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# Alteration of endothelial nitric oxide synthase expression in acute pulmonary embolism: a study from bench to bioinformatics

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**Abstract. – OBJECTIVE: This study sought to explore endothelial nitric oxide synthase (eNOS) expression in acute pulmonary thromboembolism (APE).**

**MATERIALS AND METHODS: eNOS expression in lung tissue and bone marrow-derived endothelial progenitor cells (BM-EPCs) from APE mouse models was assessed by immunohistochemistry and real-time PCR. A gene expression profile meta-analysis was performed on human venous thromboembolism (VTE) whole blood samples recorded in the Gene Expression Omnibus (GEO) repository. Significantly expressed genes were determined from the microarray data by unsupervised clustering and supervised classification. Selected sample data with significantly expressed genes were further analyzed by principal component analysis (PCA), followed by Bayesian probit regression. Key discriminate genes were further grouped and annotated using functional annotations and gene enrichments using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) software (v. 6.7).**

**RESULTS: While eNOS expression was significantly higher, serum nitric oxide levels were significantly lower in APE mice (20.42 ± 2.15 μM) compared to controls (53.50 ± 5.69 μM, $p<0.001$). eNOS mRNA and protein levels were significantly upregulated in BM-EPCs from APE mice. GEO repository data reported 3,397 upregulated and 4,173 downregulated genes (including eNOS) in VTE patients. In this regression analysis, the significant principal component PC1 and PC2 ($p<0.05$) were useful in distinguishing the VTE classification. The coefficient value of eNOS was -0.47707 in PC1 and -0.08429 in PC2, which did have some proportions on these significantly discriminated components but did not contribute significantly to the VTE classification. Functional enrichment in terms of acetylation and phosphoproteins were high.**

**CONCLUSIONS: Our findings, therefore, suggest that expression of eNOS is significantly altered in APE and may be a potential peripheral blood biomarker. Modulation of eNOS expression may be used for APE treatment.**

Key Words:
Pulmonary embolism, Endothelial nitric oxide synthase, Significance Analysis of microarrays, Principal component analysis.

## Introduction

Pulmonary embolism (PE) is a clinical and pathophysiological syndrome of the pulmonary circulation and respiratory dysfunction, which has a high rate of misdiagnosis, disability, and mortality. Deep venous thrombosis (DVT) and PE are two different stages of the same pathological process referred to as venous thromboembolism (VTE). In developed countries, PE is the third most common cardiovascular disease following coronary heart

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disease and hypertension[1,2]. In the United States, there are approximately 600,000 new cases each year, and the incidence is increasing with the aging of the population. Indeed, it was found that 6.0/10,000 of people suffered from PE per year through a study based on community[3]. In China, PE is also a disease that may be commonly seen and fatal. In recent years, 100-200 cases of PE have been diagnosed annually at the Peking Union Medical College Hospital and Beijing Chao-yang Hospital. A study of 900 autopsies showed that over 11% of cardiovascular disease cases were complicated by PE in the lung segment[1].

Nitric oxide synthases (NOSs) are a family of enzymes, the function of which is to catalyze the production of nitric oxide (NO) from L-arginine. NO has several biological functions, including modulating vascular tone, insulin secretion, and airway tone. Moreover, it is involved in both angiogenesis and neural development[4]. NO is controlled by isoenzymes: endothelial NOS (eNOS, also known as $NOS_3$), inducible NOS (iNOS), and neuronal NOS (nNOS). eNOS is mainly expressed in blood vessels, and promotes angiogenesis, maintains vascular endothelial function, and regulates vascular tone[4]. Under normal physiological conditions, a variety of cell types, including pulmonary vascular macrophages, endothelial cells, and smooth muscle cells, can produce NO in the presence of eNOS, which is necessary for blood vessel dilation, platelet aggregation inhibition, smooth muscle cell proliferation, and blood pressure regulation[5].

In the pathological process of PE, the expression and function of eNOS in the pulmonary vasculature may be altered, resulting in dysfunction of the pulmonary vascular endothelial barrier, increasing pulmonary vascular permeability and aggravating the inflammatory response. Thus, eNOS may be a key factor in repairing pulmonary vascular injury[6].

Endothelial progenitor cells (EPCs) are abundant in the bone marrow and have the capacity to migrate into the peripheral circulation from the bone marrow on ischemic stimulus[7]. It has been experimentally shown that EPCs can proliferate and home to the sites of endothelial denudation, as well as incorporate into sites of neovascularization[8,9]. EPCs may not only prevent ongoing risk factor-induced endothelial cell injury but also replace dysfunctional endothelium. They have the capability to improve the function of ischemic organs, and it's possible for them to act as a precursory biomarker for some types of ischemic diseases[10,11].

