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CORRESPONDENCE

Circulating extracellular vesicles and cytokines in congenital and acquired hemolytic anemias

To The Editor:

Extracellular vesicles (EVs) are released by several cell types and are involved in intercellular communication, as well as inflammation and coagulation. Recently, an important pro-inflammatory and pro-coagulant activity has been attributed also to red blood cell (RBC)-derived EVs (REVs), mostly in thalassemia and in sickle cell disease (SCD).[1] In the former, increased amounts of EVs, originated from oxidatively damaged RBCs and platelets, could contribute to the thromboembolic complications frequently observed in the disease. In SCD, EVs from various cellular origins (platelets, RBCs, white blood cells, endothelial cells) participate in the typical inflammation and oxidative stress, along with increased levels of pro-inflammatory cytokines and reactive oxygen species. At variance, little is known on EVs in other hemolytic anemias, such as autoimmune hemolytic anemia (AIHA), paroxysmal nocturnal hemoglobinuria (PNH)[2] and congenital hemolytic anemias (CHAs) due to membrane and enzyme RBC defects. It is known that PNH is a thrombophilic condition with more than 40% of patients experiencing thrombotic events, which are the major cause of disease-related mortality.[3] Likewise, in AIHA thrombosis occurs in 10–20% of cases and is mainly related to disease severity and previous splenectomy.[4] Regarding CHAs, the few thrombotic complications are reported in splenectomised patients with pyruvate kinase deficiency and hereditary stomatocytosis.[5]

In the present study we evaluated EV plasma levels in 116 patients with AIHA, PNH and CHAs and correlated them with retrospective and prospective clinical/hematologic features, including thrombotic complications, splenectomy and other therapies. Furthermore, we correlated EV levels with serum values of immunoregulatory cytokines. The study was approved by the Ethical Committee of Human Experimentation and was conducted in accordance with the Declaration of Helsinki and with the EU ICH GCP Guidelines. All patients gave informed consent.

Patients (63 AIHA, 13 PNH, 19 hereditary spherocytosis, three hereditary stomatocytosis, two hereditary elliptocytosis, seven congenital dyserythropoietic anemia, eight pyruvate kinase deficiency, and one pyrimidine 5′-nucleotidase deficiency) were enrolled from April 1, 2015 to October 1, 2018 and followed prospectively until March 30, 2020. Clinical and hematologic characteristics of the patients' cohort are shown in Table S1 and Figure S1. In summary, male/female ratio was 0.67 and median age 57 years (range 20–91 years). Hemoglobin levels were similar across the three groups (~11 g/dl), whilst LDH was higher in PNH and unconjugated bilirubin and reticulocyte counts greater in CHAs. Median follow-up at sampling was 93 months (range 5–1361 months). At enrolment, 48% of patients were on disease-specific treatment, 13.8% had been splenectomised (15 CHAs and one AIHA) and 12% had experienced a thrombotic episode.

The EVs were tested in peripheral blood by flow-cytometry including the following markers: annexin V-APC, anti-CD41 for platelets, anti-CD142 for tissue factor, anti-CD144 for endothelial cells, and CD235a for RBCs, as previously described and detailed in supplementary materials and methods and in Figure S2. The EV levels in all patients, subgroups and controls are shown in Figure 1 (upper panel). In general, several EVs (mostly erythrocyte-derived) were increased in patients versus controls. In detail, annV-REVs were significantlty higher in AIHA, PNH and CHAs, and annV-EVs in PNH and CHAs (all values corrected for RBC count). Concerning association with hematologic parameters, annV-EVs/RBC and REVs/RBC levels showed a negative correlation with Hb values ($r = -0.30$, $p = .001$; and $r = -0.31$, $p = .001$, respectively). In AIHA patients with LDH levels higher than 1.5xULN, several EVs were higher than in less hemolytic patients, including: annV-EVs ($p = .006$), annV-EVs/RBC ($p < .001$), annV-PEVs ($p = .006$), and annV-TEVs ($p = .03$) (Figure S3). As regards the effect of splenectomy, annV-EVs, REVs, and annV-REVs were significantly higher in splenectomised versus non-splenectomised patients, and versus controls (Figure 1, lower panel). Additionally, PEVs, annV-PEVs, and annV-TEVs were higher in splenectomised versus non-splenectomised subjects, although not significantly (Table S2). The comparison of hematologic and inflammatory markers showed increased median white blood cells (10.5 vs. 6.2 $\times 10^9$/L, $p < .001$), neutrophils (5.4 vs. 3.8 $\times 10^9$/L, $p = .03$), and platelets (470 vs. 211 $\times 10^9$/L, $p < .001$) in splenectomised versus non-splenectomised patients, whilst Hb levels and hemolytic markers were comparable. Regarding other therapies, EVs levels were similar between treated and untreated AIHA patients. At variance, PNH treated subjects displayed higher values of most investigated EVs compared to untreated ones, although not significantly (Table S3). A possible explanation may be that treatment is administered in the most severe cases, and sampling had been performed just before the next eculizumab dose (nadir of terminal complement inhibition).

