

# Extracellular vesicles modulate endothelial nitric oxide production in patients with $\beta$ -thalassaemia/HbE

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**Abstract.** Thrombosis is a significant complication in patients with  $\beta$ -thalassaemia/haemoglobin E (HbE), particularly in splenectomised patients. The endothelium is a key regulator of vascular haemostasis and homeostasis, through the secretion of various regulatory molecules. Nitric oxide (NO), produced by endothelial cells (ECs), regulates vascular functions by acting as a potent vasodilator and an inhibitor of platelet activation. Decreased NO bioavailability, a marker of vascular dysfunction, could be a contributing factor leading to thrombosis. Microparticles or medium extracellular vesicles (mEVs) are associated with thrombosis and vasculopathy in various diseases. Furthermore, elevated levels of mEVs have been observed in splenectomised patients with  $\beta$ -thalassaemia/HbE and could induce the expression of coagulation proteins, inflammatory cytokines and adhesion molecules in ECs. However, the effects of mEVs on NO regulation by ECs is currently unclear. In the present study, mEVs obtained from splenectomised patients with  $\beta$ -thalassaemia/HbE had significantly decreased NO production in human pulmonary artery ECs without affecting endothelial nitric oxide synthase expression or phosphorylation. Decreased NO production

was attributed to increased haemoglobin levels in mEVs from splenectomised patients, leading to enhanced NO scavenging. These findings highlight a mechanism whereby haemoglobin-carrying mEVs directly scavenge NO, contributing to vascular dysfunction in  $\beta$ -thalassaemia/HbE disease.

## Introduction

Thromboembolic events are a significant complication in patients with  $\beta$ -thalassaemia, particularly in splenectomised patients. These patients have an increased risk for both arterial and venous thromboembolic events affecting different organs (1). A comprehensive epidemiological study involving 8,860 patients with  $\beta$ -thalassaemia, the largest clinical investigation to date, revealed that thromboembolic events occurred in 0.9% of  $\beta$ -thalassaemia major and 3.9% of patients with intermedia  $\beta$ -thalassaemia (2). Endothelial cells (ECs) play a pivotal role in maintaining vascular homeostasis and haemostasis by producing several regulatory factors. Endothelial dysfunction in patients with  $\beta$ -thalassaemia has been reported, as evidenced by elevated levels of endothelial activation markers, such as ICAM-1, E-selectin, VCAM-1, von Willebrand factor (vWF) and thrombomodulin, in serum and/or plasma samples (3-5). Nitric oxide (NO), an important protective molecule in the vasculature, is synthesised by endothelial-nitric oxide synthase (eNOS) in the vascular endothelium, and plays a crucial role in maintaining vasomotor tone, regulating coagulation, suppressing platelet activation, cellular adhesion to the endothelium and reducing inflammation. Impaired NO bioavailability in patients with  $\beta$ -thalassaemia/haemoglobin E (HbE) has been documented (6).

Microparticles or medium extracellular vesicles (mEVs), characterized by a size in the 50-300 nm range and isolated by high-speed centrifugation at 14,000 x g (7), have emerged as

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potential mediators of EC dysfunction in  $\beta$ -thalassaemia/HbE disease. Elevated levels of mEVs have been observed in splenectomised patients with  $\beta$ -thalassaemia/HbE (8). It has been previously demonstrated that mEVs obtained from splenectomised patients with  $\beta$ -thalassaemia/HbE can induce EC activation, leading to the increased expression of tissue factor, inflammatory cytokines and adhesion molecules, and promoting leucocyte adhesion to ECs (9). Moreover, a recent proteomic analysis performed by the authors revealed significantly elevated levels of haemoglobin subunits, including  $\alpha$ -,  $\gamma$ -,  $\delta$ - and  $\beta$ -globin, (HBA, HBG, HBD and HBB, respectively) in mEVs from splenectomised patients with  $\beta$ -thalassaemia/HbE and an increase of 3-6-fold compared with those in mEVs from healthy donors (7). Haemoglobin can rapidly react with NO to form a vasodilator-inactive nitrate (10). This suggests that mEVs may play a role in modulating NO bioavailability and contributing to vascular dysfunction in patients with  $\beta$ -thalassaemia/HbE.

The present study aimed to investigate the effects of mEVs isolated from splenectomised patients with  $\beta$ -thalassaemia/HbE on NO production, NO scavenging, and eNOS phosphorylation in human pulmonary artery ECs (HPAECs). By elucidating the mechanisms through which mEVs impact NO bioavailability, the present study seeks to enhance our understanding of the pathophysiological mechanisms underlying thromboembolic complications in  $\beta$ -thalassaemia, with implications for potential therapeutic interventions.