Gene expression profiling is a powerful tool to understand the molecular basis of disease and to identify unique gene expression patterns or distinctly expressed genes, which is important for discovering potential biomarkers associated with disease classification and therapeutic responses. In this study, we investigated eNOS expression in lung tissue and bone marrow-derived EPCs (BM-EPCs) isolated from mouse models of acute pulmonary embolism (APE).

Meta-analysis based computational methods can provide general information. In this study, we analyzed microarray data of venous thromboembolism (VTE) peripheral blood samples and compared them with healthy control data using unsupervised clustering and supervised classification, and extracted the principal components from the data by Principal Component Analysis (PCA), which were further analyzed by Bayesian probit regression modeling. Also, functional annotations and gene enrichments were added in order to explore the characteristics of peripheral blood from VTE, which may be useful for generally assessing APE, for instance, acetylation and phosphoprotein.

Integrated analysis of the gene expression patterns in APE peripheral blood is essential for the accurate diagnosis of the disease, as well as patient stratification and prognosis. In this regard, theoretical and experimental data analysis cannot only provide useful indications of the APE process, but will also help in identifying potential disease biomarkers.

## Materials and Methods

### Animal Models

All of the animal experiments were put into effect in accordance with the regulations set by the local regulatory agencies and with the "Regulations for the Management of Laboratory Animals", which was published by the Ministry of Science and Technology of the People's Republic of China. All animal experiments were approved by the Institutional Animal Care and Use Committee, Capital Medical University. C57BL/6J mice served as the background for the preparation of the APE animal model.

### Assessment of eNOS Protein Expression in the Lung Tissue of the APE Mouse Model

A mouse model of APE was prepared as previously published[12]. In brief, the models of APE

were established by injecting 30 autologous thrombin in 0.4 mL saline into the right jugular vein of mice, which were randomly split into two groups, control and experimental. At designated time points after injection, mice were sacrificed, and lung tissue was harvested and fixed in 10% formalin. Lung tissue eNOS protein levels were assessed by immunohistochemical staining. Briefly, after deparaffinization and rehydration, antigen retrieval was performed according to general immunostaining protocols. After blocking with 5% bovine serum albumin, the lung sections were incubated with the anti-eNOS antibody (Beijing ZSGB Biotechnology Co., Ltd., Beijing, China) overnight at 4°C. The next step after washing was to incubate samples with secondary antibody and enable them to react for 1 h at room temperature. The expression of eNOS was visualized by a 3,3'-diaminobenzidine reaction.

### Assessment of eNOS Expression in Mouse BM-EPCs

BM-EPCs were isolated and cultured as described previously[12]. Briefly, the bone marrow cells of a mouse were aspirated through femurs and tibias. Mononuclear cells (MNCs) were isolated by the method of using density centrifugation over Histopaque-1083 (Sigma-Aldrich, St. Louis, MO, USA). The MNCs were plated onto fibronectin (Chemicon, Temecula, CA, USA)-coated six-well plates in M199 complete medium (Invitrogen, Carlsbad, CA, USA) for seven days and it's a selective medium for endothelial lineage cells. The expression of eNOS mRNA in mouse BM-EPCs was quantified by RT-PCR using a previously described method[13]. The primer sequences used for eNOS were 5'TTTGTCTGCGGCGATGT3' (forward) and 5'GTGCGTATGCGGCTTGT 3' (reverse). β-actin was used as an internal control to normalize eNOS mRNA levels (forward: CTGAGAGGGAAATCGTGCGTGACA; reverse: ATACCCAAGAAGGAAGGCTGGAAAA).

eNOS protein levels in BM-EPCs was assessed by immunofluorescence staining. Briefly, after incubation with the anti-eNOS antibody (1:200, Santa Cruz, CA, USA) the whole night at 4°C, BM-EPCs is dealt with through anti-goat IgG-Cy3 (1:200, Sigma-Aldrich, St. Louis, MO, USA). Relative quantitative analysis with Image J 1.42 software (National Institutes of Health, Rockville, MD, USA) processed images.

### Serum NO Measurement

NO serum concentrations in APE and control mice were detected using the nitrate reductase method in accordance with the instructions of the manufacturer (NO Assay Kit, Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

### Gene Array Assessment

To further evaluate the results of the current study, previously published microarray analysis gene expression data was searched, downloaded, and reanalyzed as shown in the flowchart depicted in Figure 1. Briefly, gene expression profiles were searched and downloaded from GEO repository, followed by clustering (including principal component extraction and selection) and classification, and then by gene annotation analysis.