The EVs were evaluated in patients with or without thrombosis (occurred in 14 patients before enrolment, namely 10 AIHA, two PNH, two CHAs). Venous thromboses included four pulmonary embolisms, two splanchnic thromboses, nine deep venous thromboses of the lower limbs, and two superficial thrombophlebitides; arterial episodes consisted in three strokes/transient ischemic attacks (TIA),

	All	AIHA	PNH	CHAs	Controls
Patients, N (%)	116	63/116 (55)	13/116 (11)	40/116 (34)	40
annV-EVs (N/µl)	953±698	947±739	654±594	1010±1016	746±994
annV-EVs/10^6 RBC (N)	274±258	255±268	189±189*	298±276*	162±213
REVs (N/µl)	245±209	235±158	216±184	251±356	231±142
REVs/10^6 RBC (N)	70±61	64±53	71±55*	74±101	49±32
annV-REVs (N/µl)	69±90	61±56	69±62*	98±125*	49±43
annV-REVs/10^6 RBC (N)	19±30	16±21*	24±27*	28±34**	10±9
PEVs (N/µl)	662±684	631±689	344±531	718±779	599±949
annV-PEVs (N/µl)	377±373	346±377	210±371*	439±439	399±766
TEVs (N/µl)	440±204*	425±195**	434±238	483±250*	567±265
annV-TEVs (N/µl)	41±74	36±79	27±33	64±72	56±62
EEVs (N/µl)	382±291	409±275	398±250	288±227	417±292
annV-EEVs (N/µl)	66±76	75±76	43±63	43±62	72±50

Values are expressed as median±IQR. *, p vs ctr ≤0,05; **, p vs ctr ≤0,01.

