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REGULAR ARTICLE

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Quantitative proteomics of plasma vesicles identify novel biomarkers for hemoglobin E/β-thalassemic patients

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Key Points

- Chaperones, antioxidants, iron-sequestering proteins, and cathepsin S exhibited increased abundance in thalassemic EVs.
- Haptoglobin and hemopexin are reduced in thalassemic patients' EVs, reflecting hemolysis. These could be used as clinical biomarkers.

Hemoglobin E (HbE)/β-thalassemia has a wide spectrum of clinical manifestations that cannot be explained purely by its genetic background. Circulating extracellular vesicles (EVs) are one factor that likely contributes to disease severity. This study has explored the differences in protein composition and quantity between EVs from HbE/β-thalassemic patients and healthy individuals. We used tandem mass tag labeling mass spectrometry to analyze the EV proteins isolated from the plasma of 15 patients compared with the controls. To reduce biological variation between individuals, the EV proteins isolated from randomly assigned groups of 5 HbE/β-thalassemic patients were pooled and compared with 5 pooled age- and sex-matched controls in 3 separate experiments. Alpha hemoglobin–stabilizing protein had the highest fold increase. Catalase, superoxide dismutase, T-complex proteins, heat shock proteins, transferrin receptor, ferritin, and cathepsin S were also upregulated in thalassemic circulating EVs. Importantly, haptoglobin and hemopexin were consistently reduced in patients' EVs across all data sets, in keeping with the existing hemolysis that occurs in thalassemia. The proteomic data analysis of EV samples isolated from 6 individual HbE/β-thalassemic patients and western blotting results corroborated these findings. In conclusion, we have successfully identified consistent alterations of protein quantity between EVs from HbE/β-thalassemic and healthy individuals. This work highlights haptoglobin, hemopexin, and cathepsin S as potential clinically relevant biomarkers for levels of hemolysis and inflammation. Monitoring of these plasma proteins could help in the clinical management of thalassemia.

Introduction

Thalassemia is the most common single gene disorder of red blood cells (RBCs) resulting in ineffective erythropoiesis and hemolytic anemia. The disease arises from either totally or partially impaired synthesis of the α- and/or β-globin chain. The main pathophysiology of β-thalassemia is a reduction of β-globin chain production, which causes the unpaired α-globin chains to form an irreversible hemichrome that precipitates on the erythroid membrane.[1,2] The hemichrome or availability of free iron via the Fenton reaction results in the production of reactive oxygen species (ROS) that initiate oxidative stress and cell membrane damage. Hemoglobin E (HbE) is an abnormal hemoglobin generated due to a cryptic splice site at codon 26 of exon 1 of the *HBB* gene (HBB:c.79G>A [p.Glu27Lys]). This mutation has the

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highest prevalence in Southeast Asia,[3] and compound heterozygosity for HbE and β-thalassemia is particularly common in Thailand.[3] The compound heterozygosity results in a wide range of disease severity, from intermediate to severe. Several factors play a role here, such as incomplete (β^+) or complete (β^0) loss of the β-globin chain, association with hereditary persistence of fetal hemoglobin, the degree of disparity between α- and non–α-globin chains, the starting onset and requirement for blood transfusion therapy,[4] and also extracellular vesicle (EV) production.[5,6] The numbers of EVs observed in the plasma of thalassemic patients are 4 times higher than in healthy controls; these are thought to be generated from the severe oxidative stress exposure of thalassemic red cells[7] and are also derived from platelets.[8] It is well established that these EVs are associated with increased clinically significant procoagulant activity.[9-11] This is explained by the combination of negatively charged phosphatidylserine exposure and activated tissue factor on the surface of EVs, which together initiate the coagulation cascade resulting in thrombin generation. In addition, EVs derived from platelets can induce expression of proinflammatory cytokines and chemokines. Higher amounts of platelets in the HbE/β-thalassemic patients, particularly the splenectomized patients, were also reported by Natesirinilkul and colleagues.[12]

Previous studies of the proteomics profile in EVs in the plasma released from the platelets and RBCs of β-thalassemic patients reported a higher level of proteins involved in the oxidative stress response, for example, catalase, peroxiredoxin 2 (PRDX2), and heat shock proteins 70 (Hsp70) when compared with healthy individuals.[13,14] In addition, evidence of increased RBC generation in β-thalassemia was identified, such as detection of μ hemoglobin, which is transcribed from the α gene (*HBM*) and usually expressed in cord blood reticulocytes.[15]

The close association between EV generation and pathophysiology of HbE/β-thalassemia suggests that the severity spectrum of clinical manifestations of the patients might be shown in the composition of EVs. This study aims to use quantitative tandem mass tag (TMT) coupled with sensitive nano–liquid chromatography mass spectrometry (nano–LC MS/MS) to explore the proteomic profiles of EVs to identify alterations in HbE/β-thalassemic patients' EV constituents compared with controls. The ultimate aim of such exploration is the identification of potential biomarkers that could be used to predict the severity of the disease and/or ideally to monitor requirements for blood transfusion.