### Data Retrieval and Further in Depth Data Mining

Gene expression data were collected through the GEO repository by searching for the keyword "APE" on the website "http://www.ncbi.nlm.nih.gov/projects/geo/". Criteria for data selection were described previously[14]. The data downloaded were relabeled with officially named gene symbols and sample type initials. All measurements were $\log_2$ transformed, and duplicate measurements were all collapsed by their median values. The Student's

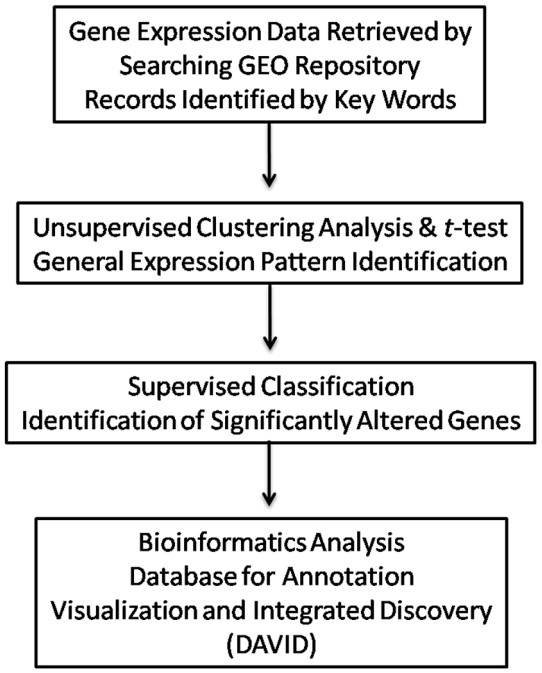



**Figure 1.** Schematic illustration of analysis of microarray data obtained from the GEO repository. Microarray data of human VTE was obtained from the GEO repository and data analysis was carried out by clustering, classification, and gene annotations.

