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A multifaceted investigation into molecular associations of chronic thromboembolic pulmonary hypertension pathogenesis

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Abstract

Purpose: Chronic thromboembolic pulmonary hypertension is characterized by incomplete thrombus resolution following acute pulmonary embolism, leading to pulmonary hypertension and right ventricular dysfunction. Conditions such as thrombophilias, dysfibrinogenemias, and inflammatory states have been associated with chronic thromboembolic pulmonary hypertension, but molecular mechanisms underlying this disease are poorly understood. We sought to characterize the molecular and functional features associated with chronic thromboembolic pulmonary hypertension using a multifaceted approach.

Methods: We utilized functional assays to compare clot lysis times between chronic thromboembolic pulmonary hypertension patients and multiple controls. We then performed immunohistochemical characterization of tissue from chronic thromboembolic pulmonary hypertension, pulmonary arterial hypertension, and healthy controls, and examined RNA expression patterns of cultured lymphocytes and pulmonary arterial specimens. We then confirmed RNA expression changes using immunohistochemistry, immunofluorescence, and Western blotting in pulmonary arterial tissue.

Results: Clot lysis times in chronic thromboembolic pulmonary hypertension patients are similar to multiple controls. Chronic thromboembolic pulmonary hypertension endarterectomized tissue has reduced expression of both smooth muscle and endothelial cell markers. RNA expression profiles in pulmonary arteries and peripheral blood lymphocytes identified differences in RNA transcript levels related to inflammation and growth factor signaling, which we confirmed using immunohistochemistry. Gene expression data also suggested significant alterations in metabolic pathways, and immunofluorescence and Western blot experiments confirmed that unglycosylated CD36 and adiponectin expression were increased in chronic thromboembolic pulmonary hypertension versus controls.

Conclusions: Our data do not support impaired clot lysis underlying chronic thromboembolic pulmonary hypertension, but did demonstrate distinct molecular patterns present both in peripheral blood and in pathologic specimens of chronic thromboembolic pulmonary hypertension patients suggesting that altered metabolism may play a role in chronic thromboembolic pulmonary hypertension pathogenesis.

Keywords

Pulmonary circulation and disease, hypertension, cardiology, lipid and lipoprotein metabolism, atherosclerosis, coagulation and fibrinolysis, thrombosis

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Introduction

Chronic thromboembolic pulmonary hypertension (CTEPH) is a disease characterized by incomplete thrombus resolution following acute pulmonary embolism (PE), accompanied by vascular intimal thickening and fibrosis. These changes result in increased pulmonary vascular resistance, right ventricular dysfunction, and ultimately death.[1–3] It occurs in 0.1–9.1% of patients after acute PE.[1]

Conditions such as inherited and acquired thrombophilias, dysfibrinogenemias, and chronic inflammatory diseases have all been associated with CTEPH; however, the molecular mechanisms underlying this disease are poorly understood.[4–6] Recently, systemic inflammation and impaired angiogenesis have also been shown to have roles in CTEPH pathogenesis and clinical outcomes.[7–9] A commonly proposed mechanism of CTEPH is impaired endogenous mechanisms for clot resolution; however, this has presently not been demonstrated.[1,4,10]

We sought to broadly characterize the molecular and functional features that predispose to CTEPH using a multifaceted approach. We hypothesized that fibrinolysis would be impaired in CTEPH patients, and incorporated functional assays of clot lysis to quantify this. We then performed exploratory analyses of peripheral blood RNA expression patterns, and detailed molecular characterization of pulmonary vascular changes in CTEPH patients, pulmonary arterial hypertension (PAH) patients, and controls. We examined gene expression profiles in both peripheral blood and pulmonary artery (PA) tissue in CTEPH patients, PAH patients, and controls and confirmed findings with protein expression in PA tissue.

Materials and methods

Clot lysis assay

After obtaining IRB approval (#142036), platelet poor plasma was collected from 14 patients with CTEPH, 14 patients with PAH, 17 patients within six months of an acute PE, and 17 controls who had no known history of thrombosis, but who were taking warfarin for atrial fibrillation. Basic clinical data including age, sex, INR level, and CTEPH patients' surgical status were collected. Only patients taking warfarin with an INR level between 2 and 4 were included in the study.

To study clot lysis rates between samples, we adapted the turbidimetric lysis assay, which has been previously described, with minor modification.[11] Because each patient was on warfarin with varying degrees of anticoagulation, we added therapeutic levels of apixaban to each citrated plasma sample to inhibit any feedback activation of the coagulation cascade by the thrombin reaction. We also repeated this experiment without adding apixaban to plasma to examine if this had any effect on clot lysis times in CTEPH patients and controls. Assays were performed in triplicate and results were averaged for each patient.

Immunohistochemistry analysis of CTEPH, PAH, and control PA specimens

Characterization of CTEPH endarterectomized tissue. IRB exemption (#141467) was obtained to study archival human PA tissue taken during pulmonary thromboendarterectomy (PEA) for patients with CTEPH. The composition of four PEA specimens from patients with CTEPH was characterized by immunolocalization of alpha smooth muscle actin (SMA), vimentin, factor VIII, and Von Willebrand factor (vWF). Tissue sections (5 μm thick) were stained according to the manufacturer's instructions.

