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Proteomic Evidence of Complement and Coagulation Pathway Dysregulation in Transfusion-Dependent Thalassemia

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ABSTRACT

Transfusion-dependent thalassemia (TDT) is characterized by chronic hemolysis, ineffective erythropoiesis, and iron overload, resulting in persistent oxidative stress and systemic inflammation. Patients with TDT are also at increased risk of thromboembolic complications, suggesting disturbance of hemostatic and immune-related pathways. However, the molecular links between hemolysis, complement-associated pathways, and coagulation abnormalities remain incompletely defined. In this study, quantitative serum proteomics was used to identify differentially expressed proteins in patients with TDT compared with healthy controls. Pathway enrichment and network analyses demonstrated coordinated alterations in proteins associated with complement regulation, coagulation pathways, lipid transport, and regulatory mechanisms. Complement component C3 and prothrombin showed increased abundance, whereas several regulatory and heme-scavenging proteins, including alpha-2-macroglobulin, hemopexin, and ceruloplasmin, were reduced in TDT patients. Selected proteins were further evaluated using ELISA validation, which supported the direction of the proteomic findings. Overall, these results identify a serum proteomic profile consistent with perturbation of complement and coagulation-related pathways in TDT in the context of chronic hemolysis and oxidative stress. This system-level view provides additional insight into thromboinflammatory mechanisms in TDT and supports further investigation of these pathways as potential biomarkers of disease severity.

Highlights

- Quantitative serum proteomics identified coordinated alterations in proteins associated with complement and coagulation pathways in transfusion-dependent thalassemia.
- Network and pathway analyses revealed interactions between complement, coagulation, and lipid transport proteins.
- Regulatory proteins involved in oxidative stress and protease inhibition were reduced in TDT patients.
- Selected proteins were validated using ELISA, supporting the proteomic findings.
- Proteomic profiling provides system-level insights into thromboinflammatory mechanisms in TDT.

1 | Introduction

Transfusion-dependent thalassemia (TDT) is a chronic hemolytic anemia driven by ineffective erythropoiesis and sustained

transfusion exposure. Over time, the combination of ongoing hemolysis and transfusion-related iron loading produces persistent oxidative stress and a systemic inflammatory state [1, 2]. Intravascular and extravascular hemolysis release hemoglobin and heme into the circulation, where they can directly injure cell membranes and the vascular endothelium and promote redox imbalance [3, 4]. At the same time, iron accumulation from repeated transfusions and increased intestinal absorption (linked to hepcidin suppression) adds further oxidative burden and contributes to progressive multiorgan injury [2, 5].

Clinical complications in TDT extend well beyond anemia. Patients face a recognized risk of thromboembolic events and features of immune dysregulation, pointing to disruption of interconnected pathways that include inflammation and hemostasis [6, 7]. The prothrombotic tendency in thalassemia has been consistently reported, and thromboembolic complications remain a major contributor to morbidity and mortality [8, 9]. Several mechanisms likely act together, including altered red cell membrane properties with increased phosphatidylserine exposure, platelet activation, endothelial dysfunction, circulating microparticles, and reduced activity of natural anticoagulant pathways (e.g., protein C, protein S, and antithrombin III) [10–12]. Splenectomy, performed in some patients to reduce transfusion requirements, may further amplify thrombotic risk through thrombocytosis and increased procoagulant activity [13, 14].

There is also growing evidence that complement activation contributes to the pathobiology of hemolytic disorders, including thalassemia [15, 16]. Reduced expression of erythrocyte complement regulators such as CD55 and CD59 has been described in β-thalassemia, which may increase susceptibility to complement-mediated injury and downstream inflammation [17, 18]. Complement activation has been observed in TDT both pre- and post-transfusion, suggesting a sustained inflammatory stimulus rather than a purely episodic response [19]. Importantly, free heme can act as a trigger for both complement and coagulation, creating a thromboinflammatory environment [6, 20]. When heme-scavenging systems are overwhelmed, and proteins such as haptoglobin and hemopexin become depleted, free heme persists and can propagate oxidative and inflammatory damage [21].