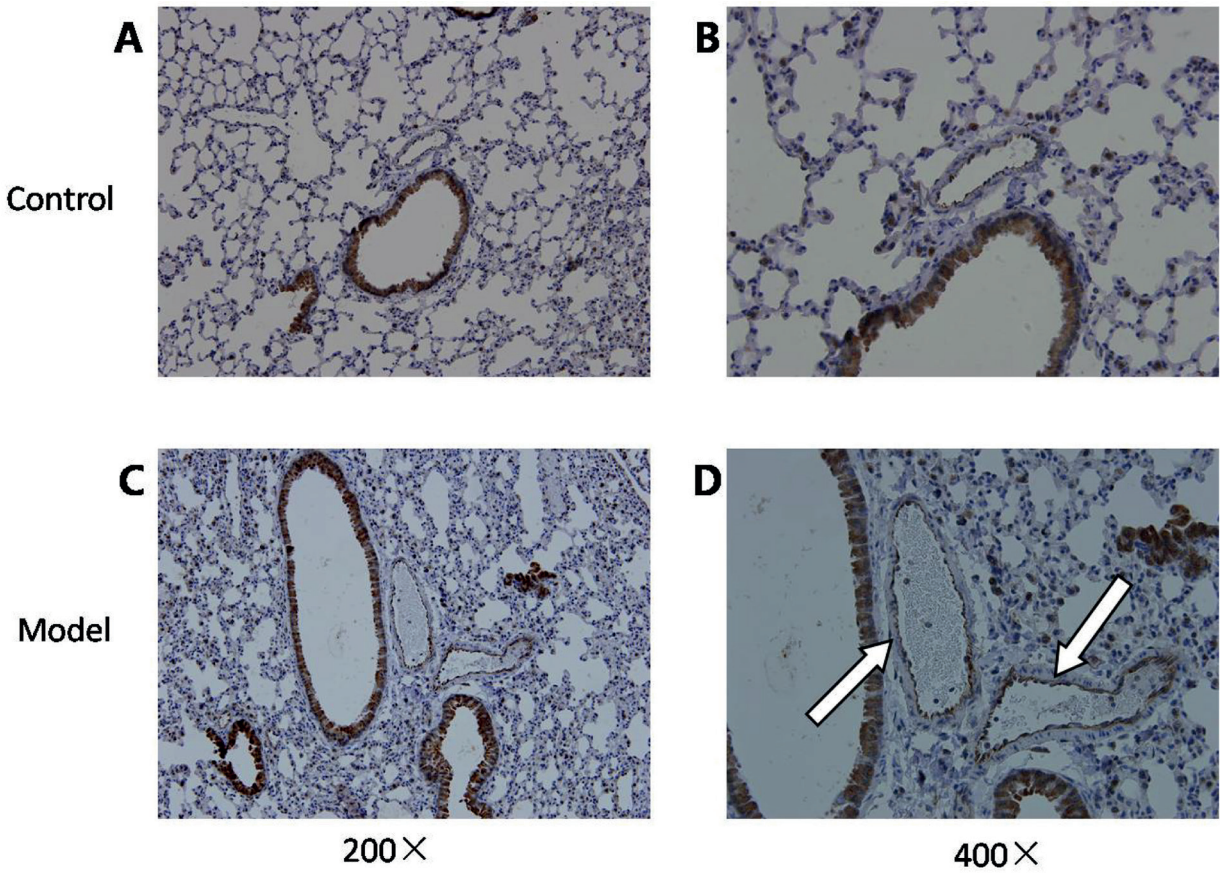



**Figure 2.** eNOS protein levels in lung tissues of APE and control mice. Lung tissues were harvested and subjected to immunohistochemical staining. A and B: Lung tissues from control mice. C and D: Lung tissues from APE mice. Arrows indicate the endothelial cell layer. Magnification: A and C: 200 ×; B and D: 400 ×.

t-test was adopted to analyze preprocessed data and differentially expressed genes ($p$<0.05), and the measurements of all samples were selected for two-way clustering (TWC) analysis. Differentiated samples with special gene expression patterns were recognized by comparing healthy controls with VTE whole blood samples, and general gene expression patterns were determined by alternately utilizing the Student's t-test and TWC analysis until no outlier samples could be recognized. The selected data were further analyzed by Principal Component Analysis (PCA) followed by Bayesian Probit Regression (BPR) analysis, which was a suitable statistical method for high dimensional data. Also, principal components with cumulative proportions of 85% were selected for BPR modeling.

Altered genes found in VTE samples relative to healthy controls were elucidated by supervised classification using the R command based software SAM 4.0 (http://www-stat.stanford.edu/~tibs/SAM/) on the sample with predicted expression patterns after unsupervised clustering[15]. False discovery rates were calculated for each altered gene. The T-statistic method predicted significantly altered genes and gave a SAM score for each altered gene. The higher the SAM scores, the more significant the distinctive gene. In this analysis, unpaired, type two was selected as the response type, and the data were centered before use. For gene annotation analysis convenience, the top 100 significantly expressed genes, including both the top 50 upregulated genes and downregulated genes, with the highest SAM scores were selected. While this number was 130 in a previous study[14], the threshold of 100 will cover the main features of the comparison and is more significant than the previously described methodology. For functional enrichment analysis, the top 100 significantly altered genes were submitted to the online software DAVID 6.7 (http://david.abcc.ncifcrf.gov/).