In addition to the above archival CTEPH tissue, control and PAH lung sections were collected under IRB protocol #9401. Immunohistochemistry was performed with the following antibodies according to the manufacturer's recommendations: IL-8, VEGF, NFκB, and ICAM-1. All immunohistochemistry samples were counterstained with Mayer's hematoxylin.

PA gene expression

RNA isolation from PA tissue. Samples of endarterectomized tissue from three patients with CTEPH who underwent PEA at Vanderbilt University Medical Center (IRB# 151082) were collected. PA samples from four idiopathic PAH (IPAH) patients were obtained from the Pulmonary Hypertension Breakthrough Initiative biobank. Control PA samples were obtained from four unmatched organ donors without known pulmonary vascular disease (IRB# 151082, VHVI Main Heart Registry). All samples were flash frozen and paraffin-embedded. RNA was isolated from these tissue samples using the Qiagen RNeasy mini kit (Valencia, CA).

RNA sequencing of PA tissue. RNA-Seq was performed on an Illumina HiSeq system with a directional mRNA library prep, SR-50, with 30 million reads. TopHat was used to align RNA-Seq reads to consensus genome sequence using the ultra-high-throughput short read aligner Bowtie2. Principal component analysis was performed with JMP, a subset of SAS, and gene ontology analyses were performed with WebGestalt.

Peripheral blood gene expression

Cultured lymphocytes. EDTA-anticoagulated venous blood was collected from patients with CTEPH and healthy controls without known cardiovascular disease (VU IRB #9401). Lymphocyte culture was performed as described previously.[12]

Microarray. RNA was isolated from lymphocytes with the use of a Qiagen RNeasy mini kit (see supplement for details). All array results have been submitted to the National Center for Biotechnology Information gene expression and hybridization array data repository (GEO, www.ncbi.nlm.nih.gov/geo/).

Analysis of peripheral blood lymphocyte gene expression. Patients with CTEPH ($n = 14$) and healthy control samples ($n = 4$) with high-quality peripheral blood lymphocytes or high-quality RNA samples were analyzed (e-Table 1). The open source software R2.13/Bioconductor2.8 was utilized for microarray analyses. Of 56,613 probe sets, 13,823 had an average expression in any group of >7 (log base 2 U), and 1517 of these had a range of expression from maximum to minimum values of >30%. These were used for an undirected principal components analysis. The top 384 genes, with a multiple-comparisons corrected p-value <0.05 for difference between controls and CTEPH, were used for a heatmap with hierarchical clustering.

Analysis of enriched gene function groups was performed with the 2010 release of WebGestalt with the use of the hypergeometric test for enrichment of either gene ontology consortium categories or Pathway Commons. Principal component analysis and hierarchical clustering were performed in JMP 10, a subset of SAS (Cary, NC).

PA immunofluorescence

Paraffin-embedded tissue sections of PEA endarterectomized tissue from CTEPH patients, and pulmonary arteries from IPAH patients and unmatched donors (controls) were collected under IRB protocols 151082 and 9401. Please see data supplement for full details of the protocol. Imaging was performed using a Nikon Eclipse Ti series confocal microscope at 10×, 20×, and 40× magnification.

PA protein expression

Protein was isolated from flash frozen human lung according to standard protocol with protease inhibitors, and concentrations were estimated by Bradford assay (see supplement for full details). Results were quantified using densitometry via ImageJ software. Results were normalized to a loading control.

Statistical methods

Continuous variables are reported as mean ± SD. Differences between continuous variables are calculated using a t-test, ANOVA, Mann–Whitney U test, or Kruskal–Wallis test, as appropriate. A p-value <0.05 is considered statistically significant for non-genetic analyses. Statistical analysis was performed using Prism version 7 for Mac, GraphPad Software, La Jolla, California, USA.

Results

Clot lysis assay

We first tested the hypothesis that patients with CTEPH have impairment in clot lysis compared with PAH patients and disease controls. Mean clot lysis time for CTEPH patients ($n = 14$), PAH patients ($n = 14$), acute PE patients ($n = 17$), and controls ($n = 17$) is shown in Figure 1 (clinical data in e-Table 2). There was no significant difference in clot lysis times across the four groups ($p = 0.072$) or between CTEPH patients and controls (485 s versus 482 s, $p = 0.26$). Clot lysis times were longer on average when apixaban was not included in the assay (971 s versus 504 s, $p < 0.0001$), but there was still no difference in clot lysis time between CTEPH and control samples (882 s versus 996 s, $p = 0.17$).