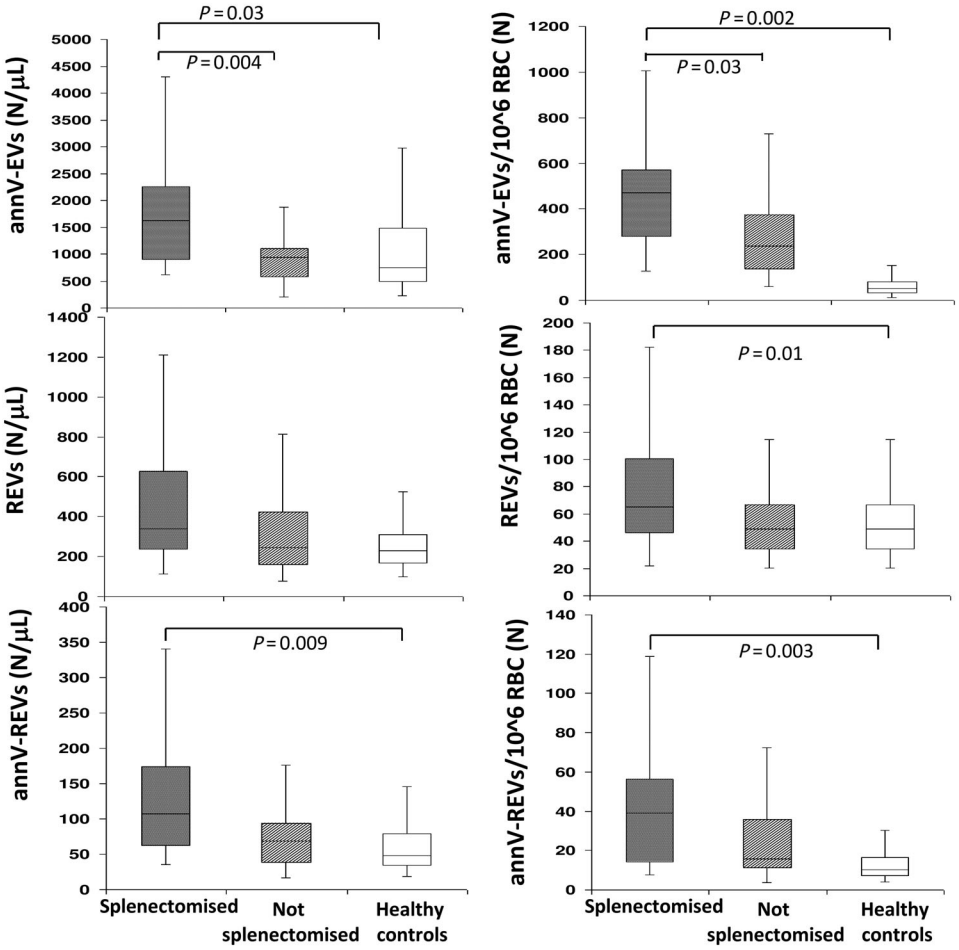

FIGURE 1 Extracellular vesicles in patients with different hemolytic anemias (upper panel) and in splenectomised and non splenectomised patients (lower panel) vs controls. AIHA, autoimmune hemolytic anemia; CHAs, congenital hemolytic anemias; PNH, Paroxysmal nocturnal hemoglobinuria. Extracellular vesicles (EVs) are expressed as number/mL or corrected for red blood cells (RBC). Single and double-stained EVs: annV-EVs identifies EVs espressing phosphatidylserine (PS); REVs indicates EVs derived from RBC; annV-REVs, double stained EVs derived from RBC; PEVs identifies EVs derived from platelets; annV-PEVs, double stained EVs derived from platelets; TEVs indicates EVs expressing tissue factor; annV-TEVs double stained EVs expressing tissue factor; EEVs represents EVs derived from endothelium; annV-EEVs, double stained EVs derived from endothelium

one myocardial infarction, and one retinal artery occlusion (five patients experienced more than one event). At the time of sampling, four patients were on oral anticoagulation, one on low molecular weight heparin and two on anti-platelet agents. Note, EV levels were not different between patients with or without a previous history of thrombosis (Table S4). During the 5-year prospective follow-up, five thrombotic events occurred (three of them in patients who already experienced thrombosis): one acute pulmonary embolism in a splenectomised patient with hereditary stomatocytosis, one subclavian vein thrombosis in a non-splenectomised AIHA, one TIA in an untreated PNH and two superficial thrombophlebitides (one splenectomised pyruvate kinase deficiency and one non-splenectomised AIHA). No clear relationship was found between EVs levels and thrombotic events, although a tendency to more elevated values were observed in subjects experiencing thrombosis. The lack of significance and predictive value may be due to the small number of events, the short follow-up, and EVs sampling not concomitant with the thrombotic event. Moreover, overexpression of EVs represents only one of the several mechanisms underlying thrombosis in the diseases investigated. Considering disease-specific treatments, two PKD patients started mitapivat, one PNH eculizumab (subsequently shifted to ravulizumab), four AIHA relapsed (one twice), and none underwent

splenectomy; two patients died for causes not related to the hematologic disease. Four patients (one AIHA and three PNH) were re-tested for EVs 3 years after the first determination. The AIHA patient was studied after a severe hemolytic crisis, and showed a reduction of annV-EVs, annV-PEVs, annV-TEVs, EEVs, and annV-EEVs, possibly related to the ongoing steroid treatment. The PNH patients displayed an increase of EVs (annV-EVs, annV-EEVs, EEVs, annV-EEVs, TEVs, annV-TEVs, EEVs, and annV-EEVs), including a subject who started therapy with complement inhibitor and one with clear increase of the PNH clone. These findings suggest that EVs levels are more affected by disease severity than by complement inhibition in PNH, in line with previous findings.[6]

