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Letter to the Editor

Red blood cells as bioindicators of cardiovascular risk in Kawasaki disease: A case report

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Kawasaki disease (KD) is a rare systemic vasculitis typical of the early childhood. It is characterized by small- and medium-sized blood vessel inflammation that can also lead to coronary artery weakening, aneurysm formation, and myocardial infarction. Therapy with immunoglobulin and aspirin aims to reduce coronary artery inflammation. However, it has been hypothesized that, notwithstanding the efficacy of the pharmacological therapy, patients with regressed KD are at increased risk to develop atherosclerosis after the acute illness.

In this work, the case of a 17-month-old child with KD was studied. The patient was recruited from the Bambino Gesù Hospital of Rome (Italy). Diagnosis of KD was based on the standard clinical criteria [1]. The study was conducted at admission of the patient (naïve patient) and at follow-up. Five age-matched healthy donors (HD) were used for each time point as controls. Informed consent by parents was obtained. This study was approved from the Institutional Review Board of the "Bambino Gesù" Hospital of Rome, Italy.

At admission, the patient reported fever since 5 days, and had urticarial, a non-purulent conjunctivitis, cheilitis and a hyper-echogenicity of coronary arteries without dilatation. Laboratory analyses showed: red blood cell (RBC) and platelet counts in the normal range ($4.26 \cdot 10^6$/μL and $334 \cdot 10^3$/μL, respectively) whereas, in comparison with HD, erythrocyte sedimentation rate (87 mm/h vs 2–20 mm/h in HD) and C-reactive protein values (CRP; 20.19 mg/dl vs 0–0.5 mg/dl in HD) were significantly higher. At eighth day, for persistent fever and for suspected KD, the patient received intravenous immunoglobulin (IVIG: two boluses of 2 g/kg) and high-dose aspirin (100 mg/kg/day). Two days after therapy the patient showed thrombocytosis (platelet number: $644 \cdot 10^3$/μL vs $150–450 \cdot 10^3$/μL) and a significantly reduced number of RBCs ($3.72 \cdot 10^6$/μL vs $4.62 \cdot 10^6$/μL). After 18 days of hospitalization, the patient was free of symptoms and either echocardiographic values or CRP levels (1.36 mg/dl) were in the normal range, but RBC count was still low ($3.35 \cdot 10^6$/μL) and thrombocytosis was still detectable (platelet number: $1.038 \cdot 10^3$/μL). The patient was discharged with the prescription of an anti-aggregating therapy with aspirin (5 mg/kg/day for six weeks). At follow-up, i.e. five months as well as five years after dismissing, the patient showed normal coronary arteries, and also RBC and platelet counts as well as CRP levels were in the normal range ($4.62 \cdot 10^6$/μL, $390 \cdot 10^3$/μL, and 0.21 mg/dl, respectively).

Considering that the major risks in KD progression are the endothelial dysfunction and cardiovascular fatal events [2], and that systemic oxidative stress together with premature aging of RBCs could play a critical role in the cardiovascular complications associated with KD [3], we decided to evaluate, in naïve patient and at follow-up (5 months and 5 years after dismissing) some peripheral blood redox-associated parameters as well as RBC redox- and aging-associated features. In particular, we focused our attention at: i) electron paramagnetic resonance-detectable production of reactive oxygen- and nitrogen-derived species (ROS) in the whole blood, measured by monitoring the oxidation rate of 1-hydroxy-3-carboxypyrrolidine (CPH) spin probe to the correspondent 3-carboxy-proxyl radical (vCP$^\bullet$) [3]; ii) plasma antioxidant power (PAP) [4]; iii) plasmatic levels of asymmetric dimethylarginine (ADMA, a specific endogenous inhibitor of the NO synthase) [3]; and iv) plasmatic levels of myeloperoxidase (MPO), a pro-oxidant enzyme [3]. In addition, by using static and flow cytometry, we evaluated RBC redox and aging markers, i.e. ROS production, total thiol content, expression of glycophorin A (GA, which is down-regulated during RBC senescence) and CD47 (thrombospondin receptor contributing to etherotypic adhesion pattern). Furthermore, externalization of phosphatidylserine (PS)

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at the outer leaflet of the plasma membrane, a marker of RBC aging and death also considered an adhesion molecule, was assessed [5]. Statistical analyses were performed by using Student's t-test. A p value ≤ 0.05 was considered as significant.

As concerns plasmatic measurements in the naïve patient, i.e. before starting IVIG and aspirin therapy, in comparison with HD, the following features were found: (i) a significantly ($p < 0.001$) higher blood CPH oxidation rate and MPO levels ($p < 0.001$); (ii) significantly ($p < 0.001$) lower levels of PAP and ADMA (Fig. 1); (iii) normal values of lactate dehydrogenase (LDH; 393 UI/L vs normal range 230–470 UI/L). Five months and 5 years after dismissing, the levels of CPH oxidation, PAP, ADMA and MPO reached values similar to those measured in plasma from age-matched HD. Conversely, increased values of plasmatic LDH were still detected at follow-up (475 UI/L after five months and 527 UI/L after five years).

