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Risk Factors for Thrombosis in Children with Cyanotic Congenital Heart Disease

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Abstract
Mechanisms of hypercoagulability in cyanotic congenital heart disease (CCHD) are not fully elucidated, but endothelial dysfunction, hemostatic abnormalities, hyperviscosity, and iron deficiency are proposed underlying factors. We aimed to evaluate the risk factors for thrombosis in children with CCHD. Forty children with CCHD and 47 matched healthy controls were enrolled. Patients were divided into: Group I; 14 (35%) with history of thrombosis and Group II; 26 (65%) without history of thrombosis. Laboratory assays included complete blood count, serum iron profile and serum thrombomodulin level. CCHD patients had higher hemoglobin and hematocrit, lower mean corpuscular volume, mean corpuscular hemoglobin, platelets, iron and thrombomodulin than the control group. Group I showed higher hematocrit and lower platelets than Group II. No significant difference in iron profile and thrombomodulin was found between the patient groups. Thrombomodulin was positively correlated to oxygen saturation in all, Group I and Group II patients. CCHD is associated with a hypercoagulable state and a multifactorial risk for thrombosis. Decreased thrombomodulin is a reliable marker for endothelial dysfunction, however not a good marker for predicting thrombosis in children with CCHD. Infections and dehydration, increased hematocrit, and platelet activation are speculated to substantially participate in the prothrombotic state encountered in CCHD.
Keywords: *cyanotic congenital heart disease; risk factors; thrombosis; thrombomodulin.*

Introduction

Cyanotic congenital heart disease (CCHD) is associated with an increased risk for thromboembolism, the precise mechanisms of which are not yet fully elucidated, but endothelial dysfunction, hemostatic abnormalities, hyperviscosity, and iron deficiency are proposed underlying factors [1].

Chronic hypoxemia in CCHD leads to maladaptive changes in blood vessel function and structure that probably contribute to impaired cardiopulmonary performance and increase the incidence of thromboembolic events [2]. Thrombomodulin (TM), a vascular endothelial cell surface receptor, acts as a critical cofactor for thrombin-mediated activation of protein C (PC) and reflects

the anticoagulant activity of endothelium [1]. It is suggested that deranged endothelial function could be an important factor in down-regulated synthesis, loss and/or internalization of TM resulting in thrombin-thrombin receptor interaction on the surface of endothelial cells, activation of various procoagulant pathways, and hence the pathogenesis of thrombosis [2]. When vascular endothelial cell injury occurs, the extracellular domain of TM is released from the endothelial cell surface by proteolytic cleavage, giving rise to multiple fragments. Quantification of soluble TM in the circulation therefore provides insight into the extent of vascular endothelial cell damage [3].

Patients with CCHD develop secondary polycythemia; a physiologic adaptive response to improve oxygen transport to peripheral tissues. However, highly increased hematocrit (HCT) impairs oxygen delivery and negates the beneficial effects of erythrocytosis, leading to symptoms of hyperviscosity and abnormalities of hemostasis [4].

Microcytic erythrocytes are produced secondary to iron deficiency in CCHD and are supposed to induce higher viscosity due to more rigidity and less deformability than normocytic erythrocytes, which may increase the risk for thromboembolic events [5].

Accordingly, the purpose of this study was to evaluate the proposed risk factors for thrombosis in Egyptian children with CCHD, as well as to assess the potential role for TM as a novel risk factor.

Materials and Methods

This study was conducted on 40 children with CCHD recruited from the Pediatric Cardiology outpatient clinic, Children's Hospital, Ain Shams University. They were 23 males and 17 females (male to female ratio, 1.4:1), their ages ranged from 2 years to 15 years (median, 3.4 years). A palliative shunt procedure was performed to all patients during the first 6 to 8 weeks of life. Forty-seven age- and sex- matched healthy children served as a control group. They were 27 males and 20 females (male to female ratio, 1.4:1), their ages ranged from 1 year to 14 years (median, 3.1 years). An informed consent was obtained from parents of the patients and controls prior to enrollment. The study was approved by the Research Ethics Committee of Ain Shams University.

Inclusion criteria comprised patients with CCHD having arterial oxygen saturation <85%, both with history of thrombosis (more than 3 months prior to enrollment) and without history of thrombosis. Exclusion criteria were patients with acyanotic congenital heart disease (ACHD) and patients with CCHD with acute illness, total corrective surgery or other surgeries (within the last 6 months prior to enrollment), patients on antiplatelet/anticoagulant therapy, iron supplementation, and known bleeding disorders as hemophilia or idiopathic thrombocytopenic purpura.

