Fetal hemoglobin and hydroxycarbamide modulate both plasma concentration and cellular origin of circulating microparticles in sickle cell anemia children

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ABSTRACT

Microparticles are cell membrane-derived microvesicles released during cell apoptosis and activation processes. They have been described as bio-markers in various vascular diseases, including sickle cell anemia, and associated with an increased risk of thrombosis. We investigated the effects of fetal hemoglobin level, a factor known to modulate the clinical expression of sickle cell anemia, and that of hydroxycarbamide treatment which reduces the frequency of vaso-occlusive crises, the canonical clinical manifestation of the disease, on both the plasma concentration and the cellular origin of circulating microparticles. Flow cytometry was used to characterize microparticles in 62 sickle cell anemia children at steady state aged 2 months-16 years; 13 of them were treated with hydroxycarbamide. In untreated children, we observed negative correlations between fetal hemoglobin levels and the absolute plasma concentration of microparticles as well as that of microparticles specifically derived from platelets, erythrocytes, and monocytes. Compared to untreated children, those treated with hydroxyurea showed lower concentrations of total microparticles as a consequence of decreased microparticles shed by platelets and erythrocytes. In conclusion, in our sickle cell patients, neonatal decline of fetal hemoglobin coincided with an increase in circulating microparticles derived from erythrocytes, platelets, and monocytes. Hydroxyurea treatment was associated with a decrease in microparticles derived from erythrocytes and platelets.

Introduction

Microparticles (MPs) are submicrometric fragments (0.1 to 1 μm) shed from the remodeling of plasma membrane in response to cell activation and apoptosis. They express high levels of phosphatidylserine (PS) on their outer leaflet together with surface markers from their cell of origin.[1] Elevated levels of MPs originating from circulating blood cells and endothelial cells have been reported in many vascular diseases associated with an increased risk of both arterial and venous thromboses. MPs have been assumed to play an important role in promoting coagulation, inflammation, and vascular dysfunction.[2,3]

Sickle cell anemia (SCA), a hemoglobinopathy resulting from the presence of sickle hemoglobin (HbS), is characterized by chronic hemolysis and recurrent vascular occlusions triggered by red blood cell (RBC) and leukocyte adhesion to the vascular endothelium. Moreover, the disease is associated to an hypercoagulable and pro-inflammatory state as well as endothelial dysfunction.[4] Previous studies reported an increase in circulating microparticle concentration in SCA adults compared to healthy controls in steady-state condition,[5,6] as well as in crisis.[7] Fetal hemoglobin (HbF) level, a widely recognized modulator of SCA severity,[8] declines rapidly during the neonatal period. Setty *et al*. previously demonstrated that a high level of HbF in children was associated with a reduced erythrocyte-derived MP level and thrombin generation.[9] Erythrocyte-derived MPs are known to expose a high amount of PS at their outer surface, thus providing an anionic phospholipid surface suitable to the assemblage of the tenase and prothrombinase complexes. Based on these overall data, MPs have been hypothesized to be both bio-effectors involved in SCA pathophysiology and witnesses of cellular activation and/or apoptosis associated with the disease.

However, no data are currently available on MPs derived from other circulating blood cells or from endothelial cells in SCA children, although these cells clearly participate in the pathophysiology of the disease. To characterize MPs during infancy and childhood in SCA patients, we determined the plasma concentration and the cellular origins of MPs in 62 SCA children by flow cytometry, together with the HbF level. In addition, because treatment with hydroxycarbamide (HC) is known to modulate the clinical course of SCA[10] through an increase of HbF level, at least partly, we also compared the MP profiles of children with and without HC-treatment.

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Design and Methods

Subjects

The study included 62 consecutive SCA children followed either at the Sickle Cell Disease Reference Centres of the University Hospital of Pointe-à-Pitre, Guadeloupe (n=41) and of the Robert Debré Mother and Child University Hospital in Paris (n=21), France. Overall, 27 boys and 35 girls between 2 months and 16 years of age were included. All children were at steady-sate, i.e. free of any acute events for one month prior to blood sampling and transfusion-free for at least three months prior to blood sampling. Among the 62 children, 49 had never received HC and 13 had been treated with HC for at least six months at the time of the study, with an average dose of 21.8±3.2 mg/kg per day. This latter group was compared to a control group composed of 26 SCA children matched for sex and age, selected from the 49 SCA children untreated by HC. All the children's parents provided their written consent before inclusion in the study which had been approved by the ethical committees from Guadeloupe and Paris.

