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ORIGINAL ARTICLE

Erythrocyte oxidative stress is associated with cell deformability in patients with retinal vein occlusion

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Essentials

- Retinal vein occlusion (RVO), characterized by blood hyperviscosity, has an unclear pathogenesis.
- We aimed to find out if hemorheological profile is altered by oxidative stress in RVO patients.
- Red blood cell (RBC) oxidative stress is associated to whole blood viscosity and RBC deformability.
- Reactive oxygen species alter RBC membrane rigidity, playing a key role in RVO pathogenesis.

Summary. *Background:* Retinal vein occlusion (RVO) is characterized by vision loss resulting from hypoperfusion and hypoxia of the retina. RVO pathogenesis is not yet fully understood, although blood hyperviscosity has been observed. Erythrocyte deformability plays a key role in determining blood viscosity, and it is critical to microvascular perfusion and oxygen delivery. It has been shown that oxidative stress-induced erythrocyte membrane fluidity alterations are linked to the progression of cardiovascular diseases. *Objectives:* To determine whether erythrocytes from RVO patients show signs of oxidative stress, and whether this condition can modify the hemorheologic profile in these patients. *Patients and Methods:* We analyzed the entire hemorheologic profile and erythrocyte oxidative stress – reactive oxygen species (ROS) production and membrane lipid peroxidation – in 128 RVO patients and 128 healthy subjects, matched for age and sex. Fluorescence anisotropy was used to evaluate the fluidity of erythrocyte membranes. *Results:* In RVO patients, erythrocyte oxidative stress was present and positively correlated with whole blood viscosity and erythrocyte deformability. Multivariate linear regression analysis after adjustment for age, cardiovascular risk factors, medications, leukocyte number and mean corpuscular volume indicated that erythrocyte-derived ROS and erythrocyte lipid peroxidation were significantly and positively correlated with erythrocyte membrane viscosity and deformability. Moreover, *in vitro* experiments demonstrated that ROS have a key role in erythrocyte membrane fluidity. *Conclusions:* Our findings indicate that erythrocyte oxidative stress plays a key role in the pathogenesis of RVO, and pave the way to new therapeutic interventions.

Keywords: blood viscosity; erythrocyte deformability; erythrocyte membrane; oxidative stress; retinal vein occlusion.

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Introduction

Retinal vascular occlusive diseases develop when terminal veins or arteries are occluded, resulting in hypoperfusion and hypoxia of the inner retina. To date, the pathogenesis of retinal vein occlusion (RVO) has not yet been defined. Central RVO and branch RVO are associated with common cardiometabolic risk factors, such as diabetes mellitus, systemic hypertension, and atherosclerotic cardiovascular disease [1,2]. In addition, alterations in fibrinolytic pathways and methionine metabolism appear to play a significant role in the pathogenesis of this disease [3–9], but the results are conflicting. An important issue to consider in the pathogenesis of RVO is that of rheologic alterations (high hematocrit, high plasma viscosity, high erythrocyte aggregation, and reduced erythrocyte

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deformability). Indeed, the capillaries of the retinal periphery are of a lower caliber than the size of erythrocytes, and only very elastic erythrocytes that can shift shape can go through them. Additionally, changes in plasma viscosity are extremely important: patients affected by hyperviscosity syndrome can show bilateral central RVO [10,11].

Erythrocyte aggregation is one of the principal determinants of blood viscosity at low shear rates (slow flow). The retinal venous circulation, which is characterized by a slow circulatory rate and a high resistance to flow, creates a favorable environment for the formation of erythrocyte aggregates. Only a few studies have investigated hemorheologic variables in RVO patients as compared with healthy controls [5,12]. In a case–control study carried out in RVO patients, we found a significant association between hemorheologic alterations and the occurrence of RVO [11]; in addition, some articles have reported a beneficial role for hemodilution therapy [12,13]. The role of oxidative stress in RVO patients has been poorly explored [14,15], and its contribution to hemorheologic abnormalities is still unknown. The objective of the present study was to determine whether erythrocytes from RVO patients show signs of oxidative stress, and whether this condition is associated with an altered hemorheologic profile.

