The Status of Procoagulant Tissue Factor-bearing Microvesicles Among Saudi Children with Sickle Cell Disease During Steady State

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Abstract:

Introduction: Sickle cell disease (SCD) is a common genetic disorders characterized by episodic occlusion of the microcirculation leading to life-threating complications, including thrombosis. Coagulation activation is a prominent feature of SCD, as shown, among others, by an increased expression of tissue factor (TF).

Objectives: To measure and compare the levels of plasma circulating tissue factor bearing microvesicles (TF-MVs) in Saudi children with SCD in steady state and in matched healthy control (MHCs).

Method: This was a prospective observational hospital-based study in which citrated whole blood was collected from 102 SCD children homozygous for sickle hemoglobin (HbSS) (aged from 2 to 18 years-old) and 51 HMCs. TF-MVs were measured using an indirect ELISA method.

Results: TF-MVs level in Saudi children with SCD is significantly elevated than in MHCs (0.82 vs 0.50 pg/ml) (P = < 0.0001).The level of TF-MVs in children below age 7 years was comparable to that in normal children.(0.76 vs 0.63 pg/ml,P 0.4). Male children with SCD showed higher levels of TF-MVs in their plasma than female children but that was not

statistically significant (0.93 vs 0.77 pg/ml) (P= 0.1).There was no statistically significant correlation between the TF-MVs level and total MVs (r=0.24) (P value 0.06).

Conclusion: This study demonstrated an elevated level of TF-MVs activity in Saudi children with SCD compared to the control. Children below the age of seven years in the study group had a level of TF-MVs comparable to that in the control group. Male children in the study group were observed to have higher level of TF-MVs than females

Key words: Tissue factor bearing microvesicles, Microvesicles, Sickle cell Disease, Children,Saudi Arabia

Introduction:

Sickle cell disease (SCD) is one of the most common genetic disorders characterized by single gene mutation, where glutamic acid is replaced by valine in the 6th position of the beta chain. This causes polymerization of hemoglobin S in low oxygen tension leading to episodic occlusion of microcirculation and increased risk infections. (1, 2). SCD is worldwide disease that affects mainly African, black- American, Arabs and those of Asian ancestry (3)

SCD is a well-known hypercoagulable state with increased risk of thrombosis in steady state and during crisis (4, 5). Blood borne tissue factor (TF) it is a physiological hypercoagulable factor which initiates the coagulation system once the blood vessel was injured (6, 7). It has been reported to circulate on MVs (8). Levels of TF-bearing MVs is found in the circulation of healthy individuals and increased in patients with various disorders (9). Many studies had reported a positive correlation of circulating cell-derived MVs to the hypercoagulable state in SCD (10). Chronic activation of endothelial cells and platelets were known to contribute to the hypercoagulability condition leading to a high incidence of thrombosis in SCD (5, 11-13).

MVs are sub-cellular small membrane vesicles (14) released during stimulation or apoptosis of different cells like platelets, red blood cells (15). MVs derived from platelets have procoagulant activity up to 100 times more than platelets (16). MVs were found in healthy people, and in patients with thrombotic abnormality (17). The procoagulant activity is enhanced in the presence of tissue factor and negatively charged phospholipids mainly phosphatidylserine present on the surface of MVs (18). Many studies had shown an increased level of TF-bearing MVs (TF-MVs) in the sickle cells. In Saudi Arabia there are no such studies. This study aimed to measure the levels of TF-MVs among Saudi children during their steady state and to determine the effect of gender and age on this level. The steady state is a point in time where the patient in question is not experiencing an acute painful crisis or any changes due to therapy (19).

Material and methods:

Patients and Blood Collection:

This was a prospective, observational, hospital based study conducted at three major general hospitals in the Southern region of the kingdom of Saudi Arabia (Al-Mahyal Assir General Hospital, Majardah General Hospital and Aseer Central Hospital) from September 6, 2014 to January 25, and 2016. The patients who met the following inclusion and exclusion criteria were taken as the study group. Inclusion criteria: Hemoglobin electrophoresis compatible with the diagnosis of sickle cell anemia, age 2-18 years, No blood transfusion in the past 6 weeks ,absence of sickle crisis for 6 weeks prior the time of sample collection and care-giver approval. Exclusion criteria were: renal disease, diabetes mellitus, bleeding disorders liver diseases and history of thrombosis and care-giver refusal. Normal children (hemoglobin AA) matched in age and sexes were taken as control group. Verbal and written consent was

obtained from caregivers of all patients. The study was approved by the research and ethical committee of King Khalid University (#2012/04/05).

