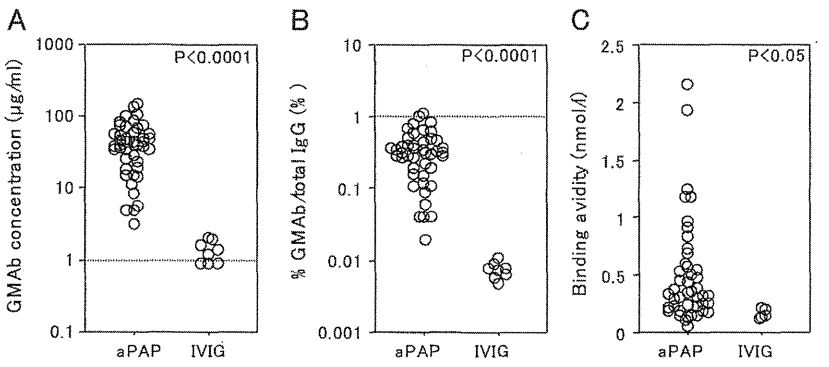

Figure 1 (A) Comparison of GMAb between patients with aPAP (n = 46) and IVIG preparations (n = 8). GMAb concentrations were significantly higher in aPAP patients than IVIG preparations (P b 0.0001, Mann–Whitney U-test). (B) Comparison of the percent GMAb per total IgG between patients with aPAP (n = 46) and IVIG preparations (n = 8). Percent GMAb per total IgG was significantly higher in the aPAP group than IVIG (P b 0.0001, Mann–Whitney U-test). (C) Comparison of the binding avidity of GMAb between patients with aPAP and IVIG preparations. The binding avidity of GMAb was significantly higher in IVIG than that of the aPAP group (P b 0.05, Mann–Whitney U-test).

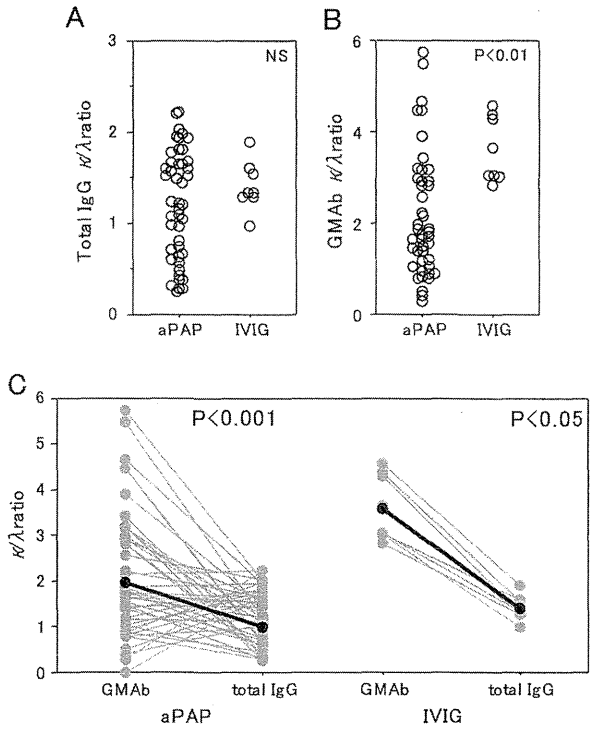

Figure 2 Comparison of the κ/λ ratio of total IgG (A) and GMAb (B) between aPAP patients and IVIG preparations. The mean κ/λ ratio in total IgG was similar between aPAP patients and IVIG preparations, whereas the κ/λ ratio for GMAbs was significantly higher in IVIG as compared to aPAP patients (P b 0.05, Mann–Whitney U-test). (C) Comparison of the κ/λ ratio as compared between GMAb and total IgG for each individual sample (gray lines) and the average (black line) in aPAP patients (left panel) and IVIG preparations (right panel). For both aPAP patients and IVIG preparations, the κ/λ ratio of GMAb was significantly higher than total IgG (P b 0.001 and P b 0.05, respectively, Wilcoxon signed-rank test).

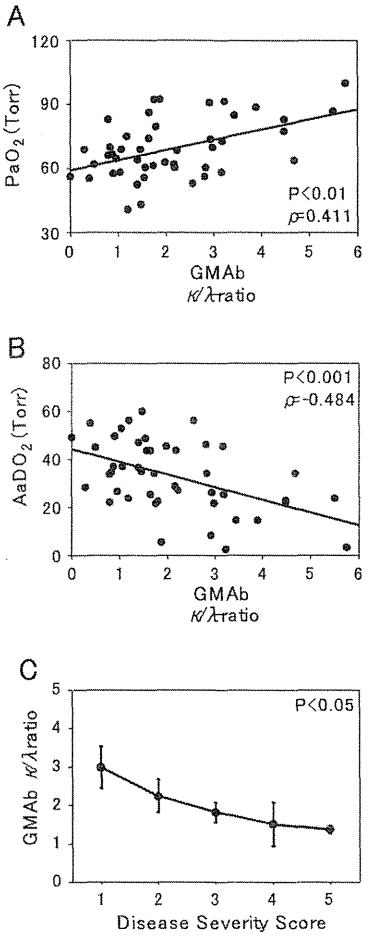

Figure 3 Correlations between the κ/λ ratio of GMAb and clinical parameters: (A) PaO_2 and (B) $AaDO_2$. Each combination was highly correlated (P b 0.01, R = 0.411; P b 0.001, R = −0.484, respectively). All data were evaluated by using Spearman's rank correlation coefficient. (C) The κ/λ ratio as a function of DSS. With increasing DSS values, the κ/λ ratio of GMAb gradually trended lower and significant differentiation was observed between the κ/λ ratios and DSS values (P b 0.05, Kruskal–Wallis test).

