Glucose Transporter-1 Distribution in Fibrotic Lung Disease

Association With [18F]-2-Fluoro-2-Deoxyglucose-PET Scan Uptake, Inflammation, and Neovascularization

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Background: [18F]-2-fluoro-2-deoxyglucose (FDG)-PET scan uptake is increased in areas of fibrosis and honeycombing in patients with idiopathic pulmonary fibrosis (IPF). Glucose transporter-1 (Glut-1) is known to be the main transporter for FDG. There is a paucity of data regarding the distribution of Glut-1 and the cells responsible for FDG binding in fibrotic lung diseases.
Methods: We applied immunofluorescence to localize Glut-1 in normal, IPF, and Hermansky-Pudlak syndrome (HPS) pulmonary fibrosis lung tissue specimens as well as an array of 19 different lung neoplasms. In addition, we investigated Glut-1 expression in inflammatory cells from BAL fluid (BALF) from healthy volunteers, subjects with IPF, and subjects with HPS pulmonary fibrosis.
Results: In normal lung tissue, Glut-1 immunoreactivity was seen on the surface of erythrocytes. In tissue sections from fibrotic lung diseases (IPF and HPS pulmonary fibrosis), Glut-1 immunoreactivity was present on the surface of erythrocytes and inflammatory cells. BALF inflammatory cells from healthy control subjects showed no immunoreactivity; BALF cells from subjects with IPF and HPS pulmonary fibrosis showed Glut-1 immunoreactivity associated with neutrophils and alveolar macrophages.
Conclusions: Glut-1 transporter expression in normal lung is limited to erythrocytes. In fibrotic lung, erythrocytes and inflammatory cells express Glut-1. Together, these data suggest that FDG-PET scan uptake in IPF could be explained by enhanced inflammatory and erythrocytes uptake due to neovascularization seen in IPF and not an upregulation of metabolic rate in pneumocytes. Thus, FDG-PET scan may detect inflammation and neovascularization in lung fibrosis.
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Abbreviations: BALF = BAL fluid; FDG = [18F]-2-fluoro-2-deoxyglucose; Glut-1 = glucose transporter-1; HPS = Hermansky-Pudlak syndrome; IPF = idiopathic pulmonary fibrosis

Fibrotic lung diseases, such as idiopathic pulmonary fibrosis (IPF) and Hermansky-Pudlak syndrome (HPS) pulmonary fibrosis, are generally progressive and lead to death from respiratory failure.[1,2] The molecular and cellular mechanisms responsible for the initiation and development of lung fibrosis are complex and incompletely understood. Pulmonary fibrosis is associated with abnormal interstitial accumulations of extracellular matrix proteins and mesenchymal cells.[3] Usual interstitial pneumonia, the pathologic hallmark of IPF, is characterized by heterogeneity, with areas of normal lung alternating with abnormal fibrotic areas. Fibroblastic foci, type 2 cell hyperplasia, and honeycombing are found within fibrotic regions in lung tissue from patients with IPF.[4] Abnormal blood vessels and lymphatics resulting from angiogenesis and lymphangiogenesis, respectively, within fibrotic areas of the lung have also been reported in IPF.[5]

HPS is an autosomal recessive disorder characterized by improper biogenesis of lysosome-related organelles.[6] Patients with any of the nine known subtypes of HPS exhibit oculocutaneous albinism and platelet dysfunction of varying severity.[7,8] Some patients with

HPS manifest granulomatous colitis, and interstitial lung disease and pulmonary fibrosis may develop in children and young adults with HPS-2.[6,9] Pulmonary fibrosis is more commonly found in adults with HPS-1 and HPS-4, which are HPS subtypes associated with abnormal biogenesis of lysosome-related organelles complex-3.[9] HPS pulmonary fibrosis and IPF share some clinical, radiographic,[1] and pathologic features.[10] Notably, however, HPS pulmonary fibrosis usually leads to death at a much earlier age than IPF.[11]

PET scan using [18F]-2-fluoro-2-deoxyglucose (FDG) is useful for diagnostic purposes to detect metabolic activity of tissues and for assessment of responses to therapy in several lung diseases, including sarcoidosis[12] and pulmonary hypertension.[8] FDG-PET scan has more limited usefulness in other pulmonary diseases, such as lymphangioleiomyomatosis.[13] FDG-PET scan was evaluated in interstitial lung diseases, pulmonary fibrosis in particular.[14] Specifically, increased radionuclide uptake occurs in areas of honeycombing compared with areas of ground-glass infiltrates, which suggests that regions of honeycombing and fibrosis may be more biologically active than previously believed.[14]