Methods

Patients and samples

Ethical approval was obtained from the Institutional Review Board Committee, Siriraj Hospital (Bangkok, Thailand). Patients and control individuals enrolled in the study provided written consent according to the Declaration of Helsinki. Peripheral blood samples were collected from 15 patients diagnosed with HbE/β-thalassemia (Table 1) and 15 age- and sex-matched controls in 3.2% citrate tubes. Circulating plasma EVs were isolated using ultracentrifugation.[16,17] Briefly, debris and platelets were removed from the plasma by centrifugation at 2000*g* for 10 minutes. Platelet-free plasma was then centrifuged first at 3000*g* for 10 minutes and then transferred to a new tube for centrifugation at 100 000*g* for 60 minutes at 4°C. The supernatant was discarded and EV pellet resuspended in phosphate-buffered saline (PBS) and stored at −20°C for shipping frozen to Bristol, United Kingdom. Long-term EV sample storage was at −80°C.

Proteomic analysis: TMT labeling and high pH reversed-phase chromatography

To reduce biological variation, EV samples were pooled together from 5 random individual samples in both the patient and the control groups, as described previously.[18-20] In addition, where sufficient sample was available, individual patient samples were analyzed alongside the pooled control samples (Figure 1B). Aliquots of 100 μg of 10 samples per experiment were digested with trypsin (2.5 μg trypsin per 100 μg of protein; 37°C, overnight), labeled with TMT reagents according to the manufacturer's protocol (Thermo Fisher Scientific, Loughborough, United Kingdom) and the labeled samples were pooled.

An aliquot of the pooled sample was evaporated to dryness and resuspended in buffer A (20 mM ammonium hydroxide, pH 10) prior to fractionation by high pH reversed-phase chromatography using an Ultimate 3000 LC system (Thermo Fisher Scientific). In brief, the sample was loaded onto an XBridge BEH C18 column (130Å, 3.5 μm, 2.1 mm × 150 mm; Waters, Elstree, United Kingdom) in buffer A, and peptides were eluted with an increasing gradient of buffer B (20 mM ammonium hydroxide in acetonitrile, pH 10) from 0% to 95% over 60 minutes. The resulting fractions were evaporated to dryness and resuspended in 1% formic acid prior to analysis by nano-LC MS/MS using an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific).

Nano-LC mass spectrometry

High pH reversed-phase fractions were further fractionated using an Ultimate 3000 nano high performance LC system in line with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Peptides in 1% (vol/vol) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (vol/vol) acetonitrile, 0.1% (vol/vol) formic acid peptides were resolved on a 250-mm × 75-μm Acclaim PepMap C18 reversed-phase analytical column (Thermo Scientific) over a 150-minute organic gradient, using 7 gradient segments (1%-6% solvent B over 1 minute, 6%-15% B over 58 minutes, 15%-32% B over 58 minutes, 32%-40% B over 5 minutes, 40%-90% B for 1 minute, held at 90% B for 6 minutes, and then reduced to 1% B for 1 minute) with a flow rate of 300 nL per minute. Solvent A was 0.1% formic acid, and solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nanoelectrospray ionization at 2.0 kV using a stainless-steel emitter with an internal diameter of 30 μm (Thermo Scientific) and a capillary temperature of 275°C.

All spectra were acquired using an Orbitrap Fusion Tribrid mass spectrometer controlled by Xcalibur 2.0 software (Thermo Scientific) and operated in data-dependent acquisition mode using a synchronous precursor selection–MS3 workflow. Fourier transform mass analyzers (FTMS)1 spectra were collected at a resolution of 120 000, with an automatic gain control (AGC) target of 200 000 and a maximum injection time of 50 milliseconds. Precursors were filtered with an intensity threshold of 5000, according to charge state (to include charge states 2-7) and with monoisotopic precursor selection. Previously interrogated precursors were excluded using a dynamic window (60 seconds ± 10 ppm). The MS2 precursors were isolated with a quadrupole mass filter set to a width of 1.2 m/z. Ion-trap tandem mass spectrometry (ITMS2)

Table 1. Characteristics and clinical parameters of the patients enrolled in the study

