Differences in the expression of DNA methyltransferases and demethylases in leukocytes and the severity of pulmonary arterial hypertension between ethnic groups

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Abstract
The loss of ten-eleven translocation (TET2) methylcytosine dioxygenase expression contributes to the pathobiology of pulmonary arterial hypertension (PAH). However, whether the expression and activity of other TETs and DNA methyltransferases (DNMTs) are altered in PAH remains enigmatic. Therefore, our objective was to determine the expression of DNMT (1, 3a, and 3b) and TET (1, 2, and 3) and their total activity. We assessed the expression of DNMT and TET enzymes in the leukocytes and their activity in extracellular vesicles (EVs). Expression of DNMT (1, 3a, and 3b), TET (2 and 3) in leukocytes, and total activity in EVs, from PAH patients was higher than in healthy controls. Additionally, we noticed there were differences in expression of these epigenetic enzymes based on ethnicity and found higher DNMT1 and lower TET2/TET3 expression in Caucasian than Hispanic/African American (combine) patients. Since loss-of-function mutation(s) and down-regulation of TET enzymes are associated with hematological malignancies and cytokine production, we determined the expression of genes that encode cytokines in samples of Caucasian and Hispanic/African American patients. Expression of IL6, CSF2, and CCL5 genes were higher in the leukocytes of Caucasian than Hispanic/African American patients, and CSF2 and CCL5 negatively correlated with the decreased expression of TET3. Interestingly, the expression of gene encoding CD34, a marker of myeloid and lymphoid precursor cells, and CD163, a monocyte/macrophage protein, was higher in the leukocytes of Caucasian than Hispanic/African American patients. Furthermore, Hispanic/African American patients having higher TET2/TET3 expression had higher pulmonary capillary wedge pressure. In conclusion, our results revealed higher DNMT1 and lower TET2/TET3 in Caucasian than Hispanic/African American patients together potentially augmented genes encoding inflammation causing cytokines, and CD34+ derived immunogenic cells, and the severity of PAH.
1 INTRODUCTION

Evidence that alteration of DNA and histone methylation or acetylation (epigenetic modifications) are associated with the development of all types of pulmonary hypertension is accumulating (Napoli et al., 2019). It has been proposed, for example, that altered methylation and acetylation marks on histones produce a persistent inflammatory phenotype in fibroblasts and an apoptosis-resistant and hyperproliferative phenotype of endothelial and smooth muscle cells from hypertensive patients and animal models (Hu et al., 2019). DNA methylation is regulated by DNA methyltransferases (DNMTs; 1, 3a, and 3b) and TET methylcytosine dioxygenases (TETs; 1, 2, and 3), DNA demethylases (Lachat et al., 2020).

In recent studies, we and others observed that differential DNA methylation and a reduction in TET2 mRNA expression may be key to the pathogenesis of pulmonary hypertension in mice and humans (Joshi et al., 2020; Potus et al., 2020). In those studies, our laboratory found that DNA methylation-dependent transcription of pulmonary hypertension-related genes and Tet2 expression is suppressed in the lungs of hypoxic mice (Joshi et al., 2020). Moreover, we observed that hypoxia-induced metabolic reprogramming contributes to the reduction in Tet2 expression, and that inhibiting glucose-6-phosphate dehydrogenase activity increases pulmonary Tet2 expression in hypoxic mice and reduces pulmonary hypertension (Joshi et al., 2020). Others have found that inherited and acquired TET2 abnormalities occur in only 0.39% of pulmonary arterial hypertension (PAH) cases, but lower expression of TET2 in mononuclear cells was detected, with elevated pro-inflammatory cytokines, in >86% of inflammation-associated PAH cases (Potus et al., 2020). Consistent with those findings, Tet2 deletion leads to increases in right ventricular (RV) pressure in 7-month-old, but not in 2-month-old, mice (Potus et al., 2020). Altogether these findings highlight an association between reduced TET2 and the pathogenesis of pulmonary hypertension in humans and mice.

While loss-of-function mutations in TET2 are associated with diverse blood cell malignancies in humans (Solary et al., 2014), loss of a single TET enzyme is not sufficient to efficiently promote malignancy and promote synthesis of pro-inflammatory cytokines (An et al., 2015). Although reduction of TET2 has been implicated as a contributing factor in the development of PAH, information regarding the expression of DNMT and other TET enzymes is differentially regulated in patients with PAH remains unclear. More so, the activity of these epigenetic writers and eraser enzymes, which are metabolically regulated, remains undetermined in PAH cases. Therefore, we assessed DNMT (1, 3a, and 3b) and TET (1, 2, and 3) expression in leukocytes as well as their activity in extracellular vesicles (EVs), submicron intact vesicles (Blair et al., 2016), isolated from plasma collected from scleroderma (scl) and idiopathic (i) PAH patients. Furthermore, whether differential expression of DNMTs or TETs influences maladaptive cytokine gene expression and severity of PAH is unknown. Therefore, we analyzed the expression of DNMTs and TETs enzyme in Caucasian, Hispanic, and African American patients with PAH. Our findings suggest that DNMT (1, 3a, and 3b), TET2, and TET3 are increased in humans with PAH. What is more, the results suggest that higher DNMT1 and lower TET2 and TET3 expression is directly correlated with increased cytokines (IL6 and CCL5) and severity of PAH in Caucasian and Hispanic/African American (combined) patients.

