Clinical and Applied
Thrombosis/Hemostasis
Volume 25: 1-6

DOI: 10.1177/1076029619828839
journals.sagepub.com/home/cat
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# Circulating Microparticles in Children With Sickle Cell Anemia in a Tertiary Center in Upper Egypt

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## Abstract

Sickle cell disease (SCD) is a genetically inherited hemolytic anemia increasingly appreciated as a chronic inflammatory condition and hypercoagulable state with high thrombotic risk. It is associated with disturbed immune phenotype and function and circulating microparticles (MPs) derived from multiple cell sources. This study was carried out to determine MPs profiles in patients with sickle cell anemia (either on hydroxyurea (HU) therapy or those with no disease-modifying therapy) and to compare these profiles with healthy children. Moreover, our study assesses the potential impact of HU on other aspects of circulating MPs. We performed a cross-sectional study on 30 pediatric patients with SCD divided by treatment into 2 groups (those receiving HU or no therapy) attending Hematology Clinic and 20 age-matched healthy children. The blood samples obtained were analyzed for MPs by flow cytometry. Sickle cell disease group with no therapy showed elevated levels of total, platelet, and erythroid MPs. In contrast, therapy with HU was associated with normalization of MPs. This study provided additional evidence that HU is an effective treatment option in pediatric patients with SCD, as it seems that it decreases the abnormally elevated MPs in those patients.

## Keywords

Sickle cell disease, hydroxyurea, microparticles, children

Date received: 19 November 2018; revised: 18 December 2018; accepted: 5 January 2019.

## Introduction

Sickle cell disease (SCD) is one of the most widespread genetic disorders all over the world. Sickle cell disease causes significant morbidity and mortality.[1] In Egypt, it has a heterogeneous distribution, and the carrier rates of sickle hemoglobin (HbS) are from 9% to 22%.[2] Among Egyptians, the majority of described globin gene haplotypes are African haplotypes, and phenotype of SCD is usually severe.[3] The disease consists of different disorders resulting from the inheritance of HbS gene either in a homozygous state or a double heterozygous state with additional abnormal hemoglobin gene. This HbS has the tendency when deoxygenated, to polymerize intracellularly into long rigid arrays called tactoids and deform red blood cells into the characteristic sickle shape;

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thus, producing variable clinical manifestations some of which include hemolytic anemia, vaso-occlusive crises, and recurrent infections with their attendant sequelae.[4]

Previous studies have reported that patients with sickle cell anemia (SCA), particularly children, have a high susceptibility to infections with higher morbidity and mortality.[5,6] The mechanisms that account for the immunocompromised state in children with SCD include impaired leukocyte functions and opsonophagocytic defect due to aberrations of complement pathway, lack of some specific circulating antibodies, and loss of both cell-mediated and humoral immunity.[7]

Moreover, growing evidence exists that SCD is a pro-inflammatory disorder with excessive immune activation. In addition to the findings consistent with immune activation in SCD, studies have established that immune activation plays a role in the pathology of SCD.[8] Previous studies showed that patients with SCD have increased cytokine levels as well as increased activation of monocytes, neutrophils, and natural killer T cells, which may have a role in pulmonary ischemia–reperfusion.[9-12] Hydroxyurea (HU) is a ribonucleotide reductase inhibitor, which raises fetal hemoglobin (HbF) in red blood cells (RBCs) of patients with SCD which decreases acute chest syndrome, as well as, the rate of blood transfusion, pain crisis, and hospitalization.[13,14] It has been widely used in pediatric sickle cell centers for these indications.[13,14] Although, previous researchers have reported that HU reduces total counts of white blood cells (WBC) and neutrophil,[15] the effects of HU on other aspects of immunity have not been fully discovered.