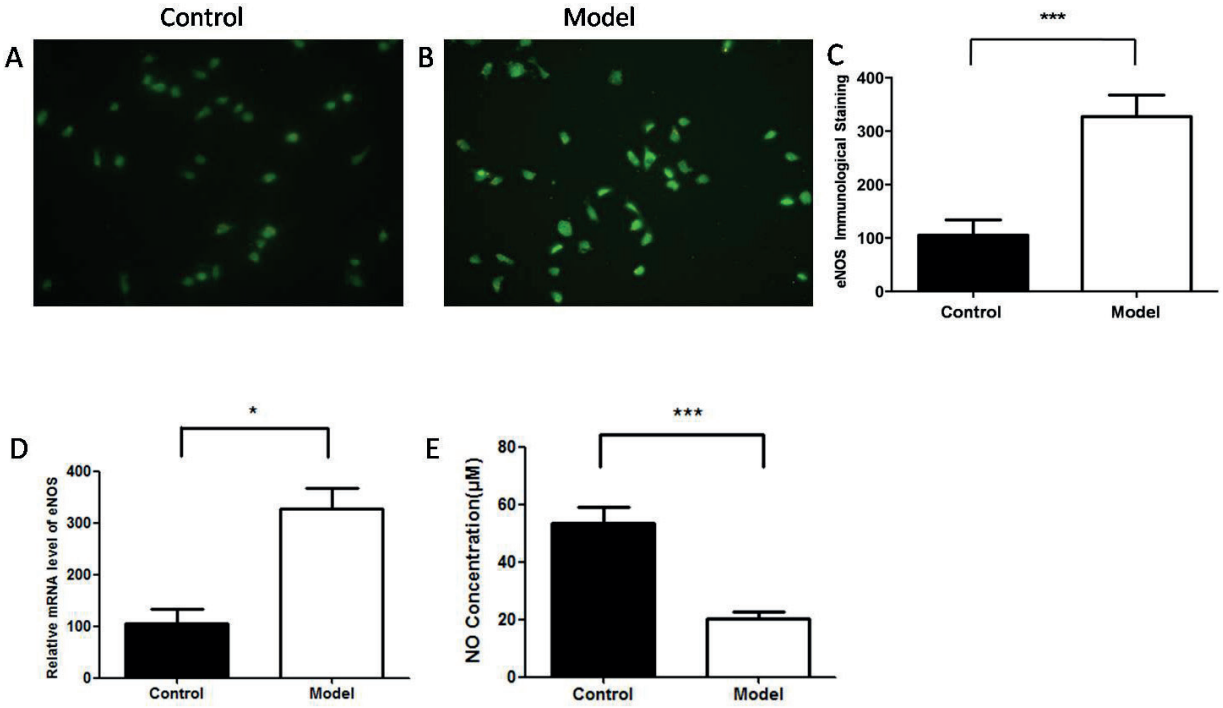



**Figure 3.** Expression of eNOS mRNA and protein in BM-EPCs, and serum NO measurements. BM-EPCs were isolated, the expression of eNOS mRNA was quantified by RT-PCR, and eNOS protein expression was assessed by immunofluorescence staining. ***A*** and ***B,*** Immunofluorescence staining of eNOS in BM-EPCs isolated from control ***(A)*** and APE ***(B)*** mice. ***C,*** Average eNOS protein expression in control (n = 3) versus APE (n = 3) groups. Vertical axis: ratio of eNOS-positive cells; horizontal axis: treatment groups. $^{***}p<0.001$. ***D,*** Expression of eNOS mRNA. Vertical axis: relative mRNA expression of eNOS versus β-actin; horizontal axis: treatment group. $^{*}p<0.05$. ***E,*** Serum NO quantification. Vertical axis: NO concentration (μM), horizontal axis: treatment groups. Data are presented as an average of eight experiments. $^{***}p<0.001$.

## Results

### *Protein Level of eNOS in APE Mice Lung Tissue*

eNOS protein levels in APE and control mice were assessed using immunohistochemistry (Figure 2). eNOS protein level was significantly increased in APE mice lung tissue (Figure 2C and 2D) compared to healthy control lungs (Figure 2A and 2B). Furthermore, eNOS immunostaining was strongly positive in pulmonary endothelial cells, bronchial epithelial cells, alveolar epithelial cells, and smooth muscle cells in lung tissue from APE mice, while it was weakly expressed in those cells in lung tissue from control mice.

### *eNOS mRNA and Protein Levels in Mouse BM-EPCs*

How eNOS mRNA expressed and protein in murine BM-EPCs was assessed. Immunofluorescence staining showed that eNOS protein was significantly increased in APE BM-EPCs (n = 3) on day one, relative to BM-EPCs from control mice (n = 3) ($p<0.001$, Fig. 3A-C). Likewise, eNOS mRNA expression in APE BM-EPCs was significantly upregulated on day one relative to controls (n = 4, $p<0.05$, Figure 3D).

In contrast to eNOS, serum NO levels were 20.42 ± 2.15 μM on day one of pulmonary embolism induction, which was significantly lower than the control group (53.50 ± 5.69 μM, n = 8, $p<0.001$, Figure 3E).

### *Microarray Data in the GEO Repository*

Since eNOS expression was significantly altered in APE mice, eNOS gene expression in PE was further investigated by searching the GEO repository database. It was found that only one entry (GSE19151) investigated gene expression modulation in human VTE. In this study, the authors analyzed whole blood gene expression in VTE patients and healthy controls. A total of 133 samples (70 samples from VTE patients and 63 controls) were investigated.

VTE gene expression data were analyzed by clustering and Student's t-test in order to explore