2 MATERIALS AND METHODS

2.1 Drugs and reagents

All chemicals and reagents were purchased from Sigma, Thermo-Fisher, R&D Systems, MyBioSource, and VWR.

2.2 Collection of blood from control individuals and PAH patients

All protocols were approved by the New York Medical College and Westchester Medical Center Institutional Review Board (Protocol #11775). Patient demographics are presented in Table 1. Blood samples were collected into heparinized tubes from PAH patients and control individuals by venipuncture and stored at 4 °C for 24 h. White blood cells (WBCs/leukocytes) were isolated as described previously (Hashimoto et al., 2020). Briefly, the cells were collected by centrifugation at 300 g for 5 min at 4 °C. The supernatant was aspirated, and the cell pellet was resuspended in 10 ml of red cell lysis buffer (0.15 M NH4Cl; 0.01 M KHCO3; 0.1 M Na2EDTA, pH 7.2–7.4) and incubated on ice for 15 min. The cells were then collected by centrifugation (1200 g), washed twice with PBS and immediately suspended in Qiazol (700 μl; Qiagen), after which total RNA was isolated and used for real-time PCR as described below. We used leukocytes for gene expression analysis, as a surrogate for lung tissue or pulmonary artery, because they are increased in blood of PAH patients and a...
previous study has measured TET expression in mononuclear blood cells from PAH patients (Potus et al., 2020).

2.3 | Quantitative real-time PCR

Real time PCR was used to analyze mRNA expression. Briefly, total RNA was extracted from lungs and human leukocytes using a Qiagen miRNeasy kit (Cat # 217004). The quality and concentration of the input RNA were measured on the Synergy HT Take3 Microplate Reader (BioTek) and cDNA was prepared using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme (Cat # 11766500, Invitrogen) for mRNA. Quantitative PCR was performed in duplicate using TaqMan™ Fast Advanced Master Mix (Cat # 44-445-57) for mRNA using a Mx3000p Real-Time PCR System (Stratagene). The primers for the qPCR were purchased from Thermo Fisher Scientific/ TaqMan. mRNA levels were normalized to internal control TUBA1A, and relative mRNA expression was reported.

2.4 | Isolation and identification of EV

Because we used the leukocytes to extract RNA for PCR, we could measure activity of epigenetic enzymes in the immune cells. Therefore, we isolated EVs from plasma collected from PAH patients and healthy controls as previously described.
Briefly, we generated platelet-rich plasma by centrifugation of samples at 1500 × g for 20 min at room temperature. The platelet-rich plasma was then used to generate platelet-free plasma (PFP) by centrifugation at 13,000 g for 10 min at room temperature. PFP was diluted 1:40 with PBS and ultracentrifuged for 60 min at 100,000 g at 4°C in an Optima XE-100 Ultracentrifuge (Beckman Coulter). The supernatant plasma was then removed, and the recovered EV pellet was dissociated in PBS. We quantitated EVs using Zeta potential analysis and identified by transmission electron microscopy (TEM).

2.5 | Measurement of DNMT and TET activity

DNMT and TET activity was measured using kits from Epigentek. DNMT and TET activity were measured in EVs isolated from plasma collected from PAH patients and healthy controls.

2.6 | Isolation of leukocytes and flow cytometry

Blood samples were collected from PAH patients. After red blood cell lysis using lysing buffer (BD Biosciences), 10⁶ cells suspended in 90 µl of buffer were treated with 10 µl of FcR blocking reagent (Miltenyi Biotec) for 10 min at 4°C and stained with 10 µl of fluorescent antibodies for 15 min at 4°C. We used fluorescein (FITC)-conjugated anti-CD34 antibody purchased from Miltenyi Biotec. Flow cytometry was performed as described before (Hashimoto et al., 2020). Negative control (without) primary antibody treated cells were used each time as validation of antibodies.

2.7 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software. Values are presented as mean ± standard error (SE). Statistical comparisons of samples were made for two groups with Mann–Whitney test. Values of p < 0.05 were considered significant.

3 | RESULTS

3.1 | Treatment and hemodynamic changes in scleroderma-associated and idiopathic PAH cases

We collected blood from eight scl-PAH and five i-PAH patients and seven healthy controls. We had four Caucasians and four Hispanics in the scl-PAH group, four Caucasians and one African American in the i-PAH group, and six Caucasians and one Indian American in the control group. Their ages ranged from 56 to 78 years in the PAH group and from 24 to 64 years in the control group (Table 1). Right atrial pressure (RAP), pulmonary arterial pressure (PAP), pulmonary capillary wedge pressure (PCWP), transpulmonary gradient (TPG), pulmonary vascular resistance (PVR), and cardiac output and index did not differ between the scl-PAH and i-PAH groups (Table 2).