All patients were subjected to full history taking and thorough clinical examination stressing on cardiac and nutritional assessment (iron and vitamin supplementation), as well as echocardio-graphy using Vivid E9 (GE Healthcare, Vingmed, Horten, Norway) for diagnosis of CCHD following a segmental sequential approach, and pulse oximetry for measurement of arterial oxygen saturation.

Venous blood samples were drawn from each patient and control in potassium ethylene diamine tetra-acetic acid (K-EDTA)-containing vacutainer tubes (BD Diagnostics, NJ, USA) for performing complete blood count (Coulter LH 750, Beckman Coulter, Inc., Fullerton, CA, USA), as well as in SST^{TM} tubes (BD Diagnostics, NJ, USA) for iron profile and TM assay. The latter samples were allowed to clot for 30 minutes followed by centrifugation for 15 minutes at 1000 ×g. Sera were collected, divided into two aliquots and stored at -20 °C.

Serum iron and total iron binding capacity (TIBC) were assayed using UniCel DxC 600 Synchron Clinical Systems (Beckman Coulter, Inc.,

Fullerton, CA, USA), whereas serum ferritin was tested on Access 2 Immunoassay System (Beckman Coulter, Inc., Fullerton, CA, USA). The diagnosis of iron deficiency was established depending upon the decrease in serum iron and ferritin, and the increase in TIBC compared to the reference ranges provided by their respective kits.

Serum TM was assayed using quantitative sandwich enzyme linked immunosorbent assay (ELISA) (Quantikine; Human Thrombomodulin Immunoassay, R&D Systems, Inc., Minneapolis, MN, USA). This kit recognizes soluble TM as well as TM complexed to thrombin, PC and thrombin/PC. A monoclonal antibody specific for TM has been pre-coated onto a microplate. Standards and diluted samples (1:10) were pipetted into the wells and any TM present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for TM was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of TM bound in the initial step. The color development was stopped and the intensity of the color was measured at 450 nm. A standard curve was constructed by plotting the log absorbance of each standard on the Y-axis against the log concentration on the X-axis. The best fitting line was drawn through the points on the graph. The sample concentration was obtained from the standard curve, multiplied by the dilution factor (×10), and expressed in pg/mL.

Statistical Analysis

Statistical analysis was performed using SPSS 17.0 statistical package. Results were expressed as mean ± standard deviation (SD) or median and interquartile range (IQR). Student *t*-test and Mann-Whitney *U*-test were used for comparing parametric and non-parametric continuous variables, respectively. Spearman Rank test was used for correlation studies. A *P* value of <0.05 was considered significant and of <0.01 was considered highly-significant in all analyses.

Results

Patients with CCHD were divided into two groups: *Group I* included 14 (35%) patients with history of thrombotic event (more than 3 months prior to enrollment). *Six* of these patients had cerebral venous thrombosis, *two* had middle cerebral artery thrombosis, *two* had right iliac venous thrombosis and *four* had shunt thrombosis. Thrombosis was reported to occur after an attack of either pneumonia or acute gastroenteritis. Group II included 26 (65%) patients with no history of thrombosis.

In comparison to controls, patients with CCHD showed a significant elevation in hemoglobin (Hb) and HCT, along with a significantly lower mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), platelets, iron and TM levels ($p<0.01$ for each). Mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), TIBC and ferritin were statistically non-significant between patients with CCHD and controls ($p>0.05$ for each) (Table 1).

On comparing Group I to Group II, Group I showed higher HCT and lower platelets than Group II ($p<0.01$ for each). However, oxygen saturation, Hb, MCV, MCH, MCHC, RDW, iron, TIBC, ferritin and TM were statistically non-significant between the two patient groups ($p\geq0.05$) (Table 1).

A positive correlation was detected between TM level and arterial oxygen saturation in all patients ($r = 0.943$, $p<0.001$), as well as in Group I ($r = 0.946$, $p<0.001$) and Group II ($r = 0.929$, $p<0.001$) patients with CCHD (Figure 1). No significant correlations were found between TM and other studied parameters in patients with CCHD ($p>0.05$).

