Thrombosis Research 174 (2019) 104–112

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Vascular and hemostatic alterations associated with pulmonary hypertension in β-thalassemia hemoglobin E patients receiving regular transfusion and iron chelation

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ARTICLE INFO

Keywords:
β-Thalassemia/HbE
Pulmonary hypertension
Nitric oxide
Hypercoagulability
Endothelial dysfunction

ABSTRACT

Introduction: Pulmonary hypertension (PH) is the commonest cardiac complication in β-thalassemia intermedia, including β-thalassemia/hemoglobin E (β-thal/HbE), and is strongly associated with splenectomy. We aimed to define the prevalence and comprehensively explore mechanisms of PH in β-thal/HbE patients receiving regular transfusion and iron chelation, which were reported to alleviate PH.

Materials and methods: β-Thal/HbE patients receiving regular transfusion and iron chelation over one year were enrolled. Patients at risk for PH were defined by tricuspid-regurgitant-jet-velocity (TRV) ≥ 2.5 m/s. Laboratory and echocardiographic variables were compared with healthy controls.

Results: There were 68 β-thal/HbE, including 31 (45.6%) splenectomized patients, and 38 controls included for analysis. PH was detected in 29 β-thal/HbE (42.6%). β-Thal/HbE with PH had a significant reduction in nitric oxide metabolites (NOx) but elevations in thrombin-antithrombin (TAT) complex, soluble thrombomodulin (*s*TM), endothelin-1 (ET-1) and flow-mediated dilation (FMD) values compared to those without PH (all, $p < 0.05$). TRV was significantly correlated with NOx, TAT, sTM, ET-1 and FMD values ($r = -0.514$, $r = 0.281$, $r = 0.313$, $r = 0.245$ and $r = -0.474$; all $p < 0.05$). Erythropoietic activity, serum ferritin, circulating total tissue factor (TF) antigen, microparticle-associated TF activity, microparticle's procoagulant activity and soluble p-selectin levels were not different between PH and non-PH subgroups. Notably, there were no significant associations between splenectomy and PH.

Conclusions: PH remains prevalent in β-thal/HbE patients receiving long-term transfusion and iron chelation. PH is not associated with splenectomy status but correlated with NO depletion, TF-independent hypercoagulability and endothelial perturbation.

1. Introduction

Pulmonary hypertension (PH) is the commonest cardiac complication in β-thalassemia, of which the prevalence evidenced by echocardiography ranges from 10–75% [1]. The pathogenesis of PH in β-thalassemia remains incompletely characterized and involves multiple pathological processes, such as chronic hemolysis, reduced nitric oxide bioavailability, iron overload and hypercoagulability [1,2]. To clearly define mechanisms underlying PH-associated with β-thalassemia is problematic due to highly diverse patient populations, which are varied in genotypic and phenotypic severities and treatment heterogeneity. Most of the previous reports investigated pathophysiology of PH in β-thalassemia patients with inadequate transfusion. Several cohorts showed that splenectomy is the strongest risk for development of PH in patients with β-thalassemia, while regular blood transfusion and iron chelation may be protective factors [3–12].

Currently, β-thalassemia hemoglobin E (β-thal/HbE) is the commonest β-thalassemia disease and accounts for approximately 50% of severe β-thalassemia worldwide [13]. Although this genotype was most prevalent in Southeast Asia, a growth in international migration has raised its global health burdens-associated with hemoglobinopathies second only to sickle cell disease (SCD) [14]. Currently, β-thal/HbE has

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https://doi.org/10.1016/j.thromres.2018.12.021
Received 19 September 2018; Received in revised form 18 November 2018; Accepted 19 December 2018
Available online 20 December 2018
0049-3848/

become the most common β-thalassemia disease identified from many newborn screening programs in the US and Europe [13]. Clinical manifestations found in β-thal/HbE are related to ineffective erythropoiesis, chronic hemolysis and iron overload. As most patients can tolerate chronic severe anemia without regular transfusion, they are usually classified as non-transfusion dependent thalassemia (NTDT) or thalassemia intermedia (TI) [13,15]. A recent cohort study demonstrated that the prevalence of several complications in non-transfused β-thal/HbE patients were comparable with those of β-TI and differed from those of β-thalassemia major (β-TM) [3,16,17]. Previously, β-thal/HbE patients received only occasional transfusion to ameliorate anemic symptoms and rarely obtained adequate iron chelation. These patients were susceptible to thromboembolic complications relating to hypercoagulability and endothelial dysfunction [18–20]. Currently, regular transfusion and iron chelation are widely adopted in practice. This may prevent or reverse the development of PH in this population. In this study, we aimed to determine the prevalence and comprehensively explore the mechanisms of PH in β-thal/HbE patients who received regular transfusion concomitant with iron chelation, which could modify natural history of disease. Our findings will provide greater insights into pathophysiology of PH and identify targets for management of persisting PH in β-thalassemia after adequate thalassemia-directed therapy.

2. Methods

2.1. Subjects

The cross-sectional study was conducted at King Chulalongkorn Memorial Hospital between May and September 2015. Adult β-thal/HbE patients aged over 18 years who had received regular red cell transfusion as defined by ≥1 units per month concomitant with any form of iron chelating agents for at least one year were eligible for the study. Enrolled patients required transfusion and recommended doses of iron chelating agents as follows: deferiprone of 50–100 mg/kg/day, deferasirox of 20–40 mg/kg/day or deferoxamine of 20–60 mg/kg/day at least 8 months per year. The transfusion target was to maintain pre-transfusion Hb ≥ 7 g/dL following the institution policy. Patients with signs and/or symptoms of decompensated cardiac failure, significant structural heart diseases, uncontrolled arrhythmias, decompensated chronic liver diseases, end stage renal disease, known risk factors for secondary PH such as chronic lung diseases, chronic smoking, connective tissue diseases, and human immunodeficiency virus infection, taking medications affecting pulmonary arterial pressure (PAP), antiplatelet or anticoagulant therapy, and pregnancy were excluded. Healthy adults aged over 18 years without any underlying diseases or taking any medications were recruited as normal controls.