3.2 | DNMT and TET expression and activity in leukocytes and EV from PAH patients

To determine whether the expression and activity of DNMTs were modified in PAH, we assessed expression of three major DNMTs (1, 3a, and 3b) in leukocytes and total DNMT activity in EVs from PAH patients and controls. Real-time PCR showed that DNMT3a and DNMT3b, but not DNMT1, expression was significantly higher in the PAH than the control group (p < 0.05; Figure 1a–c). Similarly, we measured the expression of TET enzymes. While expression of TET1 did not change, TET2 and TET3 increased in the PAH than the control group (p < 0.05; Figure 1d–f). We did not find a correlation between age and expression of DNMTs and TETs (data not shown). To determine whether there was a corresponding increase in DNMT and TET activity in PAH cases, we isolated EVs from PAH patients and controls. After using zeta potential analysis and electron microscopy to confirm the diameter and appearance of the isolated EV (Figure 2a,b), we determined their associated enzyme activity. Total activity of DNMT and TET was significantly higher in EVs from the PAH patients (Figure 2c,d).

3.3 | DNMT and TET expression in Caucasian vs Hispanic/African American (combine) PAH patients

Since lower expression of TET2 in mononuclear cells was detected in >86% of inflammation-associated PAH cases (Potus et al., 2020), our findings suggest higher TET2 expression in PAH patients with scleroderma was somewhat perplexing and surprising to us. Therefore, to determine the potential cause for the differences in the results of the present and previous studies, we analyzed/compared the expression of DNMTs and TETs between different ethnic groups. Furthermore, along those lines, we noticed the difference in their expression based on the ethnicity and hence separated the patients based on ethnicity and in Caucasian and non-Caucasian groups to determine differences in the
expression and activity of epigenetic enzymes and cytokines. In the non-Caucasian group we combined Hispanics and African American patients. Interestingly, we found higher DNMT1 and lower TET2 and TET3 expression in leukocytes of Caucasians as compared with Hispanic/African American (combined) patients (Figure 3a–c), and this was not because of differences in the number of leukocytes between Caucasian and Hispanic/African American patients (Table 3).

3.4 | Cytokine levels in Caucasian versus Hispanic/African American (combine) PAH patients

Altered DNA methylation and reduced expression of TET enzymes have been implicated to elevate inflammatory cytokines and associated with hematological disorders or malignancies (An et al., 2015; Solary et al., 2014). Furthermore, increased cytokine levels have been concurrently observed with the loss-of-function mutation in TET2 and reduction of TET2 expression in PAH patients (Potus et al., 2020). Therefore, we determined expression of IL6, CCL5, CCL2, and CSF2 genes in leukocytes of Caucasian and Hispanic/African American patients. We found Caucasian as compared with Hispanic/African American patients expressed more IL6 and CCL5 (Figure 4a,b). Next, to determine if elevation of these cytokines are dependent on altered DNMT1 and/or TET2/TET3 expression, we performed Pearson correlation analysis. Interestingly, while neither IL6 nor CCL5 depend on DNMT1 (Figure 4c,d) and TET2 (Figure 4e,f), expression of both the cytokine genes and especially CCL5 gene negatively correlated ($p < 0.05$) with TET3 expression (Figure 4g,h). Similarly, we found CSF2 expression was higher in Caucasian versus Hispanic/African American patients and CSF2 expression negatively correlated ($p < 0.05$) with TET3 expression (Figure 4i–l). CCL2 expression did not differ between Caucasian and Hispanic/African American patients.

3.5 | CD163 gene levels positively correlates with the DNMT1 expression in Caucasian versus Hispanic/African American (combine) PAH patients

Next, we determined expression of genes encoding immunogenic cell markers and found higher expression of CD163 gene in leukocyte samples of Caucasian as
FIGURE 1  Expression of DNMTs and TETs elevated in leukocytes from scleroderma-associated and idiopathic-PAH patients than their controls. Using RT-PCR, DNMT, and TET expression were determined in leukocytes (all white blood cells) isolated from peripheral blood collected from scleroderma-associated and idiopathic (scl/-i) PAH patients and their healthy controls. (a–c) Violin plot shows expression of DNMTs increased in leukocytes (all white blood cells) from scl/i-PAH patients than their healthy controls. (d–f) Expression of TET1 did not increase, but TET2 and TET3 was elevated in leukocytes of scl/i-PAH patients than their healthy controls. N = 7 in the control group and N = 13 patients in the scl/i = PH group. Comparisons were made using Student’s t-test with Welch’s correction. *p < 0.05 and ** p < 0.005

