Increased Lymphatic Vessel Length Is Associated With the Fibroblast Reticulum and Disease Severity in Usual Interstitial Pneumonia and Nonspecific Interstitial Pneumonia

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Background: Lymphangiogenesis responds to tissue injury as a key component of normal wound healing. The development of fibrosis in the idiopathic interstitial pneumonias may result from abnormal wound healing in response to injury. We hypothesize that increased lymphatic vessel (LV) length, a marker of lymphangiogenesis, is associated with parenchymal components of the fibroblast reticulum (organizing collagen, fibrotic collagen, and fibroblast foci), and its extent correlates with disease severity.

Methods: We assessed stereologically the parenchymal structure of fibrotic lungs and its associated lymphatic network, which was highlighted immunohistochemically in age-matched samples of usual interstitial pneumonia (UIP), nonspecific interstitial pneumonia (NSIP) with FVC < 80%, COPD with a Global Initiative for Obstructive Lung Disease stage 0, and normal control lungs.

Results: LV length density, as opposed to vessel volume density, was found to be associated with organizing and fibrotic collagen density ($P < .0001$). Length density of LVs and the volume density of organizing and fibrotic collagen were significantly associated with severity of both % FVC ($P < .001$) and diffusing capacity of the lung for carbon monoxide ($P < .001$).

Conclusions: Severity of disease in UIP and NSIP is associated with increased LV length and is strongly associated with components of the fibroblast reticulum, namely organizing and fibrotic collagen, which supports a pathogenic role of LVs in these two diseases. Furthermore, the absence of definable differences between UIP and NSIP suggests that LVs are a unifying mechanism for the development of fibrosis in these fibrotic lung diseases. *CHEST 2012; 142(6):1569–1576*

Abbreviations: DLCO = diffusing capacity of lung for carbon monoxide; FF = fibroblast foci; GGO = ground-glass opacity; GOLD = Global Initiative for Obstructive Lung Disease; IIP = idiopathic interstitial pneumonia; IPF = idiopathic pulmonary fibrosis; LV = lymphatic vessel; NSIP = nonspecific interstitial pneumonia; UIP = usual interstitial pneumonia; VEGF = vascular endothelial growth factor

Lymphatic vessels (LVs) have several key roles in lung homeostasis, including alveolar fluid clearance and immune-cell trafficking.¹ When LVs are injured in the setting of organ damage, new LVs originate from preexisting vessels, a process known as lymphangiogenesis, during restoration of tissue homeostasis.² In this process, lymphatic endothelial cells migrate to the site of injury and organize into new

vessels that contribute to the synthesis and formation of collagen fibers.^{3–5} New extracellular matrix supports fibroblasts and myofibroblasts, providing the structural framework for scar formation. During normal tissue healing, once homeostasis has been achieved, LVs regress and apoptosis of fibroblasts occurs.

Current concepts regarding the pathogenesis of the idiopathic interstitial pneumonias (IIPs), particularly

idiopathic pulmonary fibrosis (IPF), the clinical correlate of usual interstitial pneumonia (UIP), have centered on fibroblast and myofibroblast proliferation in the setting of alveolar epithelial damage. Aberrant healing response can follow that is heralded by the appearance of fibroblast foci (FF) and, ultimately, progressive fibrosis.⁶ The FF are proposed to represent the leading edge of injury in IPF. Importantly, the number of FF correlates with the severity of physiologic abnormalities in IPF, as well as increased mortality.^{7,8} Once FF form, they coalesce into the fibroblast reticulum, a complex structure extending from the pleura to the parenchyma, surrounded by a network of vessels originally thought to be primarily blood vessels.⁹ Notably, since blood and LVs develop in parallel and both surround the airways, it is plausible that a percentage of the vessels that surround the fibroblast reticulum are of lymphatic origin. In fact, macrophages that participate in the process of fibrosis also interact with aberrant LVs, possibly contributing to pathogenic lymphangiogenesis.^{10,11} Furthermore, damage to subpleural and interlobular LVs may adversely influence the alveolar compartment clearance, thereby contributing to disease in IPF.¹² Although emerging data suggest that lymphangiogenesis accompanies and may contribute to disease pathogenesis in fibrotic lung diseases, such as diffuse alveolar damage,¹³ its clinical relevance to other IIPs, nonspecific interstitial pneumonia (NSIP) in particular, remains to be elucidated.