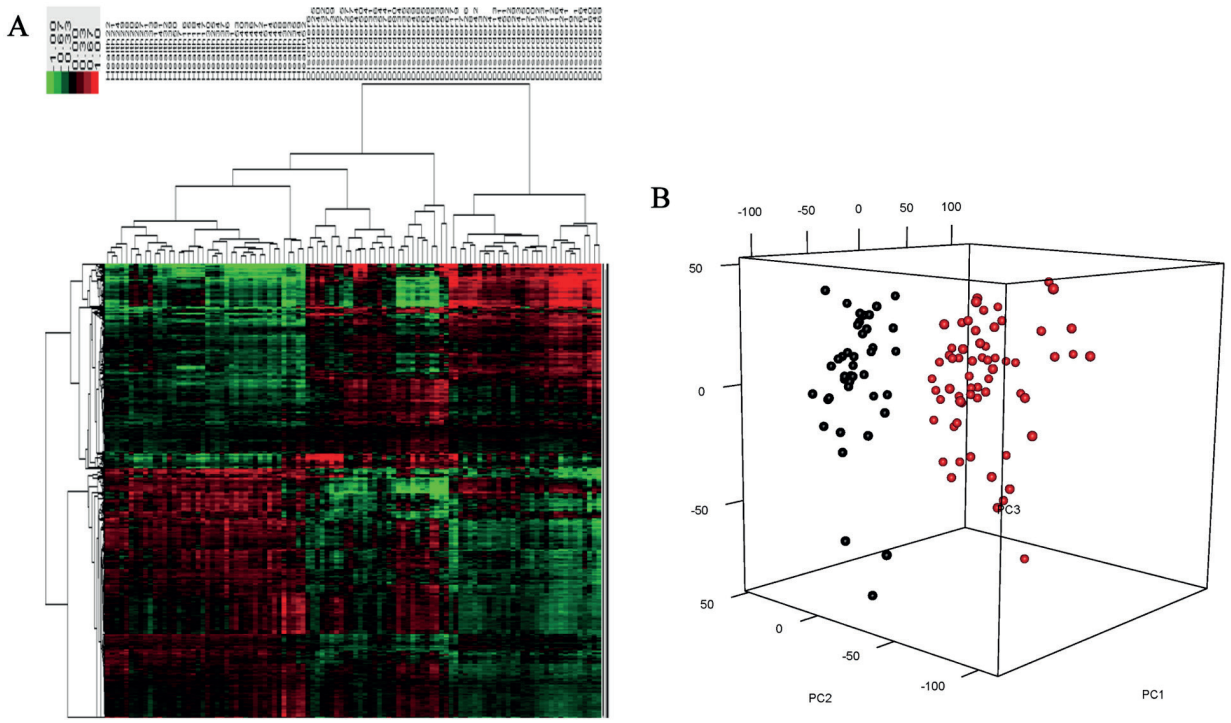



**Figure 4.** Unsupervised clustering analysis and Principal Component Analysis of the gene expression of human whole blood samples from VTE and healthy control after unsupervised TWC analysis. ***A,*** A comparison between healthy samples (n = 42) and APE samples (n = 62) was conducted. A total of 3,397 upregulated and 4,173 downregulated genes were identified. The colored bar shows the relative expression quality, where lighter green to brighter red indicates a relatively lower to relatively higher expression level. The eNOS gene was among the genes that presented significantly altered expression ($p$<0.05), and was downregulated in pulmonary thromboembolism patients. ***B,*** The unsupervised and TWC selected human whole blood samples of VTE were plotted versus healthy controls using the top two principal components of the gene expression data. Black dots were healthy samples (n=42) and red dots were VTE samples (n=62).

gene expression patterns of major clustered samples. A total of 7,570 genes were significantly altered ($p$<0.05) between VTE patients and control subjects. Of these, 3,397 were upregulated and 4,173 were downregulated in the VTE group. In contrast to the findings from the APE mice model, eNOS gene expression was significantly downregulated in VTE patients (Figure 4A, Unsupervised Clustering Results).

Gene expression data possess multi-dimensional properties. Each gene may have interactions with others, and this interaction is hidden inside measurements. Principal component analysis (PCA) is a useful method of gene expression data dimensionality reduction. Each gene has a loading coefficient with each principal component, and the contribution of each gene on sample classification can be worked out consequently. Therefore, we extracted the principal component from VTE blood data. By utilizing PCA on the selected sample data, we observed that 85% of the VTE expression data consisted of the top 30 principal components. Only PC1 and PC2 significantly contributed to the classification of VTE samples versus healthy control ($p$<0.05), but had no significant contribution to the classification of VTE. The more positive coordinate of samples on each principal component, the more disorder samples will be. The more positive coefficient value of genes on each principal component, the more upregulated contribution of genes in the disorder samples. Inversely, genes with more negative coefficient values are more downregulated in the disordered samples. Generally, coefficient values with the absolute value that up to 0.8 indicates that the gene has a contribution on the principal component. Consequently, eNOS is relative downregulated in VTE samples, which has a negative coefficient value of -0.47707 on PC1, and -0.08429 on PC2. The downregulation tendency is not obvious, but do have some (Figure 4B).

Significantly altered genes were further extracted from the dataset based on TWC results, and a clearer heat map was obtained by compa-

ring gene expression results with TWC results (Figure 5A, Supervised Classification Results). However, the how eNOS gene expressed was not necessarily changed in the Supervised Classification Results.