Pt	Sex	Splenectomy status	Transfusion interval	Hb, g/L	Hct, %	RBC, $\times 10^{12}$/L (3.9-6.01)	MCV, fL	nRBC, /100 WBC	Platelets, $\times 10^{9}$/L	WBC, $\times 10^{9}$/L	Neutrophil, %	Lymphocyte, %	Monocyte, %	Eosinophil, %	Basophil, %	Ferritin, ng/mL	Chelation
1	M	Intact	Intermittent	80	26.0	4.93	53	7	123	7.4	64.4	29.4	4.1	1.6	0.5	596	Deferiprone
2	M	Intact	Never	86	25.5	3.77	68	N/A	325	5.6	57.5	36.0	4.7	1.4	0.4	N/A*	No
3	F	Intact	Intermittent	66	19.9	3.32	60	N/A	204	6.1	47.6	45.3	5.7	1.2	0.2	392	Deferasirox
4	F	Intact	Intermittent	89	26.9	4.66	58	N/A	227	8.8	59.7	30.9	4.4	4.2	0.8	310	No
5	F	Intact	Intermittent	70	22.2	3.24	69	N/A	134	10.5	59.2	35.2	4.5	0.7	0.4	1101	No
6	M	Intact	Intermittent	49	18.5	3.21	57	34	49	7.2	36.8	57.4	4.6	0.8	0.4	335	No
7	M	Yes	Intermittent	79	27.4	4.19	65	229	858	14.9	34.3	56.3	6.9	1.2	1.3	387	Deferiprone
8	M	Yes	Intermittent	79	26.5	3.26	81	274	795	17.5	42.6	40.8	13.1	2.1	1.4	1263	Deferiprone
9	F	Yes	Regular	67	21.5	2.86	75	151	717	20.7	36.5	48.6	6.2	7.4	1.3	5328	Deferasirox
10	F	Yes	Intermittent	46	16.0	2.03	79	490	698	39.9	62.0	28.0	6.0	3.0	1.0	766	Deferiprone
11	M	Intact	Never	68	23.4	4.02	58	N/A	221	7.1	64.4	29.5	4.1	1.6	0.4	337	No
12	F	Intact	N/A	77	23.8	4.26	56	N/A	301	8.6	54.6	35.1	6.5	2.4	1.4	N/A	No
13	F	Yes	N/A	72	24.2	3.39	71	258.4	765	17.7	41.1	49.0	5.8	2.2	1.9	N/A*	Deferasirox
14	F	Yes	Intermittent	58	20.4	2.85	72	534.3	733	8.6	26.1	65.5	6.3	1.3	0.8	N/A*	Deferasirox
15	F	Intact	N/A	92	28.0	4.63	60	2	398	8.1	67.1	26.8	4.0	1.4	0.7	645	No

F, female; Hb, hemoglobin; Hct, hematocrit; M, male; MCV, mean corpuscular volume; N/A, not available; nRBC, nucleated RBC (per 100 WBC); Pt, patient; RBC, red blood cell count; WBC, white blood cell count.
*Monitor with liver iron concentration.