Materials and methods

Study population

The study population comprised 128 consecutive patients with a diagnosis of RVO who had been referred to the Thrombosis Center of the University of Florence, Italy. RVO was diagnosed in all patients within a period ranging from 1 month to 3 months before the examination, at the Department of Oto-Neuro-Ophthalmogical Surgical Sciences of the University of Florence, Italy. RVO was diagnosed by ophthalmoscopic fundus examination revealing disk swelling, venous dilatation or tortuosity, retinal hemorrhages, and cottonwool spots. The control population was composed of 128 healthy subjects selected to be comparable in age and sex with patients from the staff of the University of Florence and/or from their friends or partners. Patients and healthy subjects with a personal history of glaucoma or cardiovascular disease were excluded from the study. In order to identify symptom-free subjects and patients to exclude who were suspected of having any form of vascular disease, a detailed interview addressing personal and familial history was performed. The subjects were classified as having hypertension according to the guidelines of European Society of Hypertension/European Society of Cardiology [16], or if they reported taking antihypertensive medications, as verified by the interviewer. Diabetic subjects were defined according to the American Diabetes Association [17] or on the basis of self-reported data (if confirmed by medication or chart review). Dyslipidemia was defined according to the criteria of the ATP III Expert Panel of the US National Cholesterol Education Program [18]. Current smoking status was determined at the time of physical examination. At the time of blood sampling, all patients were receiving antiplatelet therapy (114 receiving aspirin 75–100 mg daily; 14 receiving clopidogrel 75 mg daily). All participants providedg signed informed consent; the study was approved by the local Ethics Committee, and complies with the Declaration of Helsinki.

Blood measurements

Blood samples were collected from the antecubital vein into evacuated plastic tubes (BD Biosciences, Franklin Lakes, NJ, USA) in the morning, after an overnight fast. Plasma samples were obtained by centrifuging blood at 2000 × *g* for 10 min at 4 °C. A complete blood cell count was obtained by use of the Sysmex XE-2100 hematology analyser (Sysmex, Kobe, Japan), and the fibrinogen concentration was assessed according to the von Clauss method. Whole blood viscosity (WBV) was measured at 37 °C with the Rotational Viscosimeter LS 30 (Contraves, Zurich, Switzerland). WBV was analyzed at shear rates of 0.512 s^{-1} and 94.5 s^{-1}. The erythrocyte filtration test was used to determine erythrocyte deformability with a microcomputer-assisted filtrometer (model MF4; Myrenne, Roetgen, Germany), as reported by Ramakrishnan *et al*. [19]. The erythrocyte deformability index or elongation index (EI) was estimated from a curve indicating erythrocyte filtration throughout a 10-min recording in order to determine the rheologic properties of erythrocytes when they were passed through polycarbonate filters with 5-μm micropores (Nucleopore, Pleasanton, CA, USA). The initial flow rate obtained from the microcomputer-generated curves was used for assessment of the erythrocyte EI. For the different hemorheologic variables, intra-assay coefficients of variation were < 1.6%, and interassay coefficients of variation were < 4.5% (reference values in our laboratory) [11].

Assessment of erythrocyte reactive oxygen species (ROS) generation and lipid peroxidation by flow cytometry

There are many methods for the measurement of oxidant species in cells, but the cell-permeant fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) is the gold standard for directly measuring ROS generation in cells. H_2DCFDA is responsive to hydrogen peroxide, peroxynitrite, and hydroxyl radical. Superoxide anions can also contribute to H_2DCFDA oxidation, albeit to a lesser extent [20]. The BODIPY 581/591 C_{11} fatty acid is a sensitive fluorescent reporter for lipid peroxidation, undergoing a shift from red to green fluorescence emission upon oxidation of the phenylbutadiene segment of the fluorophore. This oxidation-dependent emission shift

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enables fluorescence ratio imaging of lipid peroxidation in live cells. In general, this probe can be incorporated into cell membranes, and is highly responsive to alkoxyl or peroxyl radicals [21].

After collection, 3 μL of EDTA-anticoagulated blood samples was resuspended in 87 μL of RPMI without serum and phenol red, and incubated with anti-human glycophorin A–phycoerythrin (BD Biosciences) (10 μL) at 37 °C in the dark for 15 min, according to the manufacturer's protocol. Next, the cells were centrifuged at 650 x *g* for 6 min at room temperature, the supernatant was discarded, and the cells were washed twice in phosphate-buffered saline (PBS). To determine the level of intracellular ROS generation and lipid peroxidation, cells were incubated with H_2DCFDA (2.5 μM; Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA) and BODIPY 581/591 C11 (5 μM; Invitrogen, Thermo Fisher Scientific Inc.) in RPMI without serum and phenol red for 15 min at 37 °C, respectively. After labeling, cells were washed and resuspended in PBS, and analyzed immediately with a FACSCanto flow cytometer (Becton-Dickinson, San Jose, CA, USA). The sample flow rate was adjusted to approximately 1000 cells s^{-1}. For a single analysis, the fluorescence properties of 100 000 erythrocytes were collected. Data were analyzed with BD FACSDIVA software (Becton-Dickinson).