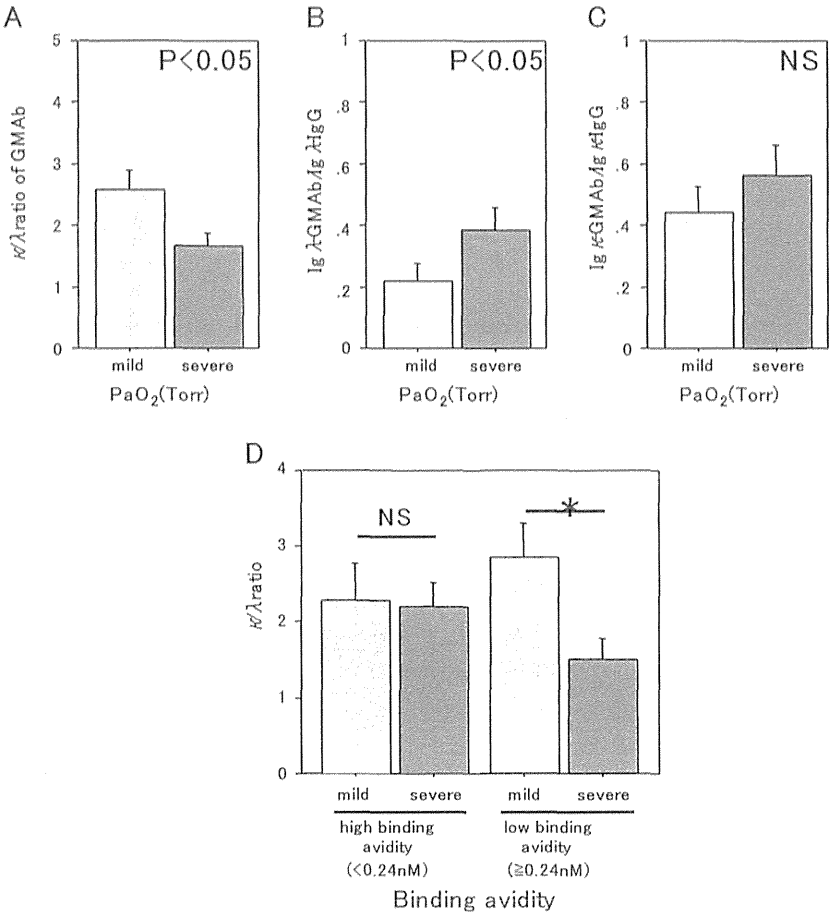

Figure 4 Comparison of the κ/λ ratio of GMAb (A) and the κ- (B) and λ-type (C) GMAb for each light-chain type of IgG between patients with high PaO_2 (mild disease) and low PaO_2 (severe disease), with a median value of 67.5 Torr. The κ/λ ratio of GMAb was significantly higher in the high PaO_2 group as compared to the low PaO_2 group (*P* b 0.05, Mann–Whitney *U*-test). The ratio of λ-type GMAb per whole λ-type IgG was higher in the severe group as compared to the mild group (*P* b 0.05, Mann–Whitney *U*-test); however, the ratio of κ-type GMAb per whole κ-type IgG did not show significant difference between high and low PaO_2 groups. (D) Comparison of the κ/λ ratio in GMAb between two groups segregated by binding avidity of GMAb, patients with high binding avidity less than 0.24 nM (the mean + 2SD for IVIG, n = 16) and patients with low binding avidity over 0.24 nM (n = 30). In the former group, the serum κ/λ ratio of GMAb was similar between severe and mild disease, whereas, in the latter, the κ/λ ratio was higher in the mild disease than that in severe disease (**P* b 0.05, Mann–Whitney *U*-test).

disease severity (i.e., the degree of hypoxemia). As previous reports and the present data indicated that the serum concentration of GMAb and the binding avidity of GMAb had no correlation with disease severity [2,18,19], the present study is the first to suggest that the light chain isotype usage in GMAb might be associated with disease severity.

GMAb has been widely accepted as the causative pathogen of aPAP [1,20] because GMAb showed consistently high concentrations in both the lungs and blood with strong binding avidities to GM-CSF, and its injection is known to reproduce PAP in nonhuman primates [21]. In the late 90s, Svenson et al. demonstrated that a high avidity but low concentration of GMAb was ubiquitously present in every batch of pharmaceutically-prepared immunoglobulin (IVIG), and such antibodies neutralized GM-CSF bioactivity [22]. This suggested the presence of GMAb in the sera of normal subjects because IVIG is the product from pooled sera of normal donors. More recently, we confirmed that a low concentration of GMAb was ubiquitously present in the sera of normal subjects, showing similar biological properties in aPAP [23]. In this regard, we first report the difference in properties of GMAb between IVIG and aPAP patients: the κ/λ ratio of GMAb is significantly higher in IVIG than that in aPAP patients.

Considering that GMAbs consistently, but not exceedingly, exist in the sera of healthy subjects, membrane $GMAb^+$ B cell clones seemed to be stimulated by intrinsic GM-CSF in healthy subjects. As κ-chain was predominant in GMAbs in IVIG, it is likely that some membrane $GMAb^+$ B cell clones in healthy subjects underwent class switching without undergoing apoptosis or receptor editing. On the other hand, increased λ-type GMAb in severely affected aPAP patients might be due to the selective expansion of λ-type membrane $GMAb^+$ B clones in the peripheral lymphatic tissues.

Recently, Wang et al. investigated 19 monoclonal antibodies against GM-CSF established from six patients with aPAP

and demonstrated that the affinity of each antibody correlated with the neutralizing capacity [24]. In this regard, it is plausible that the binding avidity might be associated with the κ/λ ratio, and thus, correlated with disease severity through the neutralizing capacity. However, our present data excluded this possibility (i.e., the binding avidity correlated with neither the κ/λ ratio nor the disease severity).

In our previous reports, the correlation coefficients between the disease severity and the serum markers of LDH, CEA, SP-A, KL-6, and SP-D were 0.29–0.53 [2]. The correlation coefficient of the κ/λ ratio with the disease severity ($\rho = -0.378$) was comparable with the coefficients described above. It is of interest for future studies to assess how closely the κ/λ ratio of GMAb correlates with the longitudinal change of disease severity and how precisely the κ/λ ratio predicts the prognosis of aPAP.

In patients with aPAP, the κ/λ ratio of GMAb was remarkably variable compared with total IgG. The variability may be partially due to the frequency of antigen stimulation, receptor editing, or clonal expansion in the secondary lymphatic organs. As λ chain usage was shown to be correlated with disease severity in this study, it is plausible to consider that some critical sequences for binding with GM-CSF are coded in the λ chain of GMAb. Moreover, as a light chain binds antigens with a corresponding heavy chain, such critical sequences may be also coded in heavy chains, especially in CDR3 regions, which are known to be major paratopes. These molecular issues remain to be investigated in future studies.