Prior to the adoption of rigorous stereologic methodologies in pulmonary research, limited data addressed the association of histopathologic and pulmonary LV abnormalities found in the IIPs with clinical phenotypes. We hypothesized that, when assessed stereologically,

increased LV length density, a marker of lymphangiogenesis, would correlate with the parenchymal components of the fibroblast reticulum: organizing collagen, fibrotic collagen, and fibroblast foci. Furthermore, we also proposed that increased LV length would closely associate with disease severity assessed by pulmonary function testing in patients with UIP and NSIP when compared with healthy control subjects. To answer this question, we used a stereologic approach to quantitatively define pulmonary LVs and histologic features of UIPs and NSIPs to establish morphometric correlations with disease severity.

MATERIALS AND METHODS

Specimens and Patient Population

Age-matched biologic specimens from patients with pulmonary disease, including UIP, NSIP, and COPD GOLD (Global Initiative for Obstructive Lung Disease) stage 0 (GOLD 0), were obtained from the US National Heart, Lung and Blood Institute multicenter Lung Tissue Research Consortium (LTRC). All patients of the LTRC had undergone an extensive, standardized, preoperative evaluation that included demographics, a detailed clinical history, serologic testing, and pulmonary function testing. Lung tissue specimens were obtained from patients undergoing surgical lung biopsy to confirm a pathologic diagnosis of IIP or to resect a solitary pulmonary nodule. Specimens were also derived from explanted lungs of patients who had disease at the time of lung transplant. In the IIP groups, six patients had a clinical diagnosis of a connective tissue disease (UIP, $n=3$; NSIP, $n=3$). Of the patients with GOLD 0 COPD ($n=14$), seven had a primary lung malignancy. The clinical, histologic, and radiographic diagnosis of the LTRC specimens can be found in e-Table 1.

The tissue analyzed did not include the malignancy. Lung tissue samples were taken from the surgical biopsy specimen that was not required for clinical decision-making. Following resection, fresh tissues were flash frozen in liquid nitrogen and stored -80°C , as well as placed in paraformaldehyde and/or formalin for further analysis. Formalin-fixed, paraffin-embedded tissue was used for this study. Anatomic sites of the specimens and biopsy type can be found in e-Table 2. The histologic specimens were reviewed by two pathologists (C. C., S. G.) who made the final histopathologic diagnosis. The clinical and demographic characteristics for each histologic subset are depicted in Table 1.

Single whole-lung samples from 15 individuals without known pulmonary disease were obtained from the International Institute for the Advancement of Medicine. Samples were flash frozen in liquid nitrogen and stored at -80°C , as well as placed in paraformaldehyde and/or formalin for further analysis. Frozen serum samples were not available in the normal group. The study was reviewed and approved by the National Jewish Health institutional review board and by the Colorado Multiple Institutional Review Board (approval number 08-0115). A detailed description of how control specimens were processed, of immunohistochemical and stereologic methods, and of serum growth factor measurement and chest CT scan assessment is provided in e-Appendix 1.

Pulmonary Function

The measurements of FVC, FEV₁, and diffusing capacity of the lung for carbon monoxide (DLCO) met the American Thoracic

Manuscript received January 4, 2012; revision accepted May 25, 2012.

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Funding/Support: This work was supported by the US National Institutes of Health/National Heart, Lung and Blood Institute [Grants HL090147, HL81151, and HL88138].

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Table 1—Demographic and Clinical Characteristics

Characteristic	Normal	GOLD 0	NSIP	UIP
Age, y	60.3 (9.2)	67.7 (10.2)	58.7 (11.2)	61.1 (6.65)
Sex, M (F)	7 (8)	7 (7)	5 (8)	13 (7)
Smoker, C/F/N	0/2/13	1/8/3	1/6/5	0/10/9
FEV ₁ , L	n/a	2.52 (0.66)	1.65 (0.55)	1.95 (0.62)
FEV ₁ , %	n/a	95.14 (13.51)	55.07 (15.35)	65.8 (20.05)
FVC, L	n/a	3.42 (0.82)	1.98 (0.74)	2.4 (0.76)
FVC, %	n/a	97.14 (11.21)	49.76 (15.89)	60.8 (16.91)
DLCO, %	n/a	84.2 (14.1)	39.9 (15.4)	41.0 (16.32)
Walking distance, m	n/a	460.76 (117.12)	243.60 (135.50)	308.04 (175.31)

Data are given as mean (SD) unless otherwise indicated. C/F/N = current/former/never; DLCO = diffusing capacity of the lung for carbon monoxide; F = female; GOLD = Global Initiative for Chronic Obstructive Lung Disease; M = male; n/a = not applicable; NSIP = nonspecific interstitial pneumonia; UIP = usual interstitial pneumonia.