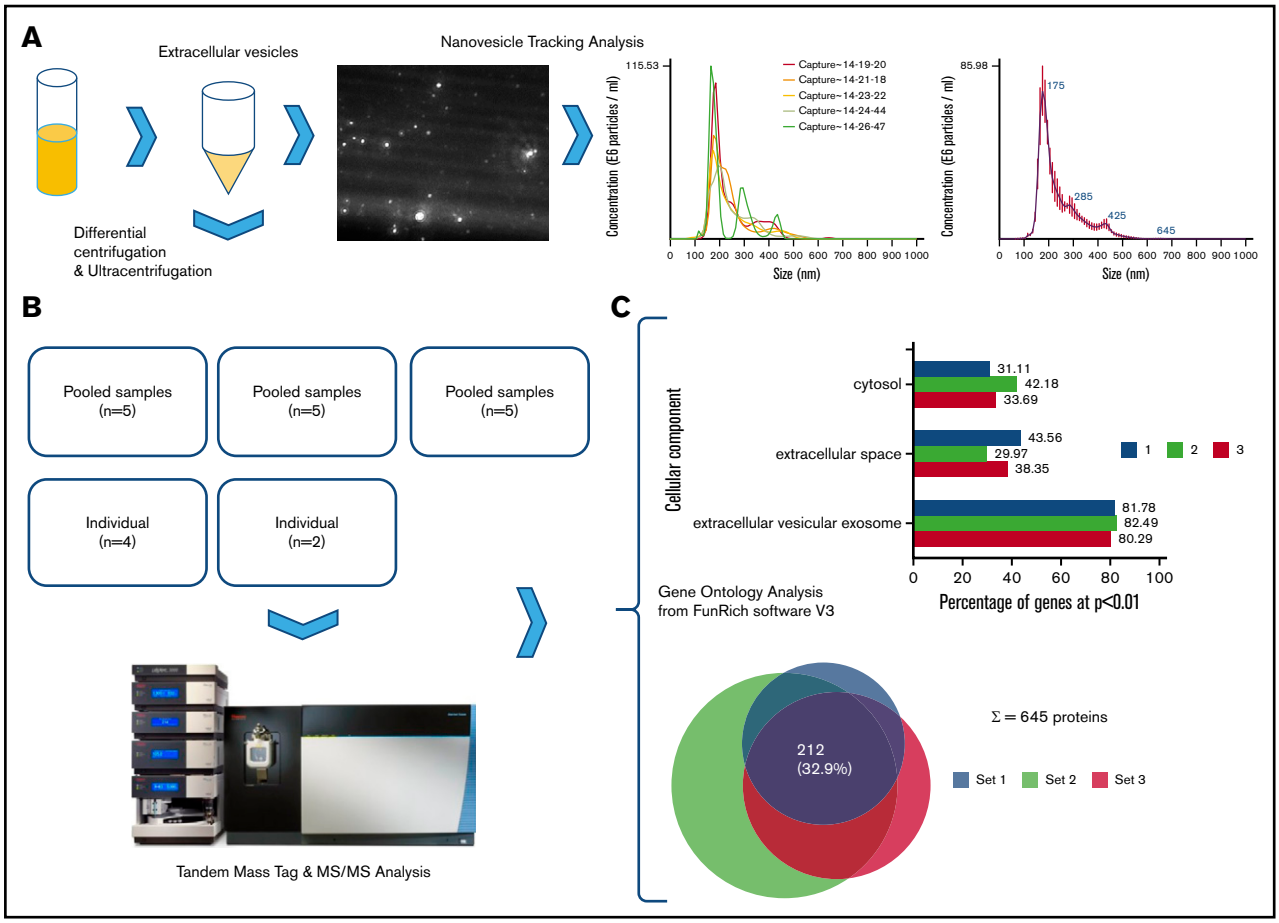

Figure 1. Flowchart of methods used for EV isolation and obtained results. From top left, EVs were isolated from 3.2% citrate plasma by differential ultracentrifugation technique. (A) EVs were analyzed using nanoparticle tracking analysis with Nanosight LM10 (Malvern Instruments Ltd). In the example shown, particle size was between 100 and 200 nm, consistent with the expected size of EVs. (B) Three sets of pooled samples with controls (n = 5) were analyzed in 3 proteomic experiments by TMT and MS/MS analysis. Additionally, 4 and 2 individual samples from the first and second sample pools, respectively, were also analyzed separately in the TMT experiments. (C) Gene Ontology analysis using Fun Rich software v3 on the 3 data sets regarding their cellular components. The majority of the identified proteins were components of EVs: 81.78%, 82.49%, and 80.29% from the first, second, and third data set, respectively. The total number of EV proteins identified was 645 EV proteins with 212 (32.9%) EV proteins common across the 3 separate experiments.

spectra were collected with an AGC target of 10 000, the maximum injection time of 70 milliseconds, and collision-induced dissociation collision energy of 35%.

For FTMS3 analysis, the Orbitrap was operated at 50 000 resolution with an AGC target of 50 000 and a maximum injection time of 105 milliseconds. Precursors were fragmented by high-energy collision dissociation at a normalized collision energy of 60% to ensure maximal TMT reporter ion yield. Synchronous precursor selection was enabled to include up to 5 MS2 fragment ions in the FTMS3 scan.

Proteomics data analysis

The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against the UniProt human database (134 169 sequences) using the SEQUEST algorithm. Peptide precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.6 Da. Search criteria included oxidation of methionine (+15.9949) as a variable modification and carbamidomethylation of cysteine (+57.0214) and the addition of the TMT mass tag (+229.163) to peptide N termini and lysine as fixed modifications. Searches were performed with full tryptic digestion, and a maximum of 1 missed cleavage was allowed. The reverse database search option was enabled, and all peptide data were filtered to satisfy a false discovery rate of 5%.