Microparticles (MPs) are complete vesicles derived from the cell membranes; their size ranges from 0.2 to 2.0 mm. Many reports suggested that MPs arise from the activation of the cell membrane or apoptosis.[16-18] In SCD, MPs derived from numerous cellular sources, including RBCs, monocytes, endothelial cells, and platelets. Microparticles were previously studied in many disorders since they mediate intercellular communications and promising as a new biomarker for disease activity.[19] Involvement of cell-derived MPs in the pathophysiology of SCD was reported in few researchers, all in adult patients. Although these studies have reported some MPs abnormalities in SCD, there is a scarcity of data in children with SCD, especially in Egypt. Consequently, we conducted this cross-sectional study for the assessment of MPs profiles in SCD children at the baseline states with no disease-modifying therapy, and with HU, in comparison with healthy children as controls.

## Patients and Methods

### Study Participants

Thirty patients with SCD were recruited from Children's Hospital, Assiut University, from May till August 2017 and were enrolled in this cross-sectional study. All patients were enrolled from Pediatric hematology outpatient clinic during routine follow-up. All were clinically stable and did not had infections or any of sickle cell crises for at least 3 weeks before sampling. We excluded children if they had significant acute sickle cell complications in the 2 weeks before the study. Twenty age- and sex-matched healthy children were enrolled in this study as controls. Ethical Committee approval was obtained from Assiut University. Children with SCD were classified into 2 groups, group 1: patients not on HU, group 2: patients on HU and the controls were classified as group 3.

Group 2 patients were categorized as being on HU therapy if the patient was taking HU for at least one year. The 2 primary indications for HU were at least 3 painful crises per year requiring hospitalization or recurrent episodes of acute chest syndrome. Hydroxyurea was adjusted at the dose of 20 to 30 mg/kg/d according to hematologic tolerance. If the treatment history was unclear, we excluded the cases from the analysis.

### Flow Cytometric Detection of MPs

All samples were collected in citrate tubes. Within 15 minutes after collection, cells were removed by centrifugation at 20°C for 20 minutes at 1550 × *g*. Then, 250 µL of plasma were centrifuged at 18 800 × *g* at 20°C for 30 minutes. The supernatant was removed after centrifugation, and the pellet was resuspended in phosphate-buffered saline (PBS) and centrifuged at 18 800 × *g* at 20°C for 30 minutes. The supernatant removed again, and MPs pellet was resuspended in PBS. Then, 5µL of MPs were diluted with 35 µL PBS containing 2.5 mM $CaCl_2$. All samples were incubated at room temperature in the dark for 20 minutes with 5 µL of fluoroisothiocyanate (FITC)-conjugated annexin V (IQ products, the Netherlands), 5 µL of PE-conjugated and 5 µL Per-CP cell-specific antihuman monoclonal antibody in each tube according to the following panel. AnnexinV/CD146 (Beckman Coulter, France)/CD45 (Becton Dickinson (BD) Biosciences, San Jose, California). Annexin V/CD15 (Beckman Coulter)/CD14 (BD Biosciences), and Annexin V/CD235 (BD Biosciences)/CD41 (EXBIO Praha, Nadsafinou, Czech Republic). The PBS/calcium buffer were added after incubation, and the samples were analyzed on FACSCaliber flow cytometry with Cell Quest software (Becton Dickinson Biosciences). Fifty thousand events were acquired. For each sample, isotype-matched negative controls (antihuman immunoglobulin G) were used. The MPs were identified according to the forward scatter of calibrating reference beads of 1.0 µm that used to calibrate the size range of MPs (Latex beads, amine-modified polystyrene, fluorescent red aqueous suspension, 1.0 µm mean particle size, Sigma-Aldrich ChemieGmbhMunich, Germany). Also by their positivity for annexin V. Total MPs had lower size than that of the reference beads and express annexin V (Figure 1). Total MPs were recorded as a percentage of the total events. The MPs subpopulation as platelet, monocytic erythrocyte, endothelial, and neutrophilic were expressed as percentages of total MPs. Platelet MPs were $CD61^+$. Erythrocyte, monocytes, and neutrophilic MPs were $CD235^+$, $CD14^+$, and $CD15^+$, respectively. Endothelial MPs was $CD146^+$ $CD45^-$.