Laboratory methods

The diagnosis of SCA (homozygous SS) was established by isoelectrofocusing (Multiphor II™ System, GE Healthcare, Bucks, UK), citrate agar electrophoresis, and cation-exchange high performance liquid chromatography (VARIANT™, Bio-Rad Laboratories, Hercules, CA, USA) and confirmed by DNA studies for most of them. Hematologic parameters were obtained with an MAXM AL automated cell counter (Beckman Coulter, Miami, FL, USA).

Isolation of microparticles

Venous blood was collected into 3.2% trisodium citrate tubes and MPs were isolated according to Nieuwland *et al.*[11] with the following modifications. In brief, MPs were extracted from freshly prepared platelet-poor plasma by centrifugation at 18,000g for 20 min at room temperature. The pellet was washed twice in working buffer (WB:10 mM HEPES pH 7.4, 136 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$) containing either 5 mM EDTA (first wash) or no EDTA (second wash). The pellet was finally suspended in WB and stored at -80°C until used.

Flow cytometry analysis

All reagents and buffers were sterile and filtered using a 0.2 μm filter. Extracted MPs (25 μL) were incubated for 30 min in the dark with 45 μL of HEPES buffer (10 mM, pH 7.4) containing 3 mM $CaCl_2$ or 3 mM EDTA (negative control) and 10 μL of fluorochrome-conjugated probes, consisting of fluorescein-isothiocyanate (FITC)-annexin-V (Beckman Coulter) and phycoerythrin (PE)-conjugated specific monoclonal antibodies (MoAbs). MoAbs included anti-CD15 (Lewis X, clone HI98, IgM), anti-CD41 (GPIIb, clone HI98, IgG1), anti-CD106 (VCAM1, clone 51-10 C9, IgG1), anti-CD14 (GPI, clone M5E2, IgG2a), anti-CD235a (Glycophorin A, clone 11E4B-7-6, IgG1) or isotype controls IgG1 (679.1Mc7), IgG2a (7T4-1F5) or IgM (G20-127). MoAbs were obtained either from Beckman Coulter or Becton Dickinson (Franklin Lakes, NJ, USA). Subsequently, 450 μL of HEPES buffer were added, and after a centrifugation at 18,000g for 20 min at room temperature, MPs were re-suspended with 450 μL of HEPES buffer before flow cytometry analysis. For absolute MP quantification, we used Flow-Count™ fluorospheres (Beckman Coulter). Flow-Count™ signal was acquired on a LogSS-LogFL3 dot-plot. Since the same volume of MPs and Flow-Count™ suspensions was added and the concentration of Flow-Count™ fluorospheres was known, the absolute count of MPs in the suspension could be determined. Plasma concentrations of MPs were obtained after correction for the dilution factor. The acquisition gate for MPs was standardized using the Megamix kit, a blend of size-calibrated fluorescent microbeads (0.5, 0.9 and 3 μm) (Biocytex, Marseille, France) according to the supplier's instructions. Sample data were acquired on a FC500 MCL flow cytometer (Beckman Coulter) using the CXP acquisition software. MPs were defined as elements smaller than 1 μm positively labeled with annexin-V. Both forward scatter and side scatter were set in a logarithmic gain and acquired at low flow rate for 5 min for all samples.

Statistical analysis

Differences in hematologic parameters and MP concentrations, expressed as median and range, between the various patient groups were assessed using the Mann-Whitney test. Categorized variables were analyzed using Fisher's exact test. Bivariate correlations were estimated by Spearman's rank correlation (ρ).