After functional enrichment analysis of the significantly altered genes, the 100 genes with the highest SAM analysis score were inputted in the online software DAVID. Further functional enrichment analysis using the term "acetylation" provided 40 of the top 100 significantly altered genes. A second term "phosphoprotein" was a hit in 66% of the genes, comprising the largest biological group within the significantly altered genes (Figure 5B, Gene Annotation Results).

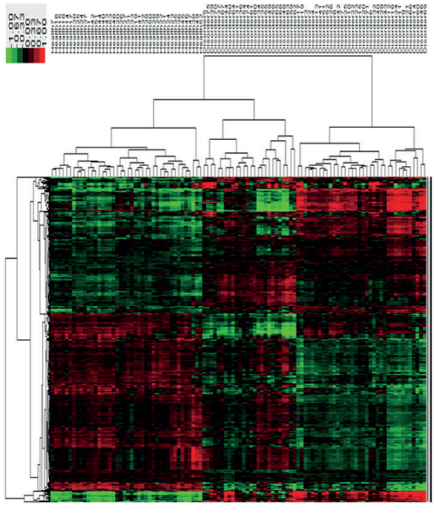
## Discussion

PE is a common but lethal disease, and is of clinical importance because of its considerable mortality and morbidity[16,17]. According to a community-based study, it is estimated that the incidence of PE is 6.0/10,000/year[3], and the mortality due to APE ranges from 7% to 11%[18]. Thrombi that is trapped in pulmonary vessels does harm to the vascular endothelium and lead to releasing proinflammatory mediators, as well as secondary pathological transformations[19,20]. As a result, concerning the pathophysiological results of PE, the PE-induced endothelial injury is critical. The current study explored eNOS gene expression in a mouse model of APE and further analyzed eNOS bioinformatics in APE using a search of the GEO database.

Based on numerous pre-experiments and histopathological results, eNOS expression in the lung was examined in the APE model on day one. It was found that eNOS expression was significantly upregulated in APE pulmonary endothelial cells on day one. This is consistent with previous findings that eNOS is mainly expressed in vascular endothelial cells and is involved in the regulation of vascular tone through NO production, which directly affects vascular function homeostasis and thus affects the initiation and manifestation of many disease processes in the circulatory system[6].

In contrast to the upregulation of eNOS in pulmonary endothelial cells, serum NO levels were significantly decreased in the APE mice model, indicating that a persistent decline of NO in the peripheral blood during APE development may upregulate eNOS expression through a feedback mechanism. Furthermore, continuous stimulation by the emboli to the endothelial cells, secondary thrombus formation, and increased blood viscosity may cause an alteration of the pulmonary

A



B

| Category | Term | RT | Genes | Count | % | P-Value | Benjamini |
|---|---|---|---|---|---|---|---|
| SP_PIR_KEYWORDS | acetylation | RT | 40 | 2.6 | 6.5E-11 | 1.2E-8 |
| SP_PIR_KEYWORDS | phosphoprotein | RT | 66 | 4.2 | 2.5E-9 | 2.2E-7 |
| SP_PIR_KEYWORDS | ribosomal protein | RT | 11 | 0.7 | 3.4E-8 | 2.0E-6 |
| SP_PIR_KEYWORDS | ribosome | RT | 8 | 0.5 | 7.7E-8 | 3.4E-6 |
| SP_PIR_KEYWORDS | ribonucleoprotein | RT | 12 | 0.8 | 1.5E-7 | 5.2E-6 |
| GOTERM_MF_FAT | structural constituent of ribosome | RT | 10 | 0.6 | 6.2E-7 | 1.6E-4 |
| GOTERM_CC_FAT | ribosome | RT | 11 | 0.7 | 6.8E-7 | 1.6E-4 |
| SP_PIR_KEYWORDS | respiratory chain | RT | 7 | 0.4 | 1.5E-6 | 4.3E-5 |
| GOTERM_BP_FAT | translational elongation | RT | 8 | 0.5 | 2.5E-6 | 2.2E-3 |
| KEGG_PATHWAY | Ribosome | RT | 8 | 0.5 | 4.7E-6 | 3.5E-4 |
| GOTERM_CC_FAT | non-membrane-bounded organelle | RT | 34 | 2.2 | 7.9E-6 | 9.1E-4 |
| GOTERM_CC_FAT | intracellular non-membrane-bounded organelle | RT | 34 | 2.2 | 7.9E-6 | 9.1E-4 |
| GOTERM_CC_FAT | cytosol | RT | 23 | 1.5 | 9.2E-6 | 7.0E-4 |
| GOTERM_CC_FAT | ribosomal subunit | RT | 8 | 0.5 | 1.3E-5 | 7.2E-4 |
| GOTERM_BP_FAT | translation | RT | 11 | 0.7 | 3.0E-5 | 1.3E-2 |
| GOTERM_CC_FAT | small ribosomal subunit | RT | 6 | 0.4 | 4.0E-5 | 1.8E-3 |