Medical history and cardiovascular examinations were taken by cardiologists. General laboratory results including complete blood count, serum blood chemistry, serum ferritin and quantity of transfusion were averaged from previous 12 months before the enrolment. Blood samples for all biomarker assays were collected and performed at Division of Hematology. Echocardiographic and hemodynamic profiles were measured and analyzed at Division of Cardiology.

Informed consent was obtained from all participants. The study was approved by the Institutional Review Board of Faculty of Medicine, Chulalongkorn University and followed the Declaration of Helsinki.

2.2. Laboratory measurement

Blood samples were obtained on the same days of echocardiography and collected in EDTA and citrate tubes as appropriate. Platelet-poor plasma (PPP) from EDTA and citrate tubes was prepared by centrifugation for 15 min at 1000*g*. Platelet-free plasma for microparticle (MP) activity measurement was prepared using PPP from citrate tubes by centrifugation for 2 min at 13,000*g*. All specimens were processed within 30 min after collection and stored at −80 °C until tested.

Kit assay tests were purchased as follows: growth differentiation factor-15 (GDF-15), soluble transferrin receptor (sTfR), total nitric oxide (NO), prostaglandin E_2 (PGE_2), endothelin-1 (ET-1), soluble thrombomodulin (sTM) and soluble p-selectin (sP-selectin) (R&D systems, Minneapolis, MN, USA); total tissue factor antigen (TFAg), microparticle-associated tissue factor activity (MP-TFA0 and microparticles' procoagulant activity (MP-PCA) (Aniara Diagnostica, West Chester, OH, USA) and thrombin-antithrombin complex (TAT) (Abcam, Cambridge, MA, USA).

Circulating NO is rapidly metabolized to nitrite and nitrate. Therefore, plasma NO levels was determined by a two-step procedure for quantification of total endogenous NO metabolites (NOx) following the kit instruction. Endogenous nitrate was converted to nitrite by nitrate reductase. The total nitrite was measured and represented plasma NO levels.

2.3. Echocardiographic assessment

Echocardiography (IE33 or EPIQ 7C, Philips, Bothell, Seattle WA, USA) was performed by cardiologists or experienced sonographers and analyzed by cardiologists using Xcelera software version 3.2. Cardiac structures and functions and PAP profiles were measured and determined according to the guideline of American Society of Echocardiography and European Association of Cardiovascular Imaging [21]. Patients at risk for PH was defined by tricuspid-regurgitant-jet-velocity (TRV) ≥ 2.5 m/s corresponding to an estimated pulmonary artery systolic pressure (PASP) ≥ 35 mm Hg, which has been used in most studies for comparison.

2.4. Assessment of endothelial function

We reviewed endothelial function from another cohort of 43 β-thal/HbE and 43 age-sex matched healthy subjects [20]. Patients and age-sex matched controls with sufficient echocardiographic data were analyzed to determine correlations between endothelial function and TRV. Endothelial function was examined using both endothelium-dependent (flow-mediated dilation, FMD) and endothelium-independent (nitroglycerine-mediated dilation, NMD) of brachial artery according to the guideline for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery from the International Brachial Artery Reactivity Task Force [22].

2.5. Statistical analysis

Normality of the data was tested using Shapiro-Wilk test. Continuous data were presented as means (± standard deviations, SD). Comparisons of continuous data and categorical data were analyzed by unpaired student *t*-test and Chi-square method, respectively, as appropriate. Correlations between variables were determined by Pearson correlation coefficient. Statistical significance was considered when a *p*-value was < 0.05. All statistical parameters were analyzed using SPSS version 22.0 for Window (Chicago, SPSS Inc.).

3. Results

3.1. Patient characteristics

Sixty-nine regularly transfused β-thal/HbE patients were screened. One patient was excluded due to rheumatic heart disease. There were 68 β-thal/HbE and 38 healthy controls for analysis. The ages (mean ± SD) were 34.1 ± 12.6, ranging from 19 to 67 years, and 37.2 ± 8.6 years, ranging from 18 to 70 years, respectively ($p = 0.136$). Females were slightly predominant in β-thal/HbE (58.8%), while males were slightly predominant in the control group (55.3%). Among 68 β-thal/HbE, 6 (8.8%) were diagnosed as diabetes mellitus,

and one (1.5%) had chronic hepatitis C. No patients had New York Heart Association class III–IV or symptoms and signs of congestive heart failure. β-Thal/HbE patients received red cell transfusion of 22.3 ± 9.6 units per year or 1.86 ± 0.80 units per month, and the mean pre-transfusion Hb was 8.1 ± 1.3 g/dL. The average regular transfusion and iron chelation duration was 8.1 ± 2.2 years, ranging from 2.2–10.6 years. Most of patients received deferiprone (82.1%) followed by deferasirox (9.0%), deferoxamine (5.9%) and combined therapy (2.9%). There were 31 (45.6%) splenectomized patients. Hematological characteristics showed that β-thal/HbE patients had significantly lower Hb levels but higher white blood cell (WBC) and platelet counts than those of controls. Serum ferritin levels were substantially elevated in β-thal/HbE patients. All β-thal/HbE patients had eGFR ≥ 60 mL/min/1.73^2. No β-thal/HbE patients had an elevation of transaminases over 1.5 times of upper normal limit.

3.2. Echocardiographic assessment

β-Thal/HbE patients had significantly higher stroke volume and enlarged right ventricular (RV) and left ventricular (LV) chambers compared to controls. Both left and right ventricular systolic functions were well-preserved, and no patients had LV ejection fraction < 55%. Although β-thal/HbE patients had statistically higher E/e′ ratios compared with controls, there were only 4 (5.9%) patients with LV diastolic dysfunction (E/e′ ratio > 14). Remarkably, β-thal/HbE patients had significantly elevated PAP compared to controls, and 29 (42.6%) β-thal/HbE were at risk for PH. No patients underwent a right heart catheterization (RHC). Among 29 having PH, 13 were splenectomized, while 16 were non-splenectomized. There were no statistical differences in demographic data, quantity of transfusion, Hb levels, WBC and platelet counts, serum ferritin levels and other echocardiographic profiles between PH and non-PH subgroups. Demographic data, basic laboratory and echocardiographic profiles were summarized in Table 1.

3.3. Biomarkers

3.3.1. Erythropoietic activity

β-Thal/HbE patients had markedly elevated GDF-15 and sTfR levels indicating substantially increased erythropoietic activity in comparison to controls. However, there were no statistical differences between these erythropoietic indices between PH and non-PH β-thal/HbE.