Results

Relationship between microparticles profiles and HbF level

Figure 1 shows representative flow cytometry dot-plots of MPs stained with FITC-annexin-V that binds to negatively charged phospholipids and thus stains MPs globally, and with PE-congugated anti-CD235a, anti-CD41, anti-CD14, anti-CD15 and anti-CD106 MoAbs which labeled MPs derived from RBCs, platelets, monocytes, granulocytes and endothelial cells, respectively. Hematologic characteristics and MP profiles of the SCA children not under HC-treatment classified into two age categories are shown in Table 1. Compared to SCA children older than three years of age, the youngest SCA children, as expected, exhibited higher HbF levels (27.5 *vs.* 7.25%; $P<0.0001$), hemoglobin concentrations (9.15 *vs.* 7.55 g/dL; $P=0.0039$), and RBC counts (3.47 *vs.* 2.7 x 10^{12}/L; $P=0.0137$), and lower reticulocyte counts (156 *vs.* 309 x 10^9/L; $P=0.035$). Lower neutrophil and platelet counts were also observed in this latter group (3.4 *vs.* 5.4 x 10^9/L, $P=0.03$; 345 *vs.* 427.5 x 10^9/L, $P=0.035$, respectively). Total MP plasma concentration was significantly lower in the youngest patient group (1,074 *vs.* 6,789 MPs/μl in patients older than 3 years; $P<0.0001$). After correction for multiple testing, we observed that the age-related increased concentration of MPs resulted mainly from an increased concentration of MPs originating from platelets (102.4 *vs.* 5,783.0 MPs/μl; $P<0.0001$) and monocytes (5.9 *vs.* 58.3 MPs/μl; $P=0.003$) while a trend was detected for those derived from erythrocytes (316.5 *vs.* 685.7 MPs/μl; $P=0.01$). No significant differences were detected for the concentration of MPs derived either from endothelial cells or from granulocytes. Since HbF is well known to inhibit HbS polymerization, the primary molecular event of SCA, we analyzed the relationship between HbF level and both the MP plasma concentration and the cellular origin of MPs in our SCA children (Figure 2). We observed negative correlations between HbF level and total MP concentration ($\rho=-0.49$; $P=0.0021$), as well as concentrations of erythrocyte-, platelet-, and monocyte-derived MPs, $\rho=-0.52$ ($P=0.0021$), $\rho=-0.54$ ($P=0.0004$) and $\rho=-0.45$ ($P=0.0054$), respectively. No correlation was detected between the level of HbF and

the concentration of granulocyte- and endothelial cell-derived MPs (ρ=0.06, *P*=0.71; ρ=-0.23, *P*=0.162, respectively) (*data not shown*). Analysis of the relationship between blood cell type count and their corresponding MP concentration revealed a positive correlation only between platelets and PLT-derived MPs (ρ=0.43, *P*=0.0061).

Effects of hydroxycarbamide treatment on the microparticle pattern

Out of the 49 SCA studied children not under HC-treatment, 26 were selected to constitute an age- and sex-matched group to compare with the group of 13 SCA children treated with HC; the MP profiles were then compared between these two groups (Table 2). Except for the mean cell volume (MCV) and the mean cell hemoglobin concentration (MCHC) values which were higher (97.4 *vs.* 79.8 fl; *P*=0.0015) and lower (32.45 *vs.* 33.6 g/100 mL; *P*=0.022), respectively, in HC-treated children compared to those of non-HC-treated children, no difference in the hematologic parameters was observed between the two groups, including Hb and HbF levels. As shown in Table 2, HC-treated children exhibited lower total plasma MP concentration compared to non-HC-treated children (541 *vs.* 8,401 MPs/μl; *P*=0.0001) (Table 2). This decrease mainly affected platelet-derived (7,436.0 *vs.* 194.3 MPs/μl; *P*=0.0056) and erythrocyte-derived (846.8 *vs.* 243.3 MPs/μl; *P*=0.0074) MPs. Although not statistically significant, concentrations of monocyte-, endothelial cell- and granulocyte-derived MPs were also reduced in the HC-treated group.

Discussion

In this study, we determined the quantitative pattern and the cellular origins of microparticles in SCA children and showed the heterogeneity of their cellular origin as well as an age-related increase in their plasma concentration following the physiological decline of HbF expression. Furthermore, for the first time to our best knowledge, we report that HC-treatment is associated with a decrease in total plasma MP concentration, affecting most-