Blood collection:

Venous blood was collected in Tri-sodium citrate and ethylene diamine tetra acetic acid (EDTA) tubes (IMPROVE, Guangzhou, Improve Medical Instruments Co., Ltd., China) from the study and control group(20). To avoid platelets activation blood samples were collected following standard laboratory guidelines (21).

TF- MVs Isolation:

Platelet-free plasma was collected by two-step separation of citrated whole blood at 1,500g for 15 minutes at room temperature then re-centrifuging the plasma for another 10 minutes at 1,500g (21). After that the plasma was immediately stored at −80°C for measuring the TF−MVs (22).

Measurement of TF-MVs in the Plasma by ELISA:

Level of procoagulant TF-MVs in plasma were measured using Zymuphen TF-MP activity kit (Aniara Diagnostica LLC, OH, USA) according to the manufacturer's instructions. The optical density were measured using ELISA plate reader (Stat fax-2100 Technology Inc. USA).

Statistics:

Graph Pad Prism 5 version 5.04 software for Windows (1992-2010 Graph Pad Software Inc., San Diego, CA, USA) was used for statistical analysis. The results were given as mean with 95% confidence interval(95% CI) and unpaired t-test was used for TF-MVs level analysis and P values were considered significant if $P < 0.05$.

Result:

A total of 102 patients on hydroxyurea for at least 3 months were enrolled as study group (31 male (30%) and 71 female (70%). 51 healthy children as control group (25 males (49%) and 26 females (51%)). The mean age in the study group was (9.0 ± 0.5 years) and in the control group was (8.8 ± 0.5 years) (P=0.05) [Table 1]. Mean age of males in the study group was (10.1 ± 1.0 years) while in the control group was (9.0 ± 0.9 years) (P=0.4). The mean age of females in the study group was (8.5 ± 0.6 years) and in the control group was (8.6 ± 0.7 years) (P=0.09) [Table 1].

The TF-MVs plasma level was significantly higher in the study group compared to the control group (0.82 pg/ml vs 0.50 pg/ml (P = < 0.0001) [Table 1]. The TF-MVs level in males in the study group was 0.93 pg/ml compared to 0.49 pg/ml in males in the control group (P = 0.0008). The TF-MVs level was also higher in females in the study group than females in the control group (0.77 pg/ml Vs 0.5 pg/ml p:0.003) [Table1] .In the study group TF-MVs mean level in males was comparable to that in females (0.93 pg/ml vs 0.77 pg/ml ,P = 0.1) Table (1) & Figure 1].

The mean level of TF-MVs in age 2 - <7 years in the study group was comparable to that in the control group; the level of TF-MVs in the study group then significantly increased with increasing age (Table 2 and figure 2).

The mean MVs level of this cohort (30.49 ± 2.84 nM) was reported before (23).There was a weak association between the levels of MVs and TF-MV (r=0.24) and the correlation coefficient was not statistically significant (P value 0.06) Figure (3).

Discussion

A preliminary report of this study was published before (24). This continuation study confirmed that the plasma levels of TF-MVs in the study group were higher compared to that in normal

children. This is similar to what had been reported in the literature (21, 25, 26). A weak and statistically insignificant correlation was observed between the Plasma MVs level and TF-bearing MVs. MVs in general were reported to be derived from erythrocytes, leukocytes, platelets and endothelial cells (8),however, in patients with SCD they were reported to be derived mainly from erythrocytes and platelets (27).On the other hand TF-MVs were reported to be derived mainly from monocytes and endothelial cell (21).This difference in origin might partly explains this lack of correlation.