Despite these clinical observations, the molecular links between hemolysis, inflammation, complement activation, and coagulation in TDT are not fully mapped. Serum proteomics offers a way to capture coordinated changes at the functional protein level in a systemic disease. Existing proteomic work in thalassemia has been relatively limited and often focused on specific subtypes (e.g., β-thalassemia/HbE) or used gel-based methods with restricted sensitivity [22]. One proteomic study in β-thalassemia/HbE reported 111 differentially expressed proteins across seven studies, with prothrombin among those repeatedly identified [22]. However, an integrated, quantitative assessment of complement and coagulation pathway perturbation in TDT remains limited.

Although individual components of oxidative stress, coagulation, and complement dysregulation have been described in thalassemia, integrated quantitative proteomic evaluation of these interrelated pathways in TDT remains limited. A systems-level understanding of protein alterations may provide important insights into mechanisms contributing to disease severity and complications.

In the present study, we performed quantitative serum proteomic analysis in patients with TDT and healthy controls using label-free liquid chromatography–tandem mass spectrometry (LC-MS/MS). Differentially expressed proteins were evaluated using pathway enrichment and protein–protein interaction (PPI) analyses to identify biological processes associated with disease mechanisms. Selected proteins were further assessed by ELISA validation, and their relationship with clinical indicators of disease severity was explored.

2 | Materials and Methods

2.1 | Study Design and Participants

This cross-sectional study was conducted at International Medical University (IMU), Malaysia. Participant recruitment and sample collection for the present analysis were carried out between January 02, 2021, and February 28, 2021. The study forms part of a PhD project approved under IMU Joint Committee on Research and Ethics (IMU-JC) Project ID IMU 431/2019, including an approved amendment to the project methodology to incorporate quantitative proteomic profiling strategies (approval letter dated October 21, 2020; approved project duration October 2020 to September 2021). The study was conducted in accordance with the Declaration of Helsinki. Written informed consent/assent was obtained from all participants (or their legal guardians where applicable).

2.1.1 | Quantitative Proteomics

Sixty-four participants were enrolled, comprising 32 TDT patients and 32 controls. TDT patients were aged 1–17 years old, further stratified by transfusion duration: Group 1 (< 5 years, $n = 10$), Group 2 (5–10 years, $n = 11$), and Group 3 (> 10 years, $n = 11$). The control group included 32 healthy volunteers who were not thalassemia carriers, had no evidence of concurrent infection, had no history of any malignant disorder, and were 1–17 years old. The sample size was estimated using the Fleiss formula for comparison of two proportions based on expected differences in biomarker detection between cases and controls reported in previous studies. Assuming $\alpha = 0.05$ and 80% statistical power, the largest calculated sample size was 32 participants per group, which was adopted for the present study.

2.1.2 | Inclusion Criteria for TDT Patients

The inclusion criteria are as follows: (1) confirmed diagnosis of β-thalassemia major or β-thalassemia/HbE requiring regular transfusions (≥ 8 transfusions/year), (2) on stable iron chelation regimen for ≥ 6 months, and (3) no acute illness or infection within 4 weeks of sampling.

2.1.3 | Exclusion Criteria

The exclusion criteria are as follows: (1) active infection or inflammatory condition, (2) malignancy, (3) autoimmune disease, (4) pregnancy, (5) recent surgery (< 3 months), and (6) transfusion within 7 days of blood sampling.

2.2 | Clinical and Laboratory Data Collection

Clinical variables recorded were age, sex, thalassemia genotype, transfusion history (frequency and duration), chelation regimen, and splenectomy status. Laboratory measurements included complete blood count, pretransfusion hemoglobin, serum ferritin, ALT, AST, bilirubin, and creatinine.

2.3 | Sample Collection and Processing

Blood (10 mL) was collected by venipuncture into serum separator tubes (BD Vacutainer), at least 7 days after the most recent transfusion to reduce potential interference from transfused proteins. Samples were left to clot for 30 min at room temperature and then centrifuged at 2000 × g for 15 min at 4°C. Serum was aliquoted into cryovials and stored at −80°C until analysis. Each aliquot was subjected to no more than two freeze–thaw cycles.

2.4 | Label-Free Quantitative LC-MS/MS Proteomics

2.4.1 | Sample Preparation and Protein Digestion

High-abundance proteins were depleted using the Pierce Albumin/IgG Removal Kit (Thermo Fisher Scientific, USA; Cat. No. 89875) according to the manufacturer's instructions, to remove serum albumin and immunoglobulin G *before* label-free LC–MS/MS. This step was performed to reduce the overall dynamic range and improve detection of lower-abundance proteins while maintaining relative protein abundances for comparative analysis. Protein concentration in the flow-through fractions was measured using the Bradford assay (Bio-Rad, USA), with absorbance read at 570 nm on a microplate reader.