**Figure 5.** Supervised classification of gene expression and gene annotation charts of the top 100 significantly altered genes. ***A,*** Hierarchical clustering presentation of the SAMs of the APE gene expression data consisting of healthy control (n = 42) and APE samples (n = 62). There were 3,012 upregulated and 3,696 downregulated genes in this classification. The colored bar shows the relative expression quality, where from lighter green to brighter red indicates a relatively lower to relatively higher expression level. ***B,*** Terms of the gene annotation were sorted by Fisher's exact test; a lower p-value indicated a greater confidence in the gene annotation. The secondary term "phosphoprotein" matched 66% of those genes, forming the largest biological group within the significantly altered genes.

vascular transmural pressure and shear forces, which may lead to the upregulated expression of eNOS. However, the downstream mechanism of the multifaceted eNOS in the pathophysiology of APE and its biological roles remain to be further investigated.

EPCs are pluripotent stem cells with the capability of differentiating into mature vascular endothelial cells, and are involved in the repair of the vascular endothelium following injury[21,22]. EPCs are activated in a variety of cardiovascular diseases, including cardiac tissue and blood vessel injury, and migrate to injured regions where they differentiate into endothelial cells and become involved in tissue repair[23]. However, when eNOS gene expression occurs due to vascular endothelial function impairment, a decline in the formation and activity of NO results, which in turn further affects other regulatory networks to inhibit mobilizing EPCs to the peripheral blood from the bone marrow[24]. Thus, the current study also investigated the expression of eNOS in primary EPC cultures. It found that eNOS mRNA and protein expression were significantly increased in extracted APE murine BM-EPCs at one day of embolization, suggesting that APE-induced EPC mobilization may involve the eNOS/NO pathway. These findings were consistent with the results reported by Dimmeler et al[25], who found that eNOS/NO was a major mediator in stimulating cell proliferation by activation of the phosphoinositide 3-kinase/Akt pathway, together with a downstream effector in vascular endothelial growth factor-induced angiogenesis.

In addition to the APE mice model, we investigated microarray data in order to further confirm the experimental results. Although only one study of eNOS expression in human whole blood samples was found in the GEO repository, we could identify the general gene expression patterns of VTE patients and found altered expression of the eNOS gene. The general gene expression patterns extracted from the raw data and the identification of significantly altered genes in VTE samples could probably describe the general genetic signature of the disease. Accordingly, significant alteration of eNOS in thromboembolism indicates that eNOS may play an important role in the disease process, although it remains to be determined if eNOS could be a potential biomarker in the peripheral blood for VTE.

Due to the limited amount of prior research regarding gene expression microarray data in the current GEO repository, we could only compare our animal experimental results with the human gene array data. The *in vivo* experimental results indicated that eNOS expression was significantly increased in APE mice. However, it was significantly downregulated according to microarray data based on human VTE blood sample data obtained from the GEO repository. A possible reason for this may be that the peripheral blood environment in humans was usually complex. While studies have shown that eNOS levels are elevated in the lungs of patients with primary pulmonary hypertension[26] and in hypoxic mice[27], the discrepancies of these findings may be due to species differences. Therefore, the mechanism remains to be further explored.

Next, using gene annotation analysis, we found that the classification term "phosphoprotein" matched 66% of the significantly altered genes, making it the largest biological classification group. Phosphorylation of eNOS can occur on serine, threonine, and tyrosine residues. The potential for multiple loci phosphorylation is important in regulating of eNOS activity[4], which is important to maintaining the health of the circulatory system[28].

## Conclusions

Through an innovative combination of computational microarray data analysis of human blood samples and bench-based experimental data analysis, the current study has demonstrated that eNOS levels increased in APE murine lung endothelial cells, but serum NO was decreased in the mice model. Moreover, the mRNA and protein expression of eNOS was significantly upregulated in BM-EPCs isolated from APE mice. Microarray data analysis of human blood samples indicated that eNOS gene expression was significantly downregulated in VTE patients. The limited alteration of eNOS in human peripheral blood VTE samples could be due to disease-related or -mediated negative feedback. Also, a more detailed classification of the VTE samples could be helpful to explore the mechanism of eNOS regulation in the disease.

Finally, additional elucidation of the role of eNOS phosphorylation in regulating eNOS activity and its downstream signal transduction pathways may help us to pry out new insights into the pathogenesis of APE-mediated endothelial dysfunction, and to develop new drugs for its treatment.

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**Conflict of interest**

The authors declare no conflicts of interest.

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