FIGURE 2  Total DNMT and TET activity elevated in extracellular vesicles from scleroderma-associated and idiopathic-PAH patients than their controls. (a, b) Extracellular vesicles were isolated from the plasma of scl/i-PAH patients and their controls and confirmed with zeta potential analysis and transmission electron microscopy. DNMT and TET activity was determined in EV isolated from plasma of 2–3 control individuals and 2–3 scl/i-PAH patients pooled together. (c, d) DNMT and TET activity was higher in EV isolated from plasma scl/i-PAH patients than the healthy control individuals. DNMT and TET activity was measured in N = 3 for controls and N = 7 for PAH patients pooled plasma. Comparisons were made using Student’s t-test with Welch’s correction. *p < 0.05
compared with Hispanic/American African patients (Figure 5a). Furthermore, expression of CD163 depended on DNMT1 (Figure 5b) but not on TET2/TET3 (Figure 5c,d). Additionally, although the expression of ITGAM, a gene that encodes integrin αM chain expressed on monocytes and macrophages, trended to be higher in Caucasian (0.10 ± 0.03) than Hispanic/African American (0.06 ± 0.01) patients, we did not find significant differences between the groups and correlation with the expression of DNMT1.

3.6 | CD34+ cells and CD34 gene levels in Caucasian versus Hispanic/African American (combine) PAH patients

CD34+ cells are precursors of immunogenic myeloid and lymphoid cells. These cells are increased in blood and lungs of PAH patients. Therefore, we determined whether these cells and the gene encoding CD34 protein are elevated in Caucasian compared to Hispanic/African American patients with PAH. As we expected, CD34+ cell numbers and the expression of the gene encoding CD34 were higher in Caucasian than Hispanic/African American patients (Figure 5e,f) Furthermore, we found expression of CD34 and CD163 positively correlated (Figure 5g).

3.7 | Correlation between epigenetic enzymes and PCWP in Caucasian versus Hispanic/African American (combine) PAH patients

To determine whether there is any difference in the severity of PAH between Caucasian and Hispanic/African American patients, we compared the hemodynamic results. Our results revealed lower (p = 0.004) PCWP (Figure 6a) and a trend toward higher PVR (Caucasian: 9.74 ± 1.49 vs. Hispanic/African American: 6.40 ± 0.89; in mmHg; p = 0.075; Table 4) in Caucasian than Hispanic/African American patients. Next, to determine whether the expression of DNMT1 and/or TET2/TET3 has any bearing on the severity of PAH in Caucasian and Hispanic/African American patients, we performed Pearson correlation analysis. We found PCWP positively correlated (p < 0.05) with the expression of TET2 and TET3 (Figure 6b,c) and not with DNMT1 (data not shown).

4 | DISCUSSION

PAH is a heterogeneous and complex angio-proliferative disease. PAH also exhibits cancer-like physiognomies, and hence some studies have characterized PAH as cancer-like disease (Culley & Chan, 2018; Leopold & Maron, 2016). One of the hallmarks of cancer is an alteration of DNA and histone methylation resulting in increased expression of oncogenes and/or decreased expression of tumor suppressor genes (Ehrlich, 2019; Samudio-Ruiz & Hudson, 2012). Increased methylation frequently silences DNA repair genes and miRs involved in cancer biology (Lakshminarasimhan & Liang, 2016). As in cancer,
differential DNA and histone methylation is associated with different sub-types of PAH in experimental models and humans (Archer et al., 2010; Joshi et al., 2020; Ke et al., 2018; Napoli et al., 2019; Potus et al., 2015, 2020). Because methyltransferases and demethylases regulate the level of DNA and histone methylation and can thus alter gene transcription (Lachat et al., 2020), we investigated expression and activity of DNMT and TET enzymes in leukocytes and EVs from PAH patients.

While DNMT1, DNMT3a, and DNMT3b expression increased in leukocytes from PAH patients as compared to healthy controls, our data showing higher DNMT1 expression and PCWP in Caucasian than Hispanic/African American PAH patients suggests Caucasian patients with higher DNMT1 appear to have more severe PAH. Therefore, this implies augmented expression and activity of DNMTs, at least partly, contributed to the development and progression of PAH. In that regard, a recent study suggests expression of DNMT3b increases and other DNMTs decrease in lungs of congestive heart disease-associated PAH, and knockdown of Dnmt3b in rats augments monocrotaline and hypoxia induced pulmonary hypertension (Yan et al., 2020). Another recent study shows an association between up-regulation of DNMT1 and down-regulation of BMPR2 expression in lungs of PAH patients (Bisserier et al., 2021). Similarly, increased DNA methylation in the lungs of hypoxic mice reduces expression of genes encoding proteins, including BMPR1B and Krebs cycle enzymes, implicated in the pathogenesis of PAH (Joshi et al., 2020). While increased DNA hypermethylation leads to suppression of genes (Sod2 and Nos3) encoding vasodilatory proteins and antioxidants in lungs of hypoxic mice, pulmonary artery SMCs of fawn-hooded rats, and endothelial cells of fetal lamb with pulmonary hypertension (Archer et al., 2010; Joshi et al., 2020; Ke et al., 2018). Furthermore, DNA hypermethylation and downregulation of miR-126 contributes to right heart failure in PAH (Potus et al., 2015), and play various roles in the regulation of genes during the development of atherosclerosis (Dong...
Caused hypermethylation and downregulation of more DNMT1 and DNMT3b enzymes methylate unmethylated and hemi-methylated CpG islands, we suggest increased DNMT3a and DNMT3b expression and activity potentially leads to de novo hypermethylation of genes in PAH patients. Furthermore, our results suggest higher DNMT1 and lower TET2 and TET3 expression in Caucasian patients caused hypermethylation and downregulation of more protective genes genome-wide, including those mentioned earlier, increasing severity of PAH.