As concerns RBCs, in the naïve patient, with respect to HD, cytometric analyses showed: i) no differences in ROS production, total thiol content and CD47 expression; ii) a significantly lower ($p < 0.01$) GA expression, and iii) a significantly ($p < 0.05$) higher percentage of cells showing PS externalization (percentage of Annexin V positive cells) (Fig. 2).

Five months after dismissing, ROS levels in RBCs were unexpectedly significantly higher ($p < 0.05$), whereas total thiol content reached values similar to those detected in RBCs from HD. In addition, GA expression, which was reduced at admission, was significantly ($p < 0.05$) increased, i.e. normal GA levels were restored. At variance, the percentage of Annexin V-positive RBCs was even increased in comparison with admission values. Conversely, CD47 expression was significantly lower ($p < 0.01$) than in HD. Thus, in few words, five months after dismissing, RBCs still appeared oxidized and aged with respect to those detected at admission or in RBCs from HD (Fig. 2).

Finally, five years after the onset of the disease, in the absence of clinical manifestations, CD47 was overexpressed (three times higher than in healthy donors). With respect to HD, no differences in ROS production, total thiol content, GA expression and the percentage of cells showing PS externalization were instead found.

Summarizing, some recommendation seems to emerge from this case report on the long-term management of patients with Kawasaki disease, i.e. that the disappearance of clinical symptoms and the normalization of some laboratory parameters should not be overestimated. In particular, the overexpression of the adhesion-related molecule CD47 on RBCs [6] and the high levels of serum LDH, recently considered a marker of thrombosis risk, should be taken into account [7]. The possibility that these laboratory data could represent critical factors or biomarkers of cardiovascular risk in patients with this rare disease cannot be ruled out. On these bases, the long term monitoring of the patient described here appears as mandatory.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology.

Conflict of interest

The authors report no relationships that could be construed as a conflict of interest.

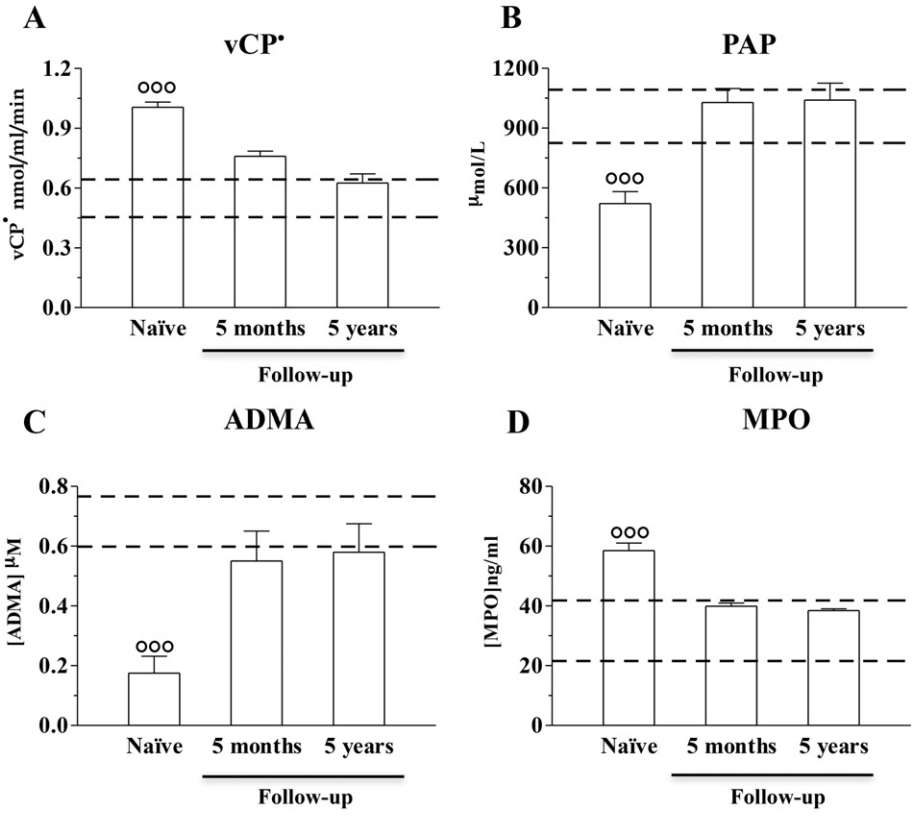

Fig. 1. Redox parameters in the peripheral blood. Histograms ± SD represent: (A) the rate of CPH oxidation to 3-carboxy-proxyl radical (*v*CP•) in the whole blood, and (B) PAP, (C) ADMA and (D) MPO levels in plasma from the KD patient were evaluated in triplicate. *v*CP• was detected by electron paramagnetic resonance, while the concentration of biomarkers was measured by using commercially available immunoassay kits. (°°°) indicates a significant difference ($p < 0.001$) in comparison with the normal range measured in 5 age-matched HD evaluated in triplicate (dashed lines). Note that, at follow-up, other values were in the normal range.