Glucose transporter-1 (Glut-1), the predominant glucose transporter in the lung, is responsible for FDG uptake.[14,15] To determine the cause of increased FDG uptake in pulmonary fibrosis, we undertook an immunohistochemical analysis of normal and fibrotic lung disease. We show that Glut-1 is predominantly expressed on the surface of erythrocytes and inflammatory cells, such as neutrophils and macrophages. These findings suggest that the enhanced FDG-PET scan uptake in pulmonary fibrosis is a function of an increase in inflammatory cells as well as an increase in lung parenchymal vasculature due to angiogenesis in fibrotic areas, rather than upregulation of the metabolic rate of fibrotic lung as previously suggested.[14]

Materials and Methods

Subject Selection

Subjects with IPF, subjects with HPS, and healthy research volunteers were enrolled in protocols approved by the institutional review boards of the National Heart, Lung, and Blood Institute (96-H-0100) and National Human Genome Research Institute (04-HG-0211). The diagnosis of IPF was established according to published criteria.[4] Subjects with HPS were diagnosed by platelet electron microscopy and genotyping; pulmonary fibrosis was diagnosed by characteristic chest CT scan findings.[1] Healthy volunteers had normal pulmonary function tests and chest radiographs and no clinical evidence of lung disease. Characteristics of subjects enrolled in these studies are listed in Table 1. BAL was performed as previously described.[5] Cytospins of BAL fluid (BALF) cells were stained with Diffquik (Siemens Healthcare Diagnostics Inc), and differential cell counts were performed (Table 1). Lung tissue specimens were obtained from clinically indicated procedures. Lung sections from subjects with IPF were obtained from explanted lungs (n = 6) or from surgical biopsies (n = 6), including a subject with IPF who underwent a medically indicated PET-CT scan and an open lung biopsy. Lung sections from subjects with HPS pulmonary fibrosis (n = 3) were obtained from explanted lungs. An array of normal lung tissue and 19 different lung tumors was purchased from US Biomax Inc (Table 2).

Glut-1 Immunostaining and Confocal Microscopy

Lung sections were deparaffinized and rehydrated in graded alcohol. Sections or cytospins were fixed with 4% paraformaldehyde and then incubated with 1% goat serum or 1% human serum, respectively, to block nonspecific antibody binding. After blocking, sections (or cytospins) were incubated with either anti-Glut-1 antibody (1:100 dilution; Abcam plc) overnight at 4°C or with phosphate-buffered saline replacing the primary antibody. After washing, the slides were incubated with the secondary fluorescein isothiocyanate-labeled goat anti-rabbit antibody (1:100 dilution; Vector Laboratories, Inc) for 1 h at room temperature. Slides were then prepared with mounting medium containing 4,6-diamidino-2-phenylindole-2-HCl (Vector Laboratories, Inc), and fluorescence was examined using a 40× oil objective. Images were collected using a Zeiss 510 laser-scanning confocal microscope (Carl Zeiss Microscopy GmbH). Consecutive lung sections were stained with hematoxylin and eosin to visualize the same fields that were imaged with laser scanning confocal microscopy. Composite figures were assembled with Adobe Photoshop software (Adobe Systems Incorporated).

Quantification of Fluorescence Intensity

To quantify fluorescence intensities, confocal images were collected using the same instrument settings for all experimental conditions and imported in Imaris (v7.1). A 100-pixel-square box was cropped from each image over erythrocytes only. Mean Glut-1 fluorescence intensity per pixel was measured and exported in Excel (Microsoft Corporation). Fluorescence intensity is expressed in arbitrary units.

Table 1—Subject Characteristics for BALF Cell Analyses

Characteristics	Healthy Volunteers (n = 5)	IPF (n = 5)	HPSPF (n = 3)	P Value
Age, mean (SD), y	55.8 (4.8)	65.2 (12.0)	40.6 (9.7)	.015[a]
Male (female)	4 (1)	4 (1)	0 (3)	…
FVC % predicted	99.6 (8.42)	87.7 (25.6)	90 (18)	.599
TLC % predicted	99.6 (9.79)	82.3 (19.0)	75.3 (24.6)	.165
D_{LCO} % predicted	105 (9.1)	53.4 (18.4)	79.3 (16.3)	.001[b]
FEV_1/FVC	77 (6.4)	82.4 (8.15)	85.5 (9.0)	.324
BAL Neutrophil, %	1.26 (0.8)	5.7 (7.4)	3.4 (2.1)	.379

BALF = BAL fluid; D_{LCO} = diffusing capacity of the lung for carbon monoxide; HPSPF = Hermansky-Pudlak syndrome pulmonary fibrosis; IPF = idiopathic pulmonary fibrosis; TLC = total lung capacity.
[a]HPSPF compared with IPF and healthy volunteers.
[b]IPF compared with HPSPF and healthy volunteers.