In addition to increased expression of DNMT (1, 3a, and 3b), our results revealed PAH patients as compared with healthy controls had higher total DNMT activity, which is driven by metabolites of one-carbon metabolism, the polyamine pathway, and the Krebs cycle (Puleston et al., 2017; Solary et al., 2014; Stover, 2009). Each of those pathways is reprogrammed in various cell types...
residing in the pulmonary artery and lungs of PAH patients and animal models (D’Alessandro et al., 2018). One-carbon metabolism and polyamine pathway metabolites (s-adenosylmethionine and spermine, respectively) are methyl-group donors and regulators of DNA methylation (Amelio et al., 2014; Puleston et al., 2017) and are elevated in lungs/fibroblasts/SMCs of PAH patients (Joshi et al., 2020).

Along with higher expression and activity of DNMT, we found increased expression of \textit{TET2} and \textit{TET3} and total TET activity in PAH patients than control individuals. These findings are somewhat perplexing because lower expression of \textit{TET2} in mononuclear cells was detected in >86% of inflammation-associated PAH cases (Potus et al., 2020) and smooth muscle cells isolated from i-PAH patients (Joshi et al., 2020). Although reasons for these differences in our results and previous studies are unclear, different source of genetic material (leukocytes vs. mononuclear cells/smooth muscle cells), methodologies (real-time PCR vs. microarray) employed to measure gene expression, disease state, and race of the patients, presumably contributed to the observed differences. \textit{TET2} expression levels were higher in Hispanic/African American than Caucasian patients who had more severe PAH. Therefore, we propose \textit{TET2} and \textit{TET3} expression and activity are increased to potentially antagonize actions of elevated DNMTs or vice versa in PAH patients.

Next, because loss-of-function mutation in \textit{TET2} and reduction of \textit{TET2} expression is associated with cytokine production in PAH cases (Potus et al., 2020), we determined the expression of genes that encode cytokines in samples of Caucasian and Hispanic/African American patients. We found higher \textit{CSF2} and \textit{CCL5} levels significantly correlated with decreased \textit{TET3}, but not with \textit{TET2} and \textit{DNMT1}, in Caucasian and Hispanic/African American patients. Increased \textit{IL6} did not correlate with \textit{DNMT1} and \textit{TET2/TET3}, indicating \textit{IL-6} expression may not be directly regulated by altered DNA methylation status in Caucasian and Hispanic/African American patients. Interestingly, augmented \textit{CD163} gene correlated with the expression of \textit{DNMT1} but not with the expression of either \textit{TETs}. Although \textit{DNMT1} is the key maintenance methyltransferase, down regulation \textit{miR-124} by increased \textit{DNMT1} facilitates M1 alveolar macrophage polarization in acute lung injury (Wang et al., 2021). Furthermore, we observed significantly higher

### Table 4: Hemodynamic results of Caucasian and Hispanic/African American PAH cases

| ID     | RAP | SPAP | DPAP | MPAP | PCWP | TPG | PVR | Fick CO | Fick CI |
|--------|-----|------|------|------|------|-----|-----|--------|--------|
| Caucasian |     |      |      |      |      |     |     |        |        |
| A^a    | 12  | 88   | 33   | 56   | 9    | 47  | 7.66| 8.63   | 4.1    |
| A5^a   | 2   | 86   | 28   | 48   | 2    | 49  | 17.56| 2.79   | 1.7    |
| B      | 1   | 58   | 13   | 31   | 4    | 27  | 5.7 | 4.73   | 3      |
| C      | 4   | 55   | 15   | 26   | 8    | 18  | 4.04| 4.45   | 2.7    |
| B1^a   | 3   | 76   | 24   | 44   | 6    | 38  | 8.59| 4.42   | 2.5    |
| J      | 15  | 86   | 27   | 45   | 6    | 39  | 12.18| 2.1    | 1.2    |
| M      | 6   | 91   | 33   | 56   | 9    | 47  | 8.27| 5.68   | 2.6    |
| O      | 13  | 83   | 35   | 51   | 11   | 40  | 8.23| 4.86   | 2.3    |
| Mean   | 7.0 | 77.9 | 26.0 | 44.6 | 6.9  | 38.1| 9.0 | 4.7    | 2.5    |
| SD     | 5.5 | 13.9 | 8.3  | 11.0 | 2.9  | 10.8| 4.2 | 2.0    | 0.9    |
| Hispanic and African American |     |      |      |      |      |     |     |        |        |
| A3^a   | 8   | 55   | 13   | 34   | 11   | 23  | 6.68| 3.44   | 1.9    |
| A4^a   | 5   | 60   | 21   | 36   | 11   | 25  | 7.39| 3.38   | 2.3    |
| B2     | 6   | 53   | 21   | 33   | 11   | 23  | 3.82| 3.88   | 2.2    |
| F      | 12  | 95   | 35   | 59   | 11   | 48  | 17.91| 2.68   | 1.87   |
| I      | 15  | 75   | 30   | 48   | 12   | 36  | 7.7 | 5.11   | 2.9    |
| Mean   | 9.2 | 67.6 | 24.0 | 42.0 | 11.2b| 31.0| 8.7 | 3.7    | 2.2    |
| SD     | 4.2 | 17.6 | 8.6  | 11.3 | 0.4  | 10.9| 5.4 | 0.9    | 0.4    |