Erythrocyte membrane preparation

Erythrocyte membranes were prepared with the method of Dodge [22], with buffer modification. Erythrocytes were hemolyzed with 20 mM Tris–HCl buffer (pH 7.4), supplemented with 1 mM EDTA and 0.01% phenylmethanesulfonyl fluoride, on ice for 15 min. The erythrocyte membranes were centrifuged at 20 000 × *g* for 5 min. The membranes were washed several times with the above-mentioned buffer until the 'white ghost' (hemoglobin-free) state was attained. All buffers were cooled to 4 °C prior to use, and the whole preparation procedure was conducted on ice. The protein concentration was estimated according to the method of Bradford [23]. The concentration of protein in the sample was read from a calibration curve in the range 50–300 μg protein mL^{-1}, with albumin from bovine serum as the standard.

Fluidity of erythrocyte membranes

The fluidity of erythrocyte membranes was measured by means of fluorescence anisotropy with two fluorescent probes: 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4′-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA–DPH). DPH is localized in the hydrophobic region near the center of the bilayer, whereas TMA–DPH is incorporated into the polar region of the erythrocyte membrane. Fluorescence anisotropy measurements were carried out with an LS-55 fluorescence spectrometer (Perkin–Elmer, Waltham, MA, USA). The excitation and emission wavelengths were 348 nm and 426 nm, respectively. The cuvette holder was temperature-controlled (37 °C). Erythrocyte membranes were diluted with buffered saline to a protein concentration of 100 μg mL^{-1}. The final concentration of fluorescent probes was 1 μM. The fluorescence anisotropy of probe X is defined as:

$$r(\mathrm{X}) = (\mathrm{Ivv} - \mathrm{Ivh} \bullet \mathrm{G})/(\mathrm{Ivv} + 2\mathrm{Ivh} \bullet \mathrm{G})$$

where I_{vv} and I_{vh} are the intensities of the fluorescence (in arbitrary units) emitted, respectively, parallel with and perpendicular to the direction of the vertically polarized excitation light, and G is the correction factor ($G = I_{hv}/I_{hh}$) for the optical system, given by the ratio of the vertically to the horizontally polarized emission components when the excitation light is polarized in the horizontal direction. X represents, respectively, DPH or TMA–DPH. According to Shinitzky [24], the fluorescence anisotropy values are inversely proportional to cell membrane fluidity. A high degree of fluorescence anisotropy represents a high degree of structural order or low cell membrane fluidity.

In vitro 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced blood oxidation

To demonstrate the key role of oxidative stress in erythrocyte alterations, an *in vitro* approach was also used. Blood samples (40 mL) from 10 control subjects were collected in Vacutainer tubes containing EDTA (0.17 mol L^{-1}). After collection, 20 mL of blood was incubated at 37 °C for 2 h in the presence of (50 mM final concentration) AAPH – a water-soluble azo compound used extensively as a free radical generator – and the remaining 20 mL of blood were maintained at 37 °C for 2 h without treatment. Following this, all experiments were performed in treated and untreated samples.

Statistical analysis

Statistical analysis was performed with SPSS for Windows (SPSS, Chicago, IL, USA) (Version 20.0). The non-parametric Mann–Whitney test for unpaired data was used for comparisons between single groups. The χ^2-test was used to test for proportions. Correlation analyses were performed with Spearman's correlation test. A logistic regression analysis was used to evaluate the risk of RVO according to the cardiovascular risk factors, erythrocyte ROS production, and erythrocyte membrane lipid peroxidation. For logistic regression analysis, odds ratios (ORs) and 95% confidence intervals are presented. A P-value of < 0.05 was considered to be statistically significant. Variables showing, in the univariate logistic regression analysis, a significant association with disease were introduced into the multivariate logistic regression model. In order to study the association between erythrocyte membrane viscosity and deformability, a linear regression analysis was performed by introducing age, sex, cardiovascular risk factors,

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medications, leukocyte number, MCV, erythrocyte ROS production and erythrocyte membrane lipid peroxidation into the linear regression model in RVO patients.

Results

The demographic and clinical characteristics of RVO patients and healthy subjects are reported in Table S1.

Hemorheologic parameters

With regard to hemorheologic profile, a significant difference in WBV between patients and controls, analyzed at shear rates of 0.512 s^{-1} (24.07 ± 3.89 versus 20.06 ± 3.27, $P < 0.001$) and 94.5 s^{-1} (4.63 ± 0.46 versus 4.09 ± 0.41, $P < 0.001$), was observed. The EI in patients differed significantly from that in controls (0.343 ± 0.026 versus 0.431 ± 00031, $P < 0.001$).

Erythrocyte oxidative stress

Flow cytometry analysis was used to evaluate erythrocyte oxidative stress. As shown in Fig. 1, erythrocytes from RVO patients had significantly higher ROS levels than those from healthy controls (685.5 ± 309.7 versus 305.0 ± 79.0, $P < 0.0001$). Moreover, erythrocyte membrane lipid peroxidation levels in RVO patients were significantly higher than those in controls (1431 ± 814 versus 784 ± 142, $P < 0.0001$). No significant difference between any of the traditional risk factors were observed.