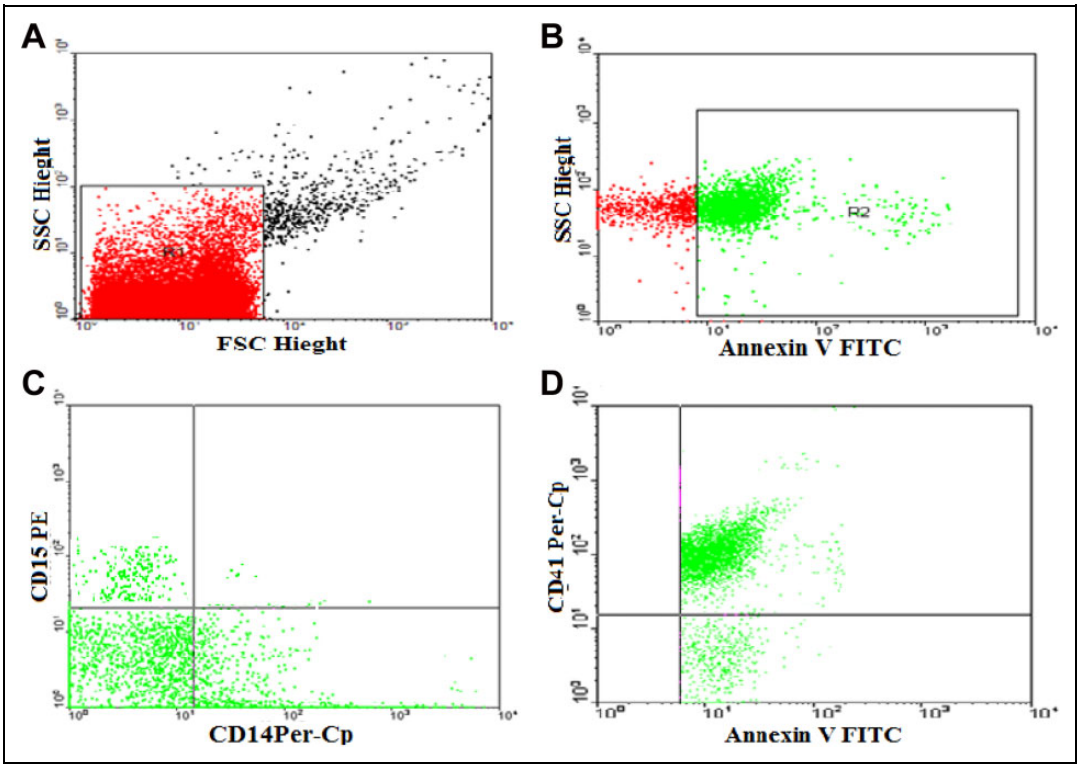


**Figure 1.** Flow cytometric analysis of microparticles. Forward and side scatter histogram was used to define the microparticles (MPs) (R1) according to the size of the reference calibrate bead (A). Events defined as MPs were then selected for their annexin V binding, determined by the positivity for annexin V (R2) (B). Then, annexin V-positive MPs (total MPs) were further examined for the expression of cell-specific antibodies as CD15, CD14, and CD41 (C, D) as an example of MPs subpopulations (not shown).

### *Statistical Analysis*

We used the statistical package for social sciences (SPSS), version 17 for data analysis. All data were expressed as mean (standard deviation, SD). Differences between the groups were studied by 1-way analysis of variance. A $P$ value of $\leq .05$ indicated a statistically significant difference. The correlations between the different studied parameters were examined by Pearson correlation coefficient.

## Results

The mean age of the patients with SCA on HU (group 2) was 10.73 (2.1) years, which is comparable to that of those without HU (group 1), 11.25 (1.6) years and to the controls (group 3) and 11.7 (2.1) years ($P = .3$). There was no statistically significant difference in the proportion of sex in the 3 groups ($P = .9$).

