High Prevalence of Antiphospholipid Antibodies in Children with Non-Transfusion Dependent Thalassemia and Possible Correlations with Microparticles

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To the editor.

Thromboembolism (TE) is one of the complications of thalassemia disease. The incidences have been reported about 0.9-4.0% in TDT and 3.9-29.0% in NTDT.1-3 TE’s etiologies in thalassemia are an abnormal expression of PS, platelet and endothelial activations, decreased nitric oxide, and splenectomy.4 In addition, expression of PS, platelet and endothelial activations, diseases and play a role in TE development.5-7 The endothelium, and leucocytes increase in thalassemia undergoing a structural change that could induce antibody formation.8 The prevalence of APAs in thalassemia has been reported mostly in β thalassemia patients with the incidence rates of 42.7% of aCL-IgG,9 16.0% of lupus anticoagulant (LA), 30.0% of aCL-IgM, and 6.0% of aCL-IgG.10 Currently, there are limited studies on APAs in NTDT. In addition, the etiology of APAs in thalassemia is still unknown. We report the positive rates of APAs in TDT and NTDT children and demonstrate the association of APAs with RBC, platelet, endothelial, and leucocyte MPs.

Patients with thalassemia disease and healthy controls who had normal hemoglobin (Hb) and Hb typing were enrolled. After obtaining written consent from parents and patients, blood was drawn for APAs – including LA (Dade Behring Siemens Healthcare GmbH, Germany), aCL and aβ2GPI antibodies (both IgG and IgM) (EUROIMMUN Medizinische Labordiagnostika AG, Germany), and MPs of RBC, platelet, leukocyte, and endothelium by flow cytometry.6 The cut-off levels were defined as >99th percentile for aCL-IgM, aCL-IgG, aβ2GPI-IgM, and aβ2GPI-IgG. Positive APA was determined by the subjects having at least one positive test result of one of the APA types according to the Sydney criteria.11

A total of 161 subjects were divided into three groups: 55 subjects with TDT, 44 subjects with NTDT, and 62 controls. TDT subjects had received regular RBC transfusions (every 3-4 weeks) to maintain a pre-transfusion mean±SD of Hb at 9.0±1.2 g/dL. After receiving RBC transfusion, their mean Hb level was 12.3 g/dL. They required mean±SD RBC transfusions of around 145.0±49.3 ml/kg/year. NTDT group who required only occasional RBC transfusion of around 4.0±11.7 ml/kg/year. As a result of regular RBC transfusion, the Hb levels in TDT and NTDT subjects in the study were similar, with higher mean corpuscular volume present in TDT than in NTDT subjects (Table 1).

The positive APA rate in all thalassemia patients as a group (23.0%) was higher than in controls (17.9%). The positive APA rate was highest in NTDT subjects (29.5%), and similar levels were shown between TDT (18.2%) and control groups (17.9%), although no significant differences were demonstrated. The LA test was positive in 14.5% of TDT subjects, 20.5% of NTDT subjects, and 12.8% of controls. When using the level cut-off of the 99th percentile in controls to determine the positivity of aCL (IgM = 9.99 U/mL; IgG = 8.46 U/mL) and aβ2-GPI (IgM = 23.45 U/mL, IgG = 3.44 U/mL) respectively, the aCL-IgM test was positive in 1.8% of TDT subjects and 1.6% of controls. The aCL-IgG test was positive in 4.5% of NTDT subjects and 3.2% of controls. The aβ2GPI-IgM and aβ2GPI-IgG were positive in 1.8% and 5.4% respectively in TDT subjects, 4.5%, and 11.4% in NTDT subjects, and 1.6% for both IgM and IgG in controls. The prolonged activated partial thromboplastin time (APTT) and prothrombin time (PT) values in thalassemia subjects, when compared to the values in controls, can be attributed to the patients with positive APAs present in the thalassemia groups, as the APTT and PT values were higher in thalassemia patients who had positive APA when compared to negative APA. It is noted that a significant difference was demonstrated only in PT values (34.1±4.2 vs. 30.9±3.7
Table 1. Demographic data and laboratory parameters of transfusion-dependent thalassemia (TDT) subjects, non-transfusion-dependent thalassemia (NTDT) subjects, and controls.