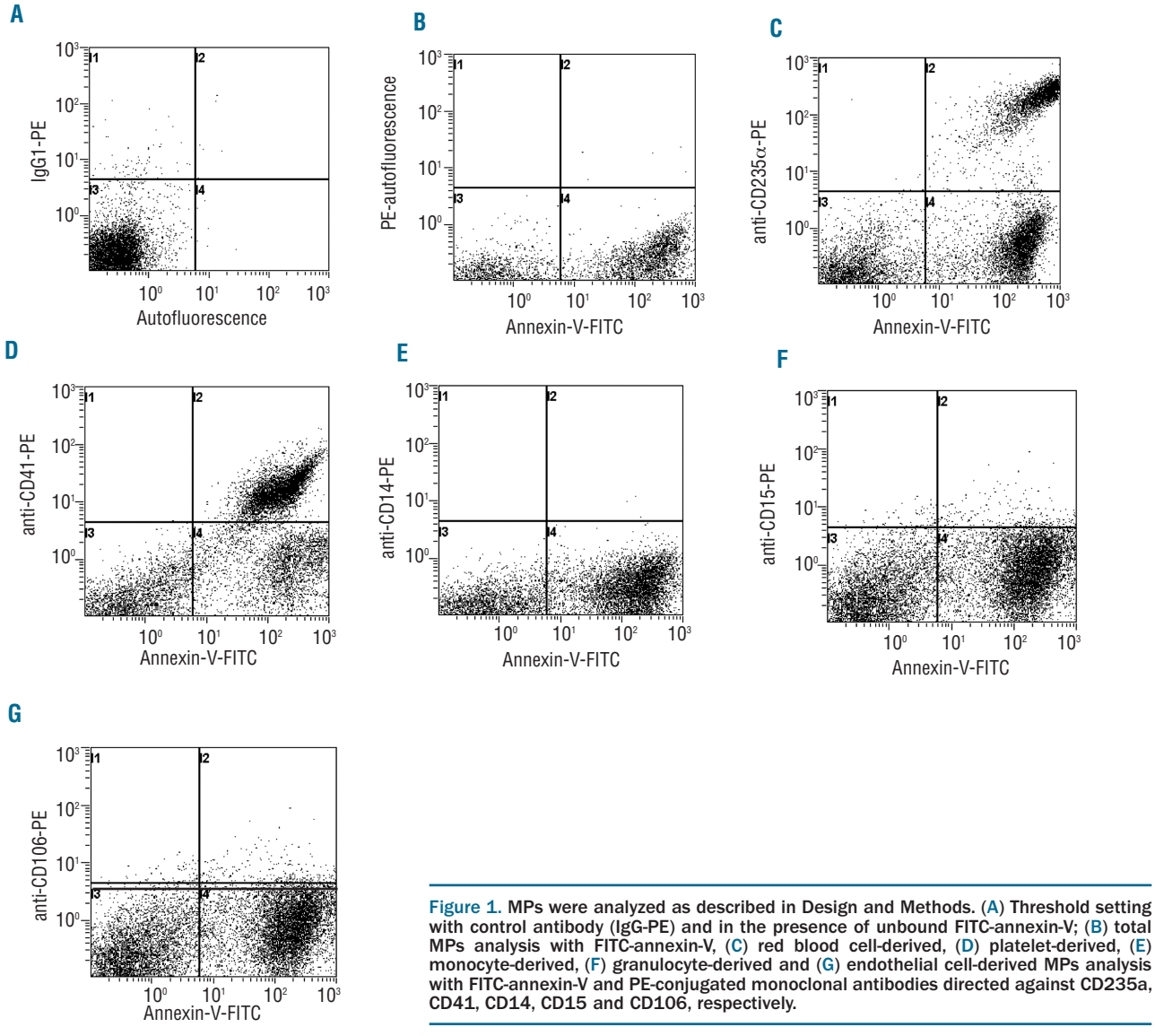

Figure 1. MPs were analyzed as described in Design and Methods. (A) Threshold setting with control antibody (IgG-PE) and in the presence of unbound FITC-annexin-V; (B) total MPs analysis with FITC-annexin-V, (C) red blood cell-derived, (D) platelet-derived, (E) monocyte-derived, (F) granulocyte-derived and (G) endothelial cell-derived MPs analysis with FITC-annexin-V and PE-conjugated monoclonal antibodies directed against CD235a, CD41, CD14, CD15 and CD106, respectively.

ly MPs derived from platelets and erythrocytes.

