

**Elevated levels of Plasma Procoagulant Microvesicles in Saudi Children in Steady-state Sickle cell
Disease**

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Abstract:

Introduction: Sickle cell disease (SCD) is a genetic disorder resulting from the presence of a mutated form of hemoglobin; known as hemoglobin S (HbS). It is characterized by various complications, including thrombosis. Increased levels of circulating procoagulant microvesicles (MVs) had been reported in this disease and many other diseases, which are causing the plasma to have prothrombotic tendency.

Objectives: This study compares the levels of circulating MVs in Saudi children with SCD in their steady state with their healthy matched controls (HMCs).

Method: Citrated whole blood was collected from 102 children homozygous for sickle hemoglobin (HbSS) (aged from 2 to 18 years-old) and 51 HMCs. MVs were measured using an indirect ELISA method.

Results: The mean level of MVs in the Saudi children with SCD is significantly higher than in HMCs (30.49 ± 2.84 vs 14.41 ± 1.68 nM) ($P = 0.0002$). Males with SCD children showed higher mean levels of MVs in their plasma than females but that was not statistically significant (36.97 ± 5.42 nM) vs (27.66 ± 3.29 nM) ($P = 0.13$).

Conclusion:

This study demonstrated a significantly high plasma level of MVs in Saudi children with SCD than the HMCs. Male children with SCD showed higher level of MVs than females and younger children had lower levels than older children.

Introduction:

Sickle cell disease (SCD) occurred due to gene mutation at the 6th position that leads to replacement of glutamic acid by valine. This will cause polymerization of HbSS in low oxygen tension. Sickling of the erythrocyte leads to chronic tissue hypoxia causing, among others, infections, sickle cell crisis and episodic occlusion of the microcirculation (1, 2). SCD has a high frequency in Africa and Asia including Saudi Arabia(3).

SCD is known as a hypercoagulable state with risk of thrombosis in steady state and during vaso-occlusion (4,5). Some studies had proposed a relation of circulating cell-derived microparticles (MPs), widely now known as microvesicles (MVs), to the hypercoagulable state in SCD(6). Endothelial cells and platelets were also known to contribute to the hypercoagulable condition causing high incidence of thrombotic events in the SCD(4, 5, 7, 8).

MVs are sub-cellular small membrane vesicles released during activation or apoptosis from different cells like platelets, red blood cells and others cells (9,10). MVs can be found in the circulation of healthy people, and in patients with diseases with known prothrombotic abnormalities (11). The increased levels of MVs are one of the important factors reported in hypercoagulability in SCD. (12) This is enhanced by the negatively charged phospholipids mainly phosphatidylserine, which is increased by the presence of tissue factor (TF) (13). Many studies were published reporting the level of MVs in the sickle cell disease (12, 14, 15) including Saudi Arabia (16).

Material and methods:

Patients and Blood Collection

This is a prospective cross-sectional hospital based study conducted in the South of Saudi Arabia in three major general hospitals (Al-Mahyal Asser , Majardah and Asser Central Hospital) from September, 2014 to January 2016. Children with sickle cell disease, confirmed by hemoglobin electrophoresis, who met the following study criteria were enrolled as study group: Age 2-18 years, had no illness related to sickle cell disease in the past 6 weeks and did not receive blood transfusion 6 weeks prior to enrollment. Patients with diabetes mellitus, renal disease liver disease or bleeding disorders were excluded from the study. Normal children (hemoglobin AA) matching the study group in sex and age were randomly selected and enrolled as control group. Verbal and written consents were obtained from caregivers of all patients. The study was approved by King Khalid University Research Ethical Committee (#2012/04/05)

Blood collection:

Venous blood was collected in Sodium citrate and ethylene diamine tetra acetic acid (EDTA) tubes (IMPROVE, Guangzhou, Improve Medical Instruments Co., Ltd., China) from the study and control group. Blood samples were collected following standard laboratory guidelines to avoid the activation of platelets (17).

Microvesicles Isolation

According to the Clinical and Laboratory Standards Institute (CLSI) (17). Platelet-free plasma was obtained by two-step separation of citrated whole blood at 1,500xg for 15 minutes at room temperature then re-centrifuging the plasma for another 10 minutes at 1,500xg. After that the plasma was immediately stored at -80°C for measuring the MVs as described elsewhere (17,18)

Measurement of microvesicles in the Plasma by ELISA

The level of plasma procoagulant MPs were measured using Zymuphen 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).(19).

Statistics:

Statistical analysis was performed with Graph Pad Prism 5 version 5.04 software for Windows (1992-2010 Graph Pad Software Inc., San Diego, CA, USA). The results were given as a mean \pm standard deviation and unpaired t-test was used for MVs analysis and P values were considered significant if $P < 0.05$.