Society standards and have been described previously.¹⁴⁻¹⁶ The % predicted value was used for statistical analysis.

Chest CT Scan

CT scans of the chest were performed and graded on 10 patients with GOLD 0 COPD, 12 patients with NSIP, and 19 patients with UIP. The patients with COPD underwent standard chest CT scan, whereas patients with IIPs underwent a high-resolution chest CT scan. A thoracic radiologist (H. S.) assessed each CT scan.

Statistics

Student *t* test, analysis of variance, and the χ^2 test were used when appropriate. The correlation of % FVC (FVC%), % DLCO (DLCO%), and the stereologic assessments was determined by Spearman rank correlation. Correlations of age with LVs and tissue morphology were estimated from 10,000 bootstrap replications.^{17,18} The relationships of stereologic assessments to FVC% and DLCO% were estimated using a general linear model. All statistical tests performed were two-sided and used a type 1 error of .05.

RESULTS

Clinical, Pathologic, and Radiographic Characteristics

The study cohort included 62 age-matched patients stratified by the following pathologic diagnoses (Table 1): UIP (n = 20), NSIP (n = 13), GOLD stage 0 (n = 14), and normal (n = 15). Severity of FVC% and DLCO% was assessed between groups. As expected, FVC% and DLCO% were lower in the UIP and NSIP cohorts compared with the GOLD 0 and normal cohorts. The NSIP cohort had a significantly lower FVC% (UIP, 60.8 [SD, 16.91]; NSIP, 49.76 [SD, 15.89]; *P* = .02), but not DLCO%, compared with the UIP group. Surgical lung biopsy sites and distribution of CT scan radiographic characteristics are described in the supplemental tables (e-Tables 2-4).

Stereologic Assessment of Lymphatic Vessels and Parenchymal Components

To determine if LVs and parenchymal (alveolar) components varied between disease states and control subjects, we used standardized stereologic techniques to quantitate the length density (the length of the LVs per volume of nonaerated lung tissue) and volume density (the volume of either LVs or tissue components relative to nonaerated tissue volume). The techniques used followed the guidelines on quantitative assessment of lung structure.¹⁹ To isolate LVs from blood vessels to perform the stereologic assessments, a double immunohistochemical stain technique was used and is shown in Figure 1. To verify that alveolar epithelial cells do not express the lymphatic marker D2-40, immunofluorescent staining was performed (e-Fig 1). To assess the stereologic relationship of LVs to the tissue, we used the following as the reference tissue: organizing collagen, fibrotic collagen, fibroblast foci, inflamed tissue, and interalveolar septa (Fig 2, e-Fig 2). LV length density was highest among patients with UIP or NSIP (Fig 3A). The LV volume density for the GOLD 0 group was highest among all groups (Fig 3B), indicating larger LVs without a significant change in length. The increased volume density of the LVs in the GOLD 0 group may be an effect of microscopic emphysema that is not clinically evident on pulmonary physiology. Stereologic assessments for emphysema were not undertaken. The volume densities of organizing collagen, fibrotic collagen, and inflammation are shown in Figure 4. There was no difference in parenchymal component volume densities between the patients with UIP and those with NSIP (Figs 4A-E). As expected, a higher

FIGURE 1. Double immunohistochemical stain of lymphatic vessels and blood vessels (original magnification $\times 400$). D2-40 was used to indicate lymphatic vessels (arrow, brown) and CD-31 was used to indicate blood vessels ($\star$, red) in a tissue section of usual interstitial pneumonia (UIP). Scale bar: 20 μ m.

FIGURE 2. Movat's pentachrome stain of UIP. A, Low power image of UIP with a fibroblast focus consisting of organizing collagen (blue) surrounded by the fibroblast reticulum consisting of fibrotic collagen (yellow) (original magnification $\times 100$). Scale bar: 100 μm . B, High power image of UIP depicting a fibroblast focus (original magnification $\times 400$). Scale bar: 20 μm . See Figure 1 legend for expansion of abbreviation.

volume density for FF was found in UIP compared with NSIP (Fig 4C). The site of the sample from either surgical biopsy or explant did not affect the length density of LVs.