The level of TF- MVs in this study might have been affected by certain factors. It was measured during steady state in patients who were receiving hydroxyurea and some of them were young children. The TF-MVs level in children below 7 years of age in this study was comparable to that in the control group. This is similar to what had been reported by Setty et al where young children were found to have normal TF-MVs (25). Microvesicles exposing tissue factor were not detected in patients with SCD in one study (27).Also they were not detected even in 21% of Brazilian patients not on hydroxyurea(28) Several studies had shown that the TF-MVs were lower during steady state than during crisis (21). The Brazilian study mentioned above (28) had shown that TF was detected in the plasma of 79% of patients with SCD not using hydroxyurea compared to 28% of patients using the drug (28). Therefore the measurement of TF-MVs in our patients while on hydroxyurea probably contributed to the reduction of the level of TF - MVs.

It had been documented that morbidity and complications is more in males than in females in the acute and chronic state of SCD (29). Baum et al had shown that males were affected with a greater rate of pain attacks than females after the age of 15 years (30). The basis for these differences was suggested to lie on the observation that the bioavailability of nitric oxide and its responsiveness are reduced in males but not females with sickle cell disease (31). TF has an important role in the activation of coagulation in both sickle cell patients and in mouse models of

SCA. This activation of coagulation significantly contributes to inflammation and vascular injury in sickle cell mice (32). What cellular sources of TF contribute to the activation of coagulation in sickle cell mice? Possible cellular sources like monocytes and lung microvascular endothelial cells have been previously reported to contribute to activation of coagulation in sickle cell mice (33). TF-positive microvesicles were observed to be higher during pain crisis episodes compared to steady-state disease. (21, 34). In this study we had shown that TF-MVs level increased with age. Males had a higher level, although not statistically significant, of TF-MVs than females. Whether this gender difference in TF- MVs level has a role in the gender differences in sickle cell morbidity and complications needs to be determined.

Limitations: Due to financial limitations we were unable to measure TF-associated MVs in a larger sample of children and to determine the origin of these MVs.This can be done in another study in the future.

Conclusion

This study had shown that plasma level of TF-MVs is higher in Saudi children with SCD compared to normal children. This difference was observed to occur after seven years of age and with the level being slightly higher in males than females. The association of elevated TF-MVs and complications of SCD needs to be explored in further studies.

Conflict of interest: All authors had none to declare. All authors agreed to the terms and conditions of Al neelain medical Journal. All authors had read the journal's authorship agreement and that the manuscript has been reviewed by and approved by all named authors.

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Table (1): Patients characteristics and TF-MVs level in the study and control group

	Number	Mean age years	P value	Mean TF-Vs(pg/ml)	P value
Study group	102	9.0±0.5	0.05	0.82 (95%: CI:0.81-0.83)	<0.0001
Control group	51	8.8±0.5		0.50 (95% CI:0.42-0.58)	
Study group Males	31(30%)	10.1±1.0	0.13	0.93 (95% CI :0.89 - 0.97)	0.1
Females	71(70%)	8.5±0.6		0.77 (95% CI = 0.76 - 0.78)	
Study group: Males	31(30%)	10.1±1.0	0.4	0.93 (95% CI:0.89 - 0.97)	0.0001
Control group Males	25(49%)	9.0±0.9		0.49 (95% CI:0.47 - 0.51)	
Study group: Females	71(70%)	8.5±0.6	0.09	0.77 (95% CI: (0.76 - .78)	0.003
Control group Females	26(51%)	8.6±0.7		0.50 (95% CI:0.48 - 0.52)	

Table(2):TF-MVS level according to age in the study and control group

Age	Mean TF-MVs level pg/ml (study group)	Mean TF-MVs level pg/ml (control group)	P value
2-<-7 years	0.72 95% CI = 0.70 - .74)	0.63 (95% CI = 0.57 - 0.68)	0.4
7-<11 years	0.83 95% CI = 0.80 - 0.86)	0.44 95% CI = 0.42 - 0.45)	0.002
11-18 years	0.91 95% CI = 0.88 - 0.94	0.49 95% CI = 0.46 - 0.52	0.003