Characterization of CTEPH endarterectomized tissue

As we did not identify alternations in clot lysis time in CTEPH patients versus controls, we next sought to

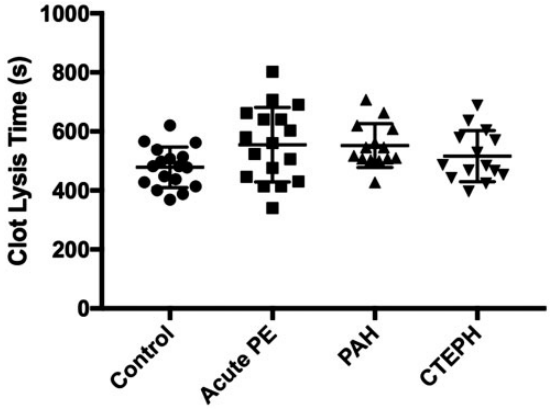

Figure 1. Comparison of clot lysis times between patients with CTEPH, acute PE, PAH, and patients treated with warfarin for atrial fibrillation with no known history of venous thromboembolic event (control).
CTEPH: chronic thromboembolic pulmonary hypertension; PAH: pulmonary arterial hypertension; PE: pulmonary embolism.

characterize CTEPH pathology, including cell type, in proximal pulmonary arterial lesions, and consider potential pathways that might be altered in CTEPH using IPAH and healthy donor pulmonary arterial tissue as controls. CTEPH endarterectomized tissue demonstrated numerically reduced, yet present, immunostaining for vWF and Factor VIII (endothelial cell markers) (Figure 2). We found that SMA staining was present in tissue from CTEPH resection. Moreover, SMA stain was less intense in CTEPH ($p < 0.05$) compared with PAH and control. In addition, vimentin, a mesenchymal cell marker, also demonstrated a trend toward a decreased expression ($p < 0.06$) in CTEPH tissue compared to controls.

Gene expression differences in PA and in cultured lymphocytes

We next sought to explore the molecular differences that may underlie pathology observed in CTEPH lesions. Using RNASeq, we studied RNA expression patterns in pulmonary arteries among the three groups (CTEPH $n = 3$, IPAH $n = 4$, control $n = 4$), and found 538 genes had at least four-fold difference in expression between CTEPH patients and controls with $p < 0.05$. Principal component analysis yielded 12 principal components; CTEPH, PAH, and control patients were reliably differentiated by the first two (Figure 3). Gene ontology analysis showed that these genes are involved