RESULTS

Increased Glucose Uptake in the Lung in IPF

A subject with a history of interstitial lung disease, suggestive of IPF, underwent a PET-CT scan because of concern about possible malignancy of a small subpleural mass. The PET-CT scan showed increased FDG uptake in the regions of honeycombing and fibrosis (Figs 1A, 1B). The patient subsequently underwent a video-assisted thoracoscopic biopsy, and the pathologic diagnosis was consistent with usual interstitial pneumonia (Figs 1C, 1D). Tissue sections from this open lung biopsy were incubated with anti-Glut-1 antibody, and immunofluorescence showed the presence of Glut-1 in erythrocytes and inflammatory cells (Fig 1E). Mesenchymal cells in fibroblastic foci were negative for Glut-1, and areas of honeycombing showed Glut-1 positivity only in erythrocytes (Fig 1F).

Glut-1 Staining in Normal and Fibrotic Lung

In normal lung tissue, Glut-1 immunofluorescence was limited to erythrocytes; no other cells were reactive with anti-Glut-1 antibody (Fig 2A). In IPF lung sections, erythrocytes were the predominant cells expressing Glut-1; some inflammatory cells were also positive for Glut-1 immunofluorescence (Fig 2D). In HPS pulmonary fibrosis lung tissue, erythrocytes and some inflammatory cells expressed Glut-1 (Fig 2B); no immunostaining was found in control tissue sections (Fig 2C). To examine the possibility that erythrocytes from patients with IPF and HPS expressed more Glut-1 on cell surface compared with erythrocytes from healthy volunteers, we measured mean fluorescence intensity of Glut-1 transporters over erythrocytes and did not find any significant difference ($P = .7$). Fibroblastic foci, which are devoid of blood vessels,[5,16] did not show any reactivity with anti-Glut-1 antibodies (Figs 2E, 2F).

In marked contrast, sections of different lung neoplasms without fibrosis showed pronounced immunoreactivity with anti-Glut-1 antibodies. Tissues from adenosquamous carcinoma, squamous cell carcinoma, and adenocarcinoma showed increased reactivity with anti-Glut-1 antibody associated with neoplastic cells (Figs 3A-3C, respectively). Further, lung tissue from bronchoalveolar carcinoma, a neoplasm that typically does not show increased PET scan uptake, exhibited anti-Glut-1 antibody reactivity with erythrocytes and not with malignant cells (Fig 3D). These findings support the contention that PET scan uptake is associated with cells expressing Glut-1.

Glut-1 Staining in BALF Cells

To determine which inflammatory cells in the lung express Glut-1, cytospin preparation of cells isolated from BALF were immunostained using anti-Glut-1 antibody. BALF cells from healthy volunteers showed evidence of Glut-1 staining associated with erythrocytes and not with inflammatory cells (Fig 4A). In contrast, cells isolated from BALF from subjects with HPS pulmonary fibrosis (Fig 4B) and IPF (Fig 4C) showed reactivity with anti-Glut-1 antibodies associated with alveolar macrophages and neutrophils.

Table 2—Lung Tissue and Cytospins Used in These Studies

Material	Lung Tissue	Cytospins
IPF lung explants	6	…
IPF biopsy	6	5
HPSPF explants	3	5
Normal lung	3	5
Lung malignancy	19	…

See Table 1 legend for expansion of abbreviations.