Protein digestion was carried out using the EasyPep Mini MS Sample Prep Kit (Thermo Fisher Scientific, USA). Depleted samples were reduced and alkylated, then digested with a Trypsin/Lys-C mixture at 37°C. Digestion was stopped as per the kit protocol, and peptides were purified using the supplied clean-up columns. Purified peptides were dried and reconstituted in 0.1% formic acid before LC–MS/MS and LC–MRM–MS analysis. To minimize run-order bias, samples were analyzed in randomized order during LC–MS/MS acquisition, and all samples were processed using identical preparation protocols to reduce potential batch effects.

2.4.2 | LC-MS/MS Analysis

Dried peptide samples were reconstituted in 0.1% formic acid prior to label-free LC–MS/MS analysis using an Agilent 6550 Quadrupole Time-of-Flight (QTOF) mass spectrometer coupled to an Agilent nanoflow UHPLC with ChipCube. All samples were processed using the same depletion and preparation workflow to minimize systematic bias. Key findings were independently validated using ELISA. The commercial ELISA kits used for quantification included human fetuin-A (AHSG) (Abcam, UK), human A2M (Abcam, UK), and human CP Assay Kit (Colorimetric) (Abcam, UK). ELISA data were analyzed using a four-parameter logistic (4PL) curve-fitting model (GainData, arigobio ELISA Calculator), and sample concentrations were calculated from the standard curves. Raw LC–MS/MS data were processed using label-free quantification workflows. Protein intensity values were normalized using total ion current normalization to minimize technical variability between runs. Proteins with insufficient or inconsistent detection across samples were excluded from downstream statistical and pathway analyses. Missing values were handled according to the default settings of the proteomics analysis software.

2.5 | Bioinformatics Analysis

2.5.1 | Pathway Enrichment and Network Analysis

Functional enrichment analysis was performed using (1) KEGG pathway analysis: Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were tested for over-representation using Fisher's exact test with Benjamini–Hochberg FDR correction ($q < 0.05$ considered significant); (2) Gene Ontology (GO) analysis: biological process, molecular function, and cellular component terms were tested using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 with FDR < 0.05; and (3) PPI networks: STRING database (Version 11.5) was used to construct PPI networks with confidence interactions (score > 0.4). Networks were visualized in Cytoscape (Version 3.9.1) with the node size proportional to fold-change magnitude and color indicating the direction of change.

2.5.2 | Correlation and Clinical Association Analysis

Spearman rank correlation coefficients were calculated between protein abundances and clinical parameters (transfusion duration, serum ferritin, and pretransfusion hemoglobin). Correlations with $|r| > 0.4$ and $p < 0.05$ were considered significant. Multiple linear regression models were constructed to identify independent predictors of protein levels, adjusting for age, sex, and splenectomy status.

2.6 | Statistical Software and Reproducibility

Data are presented as mean ± standard deviation for normally distributed variables or median [interquartile range] for non-normally distributed variables. Serum ferritin values were approximately normally distributed in this cohort and are therefore reported as mean ± standard deviation. Statistical significance was defined as $p < 0.05$. Because this study was designed as an exploratory proteomic discovery analysis, a false discovery rate (FDR) threshold of ≤ 0.10 was applied to balance the risk of false positives with the possibility of excluding potentially relevant candidate proteins during early-stage biomarker discovery. Statistical analyses, including logistic regression modeling and receiver operating characteristic (ROC) curve analysis, were performed using the Statistical Package for Social Sciences (SPSS) version 18. Model performance was evaluated using the area under the curve (AUC), sensitivity, specificity, positive predictive value, and negative predictive value.

3 | Results

3.1 | Participant Characteristics

Baseline demographic and clinical characteristics of the 64 participants are summarized in Table 1. TDT patients ($n = 32$) had a mean age of 9.5 ± 4.6 with 44% female. The mean serum ferritin was 2376 ng/mL, and the mean pretransfusion hemoglobin was 10.2 g/dL. Only one patient had undergone

TABLE 1 | Baseline demographic and clinical characteristics of transfusion-dependent thalassemia (TDT) patients and healthy controls.

