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Short title: HC decreases PS exposure on RBC-derived MPs

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Hydroxycarbamide (HC) treatment has been shown to improve the clinical course of patients with sickle cell anaemia (SCA), a haemoglobinopathy resulting from a single base substitution in the β-globin gene **(HBB)**. SCA is characterized by chronic haemolytic anaemia and recurrent vascular occlusions leading to multisystemic complications. Several beneficial biological effects of HC have been documented (Halsey *et al*, 2003) but its impact on the concentration of circulating microparticles (MPs), a subtype of extracellular vesicles **released from cytoplasmic membrane of activated or apoptotic cells** and detected at high levels in sickle plasma, remains controversial (Hebbel *et al*, 2016). The aim of the present study was to compare the MP pattern in a group of SCA patients treated by HC and followed for 2 years.

Twenty-six adult SCA patients (36.7±12.2 years, 12 males/14 females) regularly followed by the sickle cell centre of Guadeloupe were enrolled consecutively (ethical committee agreement: Academic Hospital of Pointe-à-Pitre, DRCI-CHUPAP, 230214, Guadeloupe). All patients were at steady-state at inclusion, i.e. no blood transfusion in the previous 3 months and no acute complication in the previous 2 months. Blood was collected before HC therapy and after 1, 3, 6, 12 and 24 months of treatment. Consent was obtained from all patients. SCA diagnosis was performed using standard procedures and haematological parameters obtained using an automated cell counter. **MPs were isolated from plasma and analysed using flow cytometry** (see **supplemental data**). **The procedures used allowed the detection of MPs with a diameter ranging from 400 nm to 1 µm originated from erythrocytes, platelets, granulocytes, monocytes and endothelial cells. Statistical analyses are described in supplemental data.**

HC doses were progressively increased and were stable after 6 months (Table 1). As expected, foetal haemoglobin level and mean cell volume (MCV) values increased after 1 month of treatment while haemoglobin level and haematocrit rose at 3 months. Reticulocyte count decreased after 3 months of treatment while a decline of white blood cell count was

detected after 6 months. All these haematological parameters remained stable after 6 months of HC treatment. Less consistently, a decline of platelet count was also observed.

We did not detect any significant variation of MP blood concentration in HC-treated patients (Table 2). Contrasting results have been published on the impact of HC treatment on MP concentration in SCA patients. We and others have previously described reduced plasma concentration of MPs originated from red blood cells (RBC) and platelets in HC-treated patients (Nébor *et al*, 2013; Westerman *et al*, 2008; Tantawy *et al*, 2013). Two other studies detected increased levels of MPs in treated patients (Brunetta *et al*, 2015; Piccin *et al*, 2015) while one report did not observe MP concentration variation in HC treated-patients (Kasar *et al*, 2014). Differences in pre-analytical and analytical procedures, known to significantly affect MP quantification, are unlikely to fully account for these discrepancies since the same procedures have been used in the present report and our previous publication (Nébor *et al*, 2013). It is worthwhile to notice that the previous reports were based on cross-sectional studies while MP concentrations are known to vary greatly from one patient to another one (Hebbel *et al*, 2016). Our current design, with a longitudinal follow-up of patients, considerably lowers the inter-individual variability of MP concentration and thus the related bias. To our knowledge, this is the first time that the impact of HC on circulating MPs is tested using a longitudinal study design.

We pursued this analysis by comparing the qualitative features of MPs originated from RBC, the major cellular target of HC, namely the forward scatter (FS) and the mean fluorescence intensity (MFI) of Annexin V, two parameters related to the MP size and the density of externalized phosphatidylserine, respectively. Overall, we detected significantly higher FS indexes ($p = 0.02$) and lower MFI values ($p < 0.0001$) in treated patients (Table 2). It is tempting to hypothesize that higher FS indexes are related to the HC-induced increase of RBC hydration since we detected positive correlations between FS and MCV ($\rho = 0.17$, $p = 0.03$)

and HC doses ($\rho = 0.26$, $p = 0.001$). Furthermore, an inverse relationship between HC dose and MFI was observed ($\rho = -0.44$, $p < 0.0001$), suggesting a lowering of PS density at the RBC-derived MPs surface during HC treatment. This latter qualitative modification of RBC-derived MPs may have a significant impact on their properties. Indeed, MPs may promote coagulation by providing a PS positive surface for coagulation reactions. An accelerating effect of RBC-derived MPs isolated from sickle plasma on thrombin generation has been previously described, as well as correlations between RBC-derived MP concentrations and expression of coagulation markers such as prothrombin fragment F1+2 and D-dimer (Hebbel *et al*, 2016). The lower thrombin generation in HC-treated SCA patients (**Gerotziafas *et al*, 2012**) could be related to the HC-induced decrease of PS externalization described for the first time in the present report. **Furthermore, the decrease of PS externalization could be associated with the normalization of protein C level observed in SCA HC-treated patients since RBC-derived MPs have been associated with the consumption of activated protein C (Piccin *et al*, 2015).** It has also been shown that PS externalization of RBC-derived MPs is involved in the retaining of heme on the membrane surface (Camus *et al*, 2015). **The expected lower retention of heme by RBC-derived MP from patients treated by HC could reduce nitric oxide scavenging and may partly encounter for the plasma raise of nitric oxide previously described (Piccin *et al*, 2015).** Other deleterious effects of these MPs, such as increased production of radical oxygen species by endothelial cells, increased erythrocyte adhesion to endothelial cells and endothelial cell apoptosis, were shown to be inhibited by PS saturation using Annexin V (Camus *et al*, 2015).