Association of LVs and Parenchymal Components

To assess if LVs are associated with parenchymal components, the volume and length density of LVs was correlated to parenchymal component volume density. LV length density had a strong correlation to organizing collagen volume density ($r = 0.62$, $P < .001$), and fibrotic collagen volume density ($r = 0.76$, $P < .001$) (Table 2). An inverse correlation was present with interalveolar septa tissue density ($r = -0.77$, $P < .001$). LV length also correlated with the volume density of FF and inflammation. LV volume density was not associated with any parenchymal component volume density.

Pulmonary Physiology With Stereology

The stereologic data indicate that increased lymphatics may be associated with the abnormal tissue processes involved in UIP and NSIP, leading to replacement of normal alveoli with the fibrotic process and abnormal pulmonary physiology. We investigated whether the length density of LVs and the burden of parenchymal components were associated with disease severity. A univariate analysis of the 62 samples showed significant inverse associations between FVC% and LV length density ($r = -0.63$, $P < .001$) and LV volume density ($r = 0.35$, $P = .02$). DLCO% had a strong inverse association with LV length density ($r = -0.69$, $P < .001$). The parenchymal volume density of both organizing and fibrotic collagen had a strong inverse correlation with the severity of FVC% and DLCO% (Table 3). Inter-alveolar septal tissue had a strong positive correlation with FVC% and DLCO%. Density of FF and inflammation had a weak inverse correlation.

The link between age and UIP is well described. We sought to determine if age was associated with lymphatic vessel or tissue morphometry. Age did not influence any metrics of LVs or tissue morphometry (LV length, -0.01 [95% CI, -0.20 , $+0.18$]; LV volume density, 0.07 [95% CI, -0.19 , 0.18], volume of organizing collagen, -0.18 [95% CI, -0.43 , $+0.03$], volume of fibrotic collagen, -0.09 [95% CI, -0.32 , $+0.15$], volume of inflammation, -0.12 [95% CI, -0.32 , $+0.13$], volume of inter-alveolar septa, 0.15 [95% CI, -0.09 , $+0.37$]).

Stereologic Associations With Radiographic Findings

CT scans of the chest were assessed for distribution of disease and scored based on radiographic features.

FIGURE 3. Lymphatic vessel stereology. A, Lymphatic vessel length density was found to be longer in the idiopathic interstitial pneumonias compared with control subjects. B, Lymphatic vessel volume density was found to be greater in the idiopathic interstitial pneumonias compared with the normal group and was greatest in the patients with GOLD stage 0 COPD. GOLD = Global Initiative for Obstructive Lung Disease; ns = nonsignificant; NSIP = nonspecific interstitial pneumonia. See Figure 1 legend for expansion of other abbreviation.

FIGURE 4. Total lung volume and volume densities. Lung sections were stained using Movat's pentachrome to stereologically assess the volume density of tissue subtypes. A, B, D, Organizing collagen (A), fibrotic collagen (B), and inflamed tissue (D) were also found in greatest density in the idiopathic interstitial pneumonias. C, The volume of fibroblast foci was greatest in the UIP group compared with NSIP. E, As expected, normal tissue was found to be highest in the control groups. ND = not detected; V_A = volume of alveolar septa; V_{FC} = volume of fibrotic collagen; V_{FF} = volume of fibroblast foci; V_I = volume of inflammation; and V_{OC} = volume of organizing collagen. See Figure 1 and 3 legends for expansion of other abbreviations.

Details of the distribution of radiographic features, as well as CT scan score, are in the supplementary e-Table 4. In UIP and NSIP, the radiographic abnormalities were concentrated in the lower lobes. The average CT scan score was highest in UIP and NSIP (UIP, 0.76 [SD, 0.285]; NSIP, 0.750 [SD, 0.211]) compared with GOLD 0 (0.200 [SD, 0.133]; $P < .001$). LV length density correlated with the following radiographic findings: ground-glass opacity (GGO) ($\chi^2 = 8.36$, $P = .02$), GGO/reticulation ($\chi^2 = 8.98$, $P = .03$), and reticulation ($\chi^2 = 4.06$, $P = .04$); and the average CT scan score ($r = 0.44$, $P = .004$). The parenchymal components were also found to have

strong direct correlation with the CT scan score (e-Table 5). Both FVC% and DLCO% had strong negative associations with the CT scan score ($r = -0.63$ and -0.76 , respectively; $P < .001$).