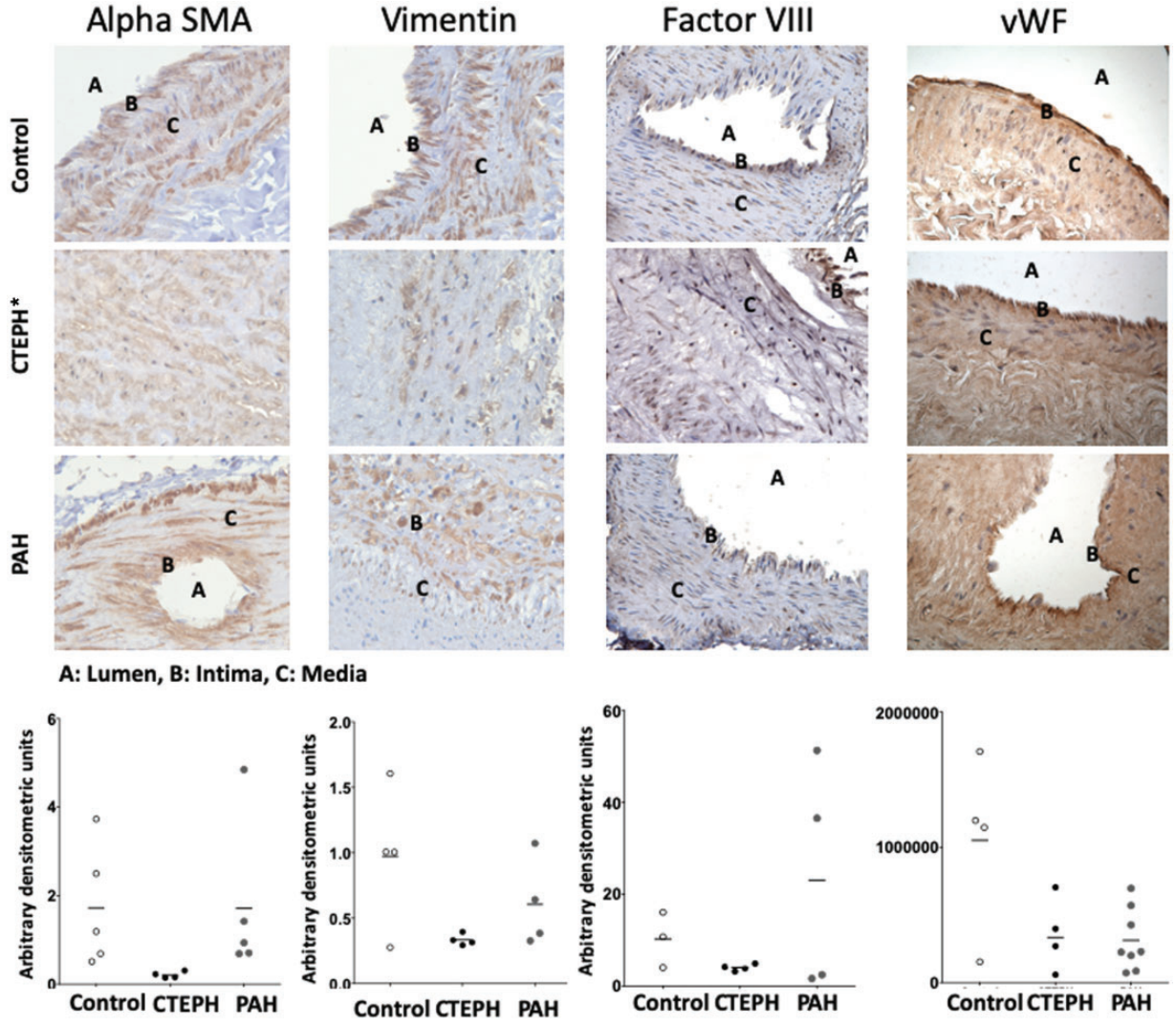

Figure 2. Alpha SMA (magnification 600×), vimentin (magnification 600×), factor VIII (magnification 200×), and vWF (magnification 400×), protein expression in control human lung PA, resected tissue from CTEPH patient, and IPAH PA. Semiquantitative densitometric analysis shows relative density of each stain in CTEPH, PAH, and controls in arbitrary densitometric units. *=$p < 0.05$ versus controls and PAH. A = lumen, B = intima, and C = media.
CTEPH: chronic thromboembolic pulmonary hypertension; PAH: pulmonary arterial hypertension; SMA: smooth muscle actin; vWF: Von Willebrand factor.

in biological processes such as response to lipid (52 genes, 1.19×10^{-9}), inflammatory response (39 genes, $P = 2.08 \times 10^{-6}$), and cell adhesion (68 genes, $p = 7.05 \times 10^{-9}$) (Table 1). The KEGG pathway that was most statistically different between CTEPH and control patients was PPAR signaling (13 genes, adjusted $P = 1.55 \times 10^{-6}$), with all genes having greater expression in controls than CTEPH patients (Table 2).

We next sought to determine if gene expression changes identified in the tissue of highest relevance may be similar in more readily available cultured lymphocytes from peripheral blood in the CTEPH and healthy controls. Three hundred and eighty-four genes were differentially expressed ($p < 0.05$) between CTEPH and controls. Undirected principal component analysis yielded 17 principal components, and the two most different principal components separated CTEPH from healthy controls (Figure 4(a)). A heatmap comparing gene expression levels between CTEPH patients and controls also showed that gene expression patterns are similar among CTEPH patients, and different from controls (Figure 4(b)). Gene ontology pathway analysis revealed multiple pathways in which genes were differently expressed between CTEPH patients and controls, including nuclear factor κB signaling, vascular endothelial growth factor receptor signaling, epidermal growth factor receptor signaling, and TNF receptor signaling (e-Table 3). In particular, inflammation appeared to be over-represented in both PA lesions and also in the peripheral blood from CTEPH patients, while the resected material also suggested metabolic pathways may be of relevance to the pathology of CTEPH.

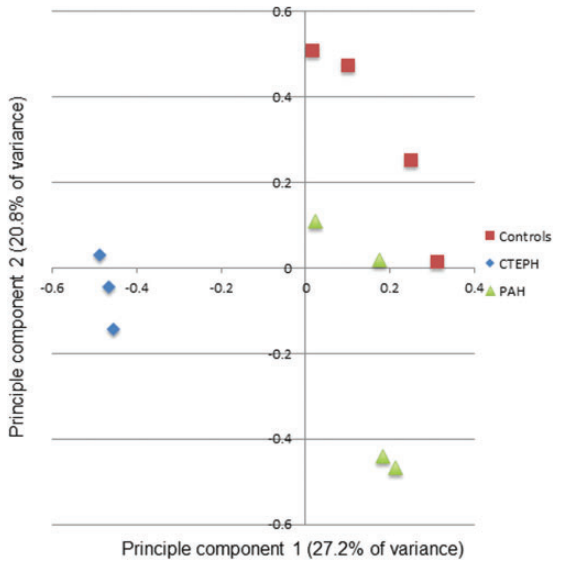

Figure 3. Undirected principal component analysis shows that gene expression patterns in PA tissue differ between patients with CTEPH, IPAH, and controls.
CTEPH: chronic thromboembolic pulmonary hypertension; PAH: pulmonary arterial hypertension.

Validation in PA tissue

Based on our PA and peripheral blood lymphocyte gene expression data, we next focused on tissue validation, particularly in the inflammatory pathway. There were no significant differences in VEGF, NFκB, or ICAM expression in pulmonary arteries from the CTEPH, PAH, and control groups (Figure 5). IL-8, however, was numerically increased in the lesions of CTEPH patients compared with controls and PAH arteries ($p = 0.05$).