| Parameter                | TDT (N=55) | NTDT (N=44) | Controls (N=62) | P-value | P-value TDT-NTDT | P-value TDT-control | P-value NTDT-control |
|--------------------------|------------|-------------|----------------|---------|------------------|---------------------|---------------------|
| Age (yr)*                | 11.6 ± 4.5 | 13.4 ± 4.9  | 13.5 ± 3.7     | 0.033   | 0.568            | 0.003               | 0.002               |
| Female:male (%)          | 38:62      | 37:63       | 39:61          | 0.969   | 0.853            | 0.0953              | 0.806               |
| Diagnosis                |            |             |                |         |                  |                     |                     |
| - HbE/β                  | 49 (89.1%) | 4 (9.1%)    |                |         |                  |                     |                     |
| - β major                | 3 (5.5%)   | -           |                |         |                  |                     |                     |
| - HbH/CS                 | 2 (3.6%)   | 12 (27.3%)  |                |         |                  |                     |                     |
| - AE Barts'/CS           | 1 (1.8%)   | 1 (2.3%)    |                |         |                  |                     |                     |
| - AE Barts*              | -          | 1 (2.3%)    |                |         |                  |                     |                     |
| - HbH                    | -          | 26 (59.1%)  |                |         |                  |                     |                     |
| RBC transfusion rate (ml/kg/yr)* | 145 ± 49.3 | 4 ± 11.7    |                | <0.001  | -                | -                   | -                   |
| Hb (g/dL)                | 9.0 ± 1.2  | 9.2 ± 1.2   | 13.6 ± 1.6     | <0.001  | 0.478            | 0.101               | 0.046               |
| MCV (μL)                 | 71.1 ± 6.6 | 60.9 ± 7.9  | 81.3 ± 8.0     | <0.001  | 0.051            | 0.487               | 0.304               |
| MCH (pg)                 | 22.7 ± 2.7 | 17.3 ± 2.3  | 26.5 ± 3.1     | <0.001  | 0.356            | 0.460               | 0.132               |
| MCHC (g/dL)*             | 31.9 ± 1.6 | 28.7 ± 2.0  | 32.6 ± 1.1     | <0.001  | 0.943            | 0.002               | 0.043               |
| Platelet (x 10^10/mL)*   | 352.6 ± 167.5 | 338.9 ± 96.1 | 317.9 ± 80.5   | 0.296   | 0.068            | 0.003               | 0.153               |
| CRP (mg/L)*              | 1 (1-24.5) | 1 (1-8.7)   | 1 (1-10.3)†    | 0.637   | 0.320            | 0.841               | 0.470               |
| Ferritin (ng/mL)*        | 1,429.2    | 103.9       |                | <0.001  | -                | -                   | -                   |

*Mean ± SD, †median (range), ‡controls n=39, APTT, activated partial thromboplastin time; CRP, C - reactive protein; CS, Constant Spring; Hb, haemoglobin; INR, international normalized ratio; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; PT, prothrombin time; RBC, red blood cell; TT, thrombin time. Genetic of thalassemia: α5, HBA2: c.427T>C; β5, HBB:c.79G>C; AE Barts'/CS; --/α5α/β5β, HbH/CS; --/α5α

sec, P=0.57, 14.2±1.2 vs. 12.8±0.7 sec, P=0.003 respectively.

Percentages of RBC, platelet, endothelial, and leucocyte MPs in the TDT and NTDT groups were significantly higher than those in the controls (Table 2).

Table 2. Percent red blood cell; RBC (Glycophorin A; GPA), platelet (CD41), endothelial (CD31, 144) and white blood cell (CD11b, CD45) microparticles (MPs) in transfusion-dependent thalassemia (TDT) subjects, non-transfusion-dependent thalassemia (NTDT) subjects, and controls.

| Percent of MPs (μL) | TDT (n=55) | NTDT (n=44) | Control (n=39) | p-value ANOVA | p-value (post hoc LSD) |
|---------------------|------------|-------------|----------------|---------------|------------------------|
|                     | Mean ± SE  | Mean adjusted ± SE | Mean ± SE  | Mean adjusted ± SE | Mean ± SE  | Mean adjusted ± SE | TDT vs NTDT | TDT vs Control | NTDT vs Control |
| GPA                 | 42.8 ± 3.1 | 43.5 ± 2.98 | 37.1 ± 2.7    | 36.9 ± 3.89   | 1.7 ± 0.5  | 1.0 ± 4.68         | <0.001*     | <0.001*       | <0.001*        |
| CD41                | 28.0 ± 1.9 | 28.2 ± 1.64 | 21.0 ± 2.0    | 21.1 ± 1.86   | 1.0 ± 0.2  | 0.6 ± 1.26         | <0.001*     | 0.004*        | <0.001*        |
| CD31                | 26.0 ± 2.0 | 25.3 ± 2.24 | 20.3 ± 2.4    | 21.2 ± 2.9    | 1.0 ± 0.2  | 0.9 ± 3.46         | <0.001*     | 0.267         | <0.001*        |
| CD144               | 19.9 ± 1.9 | 18.6 ± 1.96 | 18.3 ± 2.0    | 20.3 ± 2.5    | 0.9 ± 0.3  | 0.5 ± 3.06         | <0.001*     | 0.597         | <0.001*        |
| CD11b               | 1.1 ± 0.2  | 1.0 ± 0.28  | 1.2 ± 0.2     | 1.2 ± 0.28    | 0.3 ± 0.1  | 0.3 ± 0.29         | 0.006*      | 0.442         | 0.002*         |
| CD45                | 6.1 ± 0.8  | 6.2 ± 0.88  | 6.1 ± 1.1     | 6.2 ± 0.99    | 0.8 ± 0.1  | 0.6 ± 0.99         | <0.001      | 0.001         | <0.001         |