Quantification of Serum levels of Vascular Endothelial Growth Factor-A, -C, and -D

Serum levels of lymphangiogenic growth factors were assessed for differences between groups and for associations with LVs and lung tissue stereology and pulmonary physiology. Serum levels of vascular endothelial growth factor (VEGF)-A, VEGF-C, and VEGF-D were not statistically different between patients with UIP, patients with NSIP, and the control patients (e-Table 6). The serum growth factors were not associated with any morphometric index of LVs or parenchymal components. Serum levels of VEGF-A were inversely correlated with FVC% and FEV₁% in only the GOLD 0 group ($r = -0.86$, $P = .01$; $r = -0.82$, $P = .02$). VEGF-C did not correlate with airflow severity in any group. VEGF-D was inversely correlated with FVC% in the NSIP group ($r = -0.65$; $P = .02$). No association was found with any radiographic feature and serum levels of lymphangiogenic growth factors (e-Table 7).

Table 2—Relationship of Lymphatic Vessel to Parenchymal Component Stereology

Tissue Volume Density	LVLv		LVVv	
	r Value	P Value	r Value	P Value
Organizing collagen	0.63	<.001	ns	...
Fibrotic collagen	0.76	<.001	ns	...
Fibroblast foci	0.42	.0007	ns	...
Inflammation	0.41	.001	ns	...
Alveolar septa	-0.77	<.001	ns	...

LVLv = lymphatic vessel length density; LVVv = lymphatic vessel volume density; ns = nonsignificant.

Table 3—Relationship of Physiology to Lymphatic Vessel and Parenchymal Stereology

Variable	FVC%		FEV ₁ %		DLCO%	
	r Value	P Value	r Value	P Value	r Value	P Value
Lymphatic vessels						
Volume density	0.35	.02	0.35	.02	ns	...
Length length	−0.63	<.001	−0.59	<.001	−0.69	<.001
Tissue volume density						
Total	−0.63	<.001	−0.55	<.001	−0.61	<.001
Organizing collagen	−0.73	<.001	−0.67	<.001	−0.76	<.001
Fibrotic collagen	−0.73	<.001	−0.67	<.001	−0.76	<.001
Fibroblast foci	−0.34	.008	−0.26	.04	−0.46	.0003
Inflamed tissue	−0.53	<.001	−0.46	.0002	−0.55	<.001
Alveolar tissue	0.79	<.001	0.69	<.001	0.80	<.001

Data from univariate analysis. See Table 1 legend for expansion of abbreviation.

DISCUSSION

In our study, the length density of LVs strongly correlated with histopathologic components of tissue fibrosis in UIP and NSIP, specifically the volume density of organizing and fibrotic collagen, which are the main components of the fibroblast reticulum. Furthermore, our results demonstrate that lung LVs are longer in UIP and NSIP compared with normal and GOLD 0 control subjects when assessed relative to the abnormal tissues present in UIP and NSIP. Thus, this finding suggests that length may be considered a marker of active lymphangiogenesis in UIP and NSIP. These findings suggest that LVs are associated with the underlying pathophysiology in UIP and NSIP and support previous studies that show lymphangiogenesis occurs in response to inflammation and fibrosis in animals models.^{11,20,21} Afferent LVs may contribute to the development of fibrosis by attracting activated leukocytes expressing the chemokine receptor CCR7 by expressing the chemokine ligand CCL21, thus supporting a microenvironment conducive to fibrosis.²² In lymphangioleiomyomatosis, lymphangioleiomyomatosis cells and pulmonary macrophages are thought to be the cellular sources of serum VEGF-D.^{2,23,24} Serum levels of lymphangiogenic growth factors (VEGF-A, VEGF-C, and VEGF-D) were not elevated in the UIP and NSIP groups compared with the control groups. In UIP and NSIP, secretion of VEGF-D may be limited to pulmonary macrophages acting locally in response to injury.