## Materials and methods

**Patients and blood samples.** The present study was performed in accordance with The Declaration of Helsinki and was approved (approval no. MU-CIRB 2014/013.0502) by the Mahidol University Central Institutional Review Board, (Bangkok, Thailand). Written informed consent was obtained from all individual participants included in the present study. A total of 11 splenectomised patients with  $\beta$ -thalassaemia/HbE and 10 healthy donors were recruited into the present cohort study. The volunteers were enrolled at Nakhonpathom Hospital (Nakhon Pathom, Thailand) from January 2020 to December 2021. All patients had a DNA diagnosis of  $\beta$ -thalassaemia/HbE disease and had undergone splenectomy >5 years ago. All patients received folic acid (5 mg) daily. Patients treated with aspirin, antibiotics, anti-depressants,  $\beta$ -blockers and anti-platelets, or those with severe anaemia (Hb <5 g/dl) were excluded, and none had been hospitalised or transfused within the preceding 4 weeks. All healthy donors had a normal complete blood count, normal Hb typing and were negative for DNA diagnosis of common  $\alpha$ -globin gene deletions in Thailand, including  $\alpha$ -thalassaemia-1 both  $^{-SEA}$  (Southeast Asian deletion) and  $^{-THAI}$  (Thai deletion), and  $\alpha$ -thalassaemia-2 both  $^{-\alpha^{3.7}}$  (3.7 kb deletion) and  $^{-\alpha^{4.2}}$  (4.2 kb deletion), using multiplex gap PCR. All subjects had no evidence of concurrent infection or a history of atherosclerotic vascular disease. Venous blood samples were collected by the two-syringe technique at room temperature and were processed within 1 h (7). Haematological parameters are summarised in Table I.

**Isolation of mEVs from peripheral blood samples.** The mEVs were isolated by sequential centrifugation as described in a

previous study by the authors (11). Briefly, fresh whole blood samples collected in 3.2% trisodium citrate anticoagulant were centrifuged at 1,500 x g for 15 min at 25°C to collect platelet-poor plasma and were re-centrifuged at 14,000 x g for 2 min at 4°C to obtain platelet-free plasma (PFP). Pellet mEVs were collected after centrifugation of PFP at 14,000 x g at 4°C for another 45 min. The pellet was washed once with PBS.

**Detection and quantification of mEVs by flow cytometry (Fig. S1).** The flow cytometric analysis of mEVs followed the guidelines of the International Society for Extracellular Vesicles (ISEV) and MIFlowCyt-EV (12,13). The characterisation of  $\beta$ -thalassaemia/HbE mEVs was previously reported, as required by ISEV, using three different techniques (nanoparticle tracking analysis, flow cytometry and cryo-transmission electron microscopy) (7). The blood samples were processed within 1 h, as previously demonstrated that the absolute numbers and cellular origins of mEVs did not significantly change within 1 h of blood collection (7). Total numbers of mEVs were quantitated using flow cytometry as described in a previous study (9). Briefly, samples were stained with PE-conjugated Annexin V (BD Biosciences) and analysed using an Accuri C6 plus flow cytometer (BD Biosciences). The mEV population was determined by comparison with nano polystyrene beads of sizes 0.79 and 1.32  $\mu$ m in diameter (Spherotech, Inc.) (Fig. S1). The absolute number of mEVs was calculated using TruCount beads (BD Biosciences).

**HPAECs culture and treatment.** The primary HPAECs (cat. no. 302-05a, Cell Application) were used within 3-8 passages. HPAECs were cultured in EC growth medium (Cell Applications, Inc.) at 37°C and 5% CO<sub>2</sub>. HPAECs were plated in a 6-well-plate until they formed an 80% confluent monolayer. The cells were then treated for 10 min with 1x10<sup>6</sup> mEVs/ml or 10 ng/ml vascular endothelial growth factor (VEGF) as a positive control (14). After treatment, culture medium was kept at -80°C for the measurement of nitrite concentration, and cells were harvested for analysis of eNOS and eNOS phosphorylation by western blotting.

**Measurement of NO.** The NO production of HPAECs was determined by measuring nitrite, the stable oxidation product of NO, in the culture medium using the tri-iodide-based chemiluminescence method with a NO chemiluminescence analyser (Analyzer CLD88; ECO MEDICS AG) (15). Briefly, samples were directly injected into an acidified tri-iodide solution in the purge vessel, while purging with nitrogen connected to a gas-phase chemiluminescence NO analyser. The area under the curve was calculated using Origin 7 (OriginLab Corporation) and was converted into the amount of nitrite by comparing with a 0.005-0.025 nmol sodium nitrite (MilliporeSigma) standard curve. The change ( $\Delta$ ) in nitrite concentration in treated cells compared with untreated controls was then calculated.

**Analysis of eNOS phosphorylation.** A total of 8  $\mu$ g protein extracted with RIPA lysis and extraction buffer (cat. no. 89901; Thermo Fisher Scientific, Inc.) from HPAECs was separated by SDS-PAGE on a 10% gel. The proteins were then transferred to a PVDF membrane and blocked with 5% skim

Table I. Haematological parameters of patients with  $\beta$ -thalassaemia/HbE and normal subjects.