Table 1 shows all hematological parameters among all groups. Group 1 had significantly lower hemoglobin level compared to controls (group 3; $P < .0001$). This level was significantly increased in patients who received HU (group 2; $P < .0001$), but it was still significantly lower than the controls ($P < .0001$). Group 1 patients had significantly higher WBC counts ($P < .0001$) and absolute neutrophil counts ($P = .008$) than controls. Both counts decreased in patients who received HU, only WBCs count showed a significant decrease ($P = .028$), but still significantly higher than normal group 3 ($P = .006$). Monocytes and platelets were not significantly different between all study groups. Total lymphocytes were significantly higher in group 1 patients than the healthy controls ($P < .0001$), and their level was decreased significantly in group 2 patients ($P < .0001$), and the difference was not statically significant between group 2 and the controls ($P = .570$; Table 1).

Table 2 shows all types of circulating MPs among the 3 groups. Total MPs, platelet MPs ($CD61^+$), and erythroid MPs ($CD235^+$) were significantly higher in patients who did not receive HU (group 1) than the controls ($P = .017$, $P = .003$, $P < .001$, respectively). They decreased in patients who received HU (group 2), and this decrease was significant only in platelet MPs and erythroid MPs ($P = .049$, $P < .001$, respectively; Table 2). Endothelial MPs ($CD146^+$) was also significantly higher in group 1 patients than controls ($P = .006$), but their level was not significantly reduced in group 2 patients ($P = .64$). Microparticles of other cellular origins as monocytes ($CD14^+$) and granulocytes($CD15^+$) were not significantly different between all study groups (Table 2).

## Discussion

According to previous SCD studies it has been well established the association between SCD and raised total WBC count,[20] and some reports showed abnormal cellular immune activation

**Table 1.** Full Blood Count in Patients With Sickle Cell Disease and Controls.[a,b]

| Parameter | Group 1 (Patients Without Hydra) (11) | Group 2 (Patients on Hydra) (19) | Group 3 (Controls) (20) | *P* Value[c] | *P* Value[d] | *P* Value[e] |
|---|---|---|---|---|---|---|
| Hemoglobin (g/dL) | 7.06 (0.87) | 10.04 (1.35) | 11.91 (0.64) | <.0001[f] | <.0001[f] | <.0001[f] |
| RBCs ($10^{12}$/L) | 2.45 (0.5) | 3.33 (0.73) | 4.43 (0.62) | .002[f] | <.0001[f] | <.0001[f] |
| Hematocrit | 22.79 (4.66) | 33.13 (6.91) | 38.99 (5.8) | <.0001[f] | <.0001[f] | .011[f] |
| Platelets ($10^9$/L) | 315.2 (91.6) | 290.9 (65.9) | 271.2 (65.2) | .649 | .243 | .669 |
| WBCs ($10^9$/L) | 11.35 (2.72) | 8.92 (2.3) | 6.4 (2.34) | .028[f] | <.0001[f] | .006[f] |
| Monocytes ($10^9$/L) | 0.28 (0.08) | 0.23 (0.08) | 0.20 (0.09) | .313 | .09 | .514 |
| Neutrophils ($10^9$/L) | 5.95 (2.78) | 4.44 (2.27) | 3.38 (1.67) | .171 | .008[f] | .293 |
| Total lymphocytes ($10^9$/L) | 4.16 (0.18) | 3.16 (0.35) | 3.06 (0.29) | <.0001 | <.0001 | .570 |

Abbreviations: ANOVA, analysis of variance; RBC, red blood cells; SD, standard deviation; WBC, white blood cells.
[a]One-way ANOVA test.
[b]Data represented as mean (SD).
[c]Group 1 versus Group 2.
[d]Group 1 versus controls.
[e]Group 2 versus controls.
[f]Significant.