Result:

A total of 102 patients were enrolled as study group, all were on hydroxurea for at least three months. In addition, 51 healthy matched control (HMC) were included as a control group. There were 31 males (30%) and 71 (70%) females as HbSS patients, while there were 25 males (49%) and 26 (51%) females in the control group. The mean age in the study group was (9.00 ± 0.49 years) and in the control group was (8.77 ± 0.55 years). ($P=0.05$) (Table 1). The mean age of males in the study group was (10.06 ± 0.98 years) while in the control group was (8.96 ± 0.87 years) ($P 0.4$). The mean age of females in the study group was (8.50 ± 0.55 years) and in the control group was (8.57 ± 0.68 years) ($P 0.09$).

The mean MVs level is significantly higher in the study group (30.49 ± 2.84 nM) compared to the control group (14.41 ± 1.68 nM) ($P= 0.0002$) (Table 1 & Figure 1). In the study group the mean level in males was (36.97 ± 5.45 nM) while in females was (27.66 ± 3.29 nM) ($P 0.13$) (Figure (2)). In the control group MVs mean level in males was (21.84 ± 4.41 nM) while in the females it was (14.73 ± 1.88 nM) $P = 0.0001$. The mean level of MVs was slightly elevated in children 3 years or younger and increased after that (Table 2 & Figure 3).

Discussion:

A preliminary report of this study was published before (16). This continuation study, using more patients, confirmed the elevated plasma level of MVs (1.7-2.1 folds) in children with SCD reported in the preliminary study. These findings are in agreement with previous studies in patients with SCD or other pathological disorders (14, 20-21). The level of MVs in children 6 years and younger was mildly elevated and then increased gradually with age. This age effect was shown in Nébor et al report (15). This might be explained by the age-related reduction in hemoglobin F (HbF) levels during Childhood which was reported by many authors (15, 22).

Tantawy et al had shown a positive correlation between MVs plasma level and disease duration. The same study showed increased MVs level with older age (14). The MVs level in the study group, although elevated, might have been underestimated because of the effect of hydroxyurea. Nébor and colleagues showed that children treated with hydroxyurea exhibited lower total plasma level of MVs (15-folds reduction) compared to non-hydroxyurea treated children. They had shown that the level was lower in these patients even when their HbF level was lower than those untreated with hydroxyurea (15). The authors commented that the reduction is not mainly due to induction of HbF. Similar effect of hydroxyurea was reported by others (14). Furthermore blood collected in sodium citrate, as done in this study, gives significantly lower level of phosphatidylserine positive MVs, hence total MVs, than that collected in heparin (23).

The number of males in the study group were half that of the female's, despite that the plasma level of MVs was slightly higher in them compared to females. One of the gender-related differences in complications of SCD is hyperhemolytic crisis. This is associated with glucose 6-phosphate dehydrogenase deficiency which is considerably more likely in boys than girls (24). Furthermore high hemolytic component, present in hyperhemolytic crisis, is associated with significantly low mean Hb F level and high MVs level. (25). This

might explain partly this gender difference in plasma MVs level. A gender-related difference in the level of plasma MVs was also present in the control group.

We did not determine the cellular origin of MVs in this study as we had used ELISA

Method. ELISA method quantifies MVs but cannot tell their origin (23). Nébor and colleagues showed that Hydroxyurea lowers the plasma MVs mainly through reduction of MVs originating from platelets and red blood cells. Despite that MVs from red blood cells and platelets are the dominating ones in the studied population (15). Therefore we can assume that the cellular origin of MVs in our study is platelets, red blood cells and to a lesser extent monocytes.

Conclusion

This study clearly demonstrated elevated level of MVs in plasma of children with SCD with higher levels in older children compared to young ones. The MVs level shown in this study was not very high reflecting the reduction effect of hydroxyurea. Further studies are required to determine the cellular origin of these microparticles.

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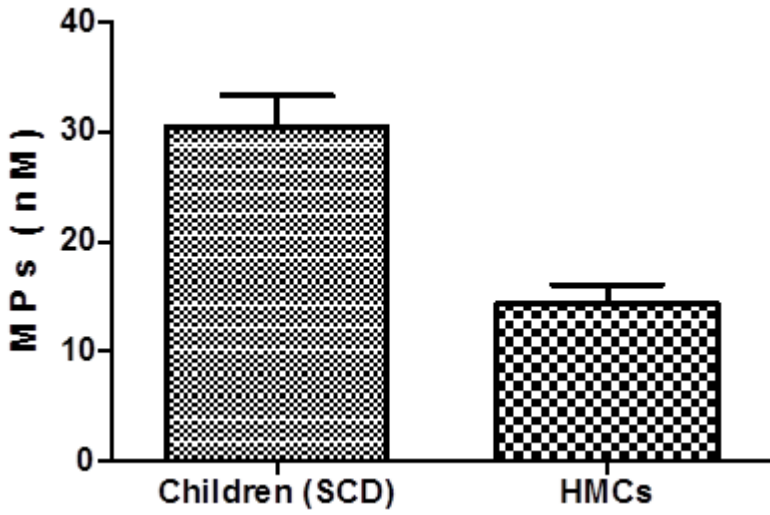