Abbreviations: CI, Fick cardiac index (L/min/m²); DPAP, Diastolic pulmonary artery pressure (mmHg); Fick CO, Fick cardiac output (liters/min); MPAP, Mean pulmonary artery pressure (mmHg); PCWP, Mean Pulmonary capillary wedge pressure (mmHg); PVR, Pulmonary vascular resistance (Woods units); RAP, Mean right atrial pressure (mmHg); SD, standard deviation; SPAP, Systolic pulmonary artery pressure (mmHg); TPG, Transpulmonary gradient.

^aResults of these patients were from a recent publication (REF #14).^b \( p < 0.005 \) versus Caucasians.
CD34+ cells and CD34 gene expression in Caucasian than in Hispanic/African American patients. Since the expression of CD34 and CD163 were positively correlated, this potentially implies increased CD34+ cells convert to CD163 gene expressing monocytes, and decreased TET3 led to the upregulation of CSF2 and CCL5 in Caucasian and Hispanic/African American patients. Indeed, the reduction of TET2 and TET3 expression is responsible for hematologic disorders/malignancies and for increasing cytokine production (An et al., 2015; Solary et al., 2014). Inflammation caused by elevated cytokines, in perivascular region of the pulmonary artery, contributes to the remodeling of the pulmonary artery and increase pulmonary vascular resistance (El Kasmi et al., 2014). This is reflected by a positive correlation between PCWP and TET2/TET3 expression. Therefore, it is not unreasonable to suggest that lower TET2/TET3 expression contributed to the severity of PAH in Caucasian than in non-Caucasian (Hispanic/African American) patients.

Although our findings clearly suggest differences in the expression and activity of epigenetic writers and erasers are potentially associated with the severity of PAH, these results should be cautiously interpreted as they could be confounded by the treatment for PAH. However, this is likely to be a minor point because there was no difference in expression of all genes, we examined, in Hispanic patients that were on treatment and one patient on no treatment. Furthermore, a small sample size in Caucasian and non-Caucasian (Hispanic/African American) precludes us from affirmatively suggesting that differences in the epigenetics between the groups is a cause of the severity of PAH, the statistically significant differences between the groups certainly hints DNA methyltransferases and demethylases must be important to eliciting the severity of PAH in Caucasian group and this must be further studied. Nevertheless, DNMT and TET activity in circulating EVs could be further developed as diagnostic markers for determining the severity of PAH in different ethnic groups. EVs are cargos that transport second messengers, genetic information, and proteins (Blair et al., 2016; Sayner et al., 2019). They are emerging diagnostic tools. Therefore, further multicenter studies in more PAH patients with diverse ethnic backgrounds are needed to identify if circulating EVs are released from the lungs or leukocytes, and to correlate the activity of epigenetic enzymes in EVs with the severity of the disease for diagnostic purposes and determining the effectiveness of the therapy in PAH patients.

In summary, our findings support the hypothesis that increased DNMT-catalyzed DNA methylation potentially contributes to the development and progression of PAH pathology in humans. Moreover, we have identified a potential association between expression and activity of increased DNMT1 and decreased TET2/TET3 and the excited inflammatory cytokine production and severity of PAH in Caucasian versus Hispanic/African American patients. Although some analyses suggest minorities may have a poor outcome in PAH, the role of ethnicity/race in PAH remains a controversial topic (Medrek & Sahay, 2018). Based on our findings, additional multicenter studies with more patients in different ethnic groups are warranted to confirm these findings and to develop new ethnicity/race-based therapies for PAH.

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AUTHOR CONTRIBUTIONS
Catherine D’Addario: performed experiments, analyzed the data, and read the manuscript. Gregg M. Lanier: Collected samples and clinical data, and read the manuscript. Christina Jacob: performed experiments, analyzed the data, and read the manuscript. Natalie Bauer: designed extra-vesicle particle isolation. Jenny L Hewes and Aritra Bhadra: performed EM and biochemical analysis of extra-vesicle particles. Sachin A. Gupte: designed the study, analyzed the data, wrote the manuscript, and organized the publication.

COMPETING INTERESTS
None.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE
This study was approved by the New York Medical College and Westchester Medical Center Institutional Review Board. A written consent was taken from each patient and control individuals prior to drawing blood.

CONSENT OF PUBLICATION
All authors have read the manuscript and have given their consent of publication.