DISCUSSION

In this report, we show that Glut-1 immunoreactivity in fibrotic lung disease localizes to erythrocytes and inflammatory cells and not fibroblasts, epithelial cells, or endothelial cells. Our data suggest that increased FDG uptake on PET scans in fibrotic lung disease is associated with Glut-1 expression by immune cells and erythrocytes and not with enhancement of
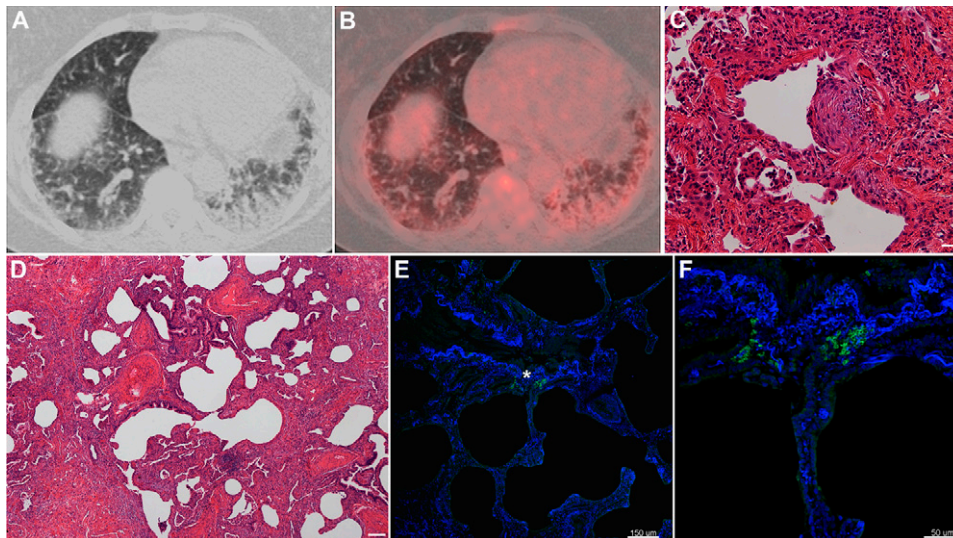
FIGURE 1. Increased glucose uptake in the lung in idiopathic pulmonary fibrosis. A, CT scan showing increased uptake in the areas of honeycombing and fibrosis. B, Axial attenuation corrected [^{18}F]-2-fluoro-2-deoxyglucose PET scan showing increased uptake in the areas of honeycombing and fibrosis. C, Hematoxylin and eosin (H&E) staining of a lung biopsy tissue section from the same patient shows a fibroblastic focus (magnification $\times$20, size bar 20 μm). D, H&E staining of a lung biopsy tissue section from the same patient shows areas of honeycombing (magnification $\times$10, size bar 100 μm). E, Immunofluorescence staining of paraffin-embedded sections of the same biopsy presented as a merged image of glucose transporter-1 (Glut-1; green) and 4,6-diamidino-2-phenylindole-2-HCl (blue) of an area of honeycombing shows Glut-1 staining associated with erythrocytes. F, High-power magnification of selected area in E (*).
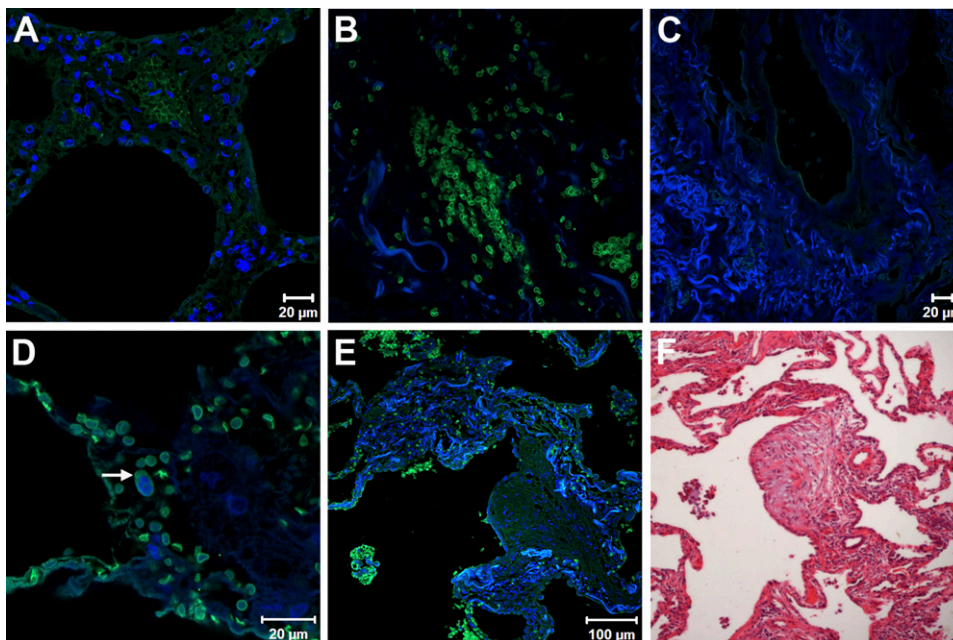