5. Conclusion

Investigation of the light-chain isotype ratio of GMAb in patients with aPAP and healthy subjects revealed that the κ chain is predominant in general, but the proportion of the λ chain increases as disease severity increases in patients with aPAP. This study may provide an important clue as to the mechanism for the production of GMAb. For future study, it is intriguing to investigate whether the disease progression is associated with the serum κ/λ ratio retrospectively and prospectively.

Conflict of interest statement

None of the authors have a financial relationship with a commercial entity with an interest in the subject of this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2013.10.002.

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Serum VEGF-D concentration as a biomarker of lymphangioleiomyomatosis severity and treatment response: a prospective analysis of the Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus (MILES) trial

Lisa R Young, MD, Hye-Seung Lee, PhD, Yoshikazu Inoue, MD, Joel Moss, MD, Lianne G Singer, MD, Prof. Charlie Strange, MD, Prof. Koh Nakata, MD, Prof. Alan F Barker, MD, Jeffrey T Chapman, MD, Prof. Mark L Brantly, MD, Prof. James M Stocks, MD, Prof. Kevin K Brown, MD, Prof. Joseph P Lynch 3rd, MD, Hilary J Goldberg, MD, Prof. Gregory P Downey, MD, Jeffrey J Swigris, DO, Angelo M Taveira-DaSilva, MD, Prof. Jeffrey P Krischer, PhD, Prof. Bruce C Trapnell, MD, and **Prof. Francis X McCormack, MD for the MILES Trial Group**

Divisions of Pediatric Pulmonary Medicine and Allergy, Pulmonary and Critical Care, Vanderbilt University School of Medicine, Nashville, TN, USA (L R Young MD); Pediatrics Epidemiology Center, Department of Pediatrics, University of South Florida, Tampa, FL, USA (H-S Lee PhD, Prof J P Krischer PhD); Department of Diffuse Lung Diseases and Respiratory Failure, Clinical Research Center, National Hospital Organization Kinki-Chuo Chest Medical Center, Osaka, Japan (Y Inoue MD); National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, USA (J Moss MD, A M Taveira-DaSilva MD); Division of Respirology, Department of Medicine, University of Toronto, Toronto, ON, Canada (L G Singer MD); Division of Pulmonary and Critical Care Medicine, Medical University of South Carolina, Charleston, SC, USA (Prof C Strange MD); Bioscience Medical Research Center, Niigata University Medical and Dental Hospital, Niigata, Japan (Prof K Nakata MD); Division of Pulmonary and Critical Care, Oregon Health and Science University, Portland, OR, USA (Prof A F Barker MD); Respiratory Institute, Cleveland Clinic Foundation, Cleveland, OH, USA (J T Chapman MD); Division of Pulmonary and Critical Care, University of Florida, Gainesville, FL, USA (Prof M L Brantly MD); University of Texas Health Science Center at Tyler, Tyler, TX, USA (Prof J M Stocks MD); Department of Medicine, National Jewish Health, Denver, CO, USA (Prof K K Brown MD, Prof G P Downey MD, J J Swigris DO); Division of Pulmonary and Critical Care Medicine, University of California, Los Angeles, Los Angeles, CA, USA (Prof J P Lynch 3rd MD); Division of Pulmonary and Critical Care, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA (H J Goldberg MD); Division of Pulmonary Biology and Perinatal Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA (Prof B C Trapnell MD); and Division of Pulmonary, Critical Care, and Sleep Medicine, University of Cincinnati College of Medicine, Cincinnati, OH, USA (Prof B C Trapnell, Prof F X McCormack MD)

Correspondence to: Dr Lisa R Young, Division of Pulmonary Medicine, Vanderbilt University School of Medicine, 2200 Children's Way, Nashville, TN 37232-9500, USA, lisa.young@vanderbilt.edu or Prof Francis X McCormack, Division of Pulmonary, Critical Care, and Sleep Medicine, University of Cincinnati College of Medicine, 231 Albert Sabin Way, Cincinnati, OH 45267-0564, USA, frank.mccormack@uc.edu.

Contributors

FXM and LRY were responsible for all aspects of the study, including concept and design. H-SL and JPK were responsible for statistical analysis. All authors contributed to data collection, intellectual content, and writing of the Article.

Conflicts of interest

LRY and FXM are coinventors on a patent for the use of VEGF-D as a diagnostic test (all potential personal royalties were waived before issuance of the patent) and members of the advisory board of the LAM Foundation. LGS received a peer-reviewed research grant from Pfizer (cosponsored with the Ontario Lung Association) for studies unrelated to this Article. All other authors declare that they have no conflicts of interest.

Summary

Background—VEGF-D is a lymphangiogenic growth factor that has a key role in tumour metastasis. Serum VEGF-D concentrations are increased in most patients with lymphangioleiomyomatosis, a rare neoplasm associated with mTOR-activating tuberous sclerosis gene mutations, lymphadenopathy, metastatic spread, and pulmonary cyst formation. We used data from the Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus (MILES) trial to assess the usefulness of serum VEGF-D concentration as a marker of severity and therapeutic response to sirolimus in patients with lymphangioleiomyomatosis.

Methods—In the MILES trial, patients with lymphangioleiomyomatosis who had forced expiratory volume in 1 second (FEV_1) of 70% or less of predicted were randomly assigned (1:1) to 12 months masked treatment with sirolimus or placebo. Serum VEGF-D concentrations were measured at baseline, 6 months, and 12 months. We used a linear regression model to assess associations of baseline VEGF-D concentrations with markers of disease severity, and a linear mixed effects model to assess the associations of VEGF-D concentrations with between-group differences in clinical, physiological, and patient-reported outcomes.

Findings—We included 42 patients from the placebo group and 45 from the sirolimus group in our analysis. Baseline VEGF-D concentrations in individual patients varied from 0·34 ng/mL to 16·7 ng/mL. Baseline VEGF-D concentrations were higher in patients who needed supplemental oxygen than in those who did not need supplemental oxygen (1·7 ng/mL [IQR 0·99–3·36] *vs* 0·84 ng/mL [0·52–1·39]; p<0·0001) and in those who had a bronchodilator response than in those who did not (2·01 ng/mL [0·99–2·86] *vs* 1·00 ng/mL [0·61–2·15]; 0·0273). Median serum VEGF-D concentrations were similar at baseline in the sirolimus and placebo groups, and fell from baseline at 6 and 12 months in the sirolimus group but remained roughly stable in the placebo group. Each one-unit increase in baseline log(VEGF-D) was associated with a between-group difference in baseline-to-12-month FEV_1 change of 134 mL (p=0·0007). In the sirolimus group, improvement in baseline-to-12-month FEV_1 occurred in 15 of 23 (65%) VEGF-D responders (ie, those in whom baseline-to-12-month VEGF-D concentrations decreased by more than they did in any patients in the placebo group) and four of 15 (27%) VEGF-D non-responders (p=0·0448).