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REFERENCES
Amelio, I., Cutruzzola, F., Antonov, A., Agostini, M., & Melino, G. (2014). Serine and glycine metabolism in cancer. Trends in Biochemical Sciences, 39(4), 191–198. https://doi.org/10.1016/j.tibs.2014.02.004
An, J., Gonzalez-Avalos, E., Chawla, A., Jeong, M., Lopez-Moyado, I. F., Li, W., Goodell, M. A., Chavez, L., Ko, M., & Rao, A. (2015). Acute loss of TET function results in aggressive myeloid
Hashimoto, R., Marsboom, G., Kim, G. H., Zhang, H. J., Toth, P. T., Svensson, E. C., Dyck, J. R. B., Gomberg-Maitland, M., Thebaud, B., Husain, A. N., Cipriani, N., & Rehman, J. (2010). Epigenetic attenuation of mitochondrial superoxide dismutase 2 in pulmonary arterial hypertension: A basis for excessive cell proliferation and a new therapeutic target. Circulation, 121(24), 2661–2671. https://doi.org/10.1161/CIRCULATIONAHA.109.916098

Bisserier, M., Mathiyalagan, P., Zhang, S., Elmastour, F., Dorfmuller, P., Humbert, M., David, G., Tarzami, S., Weber, T., Perros, F., Sassi, Y., Sahoo, S., & Hadli, L. (2021). Regulation of the methylation and expression levels of the BMP2R gene by SIN3a as a novel therapeutic mechanism in pulmonary arterial hypertension. Circulation, 144(1), 52–73. https://doi.org/10.1161/CIRCULATIONAHA.120.047978

Blair, L. A., Haven, A. K., & Bauer, N. N. (2016). Circulating microparticles in severe pulmonary arterial hypertension increase intercellular adhesion molecule-1 expression selectively in pulmonary artery endothelium. Respiratory Research, 17(1), 133. https://doi.org/10.1186/s12931-016-0445-1

Culley, M. K., & Chan, S. Y. (2018). Mitochondrial metabolism in pulmonary hypertension: Beyond mountains there are mountains. Journal of Clinical Investigation, 128(9), 3704–3715. https://doi.org/10.1172/JCI120847

D'Alessandro, A., El Kasmi, K. C., Plecita-Hlavata, L., Jezek, P., Li, M., D'Addario et al. (2015). Downregulation of MicroRNA-126 contributes to the failing right ventricle in pulmonary arterial hypertension. Circulation, 132(Suppl), 2406S–S2409. https://doi.org/10.1093/jn/132.8.2406S

Ehrlich, M. (2019). DNA hypermethylation in disease: Mechanisms and clinical relevance. Epigenetics, 14(12), 1141–1163. https://doi.org/10.1080/15592294.2019.1638701

El Kasmi, K. C., Pugliese, S. C., Riddle, S. R., Poth, J. M., Anderson, A. L., Frid, M. G., Li, M., Pullamsetti, S. S., Savai, R., Nagel, M. A., Fini, M. A., Graham, B. B., Tuder, R. M., Friedman, J. E., Eltzschig, H. K., Sokol, R. J., & Stemmark, K. R. (2014). Adventitial fibroblasts induce a distinct proinflammatory/profibrotic macrophage phenotype in pulmonary hypertension. The Journal of Immunology, 193(2), 597–609. https://doi.org/10.4049/jimmunol.1303048

Hashimoto, R., Lanier, G. M., Dhagia, V., Joshi, S. R., Jordan, A., Waddell, I., Tuder, R., Stemmark, K. R., Wolin, M. S., McMurtry, I. F., & Gupte, S. A. (2020). Pluripotent hematopoietic stem cells augment alpha-adrenergic receptor-mediated contraction of pulmonary artery and contribute to the pathogenesis of pulmonary hypertension. American Journal of Physiology: Lung Cellular and Molecular Physiology, 318(2), L386–L401.

Hu, C. J., Zhang, H., Laux, A., Pullamsetti, S. S., & Stemmark, K. R. (2019). Mechanisms contributing to persistently activated cell phenotypes in pulmonary hypertension. Journal of Physiology, 597(4), 1103–1119. https://doi.org/10.1113/JP275857

Joshi, S. R., Kitagawa, A., Jacob, C., Hashimoto, R., Dhagia, V., Ramesh, A. et al. (2020). Hypoxic activation of glucose-6-phosphate dehydrogenase controls the expression of genes involved in the pathogenesis of pulmonary hypertension through the regulation of DNA methylation. American Journal of Physiology: Lung Cellular and Molecular Physiology, 318(4), L773–L786. https://doi.org/10.1152/ajplung.00001.2020

Ke, X., Johnson, H., Jing, X., Michalkiewicz, T., Huang, Y. W., Lane, R. H. et al. (2018). Persistent pulmonary hypertension alters the epigenetic characteristics of endothelial nitric oxide synthase gene in pulmonary artery endothelial cells in a fetal lamb model. Physiological Genomics, 50(10), 828–836. https://doi.org/10.1152/physiogenomics.00047.2018