3.3.2. Activation of platelets and coagulation

MP-PCA and TAT were significantly elevated in β-thal/HbE patients, while MP-TFA, TFAg and sP-selectin levels were not significantly different between patients and controls. In addition, β-thal/HbE patients with PH had a significant elevation in TAT compared to those without PH, while other markers were not significantly different between PH and non-PH subgroups.

3.3.3. Nitric oxide metabolites, markers of inflammation and endothelial activation/injury

Plasma NOx and PGE_2 were significantly reduced in β-thal/HbE patients, while sTM levels were significantly elevated compared to controls. Plasma ET-1 levels were not statistically different between patients and controls. Among β-thal/HbE, plasma NOx levels were significantly lower in patients with PH, while sTM and ET-1 levels were significantly increased in those with PH. Although β-thal/HbE with PH had plasma PGE_2 levels lower than those without PH, the difference did not reach statistical significance.

3.4. Endothelial function

We reviewed another β-thal/HbE cohort in which endothelial functions were evaluated. There were 27 age-sex matched controls and 27 β-thal/HbE patients receiving regular transfusion and iron chelation with available FMD, NMD and TRV for analysis. β-Thal/HbE patients demonstrated significantly lower FMD, while similar NMD values compared to those of controls indicating endothelial dysfunction. Notably, 9 β-thal/HbE with PH had significantly lower FMD compared to 18 without PH indicating a greater degree of endothelial dysfunction in patients having PH.

Data on biomarker variables and endothelial functions were summarized in Table 2.

3.5. Splenectomy status

Splenectomized β-thal/HbE patients showed no statistical differences in age (33.0 ± 10.8 *vs.* 35.1 ± 14.0 years, $p = 0.506$), quantity of transfusion (1.76 ± 0.44 *vs.* 1.94 ± 1.01 units per month,

Table 1
Clinical, laboratory and echocardiographic data among β-thalassemia hemoglobin E patients and healthy control subjects (mean ± standard deviation).

Variables	Healthy control (N = 38)	β-Thal/HbE (N = 68)	*p* values	β-Thal/HbE without PH (N = 39)	β-Thal/HbE with PH (N = 29)	*p* values
Clinical characteristics						
Age (years)	37.2 ± 8.6	34.1 ± 12.6	0.136	33.9 ± 13.4	34.4 ± 11.6	0.506
Number of transfusion (units/month)	NA	1.86 ± 0.80	NA	1.93 ± 0.89	1.76 ± 0.66	0.355
Baseline laboratory						
Hb (g/dL)	13.57 ± 1.33	8.13 ± 1.33	< 0.001	8.00 ± 1.32	8.34 ± 1.33	0.255
WBC count (×10^3/μL)	6.85 ± 1.68	17.05 ± 18.31	< 0.001	15.77 ± 15.59	18.78 ± 21.61	0.406
Platelets (×10^3/μL)	260 ± 60	454 ± 215	< 0.001	474 ± 213	425 ± 217	0.253
Ferritin (ng/mL)	152.5 ± 128.9	2918.7 ± 2732.9	< 0.001	3274.4 ± 3242.3	2440.3 ± 1786.5	0.118
Echocardiographic assessment						
LVEDD (mm)	46.6 ± 3.7	50.8 ± 6.0	< 0.001	51.2 ± 5.2	50.2 ± 7.0	0.447
LVESD (mm)	27.3 ± 3.8	29.6 ± 5.9	0.043	28.9 ± 5.8	30.5 ± 6.1	0.246
SV (mL)	62.7 ± 12.9	74.7 ± 19.1	0.001	73.1 ± 20.0	76.9 ± 18.1	0.387
LVEF (%)	71.6 ± 6.9	70.5 ± 12.5	0.599	70.3 ± 12.5	70.7 ± 12.9	0.872
E/e′	8.63 ± 1.88	10.97 ± 3.07	< 0.001	10.67 ± 2.94	11.63 ± 3.44	0.337
RVD (mm)	33.9 ± 4.4	39.7 ± 6.0	< 0.001	39.8 ± 5.9	39.3 ± 6.2	0.869
S′ (cm/s)	14.3 ± 9.6	14.4 ± 2.5	0.964	13.9 ± 2.1	14.9 ± 2.9	0.540
TRV (m/s)	1.84 ± 0.35	2.48 ± 0.51	< 0.001	2.17 ± 0.23	2.89 ± 0.50	< 0.001
PASP (mm Hg)	20.2 ± 5.4	35.6 ± 12.6	< 0.001	29.1 ± 4.0	44.3 ± 14.8	< 0.001

NA = not applicable; β-thal/HbE = β-thalassemia hemoglobin E; PH = pulmonary hypertension; Hb = hemoglobin (pre-transfusion Hb for thalassemia patients); WBC = white blood cell; LVEDD = left ventricular end-diastolic diameter; SV = stroke volume; LVEF = left ventricular ejection fraction; RVD = right ventricular diameter; S′ = pulse tissue Doppler S wave; TRV = tricuspid regurgitant jet velocity; PASP = pulmonary arterial systolic pressure.

Table 2
Biomarker and endothelial function characteristics among β-thalassemia hemoglobin E patients and healthy control subjects (mean ± standard deviation).