Interpretation—Serum VEGF-D is a biologically plausible and useful biomarker in lymphangioleiomyomatosis that correlates with disease severity and treatment response. Measurement of serum VEGF-D concentrations could inform the risk–benefit analysis of sirolimus therapy in patients with lymphangioleiomyomatosis and reduce the numbers of patients needed for clinical trials.

Introduction

Lymphangioleiomyomatosis is a rare neoplasm that results in progressive cystic lung disease and respiratory failure,[1] and affects women almost exclusively. Disease can occur in two forms: sporadic lymphangioleiomyomatosis or as tuberous-sclerosis-complex-associated lymphangioleio-myomatosis.[2] In addition to round, uniform, thin-walled cysts distributed randomly throughout the lungs, patients with lymphangioleiomyomatosis can have renal angiomyolipomas and complications resulting in lymphatic infiltration and obstruction, including lymphadenopathy, cystic lymphangioleiomyomas, and chylous fluid collections in the abdomen and chest.[3] Lymphangioleiomyomatosis is deemed a low-grade metastasising neoplasm,[1,4] and spread through lymphatic channels and recurrence after lung transplantation have been noted.[5] Inappropriate lymphangiogenesis and abundant lymphatic channel formation in lymphangioleiomyomatosis lung lesions seem to be driven by expression of VEGF-C, VEGF-D, and their cognate receptor, VEGFR3.[6,7]

VEGF-D is a lymphangiogenic growth factor that has a key role in tumour metastasis.[8] Serum VEGF-D concentrations are increased in most patients with

lymphangioleiomyomatosis, but use of these concentrations as a marker of disease severity, progression, and response to treatment has not been established.[9–12] Serum VEGF-D concentrations are normal in women with other cystic lung diseases, such as pulmonary Langerhan's cell histiocytosis, emphysema, follicular bronchiolitis, lymphoid interstitial pneumonia, and Birt-Hogg-Dubé syndrome.[10,13] We have previously reported that serum concentrations of 0·8 ng/mL distinguished lymphangioleiomyomatosis from mimics that present as thin-walled cysts visible on high-resolution CT (sensitivity 73%, specificity 100%); at a cutoff of 0·6 ng/mL, diagnostic sensitivity of serum VEGF-D was 84% and specificity was 98%.[10] VEGF-D concentrations are increased in women with tuberous-sclerosis-complex-associated disease to an extent greater than that in women with sporadic lymphangioleiomyomatosis.[10] Furthermore, in a cross-sectional study of women with tuberous sclerosis complex, a serum concentration of 0·8 ng/mL effectively discriminated between patients with and without cystic changes on chest CT.[10]

The mTOR signalling pathway is constitutively activated in lymphangioleiomyomatosis because of loss of *TSC1* or *TSC2* function, resulting in dysregulated cell growth, motility, and survival.[4] Activation of the mTOR pathway is blocked by sirolimus, and several studies have shown the efficacy of mTOR inhibition in the treatment of manifestations of tuberous sclerosis complex, including angiomyolipomas,[14–16] subependymal giant cell astrocytomas,[17] and, most recently, lymphangioleiomyomatosis.[18–20] The Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus (MILES) trial[18] was an international, randomised, double-blind, placebo-controlled trial, which showed that sirolimus stabilised lung function and was associated with improvements in quality of life and functional performance. In the trial, mean serum concentrations of VEGF-D at baseline were similar in the placebo and sirolimus groups; concentrations fell during 12 months in the sirolimus group but remained stable in the placebo group.[18] We analysed data from the MILES trial to establish the usefulness of serum VEGF-D concentration as a marker of severity and response to treatment in lymphangioleiomyomatosis. Some of our results have been previously reported in abstract form.[21]

Methods

Background and study population

Our study population comprised participants in the MILES trial (who were enrolled at one site in Canada, two sites in Japan, and ten sites in the USA) who provided more than one serum sample drawn during the trial (ClinicalTrials. gov number, NCT00414648).[18] Briefly, inclusion criteria for the MILES Trial were forced expiratory volume in 1 s (FEV_1) of 70% or less of predicted (ie, moderate-to-severe lung disease based on FEV_1), a definite diagnosis of lymphangioleiomyomatosis based on compatible cystic changes on chest CT and either biopsy confirmation or a history of angiomyolipomas, tuberous sclerosis complex, or chylothorax. Late in the enrolment period, a serum VEGF-D concentration of 0·8 ng/mL or greater was added as an inclusion criterion. Participants were randomly assigned to receive dose-adjusted, double-blinded sirolimus or placebo for 1 year. At that point, sirolimus or placebo was discontinued and patients were followed up for an additional year. Only data from the first year were included in our analysis. Pulmonary function testing and patient-reported outcomes were gathered serially. The MILES trial was approved by the institutional review boards at the University of Cincinnati, Cincinnati Children's Hospital Medical Center, and all participating sites. Analysis of VEGF-D as a biomarker was prespecified in the MILES trial protocol, and VEGF-D measurements were submitted to the data coordinating centre before reporting of the primary outcome in the MILES trial.

Procedures

Serum VEGF-D concentrations were measured at baseline, 6 months, and 12 months. VEGF-D testing was done in a lab that met College of American Pathologists and Clinical Laboratory Improvement Amendments standards, by technicians masked to treatment assignment and clinical data. Reagents included in the Quantikine Human VEGF-D Immunoassay (R&D Systems, Minneapolis, MN, USA) were used in measurements. Additional methods and performance characteristics of the test are provided in the appendix. Pulmonary function testing methodology from the MILES trial has been previously reported.[18]

Statistical analysis

We used a linear regression model to assess the relation between baseline VEGF-D concentrations and results from pulmonary function tests, 6 min walk tests, and patient-reported outcomes. The Wilcoxon rank-sum test was used to test for differences in baseline serum VEGF-D concentrations between subgroups defined by the historical presence or absence of complicating disease features (eg, angiomyolipoma, pneumothorax). We used Fisher's exact test to test for differences in proportions.