Erythrocyte membrane anisotropy

Fluorescent probes were used to evaluate the fluidity of erythrocyte membranes. TMA–DPH and DPH, which are known to reflect membrane fluidity in the hydrophilic layer and in the hydrophobic core of the lipid bilayer,

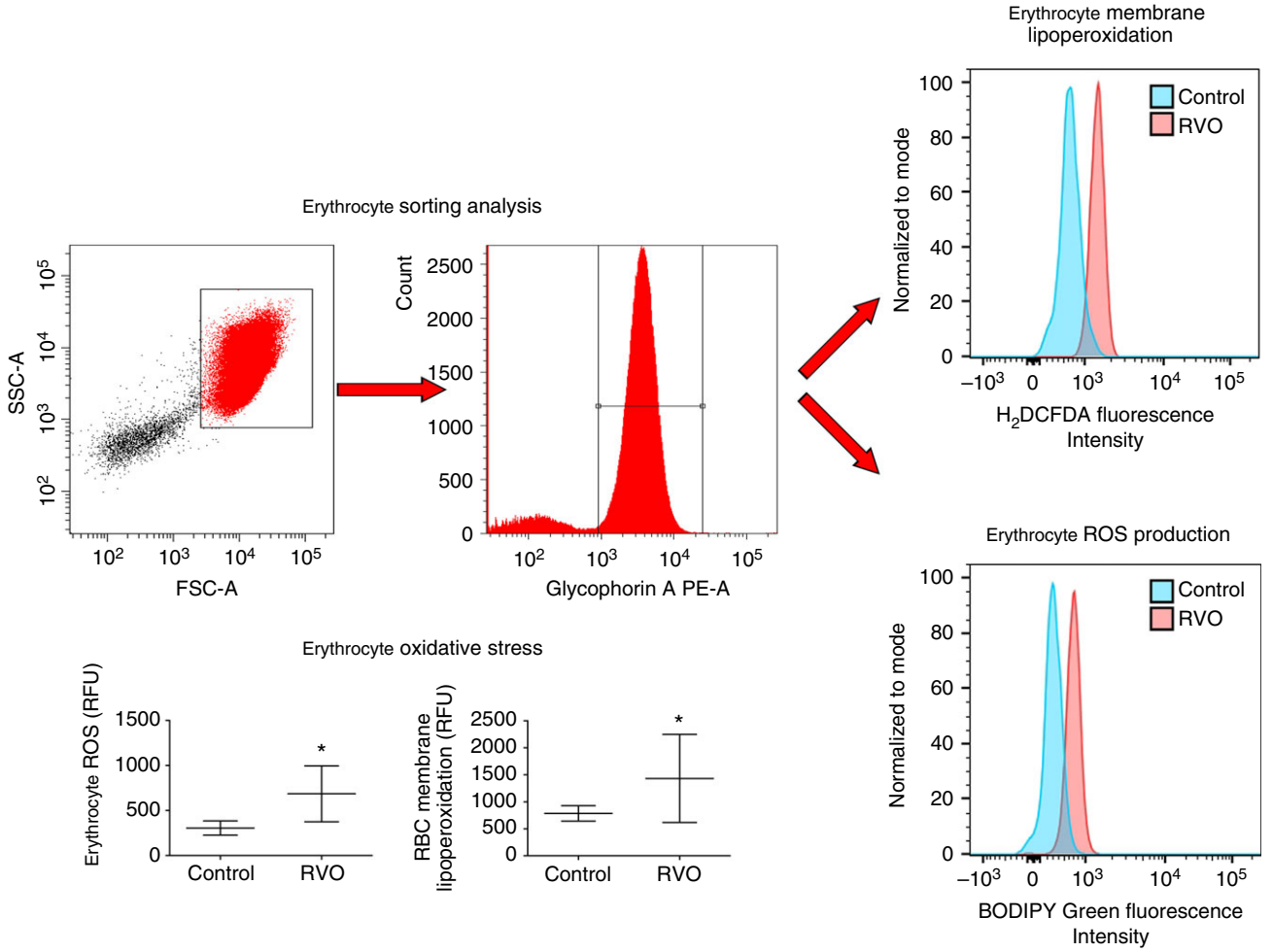

Fig. 1. Erythrocyte sorting strategies and representative fluorescence-activated cell sorting (FACS) plots of erythrocyte reactive oxygen species (ROS) production and lipid peroxidation. As reported in Materials and methods, to determine the level of intracellular ROS production and lipid peroxidation, cells were incubated with the cell-permeant fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (2.5 μM) and BODIPY 581/591 C11 (5 μM) in RPMI without serum and phenol red for 15 min at 37 °C, respectively. After labeling, cells were washed and resuspended in phosphate-buffered saline, and immediately analyzed with a FACSCanto flow cytometer. RFU, relative fluorescence units; RVO, retinal vein occlusion; PE, phycoerythrin. Group mean ± standard deviation of 128 patients or 128 controls is reported.