Given the importance of metabolic pathways in the tissue microarray in distinguishing CTEPH from PAH and controls, we selected two proteins from the PPAR signaling pathway for confirmation via immunofluorescence and Western blot in PA tissue samples in CTEPH, IPAH, and control groups. CD36 (CTEPH versus control $p = 5.5 \times 10^{-5}$) and adiponectin (CTEPH versus control $p = 1.0 \times 10^{-4}$) were chosen for having markedly different expression between the two groups, and biological plausibility for a role in CTEPH and PAH pathogenesis. Adiponectin deficiency has been implicated in rodent models of PAH, and CD36 has known roles in thrombosis and metabolism.[13–16] In CTEPH tissue, there was a striking increase in CD36 immunofluorescence, whereas immunofluorescence for adiponectin

Table 1. Gene ontology categories identified in 538 genes differentially expressed ($p < 0.05$) in pulmonary artery tissue between CTEPH patients and controls.

Gene ontology category	No. of reference genes in pathway	Observed no. of variant genes in pathways	Expected no. of variant genes in pathway	p value (adjusted for multiple testing)
Response to lipid	582	52	17.64	1.19×10^{-9}
Cell adhesion	951	68	28.82	7.05×10^{-9}
Response to hormone synthesis	723	52	21.91	7.54×10^{-7}
Inflammatory response	484	39	14.67	2.08×10^{-6}

CTEPH: chronic thromboembolic pulmonary hypertension.

Table 2. KEGG pathways identified in 538 genes differentially expressed ($p < 0.05$) between CTEPH patients and controls.

KEGG pathway name	No. of reference genes in pathway	Observed no. of variant genes in pathways	Expected no. of variant genes in pathway	p value (adjusted for multiple testing)
PPAR signaling pathway	70	13	1.73	1.55×10^{-6}
Neuroactive ligand–receptor interaction	272	23	6.71	1.73×10^{-5}
Calcium signaling pathway	177	16	4.36	0.0003
Protein digestion and absorption	81	9	2.00	0.0056

CTEPH: chronic thromboembolic pulmonary hypertension; KEGG: kyoto encyclopedia of genes and genomes; PPAR: peroxisome proliferator-activated receptor.

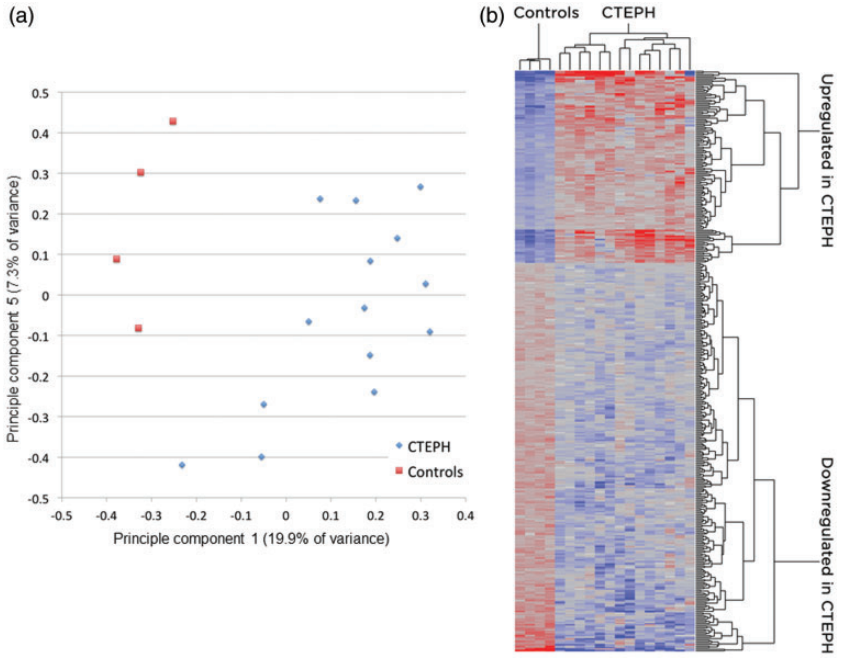

Figure 4. Undirected principal component analysis shows that gene expression patterns in peripheral blood lymphocytes differ between patients with CTEPH and controls (a). Heatmap showing gene expression is similar among CTEPH patients and differs from controls (b).
CTEPH: chronic thromboembolic pulmonary hypertension.

appeared to be modestly increased compared to control and PAH. The staining appeared strongest in endothelium and smooth muscle and increased in CTEPH compared to controls and IPAH samples (Figure 6(a)). Adiponectin staining was also present in all samples and appeared to have stronger staining in CTEPH samples compared to IPAH and controls.

Western blot indicated increased expression of adiponectin in CTEPH samples ($p = 0.01$ versus control) and IPAH samples ($p = 7.43 \times 10^{-5}$ versus control) to controls. We also found a lower ratio of unglycosylated to glycosylated CD36 in CTEPH compared to IPAH and controls (glycosylated CD36 in CTEPH versus control $p = 0.04$) (Figure 6(c)).