Overall, the MPs detected in the SCA children included in this study derived mainly from platelets (CD41+) and erythrocytes (CD235a+), and to a much lesser extent from monocytes (CD14+), endothelial cells (CD106+) and granulocytes (CD15+). This MP cellular origin distribution is in agreement with previous studies on adult SCA patients,[5,7,12] although van Beers *et al.* failed to detect the cell-specific MPs that were encountered less frequently in the present study. These discrepancies might be due to differences in the techniques used to isolate and analyze MPs, including the utilized specific monoclonal antibodies which are known parameters affecting MP detection.[13,14]

Because HbF level plays a key role in the clinical expression of SCA[8] and declines rapidly during infancy and childhood,[15] we classified our SCA children into two age groups. We used three years of age as a threshold since, at that age, the switch of fetal to adult hemoglobin, known to be delayed in SCA children, has occurred[15] and we compared the hematologic and MP parameters. Our data show that plasma MP concentration increases with age. Furthermore, we have analyzed the relationship between HbF expression and the concentration of both total and cell-specific MPs. Our results agree with those of Setty *et al.*[9] showing an inverse relationship between HbF level and erythrocyte-derived MPs. According to Allan *et al.*, MPs shed from erythrocytes could result from RBC sickling/unsickling cycles that occur in SCA patients.[7,12] Of greater importance, our results demonstrate for the first time to the best of our knowledge, a negative correlation between HbF level and the concentrations of MPs released by platelets and monocytes, suggesting that HbF, through its inhibitory effects on HbS polymerization and subsequent RBC alterations, is able to modulate platelet and monocyte activation. These relationships remain elusive but could be related to the existing link between the hemolysis-induced decrease in nitric oxide (NO) bio-availability and the resulting platelet and monocyte activation,[16] and support the proposed pivotal role of chronic hemolysis in the hypercoagulability state observed in SCA patients.[17] Further study based on the relationship between MP concentrations and hemolytic biomarker levels are warranted to clarify this point.

Hydroxycarbamide is now considered to be the main pharmacological agent to prevent several SCA complications such as painful crisis or acute chest syndrome and to improve the patient's Quality of Life. The efficacy of HC in the treatment of SCA is generally attributed to its ability to increase HbF.[9,18] However, it has been shown that HC clinical benefit may precede the increase in HbF.[19,20] HC also reduces the number of white cells, platelets and reticulocytes, each of them contributing to the SCA-associated vaso-occlusion and vascular injury.[21-23] Finally, it has been shown that HC-treatment modulates the expression of erythroid and endothelial adhesion receptors and decreases the abnormal activation status of polymorphonuclear neutrophils[24-26] in SCA children. Such observations have led to the hypothesis that HC could have additional cellular targets other than red blood cells.

In our study, HC-treatment is associated with a dramatic reduction in total MPs, with plasma MP concentrations in the group of treated children even lower than those detected in the group of non-HC-treated children under three years of age and thus with the highest level of HbF. Westerman *et al.* have previously shown that HC-treatment leads to a decrease in RBC-derived MPs.[27] We confirm and extend this result by showing that HC-treatment also affects the plasma concentration of MPs originating

Table 1. Hematologic characteristics and plasma MP concentrations of non-HC-treated SCA children according to age. All values are expressed as median and the ranges are indicated in brackets. MP concentrations are given as number of microparticles per μL of plasma. Significant *P* values are in bold. [a]:*P* values remaining significant after correction for multiple testing of MP concentration.

	Patients younger than 3 years of age	Patients older than 3 years of age	*P*
N.	13	36	–
Age (years)	1.25 (0.17 to 2.17)	10.5 (4.3 to 16)	–
Sex ratio (M/F)	6/7	17/19	0.751
Hb (g/dL)	9.15 (7.3 to 11.6)	7.55 (5.7 to 10.3)	**0.0039**
Hb F (%)	27.5 (17.1 to 62.2)	7.25 (2.6 to 19.5)	**< 0.0001**
RBC (10^{12}/L)	3.47 (2.8 to 4.8)	2.7 (2.07 to 4.8)	**0.0137**
Reticulocytes (10^9/L)	156.5 (63 to 387)	309 (84 to 588)	**0.0023**
WBC (10^9/L)	10.25 (4.48 to 14.6)	11.2 (5.3 to 17.9)	0.29
PMN (10^9/L)	3.4 (0.79 to 7.5)	5.4 (1.18 to 13.1)	**0.03**
PLT (10^9/L)	345 (251 to 642)	427.5 (252 to 743)	**0.035**
MP concentration	1,074 (202.6 to 3,097)	6,789 (568.4 to 30,640)	**< 0.0001[a]**
CD41+ MP concentration	102.4 (21.5 to 953.9)	5,783 (106.2 to 28,465)	**<0.0001[a]**
CD235a+ MP concentration	316.5 (71.25 to 2,669)	658.7 (78.72 to 4,278)	**0.01**
CD14+ MP concentration	5.9 (0.46 to 34)	58.3 (1 to 1026)	**0.0003[a]**
CD106+ MP concentration	2.82 (0 to 17)	6.71 (0 to 659.4)	**0.0285**
CD15+ MP concentration	4.3 (0 to 17)	1.3 (0 to 21.8)	0.12