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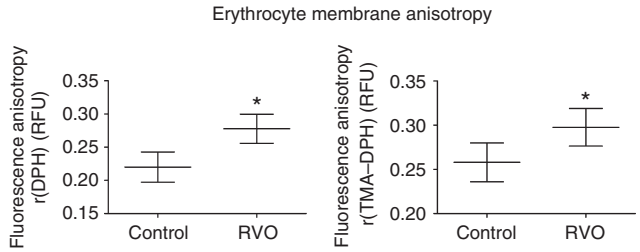

Fig. 2. Erythrocyte membrane fluidity was measured by fluorescence anisotropy with 1,6-diphenyl-1,3,5-hexatriene (DPH) and 1-[4′-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA–DPH) fluorescent probes. DPH is localized in the hydrophobic region near the center of the bilayer, whereas TMA–DPH is incorporated into the polar region of the erythrocyte membrane. A high degree of fluorescence anisotropy represents low cell membrane fluidity/high structural order. *Significant difference versus control at the $P < 0.01$ level. RFU, relative fluorescence units; RVO, retinal vein occlusion.

respectively [25,26], were applied. In Fig. 2, the analyses of fluorescence anisotropy (r) of the erythrocyte membrane are shown. DPH showed significant differences between RVO patients (0.2778 ± 0.0219, $P < 0.0001$) and controls (0.2200 ± 0.0227, $P < 0.0001$); a similar pattern was seen with TMA–DPH, with significant differences between RVO patients (0.2976 ± 0.0213, $P < 0.0001$) and controls (0.2580 ± 0.0221, $P<0.0001$). The change in fluorescent probe fluorescence anisotropy was rather small, but was statistically significant. The effect was observed with both fluorescent probes, which means that fluidity changes occurred on the surfaces of membranes as well as in the lipid core.

Correlation between investigated parameters

As shown in Fig. 3, erythrocyte-derived ROS were significantly correlated with WBV analyzed at a shear rate of 0.512 s^{-1} ($r^2 = 0.3819$, $P < 0.0001$), with WBV analyzed at a shear rate of 94.5 s^{-1} ($r^2 = 0.2948$, $P < 0.0001$), with the EI ($r^2 = 0.2155$, $P < 0.0001$), and with erythrocyte membrane anisotropy: with DPH ($r^2 = 0.4581$, $P < 0.0001$) and with TMA–DPH ($r^2 = 0.4266$, $P < 0.0001$).

Erythrocyte lipid peroxidation was significantly correlated with WBV analyzed at a shear rate of 0.512 s^{-1} ($r^2 = 0.2567$, $P < 0.0001$), with WBV analyzed at a shear rate of 94.5 s^{-1} ($r^2 = 0.2000$, $P < 0.0001$), with the EI