Characteristic	TDT patients ($n = 32$)	Controls ($n = 32$)
Age (years), mean ± SD	9.5 ± 4.6	7.2 ± 3.7
Female sex	14 (44%)	10 (31%)
Pretransfusion Hb (g/dL)	10.2 ± 1.1	13.8 ± 1.2
Serum ferritin (ng/mL)	2376 ± 840	85 ± 13
Platelets ($\times 10^3/\mu L$)	373.4 ± 25	328.2 ± 14
WBC ($\times 10^3/\mu L$)	8.2 ± 1.5	7.1 ± 1.1

Note: Data are presented as mean ± standard deviation or number (percentage), as appropriate. Group comparisons are described in the Results section.

splenectomy. Iron chelation regimens included oral monotherapy ($n = 30$, 94%) and combination oral and subcutaneous therapy ($n = 2$, 6%). Control participants ($n = 32$) had a mean age of 7.2 ± 3.7 years with 31% female.

3.2 | Comprehensive Serum Proteome Profiling

3.2.1 | Differentially Expressed Proteins

Comparative analysis identified 51 proteins with differential abundance between TDT patients and controls based on fold-change criteria (> 1.5 or < 0.67). After statistical testing with FDR correction, 13 proteins remained statistically significant ($p < 0.05$, FDR < 0.10) (Table 2) and were used for downstream pathway and network analyses. Selected proteins were validated by ELISA (Table 3). Among significantly altered proteins, five were upregulated and eight were downregulated in TDT patients. GDF-15 was not detected by LC-MS/MS, likely due to low abundance, and was therefore evaluated separately by ELISA.

3.2.2 | Pathway Enrichment Analysis

3.2.2.1 | PPI Network. STRING network analysis of the 13 significantly differentially expressed proteins revealed a highly interconnected network with 28 interactions (PPI enrichment $p = 1.2 \times 10^{-8}$), indicating coordinated dysregulation rather than independent alterations (Figure 1). Hub proteins with the highest connectivity included complement C3 (eight interactions): central node connecting complement, coagulation, and acute phase proteins; A2M (seven interactions): broad-spectrum protease inhibitor linking coagulation and inflammation; and prothrombin (six interactions): key coagulation factor connecting to fibrinogen, plasminogen, and serpins.

3.2.2.2 | KEGG Pathway Analysis. Proteins identified from the broader differentially expressed protein dataset, including interaction partners identified in STRING analysis, were further examined using Cytoscape (Version 3.9.1) to explore pathway-level relationships. Network topology analysis identified three major functional modules: [1] the coagulation cascade (F2, FGG, PLG, SERPINC1, and KNG1); [2] the complement system (C3, CFH, and C4B); and [3] lipid metabolism and transport (APOA1, APOA4, and APOE). Extensive cross talk between these modules, mediated primarily by A2M and C3, suggests integrated regulation of inflammatory and hemostatic pathways in TDT pathophysiology.

GO analysis of the 13 differentially expressed proteins was performed using the PANTHER classification system (Version 17.0). The differentially expressed proteins were mapped primarily to molecular function and biological process categories.

3.3 | ELISA Validation and Growth Differentiation Factor (GDF-15) Analysis

3.3.1 | Validation of LC-MS/MS Findings

ELISA validation of four key proteins in all 64 participants confirmed LC-MS/MS findings with strong correlations: **A2M:** $r = 0.82$ (95% CI: 0.72–0.89, $p < 0.001$), mean decrease 2.3-fold in TDT, **ceruloplasmin:** $r = 0.79$ (95% CI: 0.68–0.87, $p < 0.001$), mean decrease 1.9-fold in TDT, **AHSG/fetuin-A:** $r = 0.76$ (95% CI: 0.64–0.85, $p < 0.001$), mean decrease 1.6-fold in TDT, and **GDF-15 (**not detected by LC-MS/MS) showed consistent trends.

3.3.2 | GDF-15 as a Disease Severity Biomarker

GDF-15, a stress-responsive cytokine associated with ineffective erythropoiesis, was measured by ELISA in all participants. TDT patients showed marked elevation of GDF-15 (mean 473.8 (±42.3 pg/mL) compared to controls (mean 163.5 pg/mL), representing a 2.9-fold increase ($p < 0.001$).