Discussion

In this multifaceted approach to better understand the pathogenesis of CTEPH, we have shown that clot lysis times in CTEPH patients are similar to multiple control phenotypes. With no evidence of impaired fibrinolysis in CTEPH patients, we then sought to better characterize the tissue of interest, and found that CTEPH endarterectomized tissue has reduced expression of markers of both smooth muscle and endothelial cells, suggesting possible de-differentiation. We then explored gene expression profiles in the pulmonary arteries and peripheral blood lymphocytes of CTEPH patients and controls. We identified differences in gene

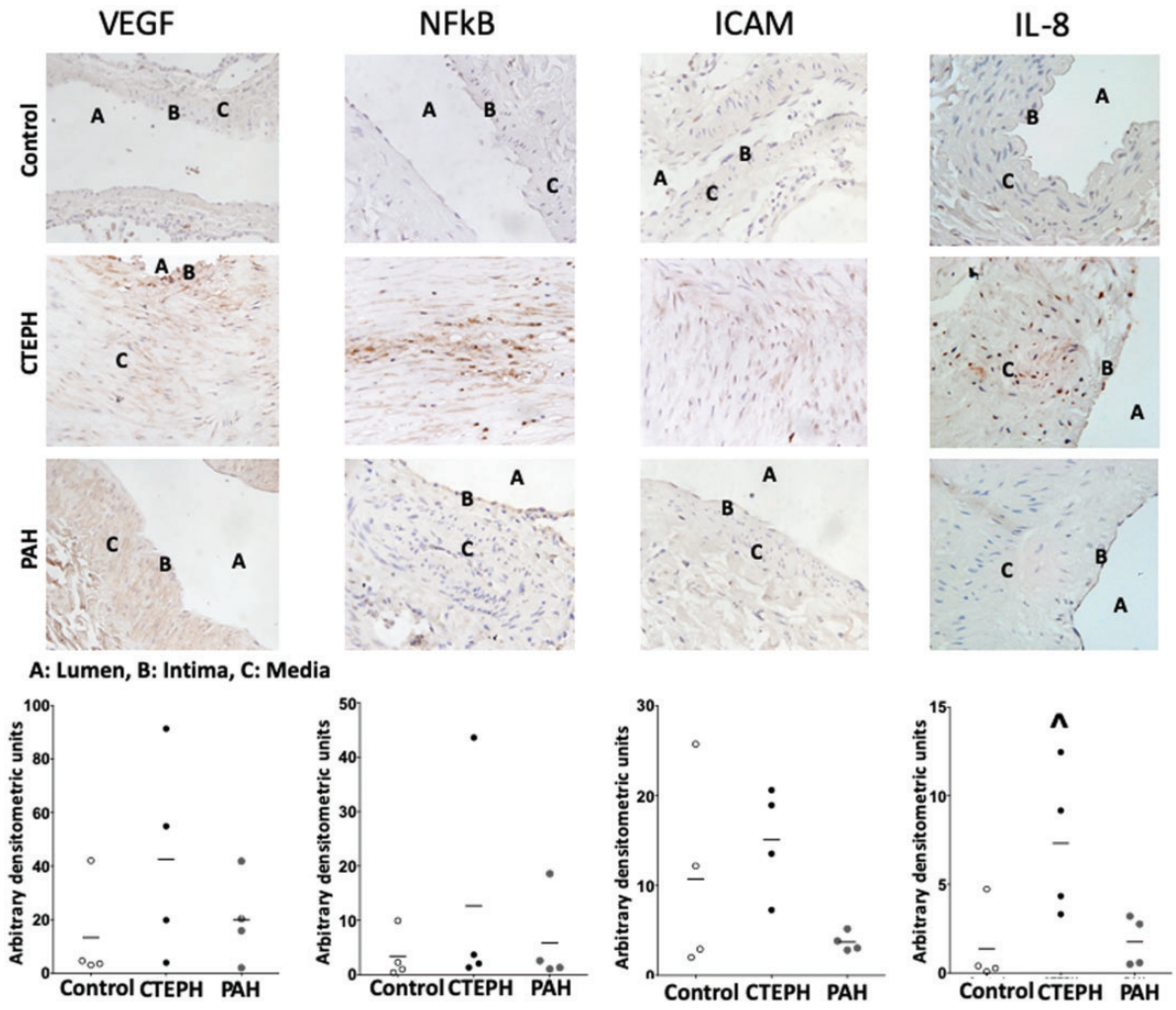

Figure 5. VEGF, NFκB, ICAM, and IL-8 protein expression in control human lung PA, resected tissue from CTEPH patient, and IPAH PA. Semiquantitative densitometric analysis shows IL-8 protein expression in numerically significant compared to control and IPAH ($p = 0.05$). Magnification 400× for all but IL-8, magnification 600×. A = lumen, B = intima, and C = media.
CTEPH: chronic thromboembolic pulmonary hypertension; ICAM: intercellular adhesion molecule; IL-8: interleukin 8; NFκB: nuclear factor kB; PAH: pulmonary arterial hypertension; VEGF: vascular endothelial growth factor.

expression patterns related to inflammation and growth factor signaling in peripheral blood which we explored using immunohistochemistry. Further, gene expression data from CTEPH endarterectomized tissue suggested significant alterations in metabolic pathways, including energy metabolism. Lastly, based on these gene expression profile differences, we performed immunofluorescence and Western blot experiments on the same tissue, and found that unglycosylated CD36 and adiponectin expression were increased in CTEPH versus controls, while glycosylated CD36 was suppressed in CTEPH.