Table 2. Comparison of hematologic characteristics and plasma MP concentrations between SCA children with and without HC-treatment. All values are expressed as median and the ranges are indicated in bracket. MP concentrations are given as number of microparticles per μl of plasma. Significant *P* values are in bold. [a]:*P* values remaining significant after correction for multiple testing of MP concentration.

	Non-HC-treated children	Children treated with HC	*P*
N.	26	13	-
Age (years)	9.6 (2 to 16)	12.6 (5.25 to 15.8)	0.32
Sex ratio (M/F)	8/18	4/9	1
Hb (g/dL)	7.6 (5.7 to 10.6)	8.15 (6.6 to 11.3)	0.34
MCV (fl)	85 (73 to 95)	97.4 (79 to 110)	**0.0015**
MCHC (g/100mL)	33.6 (31.1 to 43)	32.45 (30 to 34.5)	**0.022**
Hb F (%)	11.9 (4.3 to 25)	10.7 (5 to 24.5)	0.98
RBC (10^{12}/L)	2.72 (2.07 to 4.08)	2.62 (2.1 to 4.48)	0.27
Reticulocytes (10^9/L)	292 (81 to 465)	238 (135 to 369)	0.63
WBC (10^9/L)	11.5 (5.3 to 17.9)	9.03 (3.4 to 15.9)	0.168
PMN (10^9/L)	5.6 (1 to 13.1)	4.6 (1.25 to 8.9)	0.48
PLT (10^9/L)	428 (318 to 743)	242.5 (156 to 607)	0.28
MP concentrations	8,401 (641 to 30,640)	541 (196 to 9,550)	**0.0001[a]**
CD41+ MP concentrations	7,436 (8.3 to 28,465)	194.3 (3.9 to 7,955)	**0.0056[a]**
CD235a+ MP concentrations	846.8 (78.7 to 3,147)	243.4 (86.9 to 1,811)	**0.0074[a]**
CD14+ MP concentrations	34 (1.1 to 1,026)	21.3 (0 to 64.38)	0.07
CD15+ MP concentrations	2.73 (0 to 21.08)	0.73 (0 to 29.6)	0.13
CD106+ MP concentrations	3.2 (0 to 330)	1.02 (0 to 24.35)	**0.041**