GDF-15 levels correlated strongly with multiple disease severity parameters. Significant associations were observed with transfusion duration $r = 0.68$ (95% CI: 0.48–0.81, $p < 0.001$), serum ferritin $r = 0.52$ (95% CI: 0.28–0.70, $p = 0.002$), and pre-transfusion hemoglobin $r = -0.59$ (95% CI: −0.75 to −0.37, $p < 0.001$). Patients stratified by transfusion duration showed progressive GDF-15 elevation: < 5 years, mean 429 pg/mL; 5–10 years, mean 460 pg/mL; and > 10 years, mean 541 pg/mL (p for trend < 0.001).

3.4 | Multimarker Diagnostic Panel

To maximize diagnostic accuracy, multimarker panels were constructed using logistic regression with leave-one-out cross-validation. The optimal three-protein panel combined A2M, GDF-15, and AHSG, achieving AUC = 0.98 (95% CI: 0.95–1.00), sensitivity 94% (95% CI: 79%–99%), specificity 97% (95% CI: 84%–100%), PPV 97% (95% CI: 83%–100%), and NPV 94% (95% CI: 80%–99%). This panel performed better than any single marker and remained consistent across transfusion duration subgroups. However, given the modest sample size and the use of internal validation, these results should be interpreted cautiously and confirmed in an independent external cohort.

The logistic regression model was

TABLE 2 | Significantly differentially expressed serum proteins identified by label-free LC–MS/MS analysis in transfusion-dependent thalassemia (TDT) patients compared with controls ($p < 0.05$, FDR < 0.10).

	Accession #	Frequency (%)		Description of proteins	Gene name	*p* value	Expression	Fold change
		Cases	Controls					
1	P00450	100	100	Ceruloplasmin	CP	0.02	Downregulated	0.4
2	P00734	90	30	Prothrombin	F2	0.01	Upregulated	2.2
3	P01019	100	90	Angiotensinogen	AGT	0.01	Downregulated	0.5
4	P01023	100	100	Alpha-2-macroglobulin	A2M	≤ 0.001	Downregulated	0.4
5	P01024	95	80	Complement C3	C3	0.02	Upregulated	1.5
6	P02647	100	90	Apolipoprotein A-I	APOA1	0.01	Downregulated	0.3
7	P02763	100	100	Alpha-1-acid glycoprotein 1	ORM1	0.02	Downregulated	0.5
8	P02765	100	100	Alpha-2-HS-glycoprotein	AHSG	0.03	Downregulated	0.4
9	P02774	100	90	Vitamin D-binding protein	GC	0.02	Downregulated	0.5
10	P02790	25	90	Hemopexin	HPX	≤ 0.001	Downregulated	0.3
11	P04217	100	100	Alpha-1B-glycoprotein	A1BG	0.03	Downregulated	0.6
12	P06727	100	90	Apolipoprotein A-IV	APOA4	0.02	Downregulated	0.4
13	P19652	100	100	Alpha-1-acid glycoprotein 2	ORM2	0.01	Downregulated	0.5

Note: Fold change represents the ratio of protein abundance in TDT relative to controls (TDT/control). Values < 1 indicate downregulation, and values > 1 indicate upregulation. APOA, apolipoprotein; CP, ceruloplasmin; GC, vitamin D-binding protein; HPX, hemopexin; ORM, orosomucoid; AHSG, alpha-2-HS-glycoprotein.
Abbreviations: A1BG, alpha-1B-glycoprotein; A2M, alpha-2-macroglobulin.

$$\text{Logit}(P) = 8.42 - 1.85 \times \log_2(\text{A2M}) + 2.14 \times \log_2(\text{GDF} - 15) - 1.23 \times \log_2(\text{AHSG}), \quad (1)$$

where P is the probability of TDT diagnosis. The optimal probability threshold was 0.5. The model showed excellent discriminative performance, consistent with the ROC analysis described above.

4 | Discussion

This study shows that complement and coagulation pathways are altered in TDT, alongside reduced levels of antioxidant and heme-scavenging proteins. These findings support the clinically recognized hypercoagulable state in TDT and help clarify the mechanistic links between chronic hemolysis, oxidative stress, and thromboinflammatory complications.

The observed increase in complement component C3 suggests altered complement-related activity in TDT. This is in line with prior work showing complement activation in transfusion-dependent β-thalassemia both before and after transfusion [19]. Reduced expression of complement regulatory proteins CD55 and CD59 on erythrocytes in β-thalassemia, particularly following splenectomy, may contribute to inadequate regulation of complement activity and complement-mediated hemolysis [17, 18, 23]. Complement activation in hemolytic disorders is also increasingly viewed as a contributor to thrombosis and vascular disease, with complement acting as a key bridge between hemolysis and coagulation activation [15, 16]. However, increased total C3 levels alone do not establish complement activation, which would require measurement of activation fragments such as C3a, C5a, or terminal complement complexes. The present findings therefore support altered complement protein abundance rather than direct evidence of functional complement activation.