Immunoblotting

Protein quantification was measured using the Bradford protein assay using bovine serum albumin as a standard. EV proteins were diluted at a 1/1 volume with sodium dodecyl sulfate sample buffer. The samples were then incubated at 95°C for 3 minutes before being solubilized with 5% (wt/vol) 2-mercaptoethanol. The samples were separated on 10% to 12.5% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked for 1 hour (5% milk plus 0.05% Tween 20 in PBS [PBS-T]), then the primary antibodies were added and incubated at 4°C overnight. After sequential PBS-T washing was performed, the secondary antibody was added to each membrane and incubated for 1 hour. Chemiluminescent detection was carried out using ECL Plus reagent (Western Lightning; PerkinElmer) in Kodak Image Station 4000R.

Table 2. Proteins identified by TMT MS as having increased abundance in EVs of HbE and β-thalassemia patients compared with controls across 3 separate experiments

			First experiment		Second experiment		Third experiment	
Accession	**Gene ID**	**Description**	**Unique peptides**	**Patient/control ratio**	**Unique peptides**	**Patient/control ratio**	**Unique peptides**	**Patient/ control ratio**
Chaperone proteins								
Q9NZD4	51327	Alpha hemoglobin–stabilizing protein	6	47.40	4	43.46	5	31.70
P11142	3312	Heat shock cognate 71-kDa protein	17	2.56	29	7.44	21	4.53
P0DMV9	3303 or 3304	Heat shock 70-kDa protein 1A or 1B	17	10.65	27	13.41	24	14.02
P07900	3320	Heat shock 90-kDa protein α family class A member 1	12	4.03	21	13.54	16	4.77
P17987	6950	T-complex protein 1 subunit α	2	3.78	10	5.79	2	2.49
B3KX11	7203	T-complex protein 1 subunit γ	2	2.47	12	8.70	4	2.37
Iron metabolism								
P02792	2512	Ferritin light chain	3	15.59	5	13.59	3	11.44
P02786	7037	Transferrin receptor protein	3	13.03	2	6.52	41	20.25
Antioxidant								
P04040	847	Catalase	14	2.69	26	6.35	17	3.80
P00441	6647	Superoxide dismutase	6	2.47	9	9.72	6	2.21
P32119	7001	Peroxiredoxin 2	8	2.40	11	8.45	7	6.88
Hemoglobin								
P02042	3045	Hemoglobin subunit δ	6	7.22	6	14.51	6	9.62
RBC cytoskeleton								
P02549	6708	Spectrin α chain, erythrocytic 1	16	2.70	98	3.80	61	2.97
P16157	286	Ankyrin-1	25	2.43	53	3.39	28	3.05
Other proteins								
P25774	1520	Cathepsin S	2	3.47	4	3.89	3	3.01
P00915	759	Carbonic anhydrase 1	8	5.66	6	13.76	8	6.37
P30043	645	Flavin reductase (NADPH)	8	4.63	6	9.51	7	5.64
P37837	6888	Transaldolase	6	2.08	11	6.99	6	2.47
P26641	1937	Elongation factor 1-γ	5	2.89	12	6.39	9	4.98

Table 3. Proteins identified using TMT MS as consistently reduced in abundance in EVs of HbE and β-thalassemia patients compared with controls across 3 separate experiments

Accession	Gene ID	Description	First experiment		Second experiment		Third experiment	
			Unique peptides	Patient/control ratio	Unique peptides	Patient/control ratio	Unique peptides	Patient/control ratio
Hemoglobin and heme scavenger								
P02790	3263	Hemopexin	26	0.04	29	0.08	31	0.05
P00738	3240	Haptoglobin	19	0.05	24	0.09	19	0.14

Antibodies

Antibodies used for immunoblotting were: anti-α-hemoglobin–stabilizing protein (anti-AHSP) antibody, a gift from R. Griffiths (National Health Service Blood and Transplant [NHSBT] Filton); the anti-catalase (ab16731), anti-hemopexin (ab90947), and anti-haptoglobin (ab131236), antibodies were all obtained from Abcam (Cambridge, United Kingdom).

Statistical analysis

The association between severity of anemia in the patients represented by levels of hemoglobin and ratios of protein abundance in EV samples was analyzed by Spearman rank correlation using SPSS software version 16 (SPSS Inc, Chicago, IL).