Table 1 Comparison of Clinicopathologic Data between Patients with CCHD and Controls, and Group I and Group II Patients with CCHD[a]

Parameter		CCHD (n = 40)	Controls (n = 47)	P value	Group I (n = 14)	Group II (n = 26)	P value
Oxygen Saturation (%)	Mean ± SD Range	76.2 ± 7.4 55.0 - 84.0	-	-	74.7 ± 3.0 70.0 - 80.0	76.5 ± 8.1 55.0 - 84.0	0.116
Hb (g/dL)	Mean ± SD Range	16.1 ± 2.4 8.4 - 18.5	11.8 ± 1.2 8.7 - 13.7	0.008	16.5 ± 2.0 11.4 - 17.5	14.8 ± 2.4 8.4 - 18.5	0.05
HCT (%)	Mean ± SD Range	45.5 ± 6.1 26.3 - 52.7	36.1 ± 3.3 30.1 - 45.3	0.003	48.2 ± 3.5 42.8 - 52.7	38.9 ± 6.5 26.3 - 52.6	0.002
MCV (fL)	Mean ± SD Range	75.1 ± 9.1 52.8 - 87.0	80.7 ± 5.3 72.3 - 89.0	0.005	75.9 ± 8.3 65.5 - 87.0	74.9 ± 9.3 52.8 - 83.0	0.957
MCH (pg)	Mean ± SD Range	24.9 ± 3.8 15.2 - 30.0	27.2 ± 1.9 22.2 - 30.3	0.001	25.4 ± 3.9 19.0 - 28.7	24.8 ± 3.8 15.2 - 30.0	0.695
MCHC (g/dL)	Mean ± SD Range	32.8 ± 2.2 27.3 - 36.3	32.9 ± 1.4 29.3 - 35.7	0.892	32.6 ± 3.0 28.0 - 36.3	32.9 ± 2.1 27.3 - 36.1	0.789
RDW (%)	Mean ± SD Range	14.4 ± 1.6 12.3 - 18.1	14.4 ± 1.6 13.0 - 15.7	0.509	13.9 ± 1.8 12.9 - 15.2	14.5 ± 1.5 12.3 - 18.1	0.433
Platelets ($\times 10^3/\mu L$)	Median IQR	312.0 112.0	340.0 53.0	0.001	183.0 53.0	313.0 173.0	0.006
Iron (µg/dL)	Median IQR	30.9 23.5	48.1 38.0	0.000	30.1 9.2	32.0 27.3	0.722
TIBC (µg/dL)	Median IQR	332.0 244.8	328.2 83.3	0.554	412.0 126.4	318.0 257.8	0.091
Ferritin (µg/L)	Median IQR	38.9 58.6	33.9 42.9	0.714	38.8 18.6	42.2 102.1	0.403
TM (pg/mL)	Median IQR	3,900.0 2,200.0	5,000.0 1,400.0	0.000	4,000.0 2,000.0	3,900.0 2,350.0	0.734

CCHD, cyanotic congenital heart disease; Hb, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; TIBC, total iron binding capacity; TM, thrombomodulin; SD, standard deviation; IQR, interquartile range.

[a] Student t-test and Mann-Whitney U-test were used for comparison of parametric and non-parametric data expressed as mean ± SD and median (IQR), respectively.