Lacey, M., Baribault, C., Ehrlich, K. C., & Ehrlich, M. (2019). Atherosclerosis-associated differentially methylated regions can reflect the disease phenotype and are often at enhancers. Atherosclerosis, 280, 183–191. https://doi.org/10.1016/j.atherosclerosis.2018.11.031

Lachat, C., Bruyere, D., Etchevery, A., Aubry, M., Mosser, J., Warda, W., Herfs, M., Hendrick, E., Ferrand, C., Borg, C., Delage-Mouroux, R., Feugeas, J.-P., Guittatt, M., Hervouet, E., & Peioto, P. (2020). EZH2 and KDM6B expressions are associated with specific epigenetic signatures during EMT in non small cell lung carcinomas. Cancers (Basel), 12(12), 3649. https://doi.org/10.3390/cancers12123649

Lakshminaraisimhan, R., & Liang, G. (2016). The role of DNA methylation in cancer. Advances in Experimental Medicine and Biology, 945, 151–172.

Leopold, J. A., & Maron, B. A. (2016). Molecular mechanisms of pulmonary vascular remodeling in pulmonary arterial hypertension. International Journal of Molecular Sciences, 17(5). https://doi.org/10.3390/ijms17050761

Medrek, S. K., & Sahay, S. (2018). Ethnicity in pulmonary arterial hypertension: Possibilities for novel phenotypes in the age of personalized medicine. Chest, 153(2), 310–320. https://doi.org/10.1016/j.chest.2017.08.1159

Napoli, C., Benincasa, G., & Loscalzo, J. (2019). Epigenetic inheritance underlying pulmonary arterial hypertension. Arteriosclerosis, Thrombosis, and Vascular Biology, 39(4), 653–664. https://doi.org/10.1161/ATVBAHA.118.312262

Potus, F., Pauciulo, M. W., Cook, E. K., Zhu, N., Hsieh, A., Welch, C. L., Shen, Y., Tian, L., Lima, P., Mewburn, J., & D'Arsligny, C. L. (2020). Novel mutations and decreased expression of the epigenetic regulator TET2 in pulmonary arterial hypertension. Circulation, 141(24), 1986–2000.

Potus, F., Ruffenach, G., Dahou, A., Thebaud, C., Breuil-Bonnet, S., Tremblay, E. et al (2015). Downregulation of MicroRNA-126 contributes to the failing right ventricle in pulmonary arterial hypertension. Circulation, 132(10), 932–943. https://doi.org/10.1161/CIRCULATIONAHA.115.016382

Puleston, D. J., Villa, M., & Pearce, E. L. (2017). Ancillary activity: Beyond core metabolism in immune cells. Cell Metabolism, 26(1), 131–141. https://doi.org/10.1016/j.cmet.2017.06.019

Samudio-Ruiz, S. L., & Hudson, L. G. (2012). Increased DNA methyltransferase activity and DNA methylation following Epidermal Growth Factor stimulation in ovarian cancer cells. Epigenetics, 7(3), 216–224. https://doi.org/10.4161/epi.7.3.19273

Sayner, S. L., Choi, C. S., Maulucci, M. E., Ramila, K. C., Zhou, C., Scruggs, A. K., Yarbrough, T., Blair, L. A., King, J. A., Seifert, R., Kaever, V., & Bauer, N. N. (2019). Extracellular vesicles: Another compartment for the second messenger, cyclic adenosine monophosphate. American Journal of Physiology: Lung Cellular and Molecular Physiology, 316(4), L691–L700. https://doi.org/10.1152/ajplung.00282.2018

Solary, E., Bernard, O. A., Tefferi, A., Fuku, F., & Vainchenker, W. (2014). The Ten-Eleven Translocation-2 (TET2) gene in
hematopoiesis and hematopoietic diseases. *Leukemia*, 28(3), 485–496. https://doi.org/10.1038/leu.2013.337

Stover, P. J. (2009). One-carbon metabolism-genome interactions in folate-associated pathologies. *Journal of Nutrition*, 139(12), 2402–2405. https://doi.org/10.3945/jn.109.113670

Wang, Y., Wang, X., Zhang, H., Han, B., Ye, Y., Zhang, M., Wang, Y., Xue, J., & Wang, C. A. (2021). Transforming growth factor-beta1 promotes M1 alveolar macrophage polarization in acute lung injury by up-regulating DNMT1 to mediate the microRNA-124/PEL1/IRF5 axis. *Frontiers in Cellular and Infection Microbiology*, 11, 693981.

Yan, Y., He, Y. Y., Jiang, X., Wang, Y., Chen, J. W., Zhao, J. H., Ye, J., Lian, T.-Y., Zhang, X. U., Zhang, R.-J., Lu, D., Guo, S.-S., Xu, X.-Q., Sun, K., Li, S.-Q., Zhang, L.-F., Zhang, X., Zhang, S.-Y., & Jing, Z.-C. (2020). DNA methyltransferase 3B deficiency unveils a new pathological mechanism of pulmonary hypertension. *Science Advances*, 6(50). https://doi.org/10.1126/sciadv.aba2470

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