Variables	Healthy control (N = 38; 27)[a]	β-Thal/HbE (N = 68; 27)[a]	*p* values	β-Thal/HbE without PH (N = 39; 18)[a]	β-Thal/HbE with PH (N = 29; 9)[a]	*p* values
Erythropoietic activity						
GDF-15 (pg/mL)	387.3 ± 128.7	5950.8 ± 1649.6	< 0.001	5819.8 ± 1907.7	6126.9 ± 1231.3	0.343
sTfR (μg/mL)	2.60 ± 1.21	20.45 ± 14.33	< 0.001	21.72 ± 17.86	18.73 ± 7.33	0.288
Hypercoagulable state						
TAT (ng/mL)	5.05 ± 2.17	8.85 ± 5.05	< 0.001	8.03 ± 3.24	9.95 ± 6.00	0.046
MP-PCA (nM)	1.02 ± 0.73	3.00 ± 3.75	< 0.001	3.58 ± 4.71	2.21 ± 1.54	0.064
MP-TFA (pg/mL)	0.40 ± 0.16	0.44 ± 0.66	0.755	0.45 ± 0.80	0.42 ± 0.42	0.836
TFAg (pg/mL)	46.56 ± 20.75	52.92 ± 130.42	0.760	57.60 ± 163.26	46.62 ± 66.73	0.670
sP-selectin (ng/mL)	31.18 ± 11.32	29.75 ± 15.43	0.325	29.18 ± 15.83	30.62 ± 15.13	0.708
Nitric oxide, inflammation and endothelial activation/injury						
NOx (μmol/L)	178.2 ± 17.6	132.4 ± 32.5	< 0.001	143.2 ± 29.6	118.0 ± 31.0	< 0.001
PGE_2 (pg/mL)	1315.1 ± 682.4	674.8 ± 648.8	< 0.001	731.7 ± 683.2	598.1 ± 602.8	0.412
ET-1 (pg/mL)	1.47 ± 0.43	1.55 ± 0.73	0.468	1.37 ± 0.57	1.80 ± 0.85	0.005
sTM (pg/mL)	3084.3 ± 593.7	3969.5 ± 3487.6	0.045	3323.4 ± 1091.9	4838.4 ± 5111.1	0.026
Endothelial function						
FMD (%)	9.87	5.39	< 0.001	6.36	3.43	0.025
NMD (%)	17.77	19.96	0.514	20.49	18.84	0.612

[a] (N, N) = (other variables, endothelial function); β-thal/HbE = β-thalassemia hemoglobin E; PH = pulmonary hypertension; GDF-15 = growth differentiation factor-15; sTfR = soluble transferrin receptor; MP-TFA = microparticle-associated tissue factor activity; MP-PCA = microparticle's procoagulant activity; TAT = thrombin-antithrombin complex; NOx = nitric oxide metabolites; PGE_2 = prostaglandin E_2; ET-1 = endothelin-1; sTM = soluble thrombomodulin; FMD = flow-mediated dilation; NMD = nitroglycerine-mediated dilation.

Table 3
Echocardiographic and biomarker profiles of splenectomized and non-splenectomized β-thalassemia hemoglobin E patients (mean ± standard deviation).

Variables	Splenectomy (*N* = 31)	Non-splenectomy (*N* = 37)	*p* values
Echocardiographic assessment			
LVEDD (mm)	51.9 ± 5.7	49.8 ± 6.1	0.159
LVESD (mm)	30.9 ± 4.2	28.3 ± 7.0	0.083
SV (mL)	78.6 ± 21.4	71.2 ± 16.3	0.131
LVEF (%)	67.7 ± 13.7	73.1 ± 10.9	0.089
E/e′	10.6 ± 3.5	11.3 ± 2.8	0.555
RVD (mm)	41.2 ± 6.6	38.3 ± 5.0	0.059
S′ (cm/s)	14.3 ± 2.7	14.4 ± 2.3	0.900
TRV (m/s)	2.41 ± 0.44	2.52 ± 0.57	0.399
PASP (mm Hg)	34.0 ± 9.1	36.9 ± 14.9	0.339
Erythropoietic activity			
GDF-15 (pg/mL)	5973.2 ± 1472.9	5932.0 ± 1804.4	0.919
sTfR (μg/mL)	21.96 ± 19.20	19.18 ± 8.46	0.431
Hypercoagulable state			
TAT (ng/mg)	8.56 ± 3.97	9.09 ± 5.24	0.646
MP-PCA (nM)	2.47 ± 1.54	3.44 ± 4.87	0.289
MP-TFA (pg/mL)	0.33 ± 0.29	0.52 ± 0.86	0.232
TFAg (pg/mL)	35.47 ± 29.88	67.54 ± 174.44	0.316
sP-selectin (ng/mL)	31.94 ± 14.43	27.80 ± 16.27	0.325
Nitric oxide, inflammation and endothelial activation/injury			
NOx (μmol/L)	134.2 ± 31.5	130.9 ± 33.6	0.680
PGE_2 (pg/mL)	571.9 ± 591.8	760.9 ± 689.1	0.234
ET-1 (pg/mL)	1.54 ± 0.80	1.57 ± 0.67	0.846
sTM (pg/mL)	4090.9 ± 4036.7	3867.8 ± 3005.8	0.795

β-thal/HbE = β-thalassemia hemoglobin E; SV = stroke volume; LVEF = left ventricular ejection fraction; RVD = Right ventricular diameter; S′ = pulse tissue Doppler S wave, TRV = tricuspid regurgitant jet velocity; PASP = pulmonary arterial systolic pressure; GDF-15 = growth differentiation factor-15; sTfR = soluble transferrin receptor; MP-TFA = microparticle-associated tissue factor activity; MP-PCA = microparticle's procoagulant activity; TAT = thrombin-antithrombin complex; NOx = nitric oxide metabolites; PGE_2 = prostaglandin E_2; ET-1 = endothelin-1; sTM = soluble thrombomodulin.

$p = 0.355$) and serum ferritin levels (2460.3 ± 2092.0 *vs.* 3302.8 ± 3149.7 μg/L, $p = 0.193$) compared to non-splenectomized patients. Hb levels (8.5 ± 1.1 *vs.* 7.8 ± 1.4 g/dL, $p = 0.021$), WBC count (30.3 ± 20.2 *vs.* 5.9 ± 2.1 × 10^9/L, $p < 0.001$) and platelet count (641 ± 144 *vs.* 297 ± 114 × 10^9/L, $p < 0.001$) were significantly higher in splenectomized patients. Surprisingly, there were no statistical differences in all biomarker values and echocardiographic profiles between splenectomized and non-splenectomized subgroups (Table 3). The average interval from splenectomy to laboratory and echocardiographic assessment was 20.1 ± 9.2, ranging from 4 to 52 years. The intervals from splenectomy to evaluation of pulmonary pressure were not statistically different between PHT and non-PHT subgroups (21.1 ± 10.0 years *vs.* 19.7 ± 9.1 years, $p = 0.68$). The majority of patients were splenectomized before age of 20 years in both PHT (81.8%) and non-PHT (77.8%) subgroups. Notably, there were no significant associations between splenectomy status and PH ($\chi^2 = 0.012$, $p = 0.914$).