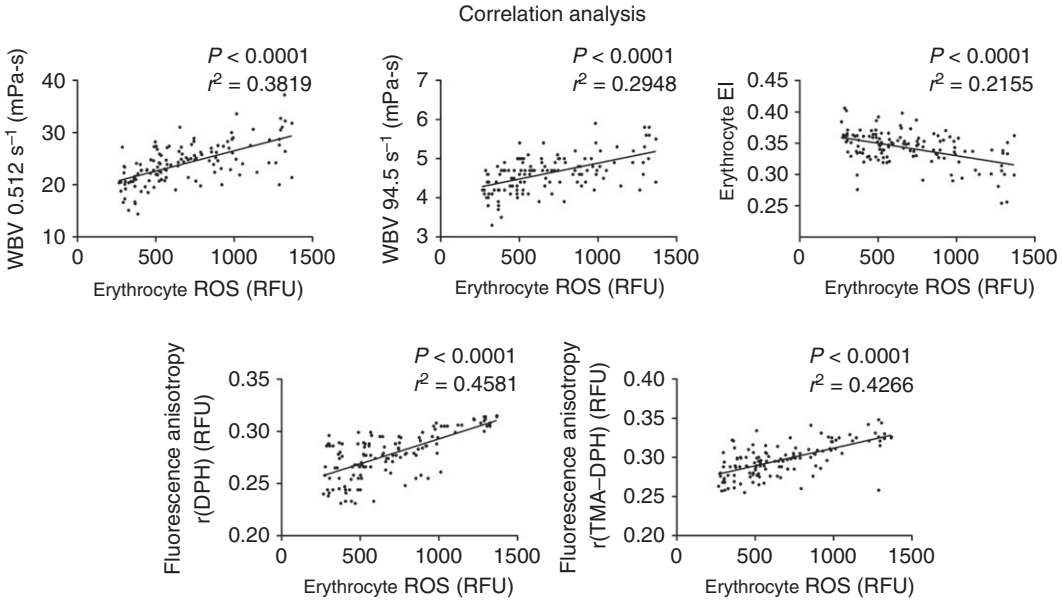

Fig. 3. Correlation analysis among erythrocyte reactive oxygen species (ROS) production, whole blood viscosity (WBV) (analyzed at shear rates of 0.512 s^{-1} and 94.5 s^{-1}), erythrocyte deformability (elongation index [EI]) and erythrocyte membrane fluidity (with 1,6-diphenyl-1,3,5-hexatriene [DPH] and 1-[4′-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene [TMA-DPH] probes) in retinal vein occlusion patients. Erythrocyte ROS production was positively and significantly correlated with WBV analyzed at shear rates of 0.512 s^{-1} and 94.5 s^{-1} ($P < 0.0001$) and with erythrocyte membrane fluidity (with DPH and TMA–DPH probes) ($P < 0.0001$). Erythrocyte ROS production was inversely correlated with erythrocyte deformability (EI) ($P < 0.0001$). RFU, relative fluorescence units.

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($r^2 = 0.3389$, $P < 0.0001$), and with erythrocyte membrane anisotropy: with DPH ($r^2 = 0.3991$, $P < 0.0001$) and with TMA–DPH ($r^2 = 0.3943$, $P < 0.0001$) (Fig. 4).

Figure 5 shows that DPH fluorescence anisotropy was significantly correlated with WBV analyzed at a shear rate of 0.512 s^{-1} ($r^2 = 0.1754$, $P < 0.0001$), with WBV analyzed at a shear rate of 94.5 s^{-1} ($r^2 = 0.1503$, $P < 0.0001$), and with the EI ($r^2 = 0.0987$, $P = 0.0003$).

TMA–DPH fluorescence anisotropy was significantly correlated with WBV analyzed at a shear rate of 0.512 s^{-1} ($r^2 = 0.1482$, $P < 0.0001$), with WBV analyzed at a shear rate of 94.5 s^{-1} ($r^2 = 0.1300$, $P < 0.0001$), and with the EI ($r^2 = 0.1313$, $P < 0.0001$) (Fig. 5).

Among clinical characteristics, no correlation between ROS production or lipid peroxidation and traditional risk factors, platelet volume, white blood cell count, mean corpuscular hemoglobin (MCH), MCH concentration, red distribution width, platelet count and fibrinogen level were found.

Regression analyses

In multivariate logistic regression analysis, after adjustment for several potential confounders, hypertension, diabetes, erythrocyte-derived ROS, erythrocyte lipid peroxidation, DPH and TMA–DPH remained significantly associated with RVO (Table 1).

Partial correlation coefficients between erythrocyte oxidative stress-related parameters (ROS production and lipid peroxidation) and erythrocyte membrane rigidity (DPH and TMA–DPH), WBV and erythrocyte deformability (erythrocyte EI), after controlling for age, sex, cardiovascular risk factors, medications, leukocyte number and MCV, were determined (Table 2).

In multivariate linear regression analysis after adjustment for age, sex, cardiovascular risk factors, medications, leukocyte number, and MCV, erythrocyte-derived ROS and erythrocyte lipid peroxidation still remained significantly and positively correlated with erythrocyte membrane viscosity and deformability (Table 2).

AAPH-induced erythrocyte membrane alterations

To elucidate the mechanisms underlying the modified hemorheologic profile in RVO patients, we performed *in vitro* experiments in which we treated blood samples from healthy subjects with the free radical generator AAPH (Fig. 6). First, erythrocyte lipid peroxidation and ROS production (Fig. 6A) were significantly increased as compared with control samples ($P < 0.001$). Moreover, a significant increase in fluorescent probe fluorescence anisotropy in oxidized samples ($P < 0.001$) was found (Fig. 6B), demonstrating the key role of oxidative stress in altering membrane rigidity. As shown in Fig. 6C, a