Similarly, the elevation of prothrombin suggests perturbation of coagulation-related pathways and supports the recognized hypercoagulable phenotype in these patients [1, 7]. Prothrombin has been reported as a differentially expressed protein across multiple independent proteomic studies in β-thalassemia, reinforcing its relevance to disease pathophysiology [22]. Clinical studies in TDT have described substantial coagulation abnormalities, including elevated D-dimer, reduced natural

TABLE 3 | ELISA validation of selected proteins in transfusion-dependent thalassemia (TDT) patients and controls.

Serum proteins	Mean conc. in cases (SEM)	Mean conc. in controls (SEM)	Fold change	Direction	*p* value
A2M	13.3(±1.5)	38.8 (±3.3)	0.34	Decreased	*≤ 0.001*
AHSG	62.7 (±3.4)	105.8 (±12.4)	0.59	Decreased	*≤ 0.001*
CP	14.27 (±2.9)	23.0 (±4.0)	0.62	Decreased	*≤ 0.001*
GDF-15	473.8 (±42.3)	163.5 (±36.6)	2.90	Increased	*≤ 0.001*

Note: Data are presented as mean concentration ± standard error of the mean (SEM). Fold change represents the ratio of mean concentration in TDT relative to controls. AHSG, alpha-2-HS-glycoprotein; CP, ceruloplasmin. The italic formatting is standard statistical notation for the variable *p* (*p* value).
Abbreviations: A2M, alpha-2-macroglobulin; GDF-15, growth differentiation factor-15; SEM, standard error of the mean.

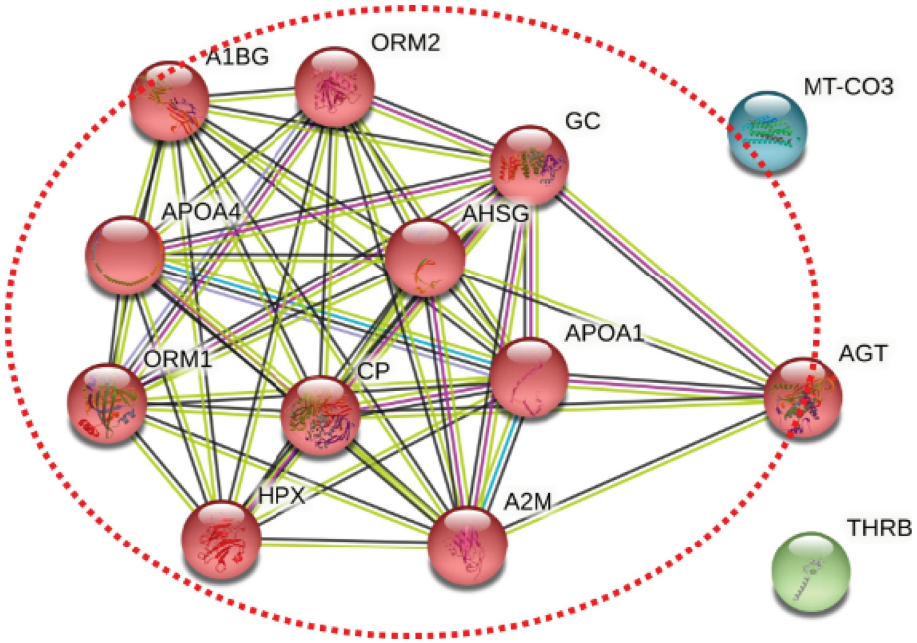

FIGURE 1 | Protein–protein interaction (PPI) network of significantly differentially expressed proteins in transfusion-dependent thalassemia (TDT) compared with controls, generated using the STRING database (Version 11.5). The network demonstrates a high degree of interconnectivity (PPI enrichment $p = 1.2 \times 10^{-8}$), indicating coordinated dysregulation rather than independent protein alterations. The node size reflects the magnitude of the fold change, while the node color indicates the direction of expression change (red, upregulated; green, downregulated). Edges represent known or predicted protein–protein interactions, with line thickness corresponding to interaction confidence (STRING score > 0.4). Central hub proteins include complement C3, alpha-2-macroglobulin (A2M), and prothrombin (F2), linking complement, coagulation, and inflammatory pathways.