Finally, given the interplay among hemolysis, coagulation and immune activation,[3,7] serum levels of different cytokines were tested in the same EV samples. Interleukin (IL)-6 and IL-10 were significantly higher in patients compared with controls. Likewise, IL-17 levels were increased, although not significantly. Tumor necrosis factor (TNF)-α and transforming growth factor (TGF)-β levels were decreased, the former significantly (Figure S4). The same pattern was observed in the subanalysis of AIHA, whilst PNH and CHAs patients showed less clear features. Regarding EVs, TNF-α positively correlated with EEVs ($r = 0.31$, $p = .001$) and REVs ($r = 0.26$, $p = .009$). Moreover, TGF-β positively correlated with annV-EVs ($r = 0.44$; $p < .001$), annV-EVs/RBC ($r = 0.40$; $p < .001$), REVs ($r = 0.26$; $p = .005$), REVs/RBC ($r = 0.25$; $p = .009$), annV-REVs ($r = 0.30$; $p = .002$), annV-REVs/RBC ($r = 0.29$; $p = .002$), PEVs ($r = 0.39$; $p < .001$), annV-PEVs ($r = 0.37$; $p < .001$), TEVs ($r = 0.19$; $p = .04$) and annV-TEVs ($r = 0.26$; $p = .006$) (Figure S5). It may be speculated that the increased levels of TGF-β, a cytokine with well-known anti-inflammatory properties, may be induced to counteract the inflammatory activity of EVs, resulting in fact in down-regulation of TNF-α. Consistently, Chen et al. demonstrated that EVs derived from human bone marrow mesenchymal stem cells increased TGF-β production and suppressed pro-inflammatory cytokines (TNF-α and IL-1β) in vitro.[8]

In conclusion, this is the first retrospective/prospective study aimed at correlating EVs levels of different cell origin with clinical/hematologic parameters and immunoregulatory cytokines in a large series of hemolytic anemias other than SCD and thalassemias. On the whole, we found that EVs were increased in patients compared with controls, and correlated with the severity of anemia and hemolytic features. As expected for diseases involving erythrocytes' destruction, this is particularly evident for RBC-derived EVs. Splenectomised patients displayed further increased RBC-derived EVs, strengthening the scavenging role of the spleen, particularly in CHAs. Increased leukocytes and platelets in splenectomised subjects may have contributed to the higher platelet- and tissue factor-derived EVs observed in these patients, possibly further fueling thrombotic diathesis. In fact, thrombosis is more frequent in splenectomised AIHAs and CHAs.[4,5] Finally, a trend was observed towards increased levels in patients who experienced thrombosis both in the retrospective and prospective follow-up. The increased levels of EVs in patients with more severe/hemolytic disease pinpoint to their pathogenic role in boosting inflammation and add insights into mechanisms of hypercoagulability in hemolytic disorders.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Wilma Barcellini conceived the study, analyzed and interpreted the data, wrote the manuscript and followed patients. Juri A. Giannotta, Bruno Fattizzo analyzed and interpreted the data, wrote the manuscript and followed patients. Anna Zaninoni, Giuliana Merati, Elena Trombetta performed the research, analyzed and interpreted the data and contributed to the manuscript. Andrea Artoni, Marco Capecchi analyzed and interpreted the data and critically revised the manuscript.

REFERENCES

1. Westerman M, Porter JB. Red blood cell-derived microparticles: an overview. *Blood Cells Mol Dis*. 2016;59:134-169.
2. Kozuma Y, Sawahata Y, Takei Y, Chiba S, Ninomiya H. Procoagulant properties of microparticles released from red blood cells in paroxysmal nocturnal haemoglobinuria. *Br J Haematol*. 2011;152:631-639.
3. Hill A, Kelly RJ, Hillmen P. Thrombosis in paroxysmal nocturnal hemoglobinuria. *Blood*. 2013;121:4985-4996.
4. Barcellini W, Zaninoni A, Fattizzo B, et al. Predictors of refractoriness to therapy and healthcare resource utilization in 378 patients with primary autoimmune hemolytic anemia from eight Italian reference centers. *Am J Hematol*. 2018;93:E243-E246.
5. Grace RF, Bianchi P, van Beers EJ, et al. Clinical spectrum of pyruvate kinase deficiency: data from the pyruvate kinase deficiency natural history study. *Blood*. 2018;131:2183-2192.
6. Weitz IC, Razavi P, Rochanda L, et al. Eculizumab therapy results in rapid and sustained decreases in markers of thrombin generation and inflammation in patients with PNH independent of its effects on hemolysis and microparticle formation. *Thromb Res*. 2012;130:361-368.
7. Zaninoni A, Fermo E, Vercellati C, Marcello AP, Barcellini W, Bianchi P. Congenital hemolytic anemias: is there a role for the immune system? *Front Immunol*. 2020;23(11):1309.
8. Chen W, Huang Y, Han J, et al. Immunomodulatory effects of mesenchymal stromal cells-derived exosome. *Immunol Res*. 2016;64:831-840.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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