We used linear models to assess whether or not the association between serum VEGF-D concentrations and pulmonary measures differed between treatment groups. Although patients were randomly assigned to either placebo or sirolimus in the MILES trial, we adjusted for age and height in the model to account for differences between participants at the North American and Japanese sites. We examined the interaction between serum VEGF-D concentrations at baseline and treatment assignment on pulmonary function tests, 6 min walk tests, or patient-reported outcomes. General linear models were used to assess absolute changes from baseline to 12 months, and linear mixed-effects models were used to assess changes with time. In the linear mixed-effects model analysis, we used the Kenward-Roger correction to adjust the degree of freedom to improve performance when data were missing.[22] Log-transformed VEGF-D concentrations were used to better fit the linear models. We deemed p values less than 0·05 to be significant. All reported p values are two-sided and unadjusted for multiple testing. We did all statistical analyses in SAS, version 9.2.

Role of the funding source

The funding sources had no role in study design, data analysis, the decision to publish, or writing of this Article. Raw data were electronically submitted to the data management coordinating centre and accessed by H-SL and JPK. The corresponding author had full access to all the data and the final responsibility to submit for publication.

Results

At baseline, serum samples were available for 87 of the 89 women with lymphangioleiomyomatosis who participated in the MILES trial (figure 1). The clinical characteristics of the trial population have been previously reported.[18] 23 (26%) participants had a baseline FEV_1 of greater than 60% of predicted, 17 (20%) had measurements between 51% and 60% of predicted, 23 (26%) had measurements between 41% and 50% of predicted, 15 (17%) had measurements between 31% and 40% of predicted, and nine (10%) had measurements less than or equal to 30% of predicted. Withdrawal during the treatment period did not differ significantly between the placebo (seven of 42 patients) and sirolimus (five of 45 patients) groups (p=0·54).

Baseline VEGF-D concentrations ranged from 0·34 ng/mL to 16·7 ng/mL. Age of participants and serum concentrations of VEGF-D were not significantly related (appendix).

Of the 79 patients with sporadic lymphangioleiomyomatosis, 54 (68%) had serum VEGF-D concentrations greater than or equal to 0·8 ng/mL, 58 (73%) had concentrations of 0·7 ng/mL or greater, and 63 (80%) had concentrations of 0·6 ng/mL or greater. Baseline concentrations were higher than 0·8 ng/mL in seven of the eight patients with tuberous-sclerosis-complex-associated lymphangioleiomyomatosis. Median baseline serum concentrations of VEGF-D did not differ significantly between patients with tuberous-sclerosis-complex-associated disease (1·95 ng/mL [IQR 0·96–5·39]) and those with the sporadic form of the disease (1·27 ng/mL [0·66–2·59]; p=0·27).

Serum VEGF-D concentrations at baseline were significantly higher in participants who used supplemental oxygen than in those who did not use supplemental oxygen (1·7 ng/mL [IQR 0·99–3·36] *vs* 0·84 ng/mL [0·52–1·39]; p<0·0001) and in those who had a positive bronchodilator response than in those who did not (2·01 ng/mL [0·99–2·86] *vs* 1·00 ng/mL [0·61–2·15]; 0·0273; figure 2). Patients with a history of pneumothorax had significantly lower concentrations than did those without such a history (p=0·0015; figure 2). Baseline concentrations of serum VEGF-D were not significantly associated with menopausal status or historical presence or absence of angiomyolipoma (figure 2).

Baseline VEGF-D concentrations were associated with markers of airflow obstruction and hyperinflation. Increased VEGF-D concentrations were positively associated with forced vital capacity (FVC), total lung capacity, residual volume, functional residual capacity, and several patient-reported outcomes, including scores on the functional performance inventory and the St George's respiratory questionnaire (table 1), which has recently been validated as a longitudinal measure in lymphangioleiomyomatosis.[23] The associations remained significant when we did sensitivity analyses that excluded either participants with tuberous-sclerosis-complex-associated disease or those who were enrolled in the MILES trial on the basis of increased VEGF-D concentrations (n=12) (data not shown).

Serum VEGF-D concentrations were similar at baseline in the placebo and sirolimus groups.[18] VEGF-D concentrations remained roughly stable with time in the placebo group but fell significantly with time in the sirolimus group (p<0·0001; appendix). Between-group differences in concentration were significant at 6 months (p=0·0123) and 12 months (0·0047). Baseline log(VEGF-D) correlated negatively (Spearman correlation −0·413; p=0·0168) with percentage change in FEV_1 during 12 months in individual patients in the placebo group and positively (0·409; 0·0087) with percentage change in FEV_1 in those in the sirolimus group (figure 3A, B; appendix). In the general linear model (which was used to compare the absolute difference between treatment groups), any one-unit increase in baseline log(VEGF-D) was associated with a 75 mL increase in FEV_1 from baseline to 12 months in the sirolimus group (p=0·0066; figure 3C) and a 59 mL decrease in the placebo group (0·0007 for between-group comparison; table 2). For FVC, any one-unit increase in baseline log(VEGF-D) level was associated with a 137 mL increase in FVC from baseline to 12 months in the sirolimus group (p=0·0152; figure 3C) and a 41 mL decrease in the placebo group (0·0003 for between-group comparison; table 2).

From the linear mixed-effects models, each one-unit increase in baseline log(VEGF-D) was associated with a 5 mL per month increase in FEV_1 (p=0·0441) and a 14 mL per month increase in FVC (0·0034) in the sirolimus group, and a 4 mL per month decrease in FEV_1 and 3 mL per month decrease in FVC in the placebo group (table 2; appendix). The slopes of FEV_1 and FVC responses as a function of baseline VEGF-D concentrations were significantly different from zero in the treatment group. The slopes of lung function responses in the placebo group were not significantly different from zero.

FEV_1 at 12 months did not improve in any of the 22 patients in the placebo group who had baseline VEGF-D concentrations of higher than 0·8 ng/mL (the cutoff previously proposed as the conservative diagnostic threshold for lymphangioleiomyomatosis[10]) but improved in four of 11 (36%) placebo group participants with baseline concentrations of less than 0·8 ng/mL (appendix). In the sirolimus group, no discrete serum VEGF-D threshold was definitively associated with improvement in FEV_1 but 18 of the 32 (56%) patients in the sirolimus group whose baseline VEGF-D concentrations were higher than 0·8 ng/mL had 12 month FEV_1 measurements that were higher than those at baseline (appendix).