LV length density had a strong inverse correlation with severity of FVC% and DLCO%. The relationship of LV length density and severity of pulmonary physiology suggests that LVs play a role in the pathophysiology of UIP and NSIP and in severity of disease. No differences were found between UIP and NSIP in LVs or parenchymal components, with the exception of FF. These findings may reflect the advanced disease in the NSIP group and may speak to a potential

common final pathway between UIP and NSIP for the development of fibrosis. LV length density tracked with radiographic findings, specifically GGO and reticulation, which are used to differentiate UIP and NSIP from other pulmonary diseases, as well as the average CT scan score.²⁵ LV length density was not found to track any score of emphysema, air trapping, or consolidation. This suggests that increased LV length is associated to disease pathogenesis and appears to be specific to UIP and NSIP.

To our knowledge, our study is the first to use rigorous stereologic approaches to quantify LVs and the morphologic changes found within lung tissue in UIP and NSIP, as described by the American Thoracic Society/European Respiratory Society official research policy statement for the quantitative assessment of lung structure.¹⁹ This study adds to the information previously described by El-Chemaly et al,¹⁰ who reported that the area of LVs inversely correlate with disease severity in UIP. Our study included normal-tissue stereologic assessments, as well as samples from the most common forms of IIP, UIP, and NSIP. Furthermore, the patients with UIP or NSIP in the present study were fully characterized clinically using pulmonary function testing and chest CT scans. LV volume density was also increased in patients with UIP compared with the normal control group, in keeping with the findings presented by El-Chemaly et al.¹⁰ In our study, LV volume density, although increased in both UIP and NSIP, was not associated with any metrics of parenchymal component volume density or any metrics of clinical severity, whereas the El-Chemaly et al¹⁰ study found a negative association between LV area and disease severity in UIP. One explanation for the discrepancy is that the samples used in the present study were less subject to sampling and orientation bias and were not limited by the usual pitfalls of randomization and lack of data pertaining to vessel length.²⁶ The increased volume density of LVs may

reflect retraction of the collagen resulting in traction on the lymphatic walls (lymphangiectasis), rather than active participation in the disease process.

A limitation of the current study is the small number of patients in each group. The limited number of patients may explain the weak association found with FF and pulmonary physiology in contrast to the strong relationship to disease severity previously reported.^{7,8} The NSIP group was found to be clinically more severe than the UIP group. This may explain the lack of differences found in the stereologic assessment of the LVs and the parenchymal components between the two groups.²⁷ Patients with mild disease were not included in the present study, thus limiting quantitative stereologic assessment of LVs or the parenchymal component in early in the course of the disease. This study confirms that changes in LVs occur in UIP and NSIP and are associated with severity of disease; however, this study does not address if these changes occur as part of the natural progress of disease, as has been suggested for angiogenesis.²⁸ To establish if LVs are necessary to the process of pulmonary fibrosis, studies should be performed that inhibit the development of LVs by using a neutralizing activity of VEGF-C and/or VEGF-D (ie, antibody traps) or receptor antagonists for the growth factors.^{29,30}

Here we show that LV length density, a marker of lymphangiogenesis, is associated with tissue burden of fibrosis in UIP and NSIP, with severity of physiologic impairment, and with radiographic features typical of these two fibrosing lung diseases. The histopathologic parenchymal elements of the fibroblast reticulum, organizing collagen, and fibrotic collagen have the strongest relationship with clinical severity. The degree to which LVs and lymphangiogenesis affect inflammation or fibrosis remains unknown, but this study suggests a role in the pathogenesis of pulmonary fibrosis.

ACKNOWLEDGMENTS

Author contributions: Dr Lara had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Dr Lara: contributed to designing and performing research, developing new analytical tools, analyzing the data, and writing the manuscript.

Dr Cosgrove: contributed to designing research, developing new analytical tools, and writing the manuscript.

Dr Janssen: contributed to designing and performing research and writing the manuscript.

Dr Huie: contributed to designing and performing research and writing the manuscript.

Dr Burnham: contributed to designing research, analyzing the data, and writing the manuscript.

Mr Heinz: contributed to performing research, developing new analytical tools, analyzing the data and drafting and approving the manuscript.

Dr Curran-Everett: contributed to designing research, analyzing the data, and writing the manuscript.

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Dr Henson: contributed to designing research, developing new analytical tools, and writing the manuscript.

Financial/nonfinancial disclosures: The authors have reported to CHEST that no potential conflicts of interest exist with any companies/organizations whose products or services may be discussed in this article.

Role of sponsors: The sponsor had no role in the design of the study, the collection and analysis of the data, or in the preparation of the manuscript.

Additional information: The e-Appendix, e-Figures, and e-Tables can be found in the "Supplemental Materials" area of the online article.

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