Results

Proteomic profiling of proteins found in EVs of HbE/β-thalassemia patients

EVs were isolated from HbE/β-thalassemic and control samples, as outlined in "Methods." To reduce biological variation between individuals, quantitative proteomics of the circulating EVs were performed across 3 separate sets of pooled patient and matched- control samples. Five plasma-derived EV samples were pooled together at the same protein concentration for each sample, and then analyzed by nano-LC MS/MS. When filtered for proteins with >1 unique peptide, we identified 685, 1127, and 859 individual proteins for the 3 individual experiments. Approximately 80% of the proteins detected in each experiment are known constituents of EVs, matching the Gene Ontology system (GO:1903561) from the AmiGO version 1.8 database (Figure 1C; supplemental Tables 3 and 4). The isolated EVs contained proteins from a mixture of cellular sources, including platelet proteins and RBC/reticulocyte proteins. Table 2 lists 19 proteins in the EV samples that were consistently more abundant in the HbE/β-thalassemia patient samples compared with the controls, across all 3 experiments. There were only 2 proteins that differentially reduced their abundance in the patient samples (Table 3) (see supplemental Tables 1 and 2 for the peptides and peptide spectrum matches of all the identified proteins with altered abundance).

Proteins that increased their abundance in the patient over control samples can be categorized according to their molecular functions as chaperone proteins, proteins involved with iron metabolism, antioxidant proteins, and erythrocyte-specific proteins (Table 2). Among these, the protein with the highest ratio difference between patients and controls was AHSP, a RBC-specific protein that prevents α-globin precipitation,[21] which exhibited between 31- and 47-fold increases in thalassemic EVs. Other chaperone proteins identified in our study were Hsp70, Hsp90, and T-complex protein 1 subunit α and γ. Three antioxidant proteins that were increased in thalassemic EVs were catalase, superoxide dismutase (SOD1), and PRDX2. Flavin reductase, a broad specificity oxidoreductase that catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH) reduction contributing to heme catabolism and provides reducing power for the release of ferritin-bound iron, was increased. Other proteins involved in iron metabolism were also increased (ferritin and transferrin), alongside carbonic anhydrase 1, transaldolase (a pentose phosphate pathway enzyme) and the erythrocyte cytoskeleton proteins spectrin and ankyrin. These data

Table 4. Hemoglobin levels and the ratio alteration of the proteins of interest in EVs from 6 HbE/β-thalassemic patients measured by TMT

Patient no.*	Hb, g/L	AHSP	HSP70	HSP71	HSP90	TCP1-α	TCP1-γ	Flavin reductase	Cathepsin S	SOD1	Catalase	PRDX2	Ferritin	Haptoglobin	Hemopexin
1	80	31.29	9.52	1.89	2.56	1.76	1.82	3.63	3.57	1.48	2.19	1.50	7.14	0.04	0.04
3	66	100.00	22.82	5.99	13.14	11.81	3.57	19.43	5.54	8.01	7.63	7.83	76.76	0.14	0.08
4	89	19.44	6.93	1.90	2.90	2.19	1.79	3.73	2.44	1.26	1.64	1.16	7.31	0.09	0.04
5	70	39.91	8.65	2.13	3.57	4.19	3.39	5.58	2.81	1.59	2.80	1.99	7.90	0.05	0.06
8	79	47.54	15.98	11.99	15.42	6.02	6.40	6.53	4.98	7.51	5.56	5.27	14.50	0.11	0.14
9	67	91.82	20.73	11.61	25.87	14.27	18.42	23.19	2.96	23.95	15.65	21.87	5.79	0.08	0.15

Ferritin, ferritin light chain; TCP, T-complex protein.
*Patient numbers are from Table 1.

strengthen the hypothesis that the circulating plasma EVs are derived in part from erythrocyte lysis.[22] Finally, an increase in the quantity of cathepsin S, a potent elastolytic protease, was detected in thalassemic EVs, which may originate from activated myeloid cells.[23]

Only 2 proteins, hemopexin and haptoglobin, were consistently and significantly reduced (12.5- to 25-fold and 7.1- to 20-fold reduction, respectively) in the patient compared with control EV samples across the 3 experiments (Table 3). These data are consistent with the pathophysiology of thalassemia, with the hemolysis causing a dramatic decrease in these hemoglobin/heme scavengers.

In addition to the pooled samples, where there was sufficient EV protein sample isolated, individual patient samples were also included within the same TMT MS experiments. Proteomics analysis of 6 individual samples across 2 separate experiments corroborated the pooled results. All of the proteins identified as having increased quantity in the pooled patient EVs had increased abundance in each individual sample, namely, AHSP, Hsp70, HspA8, Hsp90, TCP1 subunit α and γ, flavin reductase (NADPH), SOD1, catalase, PRDX2, and ferritin (see details in Table 4). Moreover, the fold increases of EV protein in the individual samples correlated well with severity of anemia of the patients. Levels of hemoglobin were used as an indicator of anemia in the patients. Ratios of antioxidant proteins, AHSP, Hsp70, and TCP1-α showed statistically significant reverse correlation with hemoglobin levels, summarized in Table 5.