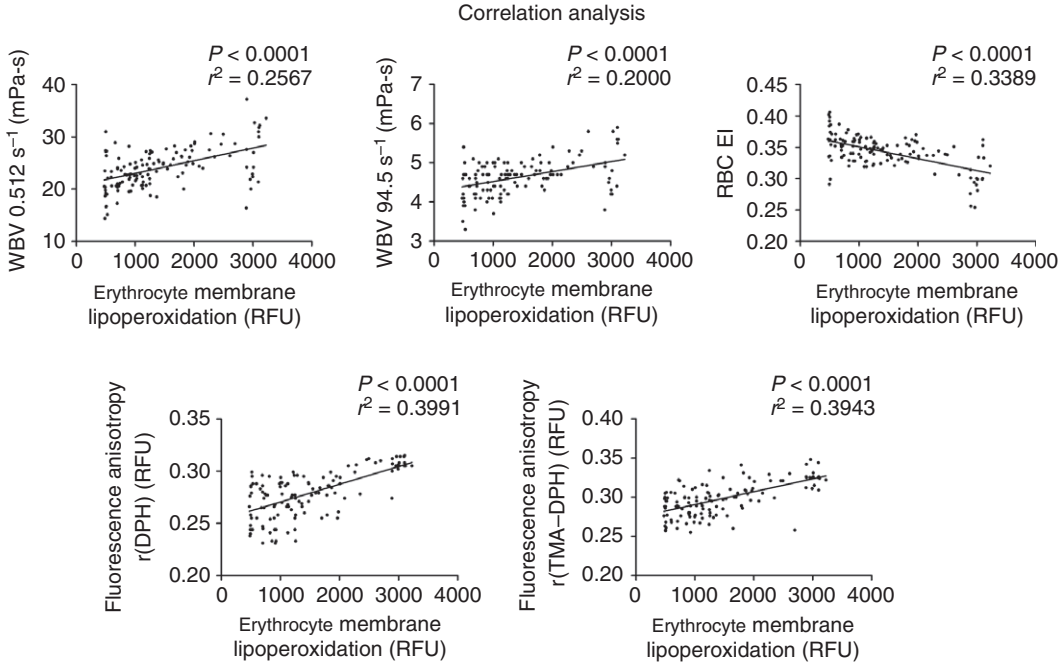

Fig. 4. Correlation analysis among erythrocyte membrane lipid peroxidation, whole blood viscosity (WBV) (analyzed at shear rates of 0.512 s^{-1} and 94.5 s^{-1}), erythrocyte deformability (elongation index [EI]) and erythrocyte membrane fluidity (with 1,6-diphenyl-1,3,5-hexatriene [DPH] and 1-[4′-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene [TMA–DPH] probes) in retinal vein occlusion patients. Erythrocyte membrane lipid peroxidation was positively and significantly correlated with WBV analyzed at shear rates of 0.512 s^{-1} and 94.5 s^{-1} ($P < 0.0001$) and with erythrocyte membrane fluidity (with DPH and TMA–DPH probes) ($P < 0.0001$). Erythrocyte membrane lipid peroxidation was inversely correlated with erythrocyte deformability (EI) ($P < 0.0001$). RFU, relative fluorescence units.

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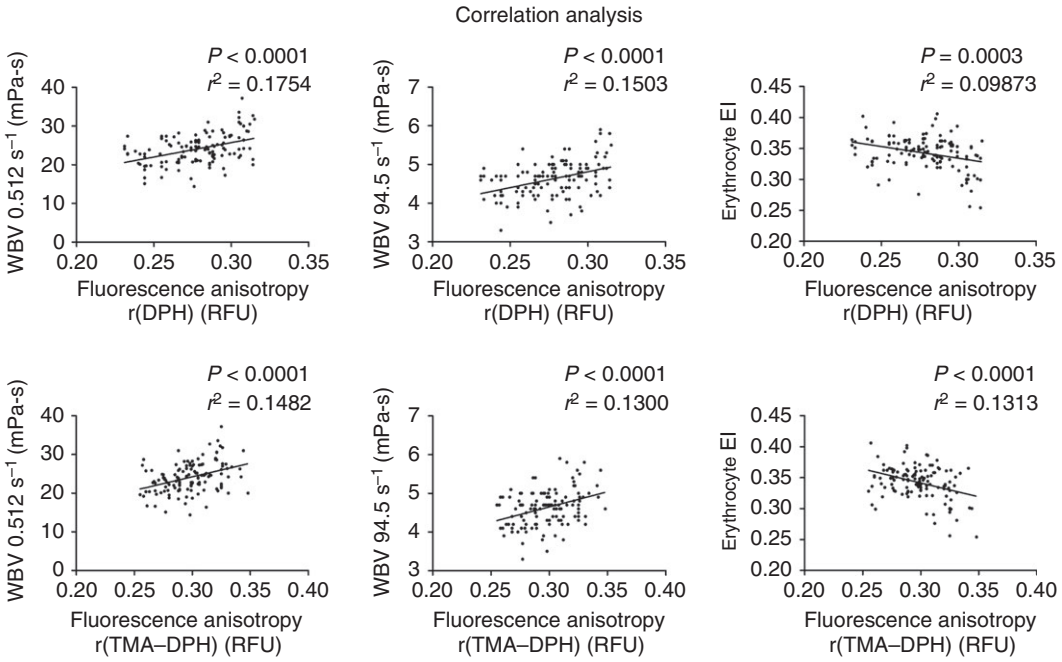