anticoagulant proteins (protein C, protein S, and antithrombin III), and increased markers of platelet activation [10, 11, 13]. Notably, the concurrent reduction in regulatory proteins such as A2M in the current study suggests weaker control of protease activity, favoring sustained rather than short-lived cascade activation. Overall, these findings indicate that complement and coagulation pathways are affected together, rather than independently, which is consistent with thrombo-inflammatory processes seen in hemolytic disorders [6]. Functional coagulation markers such as thrombin generation, D-dimer levels, or clotting time assays were not assessed in this study. Consequently, these findings should be interpreted as reflecting perturbation of coagulation-associated pathways rather than direct confirmation of coagulation pathway activation.

Chronic hemolysis is a key feature of TDT and likely links oxidative stress with changes in complement and coagulation pathways. Cell-free heme released during hemolysis is strongly pro-oxidant and can activate both complement and coagulation pathways [6, 20]. Network-based analyses of heme-triggered effects have highlighted complex interactions between heme and the coagulation system, with heme acting as a versatile modulator that promotes prothrombotic states through binding to plasma proteins and interactions with cellular components [20]. In this context, the marked reduction in hemopexin, together with decreased ceruloplasmin and A2M, suggests reduced capacity to buffer heme-driven oxidative and inflammatory signaling.

Experimental studies further support the importance of heme-scavenging pathways, showing that treatment with haptoglobin or hemopexin can reduce adverse effects associated with cell-free hemoglobin and heme and limit inflammation and organ injury in hemolysis models [21]. Reduced heme-scavenging capacity in TDT may therefore amplify complement–coagulation cross talk and contribute to a persistent thromboinflammatory state.

Oxidative stress in TDT likely contributes through several additional mechanisms beyond direct cellular injury. Chronic hemolysis can reduce nitric oxide bioavailability, promote endothelial dysfunction, and increase expression of adhesion molecules, all of which support a hypercoagulable phenotype [3, 12]. Iron overload from repeated transfusions and increased gastrointestinal absorption further increases oxidative stress via Fenton chemistry, generating reactive oxygen species that damage cellular membranes and proteins [2].

Previous proteomic studies in thalassemia have described changes in proteins related to oxidative stress, lipid metabolism, and inflammation, most commonly in β-thalassemia/HbE or in smaller cohorts [22]. A synthesis of seven proteomic studies in β-thalassemia/HbE identified 111 unique differentially expressed proteins, and ontology analysis highlighted "response to inorganic substances" as the most significant functional annotation cluster, consistent with iron overload as a major pathological consequence [22]. Prothrombin was identified in two independent studies, and carbonic anhydrase 1 and peroxiredoxin-2 were consistently upregulated across three studies, suggesting reproducible proteomic signatures in thalassemia [22].

This study builds on previous findings in thalassemia and provides a broader view by examining how complement and coagulation pathways interact in the context of disease-related stress. While our previous work examined selected individual proteins, the present study provides a systems-level analysis integrating complement, coagulation, and network-based

interactions [5]. Compared with earlier gel-based work, the current approach better captures pathway-level coordination and provides a broader view of systemic involvement. Our finding of dysregulated CP, A2M, and AHSG in TDT using quantitative serum proteomics, supported by ELISA validation, is also consistent with thalassemia plasma/EV proteomics showing reproducible disturbances in circulating proteins and pathways linked to iron overload, inflammation, and oxidative stress [24, 25].

The increase in GDF-15 observed here is consistent with its established role as a marker of ineffective erythropoiesis in thalassemia. GDF-15 is produced by erythroid precursors and suppresses hepcidin, promoting increased iron absorption despite iron overload [2]. Studies in newly diagnosed β-thalassemia major have reported markedly elevated GDF-15 with correlations to iron status measures and positive associations with serum ferritin, transferrin saturation, and hepcidin [26, 27]. GDF-15 has also been linked to cardiovascular complications in TDT, including atherosclerosis, supporting its potential as both a severity marker and a contributor to long-term outcomes [28]. In the present study, the association of GDF-15 with transfusion burden and iron overload further supports its value as an indicator of disease severity and ineffective erythropoiesis.