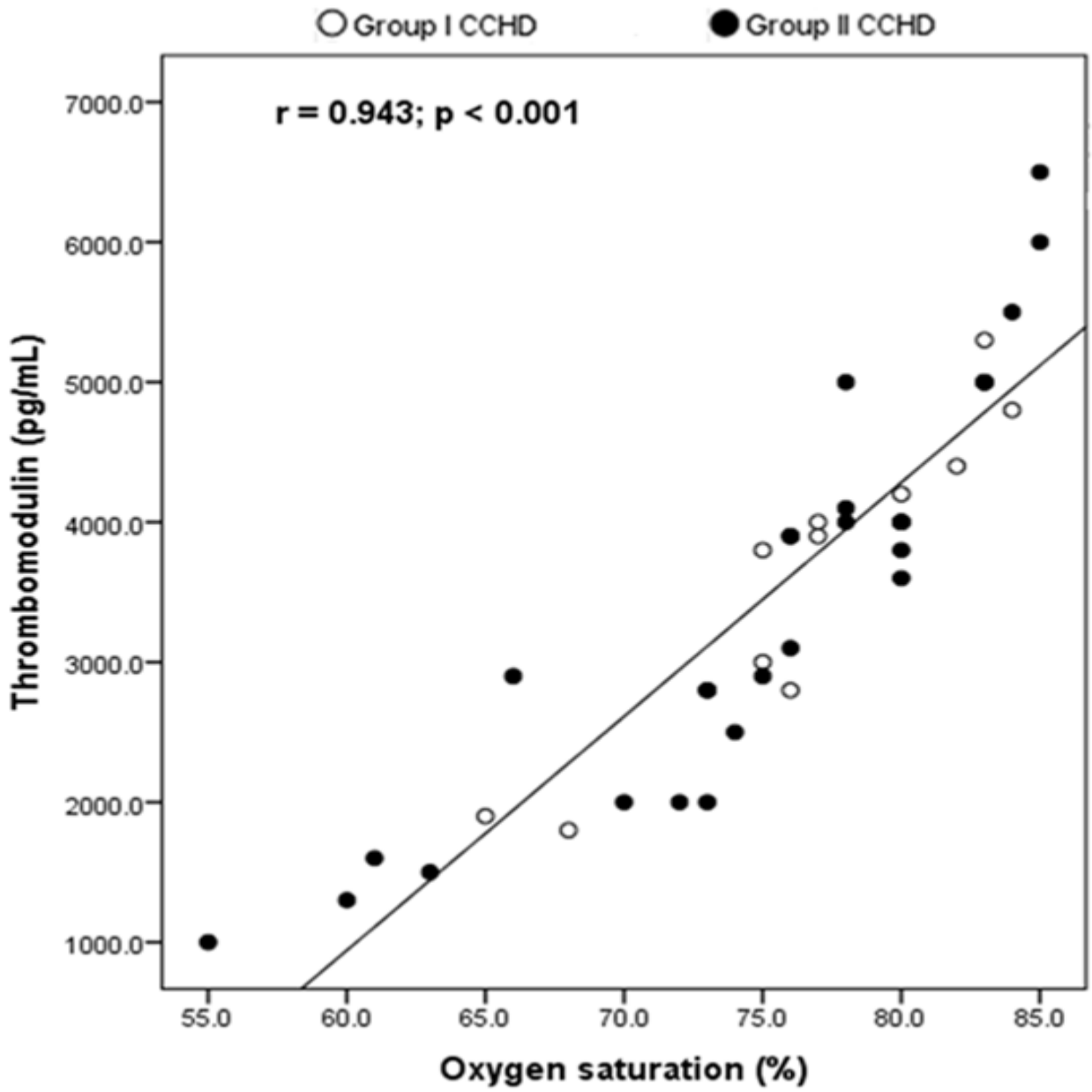

Figure 1 Positive correlation between thrombomodulin level and arterial oxygen saturation in patients with CCHD.

Discussion

Children with CCHD are at risk for clinically significant thrombosis contributing to their morbidity and mortality. The interplay between various risk factors for thrombosis in children with CCHD is complex and poorly understood. Our study showed that 35% of patients with CCHD enrolled during the study period had a history of thrombotic event. Other authors [6] reported that in patients with CCHD less than 4 years old, the risk for cerebrovascular thrombosis is increased, with the incidence varies from 1.6 to 20%. Silversides et al [7] denoted a pulmonary artery thrombus in 21% of patients with Eisenmenger syndrome.

In this study, significantly lower TM levels were found in patients with CCHD than the control group, and a positive correlation was found between TM levels and arterial oxygen saturation in patients with CCHD. These findings could be attributed to the endothelial damage caused by increased shear stress on the vessel wall due to increased blood viscosity, and/or chronic hypoxemia found in CCHD. Thus, it is likely that in patients with CCHD TM production is down-regulated as a result of chronic endothelial injury and persistent hypoxemia [1]. It is also reported that TM level initially increases with acute vascular injury but decreases with subsequent down-regulation of its production during chronic vascular injury [3], the latter being the case in our patients with CCHD.

Endothelial TM is a key component of the PC anticoagulant pathway, which facilitates the activation of PC by thrombin. Activated PC is known to inhibit clotting factors V and VIII. Therefore, TM acts as an intrinsic anticoagulant barrier between blood and endothelium, preventing blood from clotting on the internal surface of vessels [1]. Nevertheless, we could not find any significant difference in TM levels between Group I and Group II patients with CCHD. A presumable explanation could be the fluctuation of TM levels with time due to changes in the clinical status of patients; TM levels are highly likely to be confounded by multiple factors, including hypoxia, extent/severity of cardiac disease, and time since thrombosis and/or surgical procedures. In contrast, Yamashita et al [8] detected that down-regulated gene expression of TM produced by rapid atrial pacing in anesthetized rats induced a local coagulation imbalance on the internal surface of the atrial cavity, leading to atrial intramural thrombus formation. Moreover, several studies [9-11] performed on genetically-altered murine models without CCHD stated that reduced TM disturb the optimal coagulation balance and promote thrombogenesis.