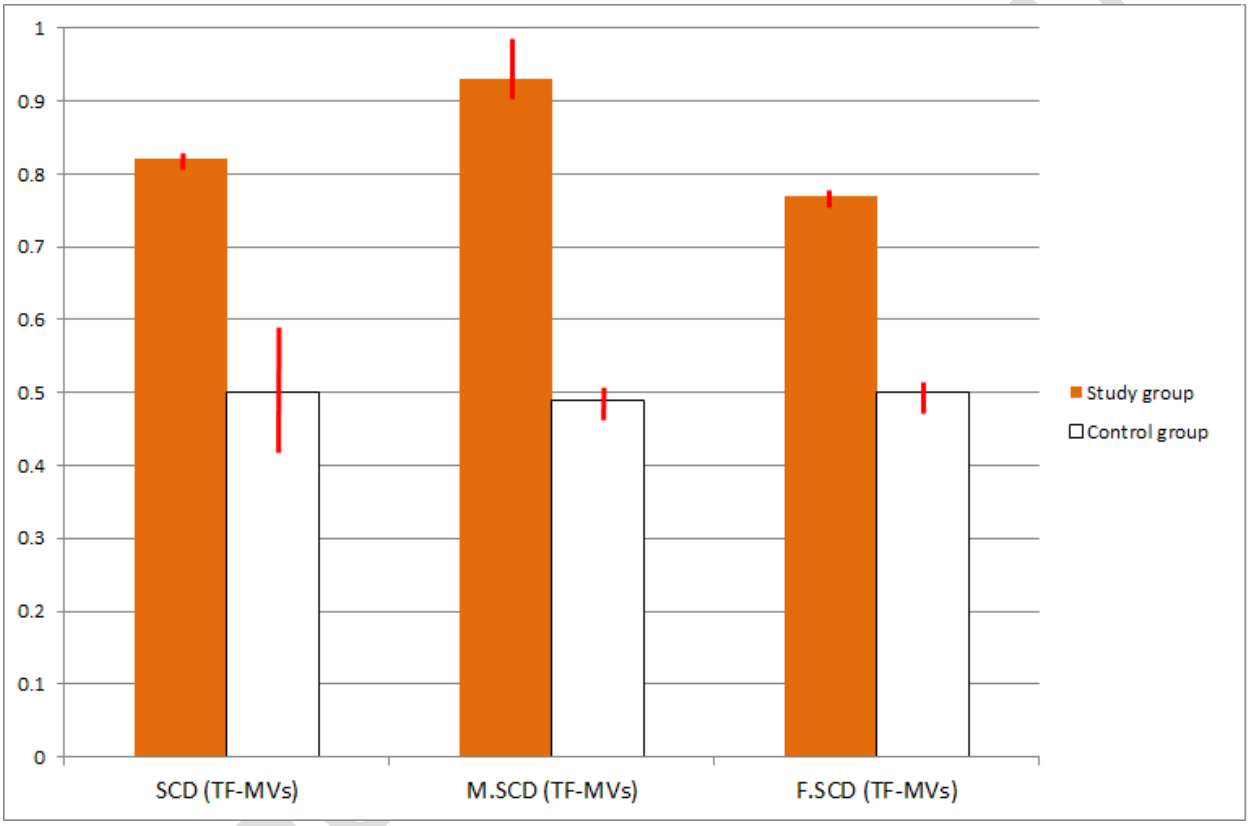


Figure (1): TF-MVs level according to gender (The top ends of the bars indicate observation means and the red line segments represent the confidence intervals surrounding them.)