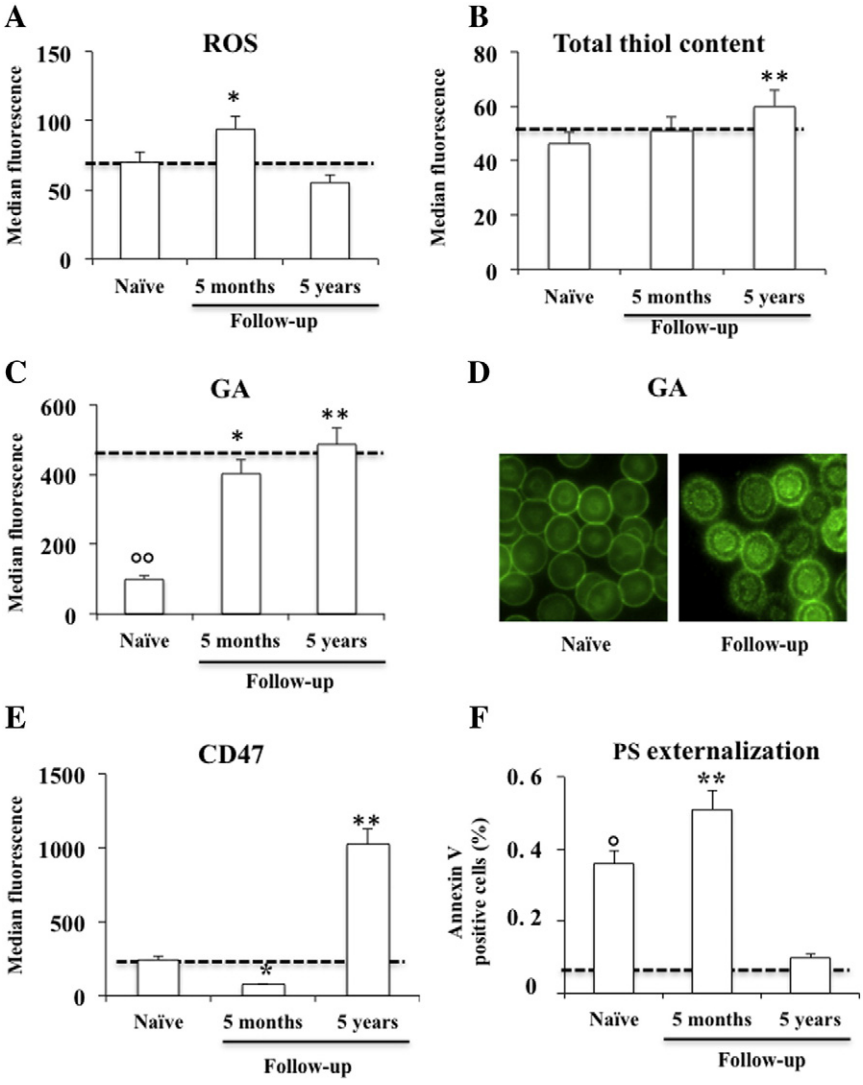

Fig. 2. Redox parameters in RBCs. Histograms ± SD of median fluorescence intensity indicate: (A) ROS production levels, (B) total thiol content, (C) GA expression levels, (E) CD47 expression levels, and (F) the percentage of RBCs displaying PS externalization. These biomarkers were evaluated in triplicate and compared to those obtained in RBCs from 5 HD (dashed lines). Note that: i) no differences were detected in naïve patient with respect to HD as concerns ROS levels, total thiol content and CD47 expression; ii) a very low GA expression (°°$p < 0.01$ naïve patient vs HD) and iii) a high percentage of RBCs with PS at the surface (°$p < 0.05$ naïve patient vs HD). To note that after 5 months: i) a significant increase of ROS levels (*$p < 0.05$ vs naïve patient); a significantly higher expression of GA (*$p < 0.05$ vs naïve patient); a very low expression of CD47 (*$p < 0.05$ vs naïve patient); and a high percentage of RBCs showing PS externalization (**$p < 0.01$ vs naïve patient) were detected. At 5 years of follow-up, with respect to naïve patient, an overexpression of CD47 (**$p < 0.01$) was detectable. Two representative micrographs obtained by static cytometry showing GA distribution in RBCs from naïve patient (left panel) and at 5 years of follow-up (right panel) are shown in (D).

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