Description	Healthy donor	Splenectomised patients with $\beta$ -thalassaemia/HbE	Reference range
Number (Male: Female)	10 (7:3)	11 (6:5)	
Age, years	37 $\pm$ 9	40 $\pm$ 9	
(Range Min-Max)	(25-47)	(28-55)	
Hb typing	A <sub>2</sub> A	EF	A <sub>2</sub> A
HbA (%)	96.7 $\pm$ 3.6	-	85-90
HbA <sub>2</sub> (%)	2.8 $\pm$ 0.2	-	2-3
HbE (%)	-	48.3 $\pm$ 8.6 <sup>a</sup>	-
HbF (%)	0.2 $\pm$ 0.1	28.6 $\pm$ 11.6 <sup>a</sup>	0.8-2.0
RBC count (x10 <sup>6</sup> / $\mu$ l)	4.8 $\pm$ 0.5	3.5 $\pm$ 0.5 <sup>a</sup>	4.2-5.4
Hb (g/dl)	14.2 $\pm$ 1.4	6.9 $\pm$ 0.8 <sup>a</sup>	12-18
Hct (%)	42.6 $\pm$ 3.8	23.7 $\pm$ 2.7 <sup>a</sup>	37-52
MCV (fl)	89 $\pm$ 3	70 $\pm$ 8 <sup>a</sup>	80-99
MCH (pg)	29 $\pm$ 1	21 $\pm$ 2 <sup>a</sup>	27-31
MCHC (g/dl)	33 $\pm$ 1	30 $\pm$ 2 <sup>a</sup>	31-35
RDW (%)	12.6 $\pm$ 0.7	33.2 $\pm$ 16.4 <sup>a</sup>	11.5-14.5
NRBCs (cells/100 WBCs)	None	184 $\pm$ 139 <sup>a</sup>	None
WBC count (x10 <sup>3</sup> / $\mu$ l)	6 $\pm$ 2	29 $\pm$ 23	4-11
Platelet count (x10 <sup>3</sup> / $\mu$ l)	280 $\pm$ 54	621 $\pm$ 184 <sup>a</sup>	150-450

Data are presented as the mean  $\pm$  standard deviation. <sup>a</sup>Significantly different when compared with healthy donor using Mann-Whitney U test at P<0.05. Hb, haemoglobin; RBC, red blood cell; Hct, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red cell distribution width; NRBCs, nucleated red blood cells; WBC, white blood cell.

milk for 1 h at room temperature. The same membrane was used to probe for total eNOS, phosphorylated-eNOS at Ser1177 and Thr495, and  $\beta$ -actin. The membrane was cut into sections before probing with the respective antibodies to  $\beta$ -actin (45 kD) and eNOS and phosphorylated-eNOS (140 kD). The membrane was incubated overnight at 4°C with primary mouse anti-total eNOS type 3 or mouse anti-phosphorylated-eNOS position Ser1177 or Thr495 antibodies, followed by incubation with the secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse polyclonal antibody or HRP-conjugated mouse anti- $\beta$ -actin antibody for 1 h at room temperature, in dark. After probing for total eNOS, the membrane was stripped and re-probed for phosphorylated-eNOS. List of antibodies is presented in Table SI. The bands on immunoblots were detected using the Enhanced Chemiluminescence Plus system (ECL plus; Bio-Rad Laboratories, Inc.). Subsequently, the films were scanned and the intensity of protein bands was measured using ImageJ software version 1.47 (National Institutes of Health). The expression of all proteins was first normalised to the individual  $\beta$ -actin protein intensity. Then, the phosphorylated-eNOS expression was normalised again to total eNOS expression.

**Measurement of potential NO scavenging.** NO scavenging by mEVs was directly measured using a NO chemiluminescence analyser, Eco Medics Analyzer CLD88 (15). A total of 2x10<sup>6</sup> mEVs from healthy donors and patients

with  $\beta$ -thalassaemia/HbE were injected into the purge vessel that contained 50  $\mu$ M NO donor, DETA NONOate (cat. no. ALX-430-014; Enzo Life Science, Inc.). The area under the curve of the potential NO scavenging peak was calculated by the Origin 7 program and was converted into amount of nitrite using the sodium nitrite standard curve.

**Analysis of  $\alpha$ -globin protein in mEVs.** An equal number of 5x10<sup>6</sup> mEVs was used for western blot analysis of  $\alpha$ -globin protein content. This approach was taken because the present study's proteomic analysis did not identify a protein with equal amounts between mEVs from healthy donors and splenectomised patients with  $\beta$ -thalassaemia/HbE that could serve as an internal control (7). The mEVs were lysed with RIPA lysis and extraction buffer (Thermo Fisher Scientific, Inc.). Total protein from an equal number of mEVs (5x10<sup>6</sup> particles) in each individual sample was loaded into each lane and separated by SDS-PAGE on a 12% gel, then, it was transferred to a PVDF membrane. The membrane was then blocked with 5% skim milk for 1 h at room temperature and incubated with a mouse anti-human  $\alpha$ -globin chain monoclonal antibody (Santa Cruz Biotechnology, Inc.), followed by incubation with an HRP-conjugated goat anti-mouse polyclonal antibody (BD Biosciences) (Table SI). Before signal detection, an ECL blotting substrate cocktail (Bio-Rad Laboratories, Inc.) was added. The films were scanned and the intensity of the protein bands was measured using ImageJ software version 1.47 (National Institutes of Health).