**Table 2.** Circulating Microparticles in Patients With Sickle Cell Disease and the Controls.[a,b]

| Percentage (%) | Group 1 (Patients Without Hydra) (11) | Group 2 (Patients on Hydra) (19) | Group 3 (Controls) (20) | *P* Value[c] | *P* Value[d] | *P* Value[e] |
|---|---|---|---|---|---|---|
| Total MPs | 77.18 (9.10) | 68.84 (10.34) | 65.70 (11.65) | .108 | .017[f] | .630 |
| Platelet MPs ($CD61^+$) | 75.09 (9.27) | 65.47 (10.70) | 61.35 (10.79) | .049[f] | .003[f] | .441 |
| Neutrophilic MPs (CD15) | 18.06 (3.34) | 17.52 (3.76) | 16.00 (5.17) | .943 | .418 | .517 |
| Endothelial MPs ($CD146^+/CD45^-$) | 22.45 (5.24) | 19.78 (10.78) | 13.00 (4.96) | .640 | .006[f] | .024 |
| Monocytic MPs ($CD14^+$) | 20.42 (2.76) | 20.56 (4.40) | 18.26 (5.21) | .996 | .411 | .256 |
| Erythroid MPs ($CD235a^+$) | 16.09 (5.77) | 9.47 (3.38) | 7.30 (2.42) | <.001[f] | <.001[f] | .175 |

Abbreviations: ANOVA, analysis of variance; MPs, microparticles; SD, standard deviation.
[a]One-way ANOVA test.
[b]Data represented as mean (SD).
[c]Group 1 versus Group 2.
[d]Group 1 versus controls.
[e]Group 2 versus controls.
[f]Significant.

in some cell types.[21,22] Hydroxyurea therapy was FDA approved more than 15 years ago to alleviate SCD manifestations, and with its use in a large-scale, multiple studies proved its beneficial effects.[23]

This study assessed MPs in pediatric patients with SCD under HU therapy and patients who are not receiving it in comparison with healthy controls.

The total leukocytic counts and the differential counts of children with SCD not on HU were significantly higher than those who received the medication, and the overall mean WBCs of $11.35 \pm 2.72 \times 10^9$/L among the patients with SCD doubles value found in healthy children ($6.4 \pm 2.34 \times 10^9$/L). Our results are similar to the findings from other studies.[24]

Regarding MPs in adult patients with SCD, many studies were done.[25-27] However, only paucity of data is available in children with SCD.[28] A previous report[29] described that all MPs from different cell origins, for example, monocytes, granulocytes, and endothelial cell-derived MPs, were found in SCD children; however, the same study found erythrocyte-derived MPs and platelet-derived MPs were the most common variety.[29] Our study showed the same findings with significantly higher numbers of platelet MPs ($CD61^+$) and erythroid MPs ($CD235^+$) in patients with SCD not on HU when compared to healthy children. The age-related decrease in HbF during childhood was linked with an upsurge in the levels of circulating MPs; predominantly that originated from monocytes and platelets and to a minor level from erythrocytes.[28] A previous study reported a positive correlation between the induction of HbF formation by HU therapy (which decreases polymerization of HbS), and the reduction in the levels of MPs, mainly those of erythrocyte and platelet source.[30] This is in agreement with our findings that children under HU therapy have reduced MPs levels in comparison to those who do not receive HU, especially platelet MPs and erythroid MPs which showed significant reductions.

This study had some limitations. First, the cross-sectional design which involves a single time point for all study population. As our findings suggest a desirable outcome of HU on

MPs phenotypes of sickle cell children, it is necessary to conduct a prospective longitudinal study to evaluate the HU treatment effects thoroughly. Besides, although we included most of our sickle cell children in this study, our sample size was relatively small in all 3 groups. Finally, although performing the research in steady-state patients allowed us to draw insights about the MPs profile of SCD itself, it is possible that patients with acute complications may show some specific changes in their MPs profile. Further studies should include patients with SCD both at steady state accompanied by patients during complications.

## Conclusion

Sickle cell disease children with no therapy showed elevated levels of total, platelet, and erythroid MPs. In contrast, therapy with HU was associated with normalization of MPs. This study provided additional evidence that HU is an effective treatment option in pediatric patients with SCD, as it seems that it decreases the abnormally elevated MPs in those patients.

### Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: All authors approved the manuscript as submitted.

### Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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