FIGURE 2. Glucose transporter-1 (Glut-1) expression in normal and fibrotic lung. A, B, Immunofluorescence staining of paraffin-embedded sections of lung tissue shown as merged image of Glut-1 (green) and 4,6-diamidino-2-phenylindole-2-HCl (blue) demonstrates Glut-1 expression in erythrocytes. A, Normal lung (n = 3; representative shown). B, Hermansky-Pudlak syndrome (HPS) pulmonary fibrosis lung (n = 3; representative shown). C, Omission of the first antibody shows complete absence of fluorescence signal in an HPS pulmonary fibrosis lung. D, Idiopathic pulmonary fibrosis (IPF) (n = 12; representative shown) lung shows Glut-1 expression associated with erythrocytes and weaker staining of neutrophils (arrow). E, Fibroblastic focus from IPF lung section demonstrates absence of Glut-1 staining of mesenchymal cells and positive Glut-1 staining of surrounding erythrocytes. F, hematoxylin and eosin image of consecutive lung sections showing the fibroblastic focus.
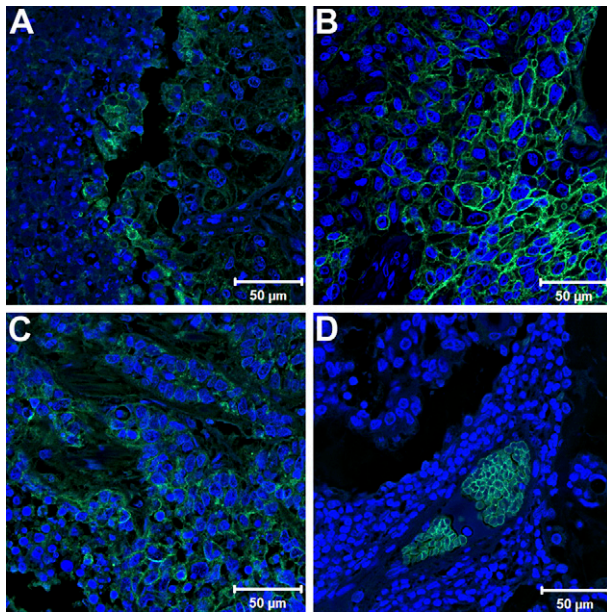
FIGURE 3. Glucose transporter-1 (Glut-1) staining of different lung neoplasms. A-C, Immunofluorescence staining of paraffin-embedded sections shown as merged image of Glut-1 (green) and 4,6-diamidino-2-phenylindole-2-HCl (blue) demonstrates positive surface Glut-1 lung tumor staining in adenosquamous carcinoma (A), squamous cell carcinoma (B), and adenocarcinoma (C). D, In contrast, sections from bronchoalveolar carcinoma show Glut-1 staining in erythrocytes, but not in malignant cells.

the metabolic rate of epithelial cells as previously postulated.

Evidence from lung tissue and BALF cell analyses show that in fibrotic lung disease, inflammatory cells express Glut-1, which could contribute to increased PET scan uptake. In contrast, Glut-1 immunofluorescence in healthy volunteers was found to be associated with erythrocytes and not with immune cells in the lung. The absence of Glut-1 expression by normal lung inflammatory cells is consistent with previous reports of inherent differences between the immune cells of patients with lung fibrosis and those of healthy control subjects.[5,17,18] In agreement with our data generated using diseased human tissue, FDG localized to lung neutrophils after IV administration in bleomycin-induced pulmonary fibrosis.[19] Taken together, these results suggest that inflammatory cells and erythrocytes are critical for FDG-PET scan uptake in fibrotic lung diseases.

Angiogenesis, or the formation of new blood vessels, is a key process in tissue repair and results from the net effects of antiangiogenic and proangiogenic factors.[20] The complex molecular signaling pathways stimulating and inhibiting angiogenesis in lung fibrosis have recently been reviewed.[21] The net result is that fibroblastic foci and areas of intense fibrosis are nearly devoid of blood vessels, and areas of honeycombing show increased vascularity.[16,22] This distribution mirrors that of vascular endothelial growth factor-A[22,23] and

hypoxia inducible factor-1α,[23] which were shown to be increased in alveolar epithelial cells and decreased in fibroblastic foci.