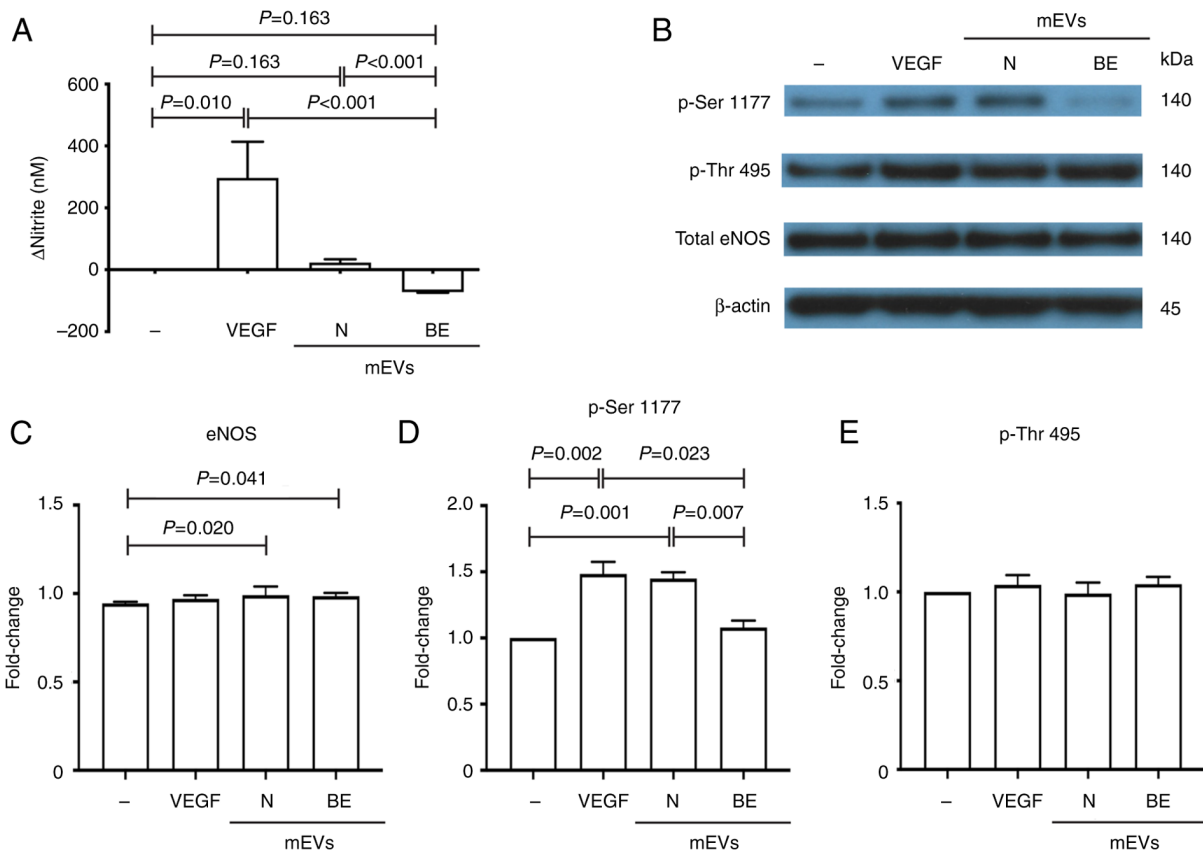


Figure 1. Splenectomised mEVs decrease HPAEC NO production without affecting eNOS expression or phosphorylation. HPAECs were treated with  $1 \times 10^6$  mEVs/ml from healthy donor ( $n=10$ ) and splenectomised patients with  $\beta$ -thalassaemia/HbE ( $n=11$ ) for 10 min. Nitrite levels in culture media were measured by a tri-iodide-based chemiluminescence method using a NO chemiluminescence analyser. The difference ( $\Delta$ ) in nitrite concentration between treated and untreated HPAECs was then calculated. Negative values indicate a reduction in nitrite levels. Expression of eNOS and its phosphorylated forms were examined by western blotting. VEGF was used as a positive control. (A) Change of nitrite levels compared with untreated cells. (B) Western blot analysis of eNOS and phosphorylated eNOS. Full length of western blot analysis is presented in Fig. S2. (C-E) Fold changes of (C) eNOS and (D) phosphorylated eNOS at Ser1177 and (E) Thr495 compared with untreated cells. Band density quantitation of eNOS and phosphorylated eNOS were performed using Image J software. eNOS and phosphorylated eNOS expression were normalised with  $\beta$ -actin and ratios of phosphorylated eNOS/total eNOS were calculated. Then, fold change of phosphorylated eNOS of treated and untreated HPAEC was then determined. Data are presented as the mean  $\pm$  S.D. Statistical analysis was performed by using a Kolmogorov-Smirnov (K-S) test, significance values have been adjusted by the Dunn-Bonferroni correction for multiple tests. MEVs, medium extracellular vesicles; HPAEC, human pulmonary artery ECs; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; Hb, haemoglobin; N, healthy subjects; BE,  $\beta$ -thalassaemia/HbE patient.