3.6. Correlations between TRV and clinical data, biomarkers and endothelial function

TRV was not significantly correlated with age and quantity of transfusion. In contrast, TRV was significantly correlated with several laboratory variables (Table 4), of which GDF-15 and NOx levels demonstrated the greatest positive and inverse correlations, respectively. Notably, TRV was significantly correlated with NOx, TAT, sTM, ET-1 and FMD values, which demonstrated significant differences between PH and non-PH β-thal/HbE.

4. Discussion

PH is the major cardiovascular morbidity and mortality found in both β-TM and β-TI [1]. However, the mechanisms underlying PH are different between β-thalassemia types. Some studies demonstrated that PH in β-TM was a result of impaired LV systolic and diastolic functions [23,24]. In contrast, β-TI-associated PH was unrelated to LV dysfunction. Furthermore, a recent study using a RHC demonstrated the 5-fold higher prevalence of PH in β-TI than β-TM approaching the prevalence of PH in SCD [25,26]. Although β-thal/HbE patients have a great diversity of clinical manifestations, PH is frequently detected in β-thal/

Table 4
Correlations with tricuspid regurgitant jet velocity as measures by Pearson correlation (N = 106).

Variables	Correlation (*r*)	*p* values
Variables showing significant differences between PH and non-PH β-thal/HbE		
NOx	−0.514	< 0.001
TAT	0.281	0.004
sTM	0.313	0.001
ET-1	0.245	0.012
FMD[a]	−0.474	< 0.001
Variables showing significant differences between controls and β-thal/HbE but no significant differences between PH and non-PH β-thal/HbE		
Hb	−0.497	< 0.001
WBC	0.203	0.039
platelet count	0.283	0.004
Ferritin	0.287	0.003
GDF-15	0.565	< 0.001
sTfR	0.336	< 0.001
MP-PCA	0.068	0.491
PGE_2	−0.180	0.066

[a] N = 54; β-thal/HbE = β-thalassemia hemoglobin E; PH = pulmonary hypertension; Hb = hemoglobin (pre-transfusion Hb for thalassemia patients); WBC = white blood cell; GDF-15 = growth differentiation factor-15; sTfR = soluble transferrin receptor; MP-PCA = microparticle's procoagulant activity; TAT = thrombin-antithrombin complex; NOx = nitric oxide metabolites; PGE_2 = prostaglandin E_2; ET-1 = endothelin-1; sTM = soluble thrombomodulin; FMD = flow-mediated dilation.

HbE patients with well-preserved LV functions resembling β-TI [3,12–16]. Notably, a large cohort study demonstrated that β-thal/HbE conferred the highest risk for PH compared to homozygous β-thalassemia and HbH disease [7]. In contrast to other previous studies, we attempted to elucidate mechanisms underlying PH in β-thal/HbE patients who received regular transfusion and iron chelation. This genotypically homogeneous population under similar therapeutic protocol would reduce multiple confounding factors and allow us to accurately define pathological processes of PH in β-thalassemia receiving long-term thalassemia-directed treatment.

The prevalence (42.6%) of PH in β-thal/HbE patients who received regular transfusion and iron chelation over one year was comparable with previous reports of β-thal/HbE with occasional transfusion, ranging from 33 to 50% [6–8]. Two recent studies demonstrated that regular transfusion with a target of pre-transfusion Hb ≥ 7 g/dL and administration of deferiprone could significantly decrease PAP in β-thal/HbE after one year of treatment regardless of serum ferritin levels [9,10]. Among 68 β-thal/HbE in this current study, there were 39 patients who obtained echocardiographic assessment from another study in 2011 and identified 21 (53.8%) with PH [27]. Although PH may be partially reversible by chronic transfusion and iron chelation, it is apparent that PH persists in this population and requires other treatment modalities.

In this study, we detected several significant alterations in laboratory and biomarker profiles-related to pathogenesis of thalassemia and PH in the homogeneous cohort of β-thal/HbE patients receiving regular transfusion and chelating therapy. TRV showed significant associations with multiple variables suggesting multifactorial processes influencing elevated PAP in β-thal/HbE. Notably, we found that NOx, TAT, sTM, ET-1 and FMD values were significantly different between PH and non-PH subgroups, and all these variables showed significant correlations with TRV.

Ineffective erythropoiesis is an initial key pathogenesis of β-thalassemia resulting in severe anemia. Elevated GDF-15 and sTfR levels, which represent an increase in erythropoietic activity in bone marrow, are significantly correlated with the degree of ineffective erythropoiesis in thalassemia patients [28–30]. Additionally, GDF-15 and sTfR levels are significantly correlated with increased PAP and poorer outcomes in idiopathic PH [31,32]. We found that Hb, GDF-15 and sTfR levels were remarkably different between β-thal/HbE and control groups. In addition, TRV was significantly correlated with GDF-15 and sTfR levels implicating the influence of ineffective erythropoiesis on elevated PAP in β-thal/HbE. However, there were no differences of Hb, GDF-15 and sTfR levels between PH and non-PH β-thal/HbE. Therefore, regular