In the placebo group, the maximum change in serum VEGF-D concentration from baseline to 12 months was 42% (mean 0·6 [SD 19·9]), and the change in VEGF-D concentration did not correlate with the change in FEV_1 during this period (Spearman's correlation 0·15; p=0·42). We therefore conservatively defined VEGF-D response in the sirolimus group as a decrease in VEGF-D concentration of more than 42% from baseline—ie, the maximum change recorded in the placebo group. Of the 38 patients in the sirolimus group for whom 12 month data were available, 23 (61%) had reductions in serum VEGF-D concentrations from baseline of greater than 42% at 12 months. Improvement in FEV_1 was noted in 15 of the 23 (65%) patients with a VEGF-D response and four of 15 (27%) without a VEGF-D response in the sirolimus group (figure 4; p=0·0448).

Discussion

We used data and samples gathered during the MILES trial to show that: baseline serum VEGF-D concentration is associated with lymphangioleiomyomatosis severity; higher baseline serum VEGF-D concentrations are associated with improvement in FEV_1 and FVC in patients taking sirolimus; and, in patients with lymphangioleiomyomatosis who are given sirolimus, a decrease in serum VEGF-D concentrations with time is associated with improvement in lung function (panel).

In our analysis, baseline serum VEGF-D concentration was a marker of gas exchange abnormalities in lymphangioleiomyomatosis (shown by the relation between increased VEGF-D concentrations and reduced diffusing capacity of the lung for carbon monoxide and need for supplemental oxygen). Furthermore, higher baseline serum VEGF-D concentrations were associated with airflow obstruction (as shown by reduced FEV_1/FVC ratio) and bronchodilator responsiveness (defined as improvement in FEV_1 or FVC of greater than 12% or 200 mL, or both, in patients after salbutamol treatment), which are markers of rapid disease progression in lymphangioleio-myomatosis.[24] Patients with higher baseline VEGF-D concentrations reported significantly greater impairment in day-to-day functioning and in some quality-of-life domains than did those with lower baseline concentrations.

We noted both similarities and differences between our findings and those of previous studies[9,11,12,25] examining the relation between serum VEGF-D concentrations and various measures of pulmonary function. Similar to Seyama and colleagues,[9] we recorded significant correlations between baseline VEGF-D concentrations and both diffusing capacity of the lung for carbon monoxide and FEV_1/FVC. However, by contrast with our findings, they did not detect an association between VEGF-D concentrations and total lung capacity or residual volume. In our previous cross-sectional study,[10] we did not show a relation between VEGF-D concentrations and age, FEV_1, or use of supplemental oxygen, whereas significant correlations were noted between VEGF-D concentrations and FEV_1 and supplemental oxygen use in this analysis of the MILES cohort.

Differences between our results and those reported in previous studies might be a result of the disparate sample sizes and non-contemporaneous nature of data collection of previous studies. We chose not to correct for multiple comparisons because the analyses were hypothesis-driven, but acknowledge that our approach increases the risk of over-interpretation. The association between high VEGF-D concentrations and decreased incidence of pneumothorax is unexplained. Similarly, the associations between high concentrations of VEGF-D and increased FVC, total lung capacity, residual volume, and functional residual capacity remain incompletely understood. Pulmonary physiological impairment in lymphangioleiomyomatosis is complex and probably a result of competing forces of obstruction (caused by narrowing of airways as a result of smooth muscle infiltration and increased compliance from destruction of elastic fibres) and restriction (related to expansion of the interstitial compartment with smooth muscle cells). How VEGF-D concentrations are related to lymphangioleiomyomatosis cell burden is unknown.

Incremental increases in baseline VEGF-D concentrations were associated with incremental benefit from treatment in this analysis of a magnitude that compared favourably with the estimated minimum clinically important FEV_1 difference of 100–140 mL for chronic obstructive pulmonary disease.[26] Collectively, our findings suggest that, when weighing the relative merits of treatment and watchful waiting in lymphangioleiomyomatosis, a high VEGF-D concentration portends an increased likelihood of therapeutic benefit in terms of FEV_1, FVC, and, perhaps, some aspects of quality of life and functional performance. Additionally, serum VEGF-D concentrations might also be useful for stratification of patients for trials and reduction of the number of patients needed to adequately power studies. The slope of FEV_1 response as a function of baseline VEGF-D concentrations was different from zero in both statistical models in the sirolimus group but not in the placebo group, suggesting that incremental increases in baseline VEGF-D concentrations were not associated with within-group lung function decline in the placebo group. Nonetheless, patients in the placebo group with VEGF-D concentrations of greater than 0·8 ng/mL at baseline had greater declines in FEV_1 at 12 months than did those whose baseline VEGF-D concentrations were less than 0·8 ng/mL. Taken together, these data suggest that baseline VEGF-D concentration is associated with disease progression and treatment responses, but the optimum VEGF-D cutoff for treatment decisions will need to be refined in future studies.

An early change in VEGF-D concentration after initiation of treatment that could be used as a surrogate for subsequent lung function response would help to do trials more rapidly. VEGF-D responses at 6 months were not significantly associated with FEV_1 responses at 12 months (data not shown). Because research serum samples were taken only every 6 months during the treatment phase of the MILES trial, the initial kinetics of the VEGF-D response are unknown. We recommend that future studies assess early biomarker responses as a potential predictor of subsequent clinically meaningful therapeutic responses, to identify more rapid surrogate endpoints for trials.

Although not a central focus of this study, our findings confirm our previously reported VEGF-D diagnostic sensitivity and proposed cutoffs, and validate a serum concentration of 0·8 ng/mL as a conservative threshold for a definitive diagnosis of lymphangioleiomyomatosis in patients with typical thin-walled cystic change on high-resolution CT.[10] Because sample handling and assay conditions can affect serum VEGF-D concentrations, normative ranges and standard operating procedures should be established in individual laboratories.[12,25]

Our study had several limitations. The median percent predicted FEV_1 was 48·5 (SD 13·8) and diffusing capacity of the lung for carbon monoxide was 43·4 (19) for patients with

lymphangioleiomyomatosis enrolling in the MILES trial,[18] consistent with moderately severe disease, although FEV_1 was well distributed (from 20–70% of predicted). By contrast, the mean FEV_1 reported for the lymphangioleiomyomatosis registry from the National Institutes of Health was 70% of predicted.[27] That a treatment trial enrolled patients with more advanced disease than did an observational study is perhaps unsurprising; however, further studies are needed to establish whether the associations and prognostic value of serum VEGF-D concentrations would also apply to patients with normal lung function or only mild impairment, or in those with chylous complications rather than diffuse lung disease as the main disease manifestation.