**Statistical analysis.** All descriptive statistics (mean and SD) were performed using Statistical Package for the Social Science (SPSS), version 17.0 (SPSS, Inc.). The distribution of all data was estimated using the one-sample Kolmogorov-Smirnov test, and the histograms were verified using P-P and Q-Q plots. All data, except the fold changes of  $\alpha$ -globin expression detected by western blot analysis, were non-parametric; therefore, the comparisons between two groups were analysed using a Mann-Whitney U test while multiple comparisons of more than two groups were analysed using a Kruskal-Wallis test, followed by the Dunn-Bonferroni. The fold changes of  $\alpha$ -globin expression were normal distribution; therefore, the comparisons between groups were performed using unpaired Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

**Results**

*$\beta$ -Thalassaemia/HbE mEVs decrease NO production in HPAECs.* The difference in nitrite concentrations between treated and untreated HPAEC samples was determined.

HPAECs treated with healthy donor-mEVs exhibited a significant increase in nitrite levels in the culture medium ( $23 \pm 10$  nM) as compared with untreated cells. An increase in nitrite was also observed in VEGF-treated HPAECs (Fig. 1A). Notably, the nitrite level in HPAECs treated with splenectomised mEVs was significantly decreased ( $-72 \pm 2$  nM) compared with that in the untreated cells. These results suggested that normal mEVs could induce NO production, while splenectomised mEVs reduced NO levels in the culture medium.

*$\beta$ -Thalassaemia/HbE mEVs have no effect on eNOS expression and activation.* The protein levels of total eNOS were not significantly different among the untreated HPAECs, and those treated with VEGF, healthy donor-mEVs or splenectomised mEVs (Fig. 1B and C). This suggested that increased or decreased NO production by mEVs did not cause changes in the levels of eNOS expression. eNOS activity is predominantly controlled via phosphorylation and dephosphorylation. For example, phosphorylation at Ser1177 activates eNOS, whereas phosphorylation at Thr495 inhibits its function (16). Consistent with the increased NO production, the level of