Immunoblotting

The alterations in abundance of catalase, AHSP, hemopexin, and haptoglobin were also confirmed in individual samples by western blot analysis. Two pairs of patients and age- and sex-matched controls were tested to represent proteins in the EVs of the patient and control groups (Figure 2). The upregulation of AHSP and catalase was demonstrated in patient samples when compared with their controls. Hemopexin and haptoglobin were markedly decreased in both cases.

Thalassemic EV plasma adsorption test

We speculated that the depletion of both hemopexin and haptoglobin from the EVs observed in this study is an indicator of their continual clearance from patient's plasma, which was in turn reflected in the amount of these proteins associated with EVs. To test this hypothesis, thalassemic patients' EV pellets were washed and then incubated for 72 hours with EV-depleted normal fresh plasma. Using western blotting, we observed that incubation of normal plasma caused the restoration of haptoglobin and hemopexin levels in patients' EVs (supplemental Figure 1).

Discussion

This study has used quantitative MS to characterize the differences between EVs produced by HbE/β-thalassemic patients and healthy controls. In both control and patient samples, the EVs ranged in size between 100 and 200 nm and ~80% of proteins were known constituents of EVs (GO:1903561) (Figure 1C), which confirms that the identity of the samples is circulatory plasma EVs. Many of the identified proteins, including platelet and erythroid proteins, were found to be common in EVs across all experiments (supplemental Table 3). We observed that antioxidant proteins, chaperone proteins, proteins involving in iron metabolism,

Table 5. Statistical correlations between altered ratios of proteins of interest and hemoglobin levels of 6 individual HbE/β-thalassemic patients

	Correlation coefficient	*P* value, 2-tailed
Chaperone proteins		
AHSP	−0.943	.005**
Hsp70	−0.829	.042*
HSPA8	−0.543	.266
Hsp90	−0.657	.156
TCP1-α	−0.829	.042*
TCP1-γ	−0.714	.111
Antioxidants		
Catalase	−0.886	.019*
SOD1	−0.886	.019*
PRDX2	−0.886	.019*
Flavin reductase	−0.829	.042*
Heme scavengers		
Haptoglobin	0.371	.468
Hemopexin	0.714	.111
Other protein		
Cathepsin S	−0.543	.266

Negative correlation coefficients denote negative correlations.
*$P < .05$ represents statistical significance of >95%.
**$P < .01$ represents statistical significance of >99%.

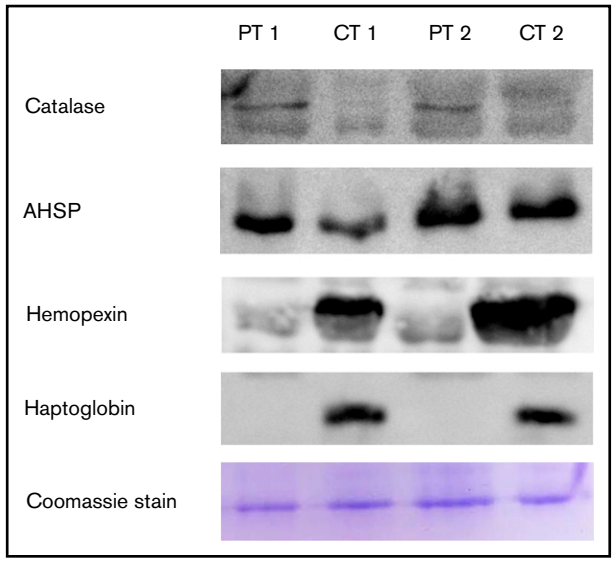

Figure 2. Western blot analysis of EV samples of 2 patients. Western blot analysis of EV samples of 2 patients (PT 1 and PT 2; which are samples of EVs isolated from patients 10 and 12, respectively) and EVs from 2 age-matched controls (labeled CT 1 and CT 2) probed using antibodies to the indicated proteins. In concordance with proteomics, the patients' EVs had an increase in abundance of AHSP and catalase when compared with control samples, and markedly decreased haptoglobin and hemopexin protein levels. An example of Coomassie-stained gel is shown to demonstrate the accuracy of loading.

hemoglobin subunit δ, cathepsin S (an inflammatory marker), and erythroid proteins were consistently increased in quantity in HbE/β-thalassemic patients across all 3 pooled samples (Table 2), and this was also observed in 6 individual samples (Table 4). Taken together, the observed alterations in protein content in the thalassemic EVs are consistent with the known increase in oxidative burden due to peripheral hemolysis reported in previous studies,[13,22,24] and this study has substantially extended the number of known proteins with an altered concentration in HbE/β-thalassemic patients' EVs.