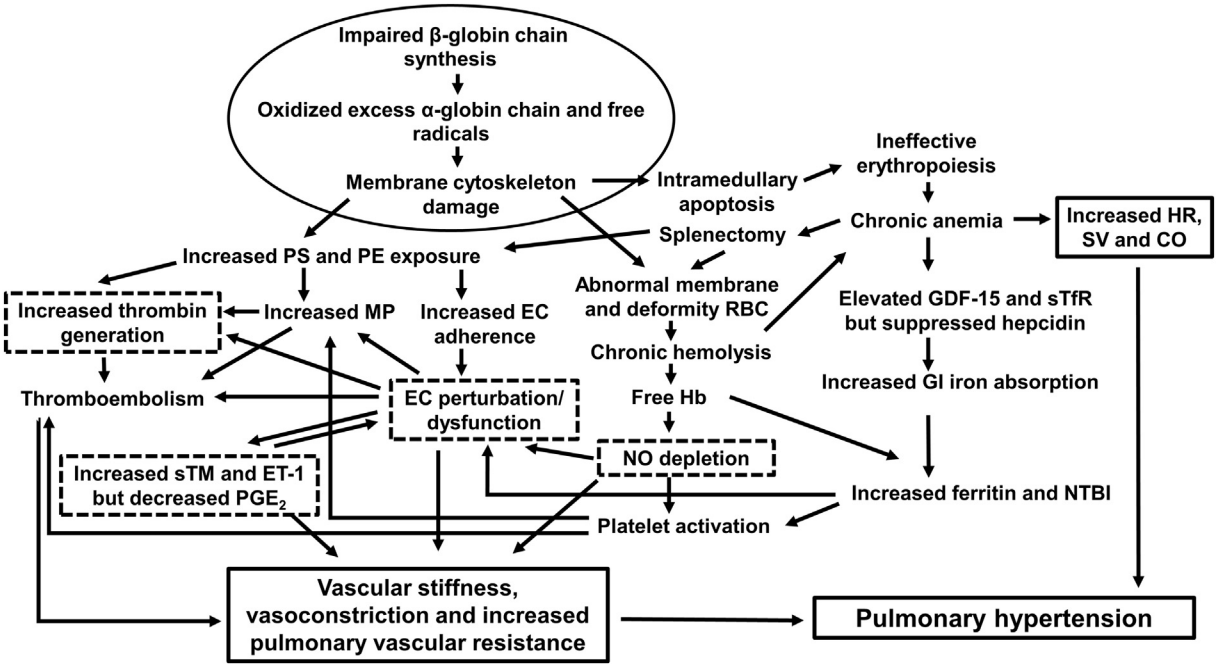

Fig. 1. Proposed pathophysiology of pulmonary hypertension associated with β-thalassemia and potential therapeutic targets for treatment in patients receiving regular transfusion and iron chelation (dashed line).
CO = cardiac output; EC = endothelial cell; ET-1 = endothelin-1; GDF-15 = growth differentiation factor-15; GI = gastrointestinal; Hb = hemoglobin; HR = heart rate; MP = microparticle; NO = nitric oxide; NTBI = non-transferrin-bound iron; PE = phosphatidylethanolamine; PGE_2 = prostaglandin E_2; PS = phosphatidylserine; RBC = red blood cell; sTM = soluble thrombomodulin; sTfR = soluble transferrin receptor; SV = stroke volume.

Table 5
Comparison of the studies on pulmonary hypertension in thalassemia syndrome.

Study	Thalassemia	Assessment	Number of patients *vs.* controls	Regular transfusion (%)/Hb (g/dL)	PH prevalence (%)	Biomarker studies	Variable-associated with PH
Uaprasert	β-E	Echo	68 *vs.* 38	100/≥7	42.6	Yes	NOx, TAT, sTM, ET-1, FMD
Taher [3]	β-TI	Echo	584 *vs.* 0	51.7/≥9	11	No	Age and splenectomy
Aessopos [4]	β-TM, β-TI	Echo	131 β-TM, 74 β-TI *vs.* 0	100 in β-TM; 0 in β-TI/≥9	0 in β-TM; 23 in β-TI	No	Not clearly defined
Aessopos [5]	β-TI	Echo	110 *vs.* 76	39.1/≥9	59.1	No	Age
Phrommintikul [6]	β-TM, β-E, HbH	Echo	13 β-TM, 40 β-E, 15 HbH *vs.* 0	0 in all subtypes	61.5 in β-TM, 45 in β-E, 20 in HbH	No	Splenectomy
Chueamuangphan [7]	β-TM, β-E, HbH	Echo	37 β-TM, 144 β-E, 43 HbH *vs.* 0	0 in all subtypes	21.6 in β-TM, 36.8 in β-E, 9.3 in HbH	No	β-Thal/HbE subtype, splenectomy
Atichartakarn [8]	β-E	Echo	110 *vs.* 0	0	37.3	Yes	Cell-free Hb, NT-pro BNP, LDH
Morris [11]	β-TM, β-E, HbH	Echo	118 β-TM, 24 β-E, 4 HbH *vs.* 0	91.5 in β-TM, 75 in β-E, 0 in HbH/≥9	33.1	No	Smoking, MRI LVEF
Singer [12]	β-TM, β-TI, HbHCS	Echo	41 β-TM, 27 β-TI, 8 HbHCS *vs.* 10	100 in β-TM, 29 in β-TI, 25 in HbHCS	12 in β-TM, 59 in β-TI, 50 in HbHCS	Yes	Platelet count, LDH
Ekwattanakit [16]	β-E, HbH	Echo	34 β-E, 23 HbH	0 in all subtypes	20.8 in β-E, 7.1 in HbH	No	Not defined
Meloni [24]	β-TM	Echo	60 *vs.* 0	100/≥9	15	Yes	Global arginine bioavailability, Hb, cardiac index, diastolic function (All were associated with TRV)
Derchi [25]	β-TM, β-TI	RHC	977 β-TM, 332 β-TI *vs.* 0	No data	1.1 in β-TM, 4.8 in β-TI	No	Age, splenectomy

PH = pulmonary hypertension; TRV = tricuspid regurgitant jet velocity; β-TM = β-thalassemia major; β-TI = β-thalassemia intermedia; β-E = β-thalassemia hemoglobin E; HbH = hemoglobin H; HbHCS = hemoglobin H Constant Spring; Echo = echocardiography; RHC = right heart catheterization; Hb = hemoglobin (pre-transfusion Hb for transfused patients); NOx = nitric oxide metabolites; TAT = thrombin-antithrombin complex; sTM = soluble thrombomodulin; ET-1 = endothelin-1; FMD = flow-mediated dilation; WBC = white blood cell; GDF-15 = growth differentiation factor-15; sTfR = soluble transferrin receptor; LDH = lactate dehydrogenase; MRI LVEF = magnetic resonance imaging left ventricular ejection fraction.

transfusion may diminish the effects of ineffective erythropoiesis on development of PH.