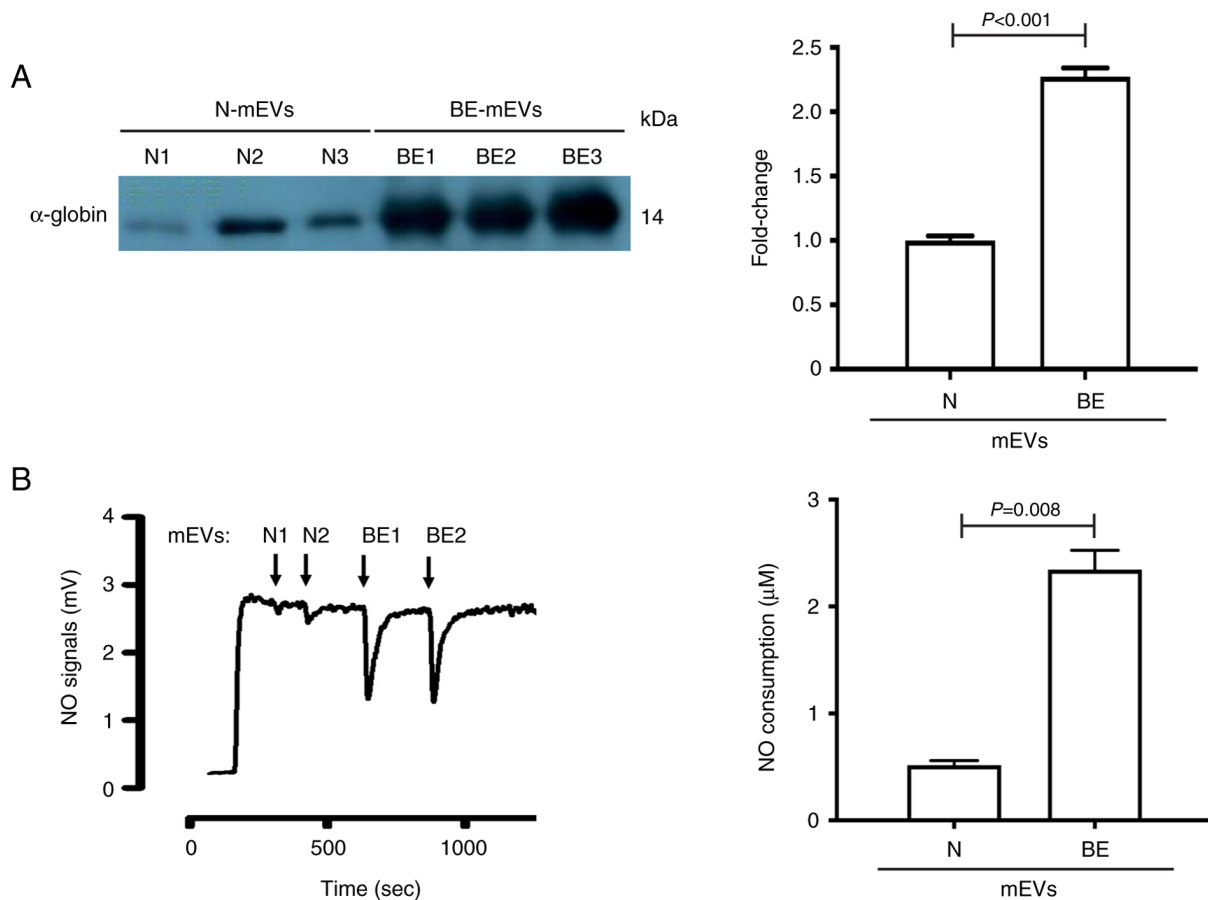


Figure 2. Increased NO scavenging by splenectomised mEVs. (A) Splenectomised mEVs carrying higher  $\alpha$ -globin protein. Western blot analysis of  $\alpha$ -globin protein from  $5 \times 10^6$  mEVs obtained from splenectomised patients with  $\beta$ -thalassaemia/HbE ( $n=3$ ) and healthy donor ( $n=3$ ). Data are presented as the mean  $\pm$  S.D. Statistical analysis was performed by using independent sample T-test. Full length of western blot analysis is presented in Fig. S2. (B) Splenectomised mEVs scavenge higher NO. A total of  $2 \times 10^6$  mEVs from splenectomised patients with  $\beta$ -thalassaemia/HbE ( $n=5$ ) and healthy donor ( $n=5$ ) were injected into the purge vessel containing  $50 \mu\text{M}$  DETA NONOate and measured by a NO chemiluminescence analyser. Data are presented as the mean  $\pm$  S.D. Statistical analysis was performed by using a Mann-Whitney U test. N, healthy donor; BE, splenectomised  $\beta$ -thalassaemia/HbE patient. NO, nitric oxide; mEVs, medium extracellular vesicles; Hb, haemoglobin; N, healthy subjects; BE,  $\beta$ -thalassaemia/HbE patient.

phosphorylated eNOS at Ser1177 was significantly increased in HPAECs treated with VEGF and normal mEVs compared with in untreated cells (Fig. 1B and D). Notably, the levels of eNOS phosphorylation at Ser1177 in splenectomised mEVs-treated HPAECs were not significantly different from those in untreated cells. Furthermore, eNOS phosphorylation at Thr495 did not exhibit significant differences among untreated HPAECs, and those treated with VEGF, healthy donor-mEVs or splenectomised mEVs (Fig. 1B and E). These results suggested that the reduction of NO in HPAECs treated with splenectomised mEVs was not caused by phosphorylation at Thr495, a negative regulatory site.

***$\beta$ -Thalassaemia/HbE mEVs can scavenge NO.*** Haemoglobin can scavenge NO (17). Additionally, proteomic analysis of  $\beta$ -thalassaemia/HbE mEVs revealed an increase in haemoglobin in splenectomised mEVs (7). The reduction of NO in the culture medium of HPAECs treated with splenectomised mEVs may be due to NO scavenging. An equal number of mEVs ( $5 \times 10^6$  particles) was used for western blot analysis of  $\alpha$ -globin protein content in mEVs, as proteomic analysis revealed no protein with equal amounts between groups that could be used as an internal normaliser. The levels of  $\alpha$ -globin

content in splenectomised mEVs were significantly higher than those in healthy donor-mEVs (Fig. 2A), confirming the previous proteomic analysis performed by the authors. The increased haemoglobin content in splenectomised mEVs could scavenge NO. Subsequently, NO scavenging by mEVs was determined by analysing the NO decay signal (Fig. 2B). Notably, NO scavenging by splenectomised mEVs ( $2.27 \pm 0.45 \mu\text{M}$ ) was significantly higher than that by healthy donor-mEVs ( $0.49 \pm 0.08 \mu\text{M}$ ) ( $P=0.008$ ).

## Discussion

NO is a crucial endothelium-derived molecule that regulates various vascular functions, including vascular tone, platelet activation and inflammation. Decreased NO bioavailability, a marker of vascular dysfunction, is a significant contributing factor to thrombosis. The present study demonstrated that mEVs obtained from splenectomised patients with  $\beta$ -thalassaemia/HbE exhibited elevated haemoglobin content and enhanced NO scavenging, leading to decreased NO levels.

Clinically, diminished NO production is a hallmark of endothelial dysfunction, contributing to the development

of atherosclerosis and thrombosis. Individuals with high altitude-related excessive erythrocytosis (EE) or permanent spinal cord injury (SCI) are at increased risk of cardiovascular comorbidities, including endothelial dysfunction, hypertension, coronary artery disease and thrombosis. NO production has been significantly reduced in human umbilical vein ECs (HUVECs) treated with mEVs from individuals with EE or SCI. In both conditions, this reduction was not due to decreased eNOS expression but was attributed to impaired eNOS activation, with reduced phosphorylation at Ser1177 and increased phosphorylation at Thr495 (18,19). By contrast, while mEVs from EE, SCI and splenectomised  $\beta$ -thalassaemia similarly reduced EC NO production, the present study revealed that splenectomised mEVs reduced NO production in HPAECs without affecting eNOS expression or phosphorylation, suggesting a different mechanism. This reduction is due to direct NO scavenging by the high haemoglobin content of splenectomised mEVs, a mechanism distinct from the impaired eNOS activation observed in EE and SCI, and has important implications for understanding the unique vascular complications in patients with  $\beta$ -thalassaemia.