Our finding that clot lysis times are similar between CTEPH patients and multiple controls suggests that dysfibrinogenemias and impaired endogenous clot resolution are likely not the primary mechanism in the pathogenesis of CTEPH. Previous studies have reported cases of dysfibrinogenemias in CTEPH cohorts; however, it should be noted that in these cohorts the majority of patients had no identifiable fibrinogen mutations, and the extent to which these mutations contribute to the overall pathogenesis of CTEPH is unclear.[5,6,17] These studies also used purified fibrin extracted from patients, rather than a clot lysis assay, a functional assay we have used in our experiments. The adapted turbidimetric assay may provide a better model of endogenous thrombus formation and thrombolysis. This assay also has limitations, however, as it cannot account for the role that circulating cells such as platelets, or the vascular endothelium, play in the fibrinolytic process. Given our consistent findings, and the relatively low prevalence of fibrinogen

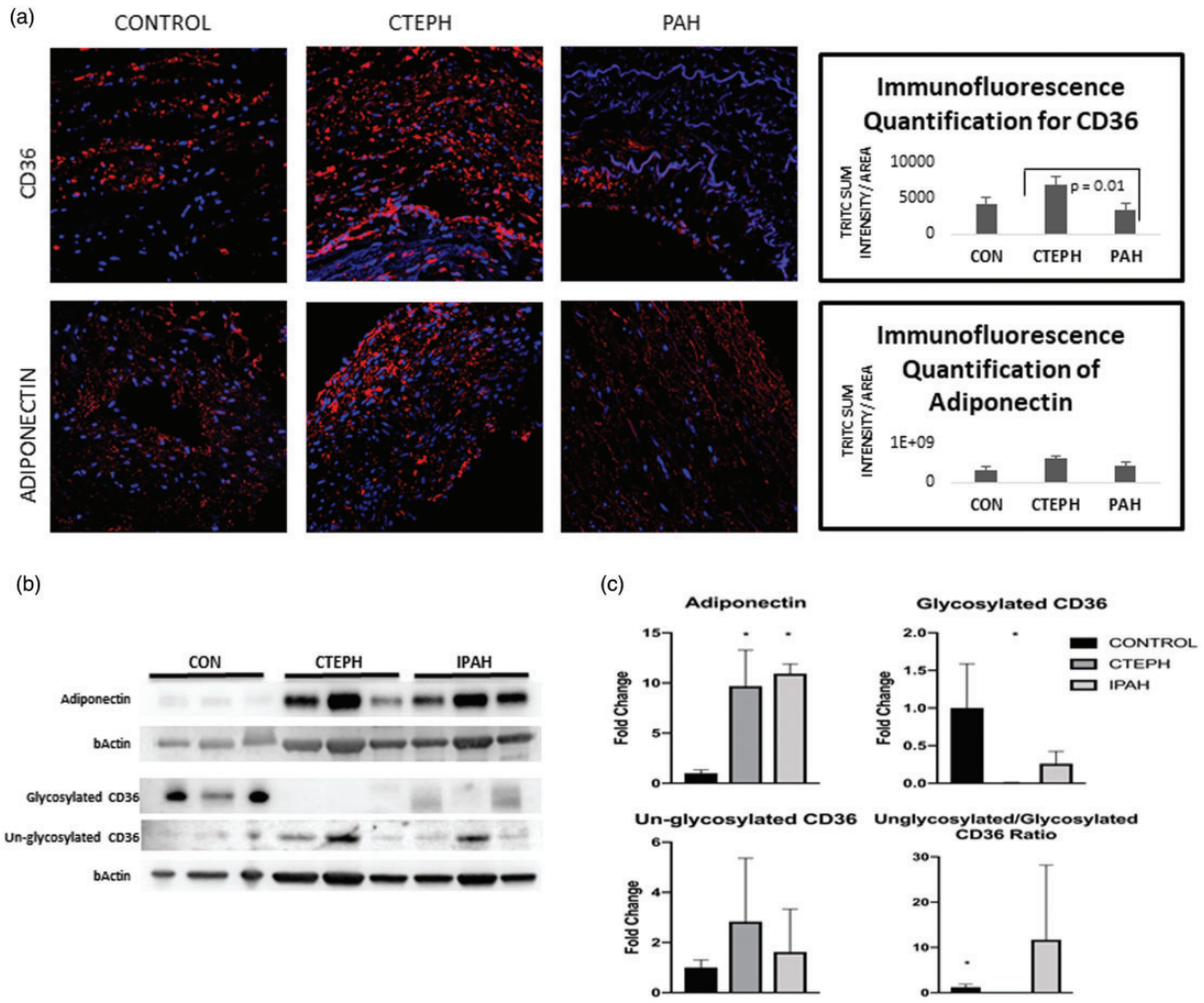

Figure 6. Immunofluorescence staining for CD36 and adiponectin in PA samples from controls, CTEPH, and IPAH patients (a). Representative Western blot for CD36 and adiponectin among the same groups (b). Relative protein expression of adiponectin, unglycosylated CD36, and glycosylated CD36 and ratio of unglycosylated to glycosylated CD36 from Western blots (c). *=$p < 0.05$ versus controls.
CTEPH: chronic thromboembolic pulmonary hypertension; IPAH: idiopathic PAH; PAH: pulmonary arterial hypertension.

mutations among CTEPH patients, our data suggest that other risk factors and mechanisms likely play a larger role in CTEPH pathogenesis.