Missing data and withdrawals may have affected results, although the proportion of missing data was small, withdrawals did not differ substantially between the sirolimus and placebo groups during the treatment period, and no significant difference was noted in VEGF-D concentrations between patients who withdrew early and 12 month trial completers. The MILES trial analysis of VEGF-D is confounded by the use of VEGF-D as both a trial eligibility criterion and an outcome measure for 12 of the 87 (14%) patients, and by the heterogeneity introduced by inclusion of patients with tuberous-sclerosis-complex-associated lymphangioleiomyomatosis who might have a different disease course from those with sporadic lymphangioleiomyomatosis.[17] However, patients for whom VEGF-D concentration was an eligibility criterion and those with tuberous-sclerosis-complex-associated lymphangioleiomyomatosis were equally distributed between the sirolimus and placebo groups, and sensitivity analyses showed that the key analyses remained significant when these individuals were excluded. Because of the small numbers of patients with tuberous-sclerosis-complex-associated lymphangioleiomyomatosis in the MILES trial, future studies are needed to establish whether serum VEGF-D concentrations correlate with pulmonary disease severity in that population. Furthermore, the reason why normal serum VEGF-D concentrations are noted in some patients with lymphangioleiomyomatosis remains unknown and we are optimistic that future studies will might provide insight into disease phenotypes and pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Panel: Research in context

Systematic review

In 2009, we searched PubMed, Embase, Scopus, and Web of Science with the terms "lymphangioleiomyomatosis and biological markers/biomarkers", "LAM cells in blood/urine", "VEGF-D", "metalloproteinase", "osteopontin", "prognosis/prognostic", "severity", "predictor/prediction", "survival analysis", "score", "forecasting", "disease progression", "pneumothoraces", "chylous effusions", "angiomyolipomas", "sporadic *vs* TSC-lymphangioleiomyomatosis", "postmenopause", and "cyst size". When the Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus (MILES) trial closed and was reported in 2011, only three original studies[9,11,13] of VEGF-D in lymphangioleiomyomatosis had been published. Therefore, we concluded that analysis of VEGF-D as a prognostic and predictive biomarker of pulmonary disease progression and treatment response was needed because no prospective, randomised interventional studies had been done in patients with lymphangioleiomyomatosis. On March 1, 2013, we repeated our search strategy in PubMmed and identified additional reports of VEGF-D as a diagnostic biomarker and in patients with angiomyolipomas associated with tuberous sclerosis, but did not identify any other randomised studies in which the relation between VEGF-D concentrations and pulmonary treatment response in lymphangioleiomyomatosis was assessed.

Interpretation

We used the MILES trial data to show that baseline serum VEGF-D is associated with lymphangioleiomyomatosis severity. In the MILES trial, high baseline serum VEGF-D concentrations were associated with improvement in forced expiratory volume in 1 s (FEV_1) and forced vital capacity in patients given sirolimus, whereas a decrease in serum concentrations were associated with improvements in lung function. These findings suggest that serum VEGF-D concentrations can inform the risk–benefit analysis of sirolimus treatment in patients with lymphangioleiomyomatosis. Whether these results would apply to patients who were excluded from the trial (including those with FEV_1 >70% of predicted, those with chylous complications impairing lung function, or those who have undergone lung transplantation) is unknown. Future studies are needed to establish why serum VEGF-D concentrations are normal in some individuals with lymphangioleiomyomatosis, and whether VEGF-D-driven lymphangiogenesis is a promising therapeutic target.

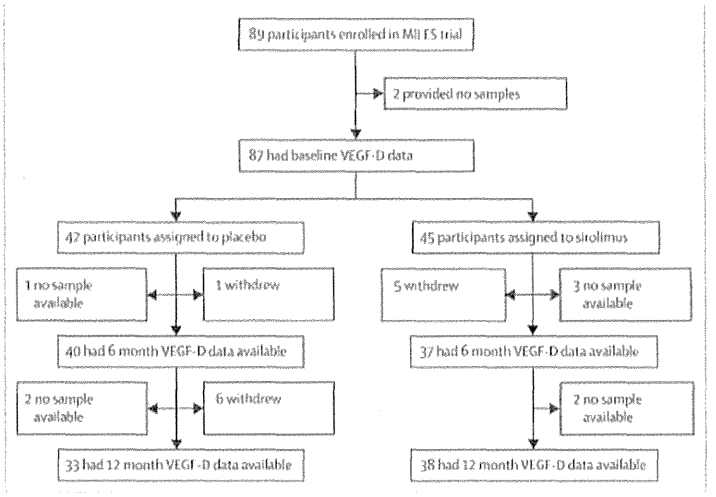

Figure 1. Study population

MILES=Multicenter International Lymphangioleiomyomatosis Efficacy of Sirolimus.