Hypercoagulability is a well-recognized pathological state in thalassemia syndromes. Thromboembolic events are highly prevalent in β-thalassemia and most frequently found in β-TI. Mechanisms of prothrombotic state in β-thalassemia have been extensively investigated. Ineffective erythropoiesis together with chronic hemolysis and iron overload results in an increased expression of anionic phospholipids on red blood cell (RBC) membranes, nitric oxide depletion, increased activation of several cellular elements as well as coagulation system and generation of MPs, while suppression of natural anticoagulants, leading to an increased risk of thrombotic complications [33,34]. Increased anionic phospholipid exposure, in particular phosphatidylserine (PS), on thalassemic RBCs results in an enhancement in aggregability of RBCs and thrombin generation [35–37]. Additionally, hypercoagulable state and thrombotic burdens are more profound in splenectomized patients due to a substantial increase in circulating abnormal cellular elements, especially damaged RBCs and reactive platelets, which increase susceptibility to thrombotic and vascular complications [37,38].

MPs are small membrane vesicles derived from various activated cell types, and their procoagulant activity is increased by the presence of anionic phospholipid and/or TF [39]. Several studies demonstrated important roles of elevated procoagulant activity of MPs in development of several thrombotic and vascular disorders including idiopathic PH [39,40]. Flow cytometric assessment demonstrated a significant elevation in MPs primarily derived from RBCs, platelets and endothelial cells in β-thalassemia patients and their relations to PH and thrombotic events [41,42]. However, the procoagulant activity of MPs has not yet been directly evaluated in thalassemia patients. In this study, significantly increased TAT and MP-PCA levels remained detectable in regularly transfused β-thal/HbE patients, while MP-TFA, TFAg and sP-selectin levels were not different from healthy controls. Increased thrombin generation in β-thalassemia was consistently found in several reports suggesting increased coagulation activation [33]. Interestingly, we have first demonstrated a significant increase in MP-PCA representing MP-dependent thrombin generation in β-thal/HbE patients. However, MP-TFA and TFAg levels were not significantly elevated suggesting hypercoagulability in β-thal/HbE with chronic transfusion was independent of circulating TF. Furthermore, sP-selectin levels that reflected platelet activation were not significantly increased compared with controls and did not differ between splenectomized and non-splenectomized subgroups as observed in previous reports which patients were mostly on non-regular transfusion [43,44]. Remarkably, TAT was the only prothrombotic variable significantly different between PH and non-PH subgroups and was significantly correlated with TRV. Several reports demonstrated benefits of transfusion in a reduction in activation of platelet, coagulation and inflammation as well as amelioration of PAP in β-thalassemia patients [45–47]. Our findings suggest a correlation between persistent TF-independent coagulation activation and PH in well-transfused β-thal/HbE patients.

Chronic hemolysis is considered as a key mechanism of PH in β-thalassemia. Free Hb acts as a potent NO scavenger which rapidly depletes circulating NO [48,49]. NO is a soluble gas continuously synthesized and released mainly from endothelium and plays crucial roles in maintaining vascular homeostasis, including vasodilation, inhibition of platelet activation and aggregation, suppression of leukocyte migration and various cellular adhesion to endothelium and regulation of local cell proliferation [50]. NO depletion has been documented in thalassemia patients as a consequence of hemolysis and impairment of endothelial NO synthesis secondary to endothelial dysfunction [20,51–53]. Here we further confirmed NO depletion and its significance in PH in β-thal/HbE patients. NOx levels were significantly reduced in β-thal/HbE patients and greater depleted in β-thal/HbE having PH. Furthermore, NOx levels showed the strongest inverse correlation with TRV suggesting their causal relation.

PGE_2 is the most abundant eicosanoid in humans and is produced by

most cell types including endothelial cells. PGE_2 has multiple biological properties including vasodilation, smooth muscle relaxation and modulation of inflammation [54]. Patients with SCD had strikingly elevated ET-1 and PGE_2 levels at the onset of acute vaso-occlusive crisis, and their levels were reduced within 1–3 weeks after discharge but remained significantly higher than healthy controls reflecting persistent microvascular ischemia and chronic inflammation in SCD [55]. Interestingly, we previously demonstrated a significant reduction in PGE_2 levels in association with endothelial dysfunction in another well-transfused β-thal/HbE cohort suggesting impaired endothelial PGE_2 synthesis that was diverse from pathophysiological changes in SCD [20]. In this study, β-thal/HbE patients had significantly decreased PGE_2 levels compared to controls. Although PGE_2 levels in β-thal/HbE with PH was lower than in those without PH, their differences did not reach statistical significance. In addition, there were no significant correlations between PGE_2 levels and TVR. Therefore, PGE_2 may involve in the development of endothelial dysfunction, but its role in pathogenesis of PH could not be demonstrated in this study.

ET-1 is the most potent endogenous vasoconstrictor, which is mainly synthesized and secreted by endothelial cells. Recently, a large African American cohort study demonstrated an association of elevated ET-1 levels with PH, heart failure and mortality, with the highest risk in the subgroup having PH and high ET-1 levels [56]. Although ET-1 was remarkably elevated in SCD patients, its levels were slightly increased in pediatric β-thal/HbE patients and returned to normal values within one week after transfusion [57]. In this study, we found that ET-1 levels in adult β-thal/HbE patients did not differ from healthy controls. However, ET-1 levels were significantly elevated in β-thal/HbE having PH compared to either β-thal/HbE without PH or controls. Additionally, TRV was significantly associated with ET-1 levels. These findings suggest a significant role of ET-1 in development of PH in β-thal/HbE patients.

TM is a membrane protein distributed in broad cell and tissue types. On endothelial surface, its primary function serves as a cofactor of thrombin to convert a natural anticoagulant protein C to activated protein C which inhibits coagulation factor V and VIII as well as stimulates intracellular signaling to prevent apoptosis and suppresses inflammation on endothelium [58,59]. Circulating TM represents detached TM from endothelial surface through endothelial cell injury and/or activation. Several studies demonstrated an association between elevated sTM and several cardiovascular disorders and organ failures [60,61]. In this cohort, sTM levels were significantly elevated in β-thal/HbE patients and greater elevated in the PH subgroup. A significant association between sTM and TRV was observed and suggested the risk for PH in β-thal/HbE with elevated sTM levels.