These proteomic findings may also have clinical relevance for risk stratification and monitoring of thromboinflammatory complications in TDT. Hypercoagulability is now recognized as a core feature of thalassemia, with thromboembolic events contributing substantially to morbidity across thalassemia phenotypes [6, 9]. Recent consensus guidance emphasizes the importance of ongoing assessment of both thrombotic and bleeding risk in TDT and the use of preventive strategies where appropriate [8].

Splenectomy remains a major risk factor for thrombosis, with meta-analyses reporting thrombosis occurring 7.59 times more frequently in splenectomized compared with non-splenectomized thalassemia patients [14]. The proteomic changes observed in this study, particularly reduced regulatory proteins alongside elevated procoagulant factors, may help identify patients at greatest risk of thromboembolic complications. While these findings are exploratory, they support further evaluation of selected proteins as candidate biomarkers for thrombotic risk stratification.

The impact of emerging therapies on thrombotic risk also warrants careful study. Recent evidence suggests that agents such as luspatercept may influence hypercoagulability, although the mechanisms and clinical implications are not yet fully defined [6]. Defining proteomic signatures associated with different treatment exposures may support personalized care and help guide strategies aimed at reducing thromboinflammatory complications.

Finally, longitudinal studies and independent external cohorts will be needed to define the prognostic value of the proteomic alterations identified here and to clarify the influence of key clinical variables, including transfusion burden, iron overload, chelation therapy, and splenectomy status. Integrating proteomics with clinical outcomes, imaging, and complementary biomarkers may offer a more complete framework for understanding disease progression and treatment response in TDT.

5 | Limitations

This study has several limitations. First, the cross-sectional design limits causal interpretation, and some proteins showed relatively small fold changes despite reaching statistical significance. Second, depletion of high-abundance proteins can affect quantitative estimates; however, the direction and relative differences were supported by independent ELISA validation. Third, variability may have been introduced by heterogeneity within the control group and by the lack of stratification according to the chelation regimen. Although the sample size was sufficient for proteomic discovery, it limited more granular subgroup analyses based on clinical factors such as splenectomy status, transfusion frequency, and severity of iron overload. In addition, we did not include functional assays of complement or coagulation activation, which would have strengthened mechanistic interpretation. These considerations are important when interpreting the results. Nevertheless, the overall agreement between the proteomic findings, pathway analyses, and ELISA validation supports the biological relevance of the observed alterations.

6 | Conclusions

These findings are consistent with a thromboinflammatory and oxidative stress-associated biological milieu in TDT and highlight coordinated alterations in complement- and coagulation-related pathways. The increase in complement C3 and prothrombin, together with reduced hemopexin and A2M, is consistent with a persistent thromboinflammatory and oxidative stress state in TDT.

GDF-15 also emerged as a strong marker of disease severity, with clear associations with transfusion burden and iron overload. Although these results provide useful biological insights and highlight candidate biomarkers, longitudinal and multicenter studies will be needed to confirm prognostic value and clarify therapeutic relevance.

Author Contributions

Afshan Sumera: conceptualization, formal analysis, validation, investigation, data curation, project administration, and writing–original draft.

Ammu K. Radhakrishnan: methodology, supervision, project administration, and writing–review and editing.

Abdul Aziz Baba: resources, supervision, writing–review and editing, and funding acquisition.

Soon Keng Cheong: resources, supervision, and writing–review and editing.

Afshan Sumera had full access to all of the data in this study and takes complete responsibility for the integrity of the data and the accuracy of the data analysis.

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Disclosure

All authors have read and approved the final version of the manuscript.

Ethics Statement

This study was conducted in accordance with the Declaration of Helsinki and the Malaysian Good Clinical Practice Guideline. Written informed consent was obtained from all participants or their legal guardians, with assent obtained from minors where appropriate.

Ethical approval was granted by the International Medical University Joint Committee on Research and Ethics (IMU-JC Project ID IMU 431/2019, including the approved amendment dated October 21, 2020) and the Medical Research & Ethics Committee Malaysia (MREC KKM/NIHSEC/P20-2360(12), dated December 09, 2020). The IMU-JC approval represents institutional ethical clearance, while the MREC approval represents national-level regulatory approval from the Ministry of Health Malaysia.

The study forms part of a larger approved PhD project under the same ethics protocol. All recruitment and sample collection for the present analysis occurred after ethics approval (January 02, 2021–February 28, 2021).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request. The data are not publicly available due to privacy or ethical restrictions.

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