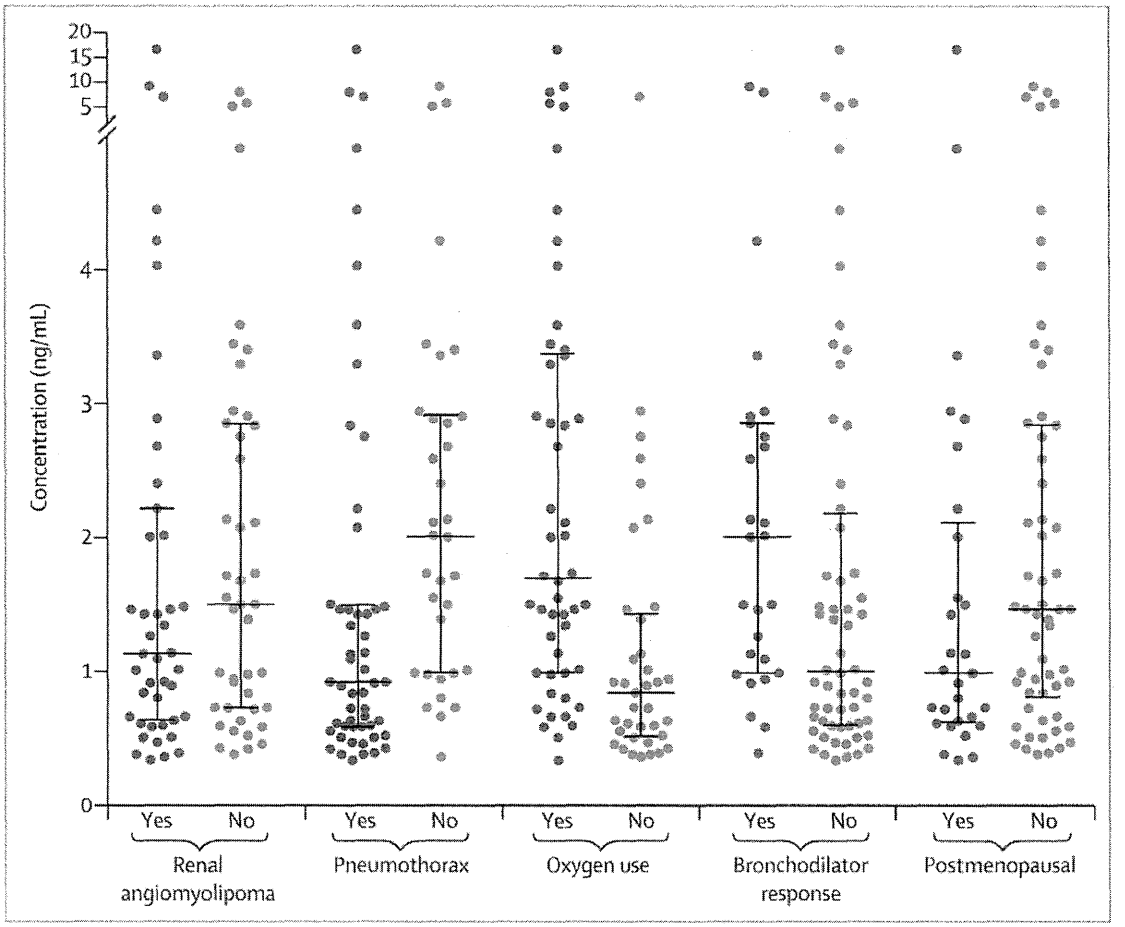

Figure 2. Relation between serum VEGF-D concentrations and disease features at enrolment

Note that the y-axis is broken and that the scale changes. Serum VEGF-D concentrations at baseline were significantly higher in participants who used supplemental oxygen than in those who did not use supplemental oxygen ($p<0{\cdot}0001$) and in those who had a positive bronchodilator response than in those who did not (0·0273). Patients with a history of pneumothorax had significantly lower baseline concentrations than did those without such a history (0·0015). Baseline concentrations were not significantly associated with menopausal status or historical presence or absence of angiomyolipoma.

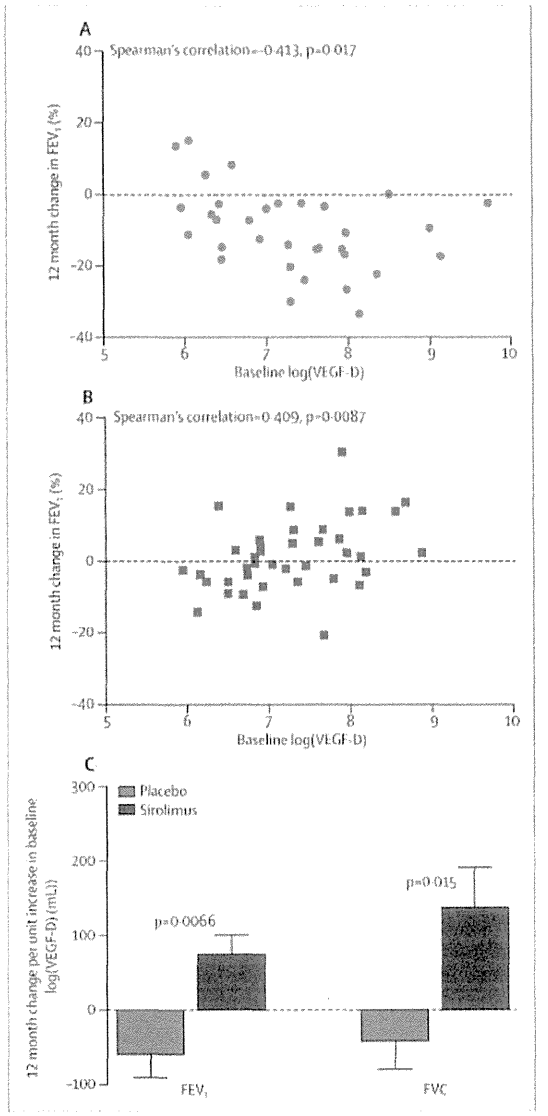

Figure 3. Relation between baseline log(VEGF-D) and 12 month change in FEV_1 for individual participants in the placebo (A) and sirolimus (B) groups, and expected change from baseline in FEV_1 and FVC (in mL) on the basis of each one-log-unit change in baseline log(VEGF-D) (C)
FEV_1=forced expiratory volume in 1 s. FVC=forced vital capacity.

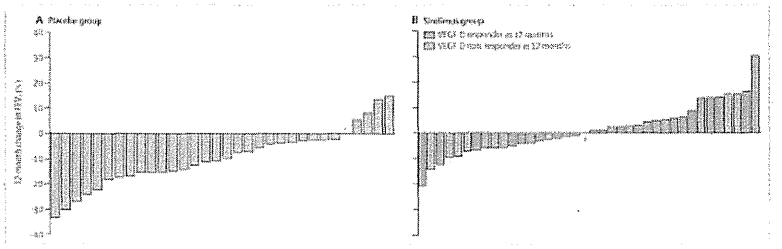

Figure 4. Distribution of baseline-to-12-month change in FEV_1 in participants in the placebo group (A), and relation between VEGF-D response and baseline-to-12-month change in FEV_1 in the sirolimus group (B)

Data are shown for all patients who had both VEGF-D concentrations and FEV_1 data at the 12 month study visit (one patient in the placebo group did not have a FEV_1 measurement at 12 months). Participants in the sirolimus group were stratified on the basis of VEGF-D response, which was defined as a decrease of greater than 42% in VEGF-D concentrations from baseline to 12 months—ie, the maximum change noted in the placebo group. FEV_1=forced expiratory volume in 1 s. *Denotes a participant who did not have a change in FEV_1 at 12 months. †Denotes a VEGF-D nonresponder whose FEV_1 decreased by 0·55% at 12 months.