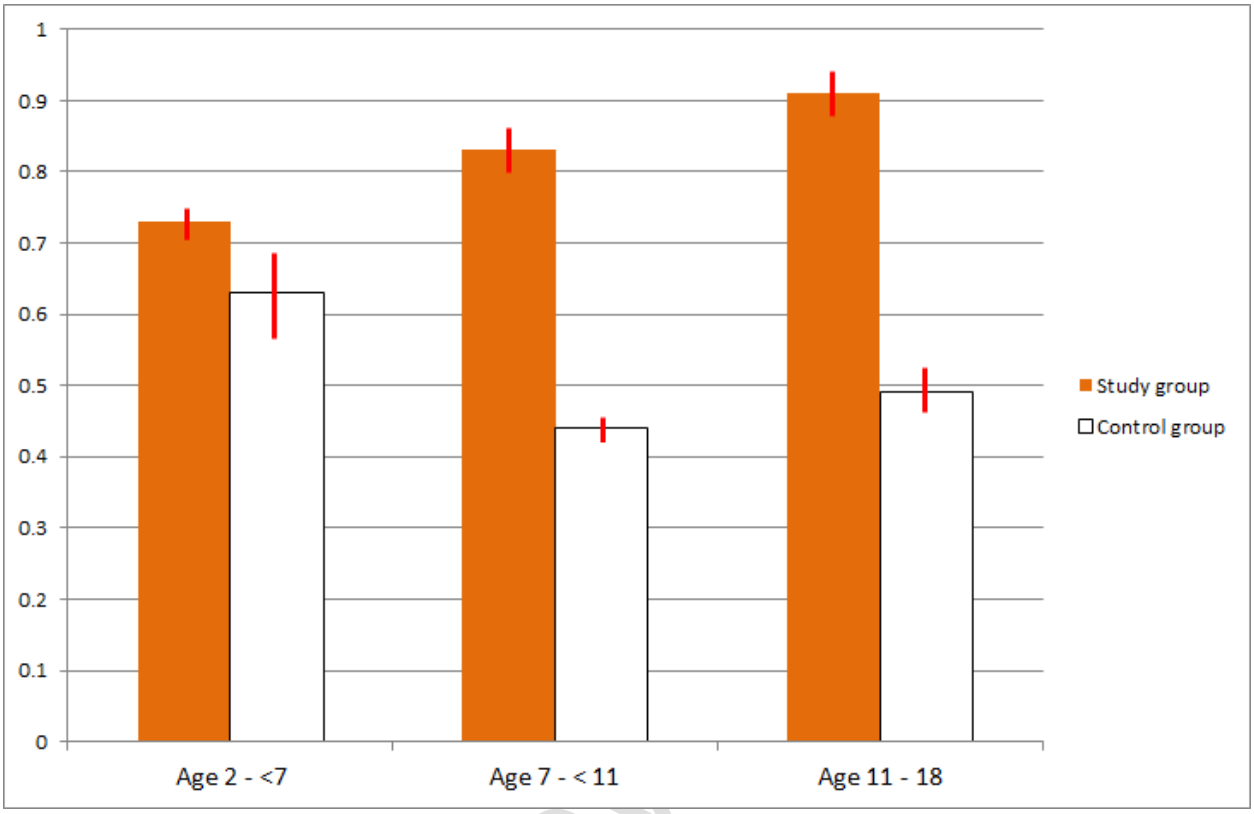
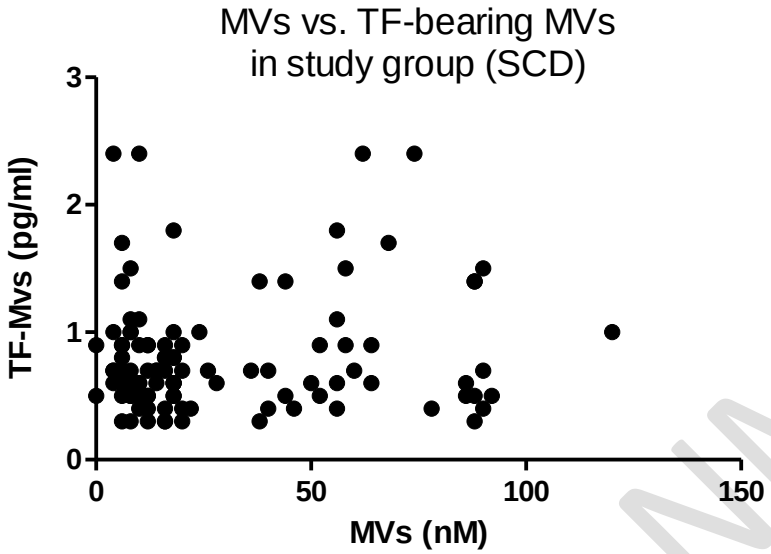


Figure (2): TF-MVs level according to age(The top ends of the bars indicate observation means and the red line segments represent the confidence intervals surrounding them.)



Number of XY Pairs	102
Pearson r	0.1160
95% confidence interval	-0.08029 to 0.3037
P value (two-tailed)	0.2455

Figure (3): Correlation between procoagulant microvesicles and tissue factor-bearing microvesicles in the study groups (SCD).