Fig (1):Mean plasma MVs level in the study group and the control group (P .0002)

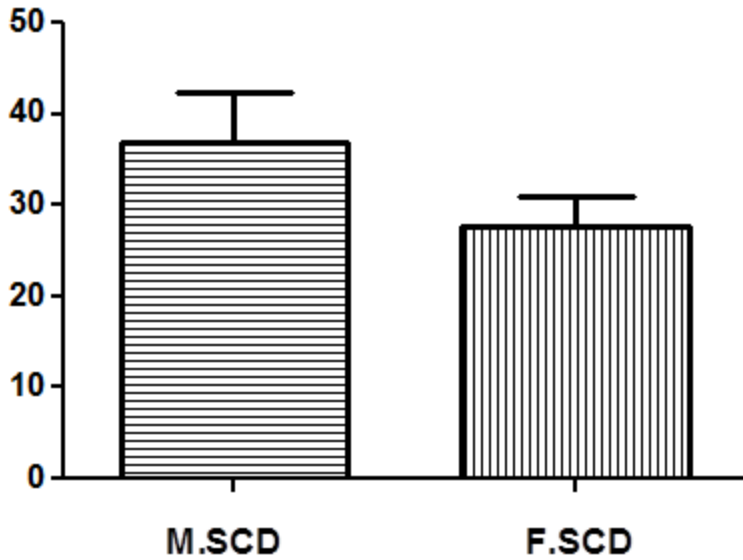


Fig (2): Mean plasma MVs level in the study group based on gender (P 0.13)

M.SCD=Males with SCD

F.SCD=Females with SCD

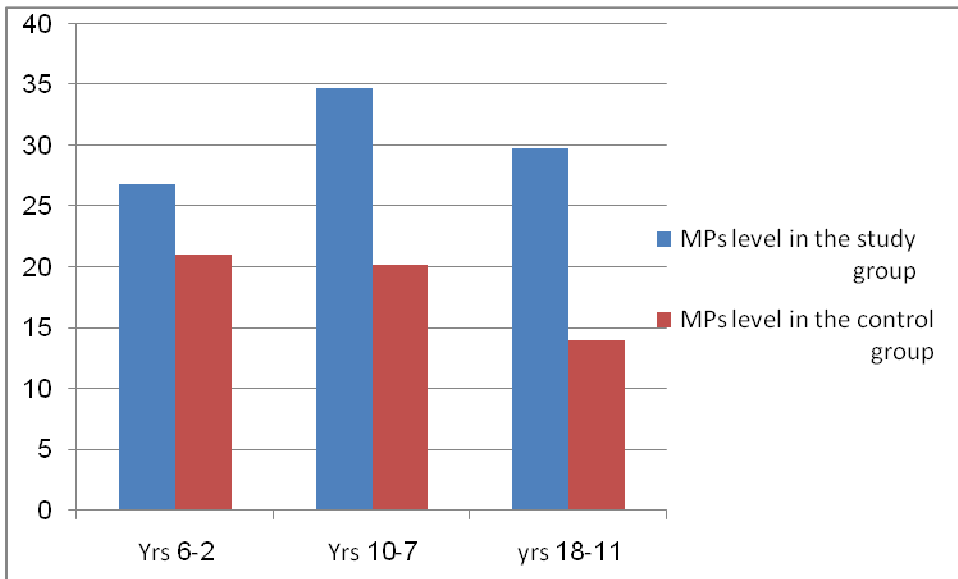


Fig (3): Mean plasma level of MVs according to age group

Table (1): Characteristics of patients in the Study and control groups

	Total No of patients	Mean age(years)	No of males	No of females	Mean MVs level
Study group	102	9.00 ± 0.49	31 (30%)	71 (70%)	30.49 ± 2.84nM
Control group	51	8.77 ± 0.55	25 (49%)	26 (51%)	14.41 ± 1.68nM

Table (2): Mean plasma MVs level (nM) in study and control group based on age.

The number of patients is indicated in brackets

Age in years	2 - 6	7 - 10	11 - 18
Study group:	26.76 ± 4.92	34.67 ± 5.65	29.75 ± 4.28
MVs level	(29)	(33)	(40)
Control group	21.09 ± 6.19	20.18 ± 3.79	14.06 ± 3.26
MVs level	(11)	(22)	(18)