Patients with CCHD in this study had significantly higher Hb and HCT levels in comparison to controls; Group I had higher HCT than Group II. Our patients mentioned that thrombosis was preceded by an attack of pneumonia or acute gastroenteritis which seemed to be one of the predisposing factors for thrombosis. Dehydration precipitated by repeated infections and fevers leads to hemoconcentration that is reported to precipitate or aggravate symptomatic hyperviscosity which could potentially increase the risk for a sudden thrombotic stroke; dehydration must be corrected before resorting to phlebotomy in CCHD with a HCT of >65% [12]. Thrombosis was stated as the major consequence of hyperviscosity, because the increased red cell mass and the abnormal platelet functions induce sludging in the vasculature [5]. Unlike our results, a previous study [1] had employed adult patients with CCHD and failed to demonstrate high HCT as a risk factor for thrombosis in CCHD.

Our study revealed significantly reduced platelet counts in patients with CCHD compared to the control group, as well as in Group I than Group II patients. Four main pathogenetic mechanisms are suggested to be potentially responsible for thrombocytopenia in CCHD: (i) decreased platelet production, (ii) decreased megakaryocyte production, (iii) increased platelet destruction, and (iv) increased platelet activation. It has been hypothesized that the pathogenesis of thrombocytopenia in CCHD reflects the right-to-left shunts that deliver whole megakaryocytes into the systemic arterial circulation, bypassing the lungs where megakaryocytic cytoplasm is fragmented into platelets, thus reducing platelet production [13].

Elevated P-selectin expression and platelet microparticles were found on activated platelets in patients with CCHD proposing an important role for platelet activation in thrombus formation. The platelet activation observed in patients with CCHD could be attributed to increased shear stress due to hyperviscosity, and/or endothelial dysfunction but the precise mechanisms remain unclear [14]. This might explain the co-existence of thrombocytopenia, probably secondary to platelet activation, with thrombosis in our studied patients.

We found significantly lower MCV, MCH and serum iron in patients with CCHD compared to controls. Thirty-four out of forty (85%) studied patients had iron deficiency. Various studies advocated the prevalence of iron deficiency in CCHD as more than one-third [15], 52.2% [16], and 63.6% [17]. The higher rate of iron-deficient patients with CCHD found in our study could be due to the more prevalence of nutritional iron deficiency among Egyptian children due to decreased iron intake, added to the functional anemia consistently found in CCHD. The possible causes of iron deficiency in CCHD are; increased iron consumption through increased erythropoiesis, inappropriate venesections, hemoptysis, bleeding from arteriovenous malformations or collateral vessels, abnormal hemostasis, limited dietary intake or absorption, and use of anticoagulants and antiplatelets [18].

On the other hand, our study revealed comparable differences in MCV, MCH and serum iron between Group I and Group II patients with CCHD. By contrast, other authors [19] reported that iron deficiency may contribute to a hypercoagulable state by many mechanisms including; affection of the blood flow patterns within the vessels due to reduced deformability and increased viscosity of the resulting microcytic red cells, decrease in the antioxidant defense with increase in oxidant stress which may result in a tendency towards platelet aggregation, as well as the secondary thrombocytosis attributed to the release of the inhibitory effect on thrombopoiesis maintained by normal iron levels; the latter not being the case in our patients. Moreover, iron-deficient erythrocytosis in patients with CCHD less than 4 years old was reported to predispose cerebrovascular thrombosis in the intracranial venous sinuses [6].

Although the precise mechanisms for the hypercoagulable state existent in CCHD are still not fully clarified and are proposed to be multifactorial, yet this study explicitly addressed, helped understanding and provided further insights on the role for the proposed risk factors for thrombosis in children with CCHD, as well as assessed the potential role for TM as a novel risk factor.

Conclusion

We finally concluded that decreased TM can be considered a reliable marker for endothelial dysfunction; however, not a good marker for predicting thrombosis in children with CCHD. Infections, fevers and dehydration, along with increased HCT, and platelet activation are speculated to collectively interplay and substantially participate in the prothrombotic state encountered in CCHD.

Larger scale prospective follow-up studies are warranted to further explore other potential candidate risk factors for thrombosis in CCHD such as protein C, protein S, antithrombin, factor V Leiden, prothrombin mutation 20210, methylenetet-rahydrofolate reductase mutation, activity of clotting factors, and platelet activation markers for prediction, early detection, prevention and treatment of thrombosis in children with CCHD.

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