Erythrocytes, which express Glut-1 in normal and fibrotic lung, are not reported to express vascular endothelial growth factor-A or hypoxia inducible factor-1α.[24] Glut-1 is the only glucose transporter expressed at the surface of erythrocytes.[25] Further, Glut-1 in erythrocytes is not translocated and is consistently present on the cell surface.[3] Our data showing similar Glut-1 fluorescence intensity on the cell surface of erythrocytes in healthy and fibrotic lungs suggest that increased Glut-1 expression in fibrotic lung disease is due to changes in vascular development and increased erythrocyte availability. Overall, these changes lead to enhanced FDG uptake in the areas of honeycombing seen on PET-CT scans in patients with pulmonary fibrosis.

Glut-1 expression was reported in hepatic fibrosis. In cirrhosis, Glut-1 staining was similar to normal liver. Specifically, immunoreactivity for Glut-1 was found in endothelial cells of portal arteries and veins and not hepatocytes.[26] Our finding of increased Glut-1 immunostaining associated with inflammatory cells and not with the endothelial or epithelial cells of lung fibrosis contrasts markedly with a published report of
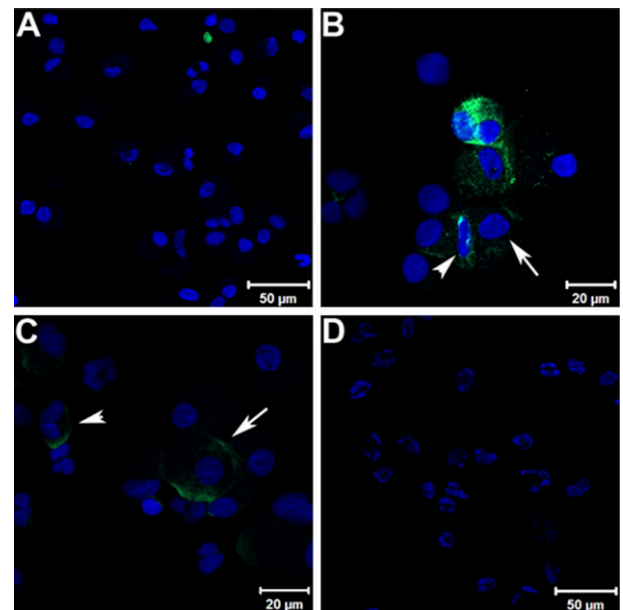

FIGURE 4. Glucose transporter-1 (Glut-1) staining of BAL cell cytospins. A, Representative field of cytospin slide stained for Glut-1 from a healthy volunteer, showing positive staining of an erythrocyte but not inflammatory cells. B, Representative field of cytospin slide stained for Glut-1 from a patient with Hermansky-Pudlak syndrome pulmonary fibrosis, showing positive alveolar macrophages (arrow) and neutrophil (arrowhead). C, Representative field of cytospin slide stained for Glut-1 from a patient with idiopathic pulmonary fibrosis (IPF), showing positive alveolar macrophages (arrow) and neutrophils (arrowhead) (n = 5 for each group; representative case shown). D, A negative control from a patient with IPF.

Glut-1 expression in pulmonary hypertension.[8] In that disorder, Glut-1 is found in pulmonary artery endothelial cells and smooth muscle cells but not inflammatory cells.[8] Thus, although the glycolytic rate in pulmonary hypertension may be increased, and PET scans may be a useful method to follow disease activity and response to therapy,[8] the usefulness of PET scans in managing patients with pulmonary fibrosis remains unclear and should be addressed.

It is possible that other Glut transporters could be involved in FDG uptake.[27] We found that lung malignancies known to be PET scan avid (eg, adenocarcinoma, squamous cell carcinoma) show increased Glut-1 expression, and a cancer that is recognized as non-PET avid (ie, bronchoalveolar carcinoma)[28-30] shows a marked absence of Glut-1 expression. Moreover, our results suggest that Glut-1 is an important determinant of FDG uptake in PET imaging of the fibrotic lung. In addition, FDG-PET uptake in fibrotic lung diseases appears to be driven by inflammatory cells and erythrocytes within regions of neovascularization and not by an increased metabolic rate of epithelial cells. Further studies are needed to determine the usefulness of FDG-PET in the clinical management of patients with fibrotic lung diseases. As the role of blood vessel formation in pulmonary fibrosis is further defined, FDG-PET may be a potential modality to noninvasively measure inflammation and/or erythrocyte binding as a surrogate for monitoring abnormal blood vasculature formation in fibrotic disorders of the lung.

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