Our proteomic analysis of  $\beta$ -thalassaemia/HbE mEVs showed an increase in all haemoglobin subunits in splenectomised mEVs, which was validated in the present study by western blot analysis of  $\alpha$ -globin protein. Haemoglobin in the ferrous redox state, such as oxyhaemoglobin (HbO<sub>2</sub>), rapidly and irreversibly reacts with NO via a dioxygenation reaction, producing inactive vasodilator nitrate and methaemoglobin (metHb), as illustrated by the reaction:  $\text{NO} + \text{HbO}_2 \rightarrow \text{NO}^3 + \text{metHb}$ . This reaction occurs at a rate  $\sim 1,000$ -fold faster with cell-free haemoglobin compared with haemoglobin contained within red blood cells (RBCs). Furthermore, RBC-derived mEVs scavenge NO in a manner that more closely resembles cell-free haemoglobin, with a reaction rate only 2.5 to 3-fold slower than that of cell-free haemoglobin, attributed to their increased surface area compared with RBCs (17). Blood bank storage of human RBCs leads to significant haemolysis and the release of mEVs, resulting in an increased rate of NO scavenging that increases with the duration of storage. Infusion of plasma from stored human RBCs into rat circulation can induce notable vasoconstriction, and this effect is correlated with elevated levels of haemoglobin-carrying mEVs released during storage (17).

Plasma NO levels of patients with  $\beta$ -thalassaemia/HbE are significantly decreased. The flow-mediated dilation, a test which represents vascular dilation mediated by EC-derived NO response to the shearing force, has been reported to be significantly correlated with plasma NO levels in patients with  $\beta$ -thalassaemia/HbE (6). Furthermore, decreased local vasodilators, such as NO, and other factors, including endothelial dysfunction, platelet activation and a hypercoagulable state, contribute to pulmonary hypertension, a life-threatening complication in  $\beta$ -thalassaemia. Administration of inhaled nebulized sodium nitrite can rapidly decrease pulmonary artery pressure in patients with  $\beta$ -thalassaemia with pulmonary hypertension, as measured by echocardiography and right heart catheterization (20). Additionally, combined treatment of sildenafil and inhaled nebulized nitrite at 30 mg sodium nitrite twice a day for 12 weeks resulted in decreased mean pulmonary artery pressure and an increase in the 6-min walk distance (21).

This suggests the importance of NO bioavailability in patients with  $\beta$ -thalassaemia/HbE. The reduced NO bioavailability due to increased haemoglobin content in the splenectomised mEVs could have multiple effects related to Virchow's triad, endothelial injury, hypercoagulability and stasis of blood flow. First, reduced NO can contribute to endothelial dysfunction, as NO plays a crucial role in maintaining endothelial function by inhibiting platelet activation and inflammation. Second, low NO levels can exacerbate a hypercoagulable state by impairing the natural anticoagulant mechanisms and promoting platelet aggregation. Finally, inadequate NO-induced vasodilation can lead to stasis of blood flow, further increasing the risk of clot formation. Collectively, these factors suggest that the impaired NO bioavailability due to haemoglobin scavenging in splenectomised mEVs could contribute to thrombosis by influencing all components of Virchow's triad.

Endothelial dysfunction in patients with  $\beta$ -thalassaemia is multifactorial, with contributors including iron overload, oxidative stress, chronic inflammation, reduced NO bioavailability and increased mEVs. Iron-induced oxidative stress can damage ECs, reduce NO bioavailability and trigger inflammation. Increased oxidative stress, as indicated by decreased total glutathione levels and increased basal production of superoxide radicals in patients with  $\beta$ -thalassaemia/HbE, has been reported to be correlated with impaired endothelial function, as demonstrated by significantly elevated basal forearm blood flow tests (22). Moreover, elevated levels of pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-6, have been observed in patients with  $\beta$ -thalassaemia (23,24). Circulating mEVs are linked to increased aortic stiffness (25), and promote endothelial expression of tissue factor, inflammatory cytokines and adhesion molecules (9).

EVs are associated with the pathology of various conditions, including genetic diseases, infections, cancer, metabolic syndrome and traumatic injuries. In type 2 diabetes, EVs play a crucial role in inter-organ communication, with EVs derived from organs, such as the liver, adipose tissue and pancreas, carrying bioactive molecules, including microRNAs and proteins, that impact insulin signalling pathways and glucose metabolism, thus contributing to insulin resistance and  $\beta$ -cell dysfunction (26). EVs also play a dual role in viral infections by promoting virus dissemination and triggering immune responses. They facilitate virus-host interactions by transferring both viral and host-derived proteins and RNAs, a mechanism observed in several emerging and re-emerging viruses, including SARS-CoV-2, dengue, Ebola and Zika viral infectious diseases (27). In traumatic brain injury, EVs have been shown to induce endothelial dysfunction by disrupting the endothelial barrier and promoting vascular leakage. This is mediated by EVs carrying high mobility group box 1, which triggers a cascade of inflammatory responses. Additionally, vWF on the surface of EVs facilitates their interaction with ECs, further amplifying endothelial dysfunction (28). In sickle cell disease, EVs play a critical role in endothelial activation, promoting RBC adhesion and contributing to microvascular stasis. RBC-derived EVs have been shown to activate microvascular ECs, leading to increased vWF expression and enhanced RBC adhesion under microfluidic conditions (29).