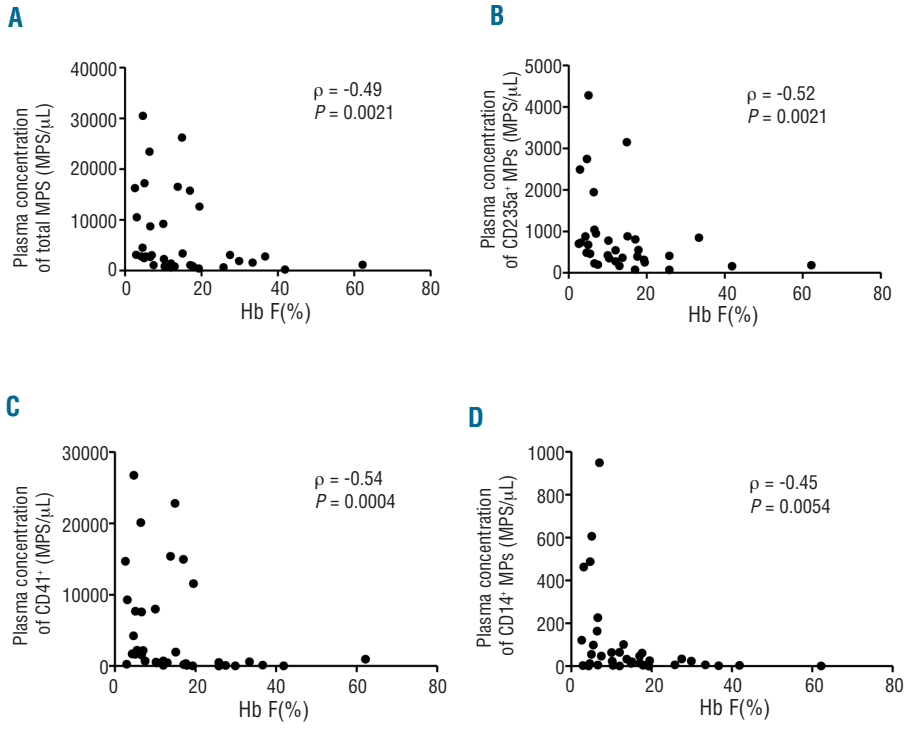

Figure 2. Figure (A) represents correlation between HbF level and total MP concentration. (B-D) Correlations between the HbF level and erythrocyte-, platelet-, and monocyte-derived MP concentrations, respectively.

from platelets. This reduction is, at least partly, in agreement with the known pleiotropic effect of this molecule and raises several issues regarding its cellular targets.

The decrease in erythrocyte-derived MP concentration observed in the HC-treated group was expected and is probably related to the HC-induced inhibition of HbS polymerization, via increased HbF, and thus to the resulting decrease in RBC sickling. However, no inter-group difference in HbF level was observed in our study that may have lacked statistical power. In contrast, higher MCV and lower MCHC values were detected in the HC-treated children, indicating a better RBC hydration, a well-known effect of HC. It is, therefore, tempting to speculate that the HC effect on erythrocyte-derived MP concentration could also be related to a better RBC hydration, a known condition associated with an increase in the HbS polymerization delay-time.

The decrease in MP originating from platelets in the HC-treated group could be linked to an HC-induced reduction in the platelet count, although this was not observed in our limited series of patients. Alternatively, HC is a donor of nitric oxide (NO)[28] and NO is known to inhibit platelet activation via the activation of guanylate cyclase and the accumulation of platelets cGMP.[29,30] In SCA patients, platelets are abnormally activated[31,32] and thus it is possible that through the NO pathway HC reduces the level of platelet activation and thereby the number of platelet-derived MPs.

Although not significant, the lower concentration of endothelial cell-derived MPs in HC-treated children observed in our study may suggest an overall lower activation of endothelial cells directly mediated by HC. This could be supported by the observation that, *in vitro*, HC decreases the expression of VCAM-1, a paradigmal marker of endothelial cell activation, at the surface of endothelial cells in culture.[25] Alternatively, the decrease in MPs shed by endothelial cells in our treated children may reflect a lower injury of these cells indirectly mediated by HC through its overall inhibiting effect on RBC alterations and vaso-occlusion. Clearly further studies are warranted to clarify the HC-induced effect on both platelets and endothelial cells.

The small number of patients included in the present report is clearly one of the limitations of our study. The study may have lacked the statistical power necessary to detect those differences in the concentration of MPs shed by monocytes and granulocytes which could have been expected from the documented HC-induced decrease in the leukocyte count and in the activation level of neutrophil granulocytes.[23,33]

The negative relationship between HbF expression and plasma concentration of MPs, as well as the reduced MP concentrations induced by HC-treatment, may be significant factors in the beneficial effect of these two conditions. Indeed, MPs are not only bio-markers of cellular processes such as apoptosis and activation, but also bio-effectors of various physiological and pathophysiological pathways. For example, several studies have documented the procoagulant properties of circulating erythrocyte-derived MPs isolated from SCA patients.[5,9] A recent study from Donadee *et al.*[34] also showed that erythrocyte-derived MPs obtained from aged stored human red cell units scavenge NO as efficiently as free hemoglobin, illustrating the need for further studies on the impact of these cellular elements on the pathophysiological mechanisms of SCA.

In conclusion, our study describes an age-related increase in total MP concentration in SCA children and negative correlations between HbF level and plasma concentrations of platelet- erythrocyte-, and monocyte-derived MPs, suggesting a link between HbF level and both platelet and monocyte activation in SCA children.

Moreover, our data provide evidence that HC-treatment is associated with a decrease in total MP concentration, affecting mostly those derived from platelets and RBCs, and thus suggest that, like erythrocytes, platelets might also be targets of HC.

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