Endothelial dysfunction is an early systemic pathological change prior to development of atherosclerosis and symptomatic cardiovascular disorders. Impaired nitric oxide bioavailability is the crucial evidence representing endothelial dysfunction [62]. We recently reported a significant correlation between NO depletion and endothelial dysfunction in β-thal/HbE [20]. In this study, we observed an association of TRV with NO depletion as well as elevated ET-1 and sTM levels which highly suggested endothelial dysfunction in these patients. Therefore, we anticipated a significant correlation between TRV and endothelial dysfunction. FMD values represent an endothelium-dependent vasodilation mediated by endogenous endothelium-derived NO response to the shearing flow. Impaired NO bioavailability is confirmed by NMD indicating normal vascular smooth muscle function in response to exogenous NO from nitroglycerine administration. From our recently published cohort [20], endothelial dysfunction was detected in β-thal/HbE patients with the greatest impairment in the PH subgroup. In addition, TRV was significantly correlated with FMD values. Therefore, endothelial dysfunction appears to be an early vasculopathic event before development of PH in β-thalassemia.

Surprisingly, we did not find significant differences in all clinical variables, biomarker values and echocardiographic profiles between splenectomized and non-splenectomized β-thal/HbE. Although Hb levels, WBC counts which were substantially increased due to a marked elevation in circulating nucleated RBCs, and platelet counts were significantly higher in splenectomized patients, these parameters were not significantly associated with pulmonary pressure. Splenectomy has been assumed to be the strongest risk factor for PH in β-thalassemia [3,6,7]. However, several reports did not confirm this observation, especially in regularly transfused patients [5,9,12,24]. The main mechanisms of how splenectomy contributes to the development of PH in thalassemia patients mainly rely on the evidence of hypercoagulability [1,2]. Circulating RBCs from splenectomized β-thalassemia patients show significantly increased PS exposure causing an increase in red cell aggregation as well as thrombin generation [35,37,38]. Additionally, splenectomized thalassemia patients have significantly elevated platelets which showed increased reactivity to various platelet agonists [47]. Red cell transfusion supplies normal RBCs, while suppresses ineffective erythropoiesis resulting in a reduction in circulating PS-exposed RBCs which in turn ameliorates red cell aggregation and attenuates platelet, coagulation and inflammation activation both *in vitro* and *in vivo* [35,45–47]. Notably, we did not observe any statistical differences of TAT, MP-PCA, MP-TFA, TFAg and sP-selectin between splenectomized and non-splenectomized subgroups in this cohort in which patients had received regular transfusion and iron chelation. Taken together, these findings suggest that chronic transfusion concomitant with iron chelation could abrogate deleterious effects of splenectomy, including hypercoagulability, in β-thal/HbE.

Collectively, we observed significant differences in NOx, sTM, ET-1, FMD and TAT values between PH and non-PH β-thal/HbE subgroups representing endothelial perturbation and increased thrombin generation. As circulating MP-MCA, MP-TFA and TFAg as well as sP-selectin were not different between PH and non-PH subgroups, microparticles and tissue factor were unlikely to be involved. We hypothesized that increased coagulation activation in regularly transfused β-thal/HbE was secondary to increased thrombin generation on activated endothelial cell surface. Previous studies reporting endothelial dysfunction in pediatric β-thal/HbE patients suggested that vasculopathy in β-thal/HbE patients had started since early life [63,64]. Therefore, intervention to prevent development of PH in β-thalassemia patients may require early implementation since childhood.

Currently, management of hemoglobinopathy-associated PH is based on evidence primarily derived from idiopathic PH, case series, case reports and expert opinion [1,2]. Transfusion and iron chelation are generally recommended as the first-line approach but the current study demonstrates that PH persists despite this treatment. On the basis of our findings, restoration of NO bioavailability, inhibition of ET-1 and anticoagulation are reasonable therapeutic options for future studies. Although there are several approved PH-specific agents, the long-term outcomes, especially a survival benefit, remain unsatisfying. Novel treatments are required for improving outcomes in β-thalassemia-associated PH. As we demonstrate that endothelial dysfunction is central to the pathophysiology of PH, treatment to improve endothelial function or regenerate functional endothelium is interesting. There is growing evidence supporting this concept, and several strategies targeting endothelial function are being investigated in both early phase clinical studies and animal models [65,66].

Taken together, we propose a new mechanistic model of β-thalassemia-associated PH and potential therapeutic targets for PH in patients receiving regular transfusion and iron chelation (Fig. 1).

There were some limitations in our study. No patients underwent a RHC to confirm PH. However, a RHC is not suitable for screening clinically asymptomatic PH. In addition, echocardiography demonstrated reliable diagnostic performance and a good correlation to RHC in meta-analysis and a large patient population [67,68]. Therefore, most studies have employed echocardiography for investigating PH-associated with thalassemia syndromes [3–12,16,24]. We investigated the mechanisms underlying PH in β-thal/HbE patients to reduce

genotypic confounders, but this homogeneous population may limit the generalization to different β-thalassemia populations. However, several studies demonstrated common phenotypes, including hypercoagulability and PH, among β-thal/HbE and other β-TI patients indicating overlapped mechanisms underlying their complications [3–12,16–18].

Epidemiologic data and biomarker profiles among studies were summarized for comparison (Table 5). In this current study, PH remained common in regularly transfused β-thal/HbE patients. Although these patients did not receive hypertransfusion to maintain pre-transfusion Hb above 9–10.5 g/dL to normalize ineffective erythropoiesis [69], several studies suggested that the protective effect of transfusion for PH may depend on the onset of transfusion rather than Hb levels [4,9,11,12,24,25]. Studies in β-thal/HbE demonstrated that regular transfusion with a target pretransfusion Hb ≥ 7 g/dL could reduce PAP and hypercoagulability [9,45]. In contrast, PH remained detectable in thalassemia patients receiving hypertransfusion but was absent or uncommon in a subgroup which regular transfusion had been initiated since early childhood [4,11,12,24]. This emphasizes the necessity of early intervention for prevention of PH in thalassemia syndrome.

In conclusion, PH remains prevalent in β-thal/HbE patients receiving long-term transfusion and iron chelation. In these patients, PH is not associated with splenectomy status. NO depletion, circulating TF-independent hypercoagulability and endothelial dysfunction are significantly associated with PH in this population. Our findings identify pathophysiologically relevant biomarkers and novel potential targets for treatment of PH arising in β-thalassemia despite appropriate transfusion and chelation therapy.

Conflicts of interest

The authors declare no relevant conflicts of interest.

Acknowledgements

The study was granted by the Ratchadapiseksompotch Fund, Faculty of Medicine, Chulalongkorn University, Thailand.

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