*After adjusted by RBC, Hb and age, †adjusted by WBC, age and gender, ‡adjusted by platelet, age and gender.
and aCL-IgM of 6.0%\textsuperscript{9,10,12} Our study demonstrated that subjects with positive LA in the TDT group consisted of 14.5% and aCL-IgM of 1.8%. The differences between the positivity rates in APAs may be due to several factors, including the diversity of the population among the studies, the disease severity, treatment plan (e.g., regular RBC transfusions), antibody detection methods, and differences in cut-off value for positivity. The other types of APAs –αβ2GPI-IgM and IgG – were also included in the present study but did not feature any previous studies\textsuperscript{9,10,12} The rates of positive αβ2GPI-IgM and IgG in the TDT group were 1.8% and 5.4%, respectively, which were higher than the aCL-IgM and IgG positivity rates. In the NTDT group, the rates of all positive APAs (29.5%) were higher than those in the TDT group (18.1%), although there was no statistically significant difference demonstrated. Higher APA positivity rates in NTDT subjects were also found for individual antibodies, including LA, aCL-IgG, αβ2GPI-IgM, and IgG antibodies. To our knowledge, there have been very few studies that have reported on positive APAs in NTDT subjects, particularly in children.

MPs were higher in the TDT and NTDT groups when compared to those levels in controls. All MPs levels (except for platelet MPs) between TDT and NTDT groups were not significantly different. The similar MPs levels in TDT subjects, despite more severe symptoms, may be related to the regular RBC transfusions received by TDT subjects, and that can reduce the amount of abnormal PS surfaces exposed. This hypothesis is supported by a study by Atichartakarn et al.\textsuperscript{13} The study enrolled severe splenectomized thalassemia with pulmonary hypertension subjects. After receiving RBC transfusions, the amount of PS surfaces exposing RBC was reduced in those subjects. The report suggested that the reduction of erythropoiesis and PS exposing cells' dilution was due to RBC transfusion.\textsuperscript{13} In addition, platelet activation was reduced after regular RBC transfusion.

Our study also demonstrated statistically significant correlations between αβ2GPI-IgG and leucocyte (CD11b) MPs and aCL-IgM level and endothelial (CD31) MPs, although the correlations were not strong. These findings point to the likelihood that APAs in thalassemia subjects may be related to PS's expression. The sites of PS expressed surfaces are where glycoproteins, such as β2GPI, bind to anionic PS surfaces. After binding to PS surfaces, β2GPI changes the conformation of the molecule and induces antibody formation.\textsuperscript{8} Even stronger correlations may be demonstrated in older subject age groups because the antibody formation may require time after exposure to the PS expressed apoptotic cells.\textsuperscript{14} In this study, the lower positive APA rate in the TDT group compared to the NTDT group may be related to the regular RBC transfusion, which may reduce the exposure to PS expressed MPs, especially early after transfusion. In addition to regular RBC transfusions, deferasirox has been reported to improve immunological response, possibly from the iron chelator's direct action and the reduction of free iron radicles.\textsuperscript{15} All TDT patients in the present study received iron chelation, which was started when serum ferritin levels reached more than 1,000 ng/mL. Deferiprone was the most prescribed medication in the present study, accounting for 76.4% of TDT subjects. The strengths of the present study were that it demonstrated a high prevalence rate of APAs, especially in thalassemia patients who received an occasional transfusion, and to our knowledge, the correlations of APAs to MPs was first demonstrated.

In summary, high APA positive rates, associated with high MPs, were demonstrated in a pediatric population with thalassemia disease, especially NTDT. This suggests that MPs may play a role in APA development. Further larger cohort and basic research studies are required to confirm these results, better understand the occurrence of APAs in this population, and demonstrate the risk of TE-linked APA presence in thalassemia subjects.

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