The observed increased abundance of antioxidant and chaperone proteins in thalassemic EVs was also observed by Ferru et al, who detected alterations of Hsp70, PRDX2, and catalase.[13] In our study, we detected these proteins in at least twofold greater abundance in thalassemic EVs when compared with EVs from control individuals. The presence of these antioxidant proteins likely reflects the stress-response mechanism in thalassemic erythrocytes, and we propose this could be a result of either EVs shedding from the viable erythrocytes or being generated from red cell lysis. This is also consistent with the detection of RBC-specific proteins in EVs here by us and by other studies.[22]

Chaperones are another group of proteins that exhibited an increased abundance in the patients' EVs. These proteins facilitate the refolding of damaged proteins resulting from oxidative stress in erythrocytes.[25] AHSP, a specific erythroid chaperone significant in erythropoiesis and exclusively binding to the α-hemoglobin chain, has the greatest fold differences in patients' EVs, consistent with the known disturbance in β-globin in thalassemic erythrocytes. Several genotype-phenotype studies exploring the association between the *AHSP* gene and severity of thalassemia could not identify any correlation.[26-28] However, Bhattacharya et al[22] reported an increase of AHSP expression in thalassemic erythrocytes, which likely represents the original the source of AHSP for EVs.

The proteomics also identified a higher quantity of ferritin and transferrin receptor in EVs, 2 crucial iron-binding molecules. Transferrin receptor is known to be lost during reticulocyte maturation, suggesting that these may also be a source of EVs and raised ferritin correlates with the increased iron status of the patients (Table 1). This study focused on nontransfusion-dependent thalassemic patients who develop iron overload due to increased iron absorption and acceleration of iron released from the reticuloendothelial system.[29] The mean serum ferritin in our study is 1035 ng/mL (normal value <300 ng/mL, from 10 of 15 patients) with some patients requiring chelation. The heightened iron level observed in these patients represents an important source of oxidative stress in thalassemic erythrocytes, which may explain why EVs from thalassemic patients have more iron-binding substances and antioxidant proteins in the plasma than healthy individuals.

Haptoglobin and hemopexin are decreased in our patient EV samples, and both are free hemoglobin and free-heme scavenging plasma proteins, respectively. Free hemoglobin and hemin, ferric hemoglobin, can unleash an oxidative catastrophe to the vascular endothelium and parenchymal tissue.[30] These proteins bind these toxic substances and transport them to the reticuloendothelial tissue to be eliminated.[30,31] Importantly, we demonstrated that circulating

plasma EVs flexibly adsorb haptoglobin and hemopexin, thus indirectly reflecting the availability and concentration of these proteins in the plasma.

Overall, this report has undertaken the most detailed proteomic study to date, describing the constituents of circulating EVs of HbE/β-thalassemic patients, and providing quantitative differences of protein expression in EVs in comparison with age- and sex-matched healthy individuals. When compared with the pathophysiology of the disease, the observed proteomic changes typify the protective mechanisms used by the thalassemic patients. Antioxidants, iron-sequestering proteins, and chaperones were the predominant proteins that exhibited an increased abundance in thalassemic EVs. We also report for the first time that the quantity of haptoglobin and hemopexin, the free hemoglobin and heme-eliminating proteins, are reduced in thalassemic patients' EVs. Furthermore, the alteration of levels of these proteins correlated with hemoglobin levels of the patients (Tables 4 and 5). As far as we are aware, these plasma proteins are not routinely tested for in the plasma of thalassemic patients. Similar reductions in haptoglobin and hemopexin were reported recently in the plasma of pediatric patients with sickle cell disease[32] and were proposed as potential biomarkers of clinical severity of hemolysis in these patients. Thus, we have shown that these plasma markers are also applicable for HbE/β-thalassemic patients, where a deficit in haptoglobin and hemopexin availability reflects the severity of systemic hemolysis. Finally, we have also detected the altered levels of cathepsin S, a potent elastolytic protease that could be useful as an inflammatory plasma marker to monitor the degree of inflammation in thalassemia.[23] Future studies to evaluate the clinical application of these plasma biomarkers for monitoring the severity of thalassemia and transfusion requirements are now required.

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Authorship

Contribution: J.K. carried out the experiments, performed the analysis, prepared the figures, and wrote the manuscript; J.K., V.K.C., and A.M.T. designed the experiments; N.S. and J.K. recruited all of the research participants; M.C.W. and K.J.H. set up the MS protocol and performed the proteomics; and J.K., V.K.C., and A.M.T. wrote the manuscript.

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