Altogether, these data strongly suggest that a decrease of PS density at the membrane of RBC-derived MPs may affect their functional properties. Further studies are warranted to determine their functional biological consequences.

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Haematological parameters of SCA patients before and during HC treatment

	Before	1 month	3 months	6 months	12 months	24 months
HC dose (mg/kg/d)	-	9.9 ± 3.3	14.3 ± 5.5	16.3 ± 5.8	16.3 ± 5.6	15.9 ± 5.3
HbF (%)	5.9 ± 4.1	7.0 ± 4.2 ***	10.85 ± 5.5 ***	13.4 ± 7.75 ***	15.2 ± 7.6 ***	14.5 ± 5.8 ***
Hb (g/dL)	8.07 ± 1.0	8.1 ± 0.9	8.6 ± 0.9 ***	8.8 ± 1.1 ***	8.9 ± 1.0 ***	9.0 ± 0.8 ***
Hct (%)	22.6 ± 3.3	22.7 ± 2.7	24.4 ± 2.7 **	24.9 ± 3.5 **	26.2 ± 4.4 **	26.5 ± 2.5 ***
MCV (fl)	83.5 ± 6.8	89.9 ± 8.6 ***	95.7 ± 10.9 ***	100.4 ± 12.1 ***	101 ± 12.7 ***	101.6 ± 10.7 ***
RET (10^9/L)	261 (222-385)	219 (159.5-268)	131 (109-190) **	126 (81-174) **	125 (80-143) **	121 (91-195) ***
WBC (10^9/dL)	8.8 (7.8-11.1)	8.25 (6.35-9.2)	8.25 (6.35-9.2)	6.1 (4.78-7.83) ***	5.7 (4.85-7.13) ***	5.9 (5.03-7.5) ***
PLT (10^9/dL)	392 ± 142	341 ± 143 *	343 ± 173	287 ± 111 **	293 ± 108 **	355 ± 122

Values, expressed as means and standard deviations or medians and interquartile ranges, were compared between groups using ANOVA or Friedman test when appropriated. Hb: haemoglobin; HbF: foetal haemoglobin; Hct: haematocrit; MCV: mean cell volume; RET: reticulocyte; WBC: white blood cell; PLT: platelet. Different from before treatment: *$p<0.05$, **$p<0.01$, *** $p<0.001$.

Qualitative and quantitative blood MP patterns before and during HC treatment

	Before	1 month	3 months	6 months	12 months	24 months
RBC-MP (MP/µl)	1,375 (467-2,505)	670.6 (444-1,164)	962.1 (449-2,085)	732.5 (393-1,314)	876.5 (471-1,442)	1,296 (615-1,914)
PLT-MP (MP/µl)	7,010 (5,410-10,712)	8,810 (5,592-13,301)	10,595 (5,608-18,616)	8,858 (5,824-14,697)	9,678 (6,335-17,645)	12,977 (7,870-18,511)
Mono-MP (MP/µl)	31.6 (0-147.5)	0.765 (0-2.81)	0.73 (0-57.2)	8.11 (0-92.3)	8.11 (0-70.3)	2.91 (0-32.4)
PNN-MP (MP/µl)	137.3 (25-491)	193.2 (47-531)	244.4 (106-661)	171.4 (16-435)	483.7 (193-723)	211.9 (33-619)
End-MP (MP/µl)	64.1 (0-268)	38.1 (0-111)	27.3 (0-153)	56.7 (15-190)	29.95 (1-115)	15.3 (0-68)
RBC-MP FS index	6.9 (6.4-7.2)	7.4 (6.9-8.0)	7.6 (7.0-8.5) *	7.9 (6.85-9.1) *	7.6 (7.4-8.7) **	7.7 (7.2-8.2)
RBC-MP AV MFI	291 (151-443)	277.5 (180-377)	247.5 (119-345)	136.4 (60-276)	78.95 (36-190) ***	30.4 (19-129) ***

Values, expressed as means and standard deviations or medians and interquartile ranges, were compared between groups using Friedman test. Red blood cell: RBC; PLT: platelet; Mono: monocyte; PNN: polynuclear neutrophil; End: endothelial. FS: forward scatter; MFI: mean fluorescence intensity. Different from before treatment: *$p<0.05$, **$p<0.01$, *** $p<0.001$.