Our characterization of CTEPH endarterectomized tissue suggested significant differences between CTEPH and PAH as well as controls, despite the known similarity between CTEPH and PAH histopathologically.[18,19] There was generally reduced expression of all markers of endothelial and smooth muscle cells. These are likely similar to mesenchymal progenitor cells, and "endothelial-like," "smooth muscle-like," and "myofibroblast-like" cells described in previous descriptions of CTEPH histopathology.[18,20,21] Spindle cell carcinomas and other similar tissues have been described to be vimentin positive; however, CTEPH endarterectomized tissue, while morphologically similar to spindle cells, did not have significantly enhanced vimentin staining.[22] This mesenchymal cell marker may be used to identify fibroblasts as well, and this cell type does not appear to be enriched in CTEPH. Taken together, CTEPH cells appear to have reduced, but not absent, markers of endothelial and smooth muscle cells, suggesting possible de-differentiation of cell type within these lesions.

Chronic inflammatory conditions such as inflammatory bowel disease and malignancy, and infections such as osteomyelitis have all been associated with increased risk for CTEPH.[4] Furthermore, recent studies have demonstrated the accumulation of neutrophils and macrophages in PEA specimens from CTEPH patients, and evidence of enhanced systemic inflammation, as demonstrated by increased pro-inflammatory cytokines

in serum.[7] We found that IL8 expression is numerically higher in CTEPH PA lesions than in PAH or control in our studies. Interestingly, IL-8 has been shown to be increased in peripheral blood of patients with CTEPH previously, though direct enhancement in the PA has not been described.[23]

Alterations in certain metabolic pathways, particularly insulin resistance and fatty acid metabolism, have shown repeatedly in both PAH and pulmonary hypertension associated with heart failure.[24–26] The PPAR pathway has been implicated in this, and even targeted for potential therapeutic intervention in animal models.[27,28] CD36, in particular, has multiple potential mechanisms for contributing to CTEPH pathogenesis. CD36 is a receptor for thrombospondin in platelets, endothelial cells, monocytes, and various other human cell lines, and plays a role in mediating endothelial cell antiangiogenic response.[29] CD36 glycosylation is also known to increase fatty acid uptake, and glycosylation is necessary for trafficking to the plasma membrane.[16,29,30] Thus, the predominance of unglycosylated CD36 in CTEPH and PAH is potentially mechanistically related to the multiple metabolic derangements seen in these diseases, particularly insulin resistance and lipid metabolism.

Our study has several limitations. First, it is unclear what an appropriate tissue control for pathologic examination of CTEPH is, given that CTEPH endarterectomized tissue does not have a clear correlate in non-diseased lungs or other disease states. We chose to use PA tissue from unmatched donors and PAH patients as the best available control, but fully recognize that there is no true pathologic correlate of PEA endarterectomized tissue from the PA. Also, we were only able to study proximal tissues resected during PEA, not distal arteries or arterioles, which may have different pathologic manifestations of the disease. These differences may partly account for differences in expression patterns; however, the tissue validation made this less likely. This study is also limited by the number of available pathology specimens, given the relative rarity of the disease, especially PAH pulmonary arteries and the PEA CTEPH samples. Additionally, while the use of peripheral blood lymphocytes for gene expression profiling is a convenient due to their easy accessibility, this is not a comprehensive evaluation of the cell types that may have altered gene expression in CTEPH, and does not allow for detection of all inflammatory markers that may be altered in the disease.

In conclusion, the results from our multifaceted approach to better characterize CTEPH pathogenesis suggest that thrombophilias and impaired fibrinolysis may have less of a role in CTEPH pathogenesis than previously suspected, and alterations in systemic inflammation and metabolism may have a larger, and previously underappreciated role. Whether these changes in inflammation and metabolism are causative, or are sequelae of CTEPH cannot be determined from the current study, but certainly merit further investigation.

Contributorship

Study concept and design: DTM, YRS, TSA, JDW, ARH; Acquisition of data: DTM, JDW, MHT, NLF, DG, AM, ARH; Analysis and interpretation of data: SJH, DTM, JDW, MHT, DG, AM ARH; Drafting of the manuscript: SJH, DTM, ARH; Critical revision of the manuscript for important intellectual content: SJH, DTM, YRS, TSA, JDW, MHT, EDA, ARH; Statistical analysis: DTM, JDW, ARH; Study supervision: DTM, ARH.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

This study was approved by the Vanderbilt University Internal Review Board (IRB #s provided within text).

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Supplemental Material

Supplemental Material for this article is available online.

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