In splenectomised  $\beta$ -thalassaemia, mEVs play a significant role in promoting vascular complications. Although



no significant difference has been detected in angiogenesis when HUVECs are incubated with mEVs from patients with  $\beta$ -thalassaemia/HbE compared with those from healthy controls (30), EVs from patients with  $\beta$ -thalassaemia have been correlated with platelet factor 3-like activity and prothrombinase complex activity (8). mEVs from splenectomised patients can induce platelets, leading to platelet activation, platelet aggregation and platelet-neutrophil aggregation (31). Furthermore, consistent with previous studies showing that patients with  $\beta$ -thalassaemia/HbE exhibit endothelial dysfunction, the current study revealed that splenectomised mEVs induce EC activation, resulting in increased expression of tissue factor, inflammatory cytokines and adhesion molecules, promoting leucocyte adhesion to ECs (9). Importantly, the present study further expands on prior findings by demonstrating that splenectomised mEVs contribute to EC dysfunction by decreased NO bioavailability in patients with  $\beta$ -thalassaemia/HbE through direct NO scavenging due to their high haemoglobin content. This mechanism is similar to that observed in mEVs from stored human RBCs in blood banks, where prolonged storage leads to increased hemolysis and a heightened rate of NO scavenging. However, this mechanism is distinct from the impaired eNOS activation observed in other conditions, such as high-altitude-related EE or SCI.

A limitation of the present study was the relatively small number of patients recruited. However, the variation within each group was minimal, and statistically significant differences between the patient and healthy donor groups were observed. Additionally, the two groups were distinct in their characteristics. Ethical considerations also guided the decision to limit the number of participants. Another limitation was the exclusion of non-splenectomised patients from the study. Non-splenectomised patients typically exhibit a lower incidence of thrombotic events, and mEVs isolated from these patients tend to induce EC activation to a degree between that observed in normal controls and splenectomised patients. To avoid intermediate data and to provide a clearer comparison, the current study focused on the two ends of the spectrum of EC activation, specifically using mEVs from healthy donors and splenectomised patients. While the present study demonstrated reduced NO bioavailability, a limitation was the absence of functional assays, such as endothelial-dependent vasodilation, endothelial cell migration, or tube formation assays, to directly assess the physiological impact of these changes. Although previous studies have shown that mEVs from patients with  $\beta$ -thalassaemia/HbE do not promote angiogenesis in HUVECs compared with healthy controls (30) and that NO donors improve pulmonary hypertension in patients with  $\beta$ -thalassaemia (20,21), functional assays would have provided a more comprehensive understanding of the observed NO reduction by mEVs. The findings in the present study were based on *in vitro* models, and further studies using animal models or *ex vivo* blood vessels will be necessary to validate these results.

Future research focusing on elucidating the detailed molecular mechanisms by which mEVs from splenectomised patients with  $\beta$ -thalassaemia/HbE induce endothelial dysfunction, and the downstream effects. This may provide insights into vascular complications and could shed light on novel alternative therapies. Furthermore, given the proven benefits of NO donors for patients with  $\beta$ -thalassaemia with pulmonary

hypertension, investigating the impact of circulating mEV levels on improvements from NO donor treatment could provide valuable insights into therapeutic strategies. Exploring the correlation between pulmonary hypertension improvement and mEV levels following NO donor treatment is warranted. Additionally, studies targeting mEV production or uptake could provide new avenues for preventing vascular complications in  $\beta$ -thalassaemia. Longitudinal studies in patients would also be beneficial to determine how mEV levels correlate with disease progression and the risk of thrombotic events, ultimately aiding in the development of predictive biomarkers.

In summary, vascular complications, such as cardiovascular alterations, pulmonary arterial hypertension and thromboembolic events, are notable complications in splenectomised patients with  $\beta$ -thalassaemia. It has been previously shown that splenectomised mEVs are one of the factors that contribute to vascular complications via several mechanisms. The present study demonstrated that splenectomised mEVs could decrease NO bioavailability in the patients. These findings highlight the significant implications of splenectomised mEVs in the pathogenesis of thromboembolism in  $\beta$ -thalassaemia disease. The present study emphasizes the association between splenectomy and thrombotic complications, reinforcing the need for careful consideration before performing a splenectomy in these patients.

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### Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

### Authors' contributions

KPh performed the experiments, the analysis and interpretation of the data and drafting the manuscript. WK and NP performed the isolation of mEVs, flow cytometry and analysis

of the data. TS performed the NO analysis. KPat and SF contributed to specimen collection. KPat and NS contributed to the concept of the present study and interpretation of the data. PC contributed to the concept of the present study, design of the experiments, analysis and interpretation of the data, and drafting the manuscript. SS was the principal investigator and takes primary responsibility for the concept and design of the project, the analysis and interpretation of the data, drafting and editing the manuscript. All authors read and approved the final version of the manuscript. SS and PC confirm the authenticity of all the raw data.

### Ethics approval and consent to participate

The present study was performed in accordance with the Helsinki Declaration and was approved (approval no. 2014/013.0502) by the Mahidol University Central Institutional Review Board (Bangkok, Thailand). Written informed consent was obtained from all individual participants included in the present study.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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