Fig. 5. Correlation analysis among erythrocyte membrane fluidity (with 1,6-diphenyl-1,3,5-hexatriene [DPH] and 1-[4′-(trimethylammonium) phenyl]-6-phenyl-1,3,5-hexatriene [TMA–DPH] probes), whole blood viscosity (WBV) (analyzed at shear rates of 0.512 s^{-1} and 94.5 s^{-1}) and erythrocyte deformability (elongation index [EI]) in retinal vein occlusion patients. Erythrocyte membrane fluidity of the hydrophobic region of the bilayer (DPH probe) was positively and significantly correlated with WBV analyzed at shear rates of 0.512 s^{-1} and 94.5 s^{-1} ($P < 0.0001$) and inversely correlated with erythrocyte deformability (EI) ($P = 0.0003$). Erythrocyte membrane fluidity of the polar region of the bilayer (TMA–DPH probe) was positively and significantly correlated with WBV analyzed at shear rates of 0.512 s^{-1} and 94.5 s^{-1} ($P < 0.0001$) and inversely correlated with erythrocyte deformability (EI) ($P < 0.0001$). RFU, relative fluorescence units.

significant difference in WBV analyzed at shear rates of 94.5 s^{-1} and at 0.512 s^{-1} was observed between treated and untreated blood samples ($P < 0.001$). Similar results (untreated versus treated blood, $P < 0.001$) were obtained when the EI was measured (Fig. 6D).

Discussion

For the first time, a marked increase in erythrocyte oxidative stress in RVO patients as compared with control subjects has been observed. We found that, in erythrocytes from RVO patients, ROS production and membrane lipid peroxidation were significantly increased, and that these parameters were positively correlated with WBV and erythrocyte deformability, thus contributing to the altered hemorheologic profile in these patients. Moreover, our *in vitro* experiments indicate that ROS have a key role in erythrocyte membrane fluidity.

Oxidative stress, which occurs when the level of ROS overwhelms antioxidant defense systems, is closely associated with several chronic and acute disorders [27,28]. The erythrocyte, during its lifetime, is particularly exposed to oxidative stress, owing to the high content of membrane polyunsaturated fatty acids and to the auto-oxidation of hemoglobin within the cell [29].

In RVO patients, signs of oxidative stress, such as enhanced plasma lipid peroxidation (increased thiobarbituric acid reactive substance levels) and decreased antioxidant activity of paraoxonase, have been reported [30]. However, the oxidative stress-related erythrocyte alterations in RVO patients have never been explored. Biological membranes generally contain considerable amounts of highly unsaturated lipids and cholesterol. A prevalence of polyunsaturated fatty acids, however, makes the membrane phospholipids potentially susceptible to oxidation. This process disturbs the bilayer structure, modifies membrane properties such as membrane fluidity, alters the physiologic functions of cell membranes, and contributes to cell membrane damage [31,32]. ROS exert their damaging effects on erythrocyte membrane polyunsaturated fatty acids, resulting in the production of malondialdehyde, which can cause progressive echinocyte transformation and increased membrane rigidity. This accounts for reduced erythrocyte flexibility and to microcirculatory disorders. The deformability of erythrocytes resulting from their membrane flexibility is a factor in maintaining normal blood flow in the microcirculation, allowing their transit through capillaries whose lumen is narrower than the cell diameter [33]. A key feature of microcirculatory hemodynamics is the ability of erythrocytes to assume a parachute-like shape in small capillaries, and this is critically dependent on their bending and shear moduli. The deep changes induced in the main structural components of erythrocytes hamper cell

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Table 1 Univariate and multivariate logistic regression analyses for retinal vein occlusion; analyses were adjusted for age and sex

	Univariate analysis (controls, $n = 128$; patients, $n = 128$)		Multivariate analysis (controls, $n = 128$; patients, $n = 128$)	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Age	1.01 (0.98–1.02)	0.954	–	–
Sex (F versus M) M = 70; F = 58	0.85 (0.52–1.40)	0.531	–	–
Hypertension Patients, $n = 62/128$ Controls, $n = 22/128$	4.53 (2.54–8.05)	< 0.001	9.22 (3.04–27.97)	< 0.001
Dyslipidemia Patients, $n = 59/128$ Controls, $n = 46/128$	1.52 (0.92–2.52)	0.099	–	–
Diabetes Patients, $n = 16$ Controls, $n = 2$	4.53 (2.54–8.05)	< 0.001	13.74 (2.40–78.84)	0.003
Smoking habit Patients, $n = 34/128$ Controls, $n = 22/128$	1.74 (0.95–3.19)	0.071	–	–
Erythrocyte-derived ROS*	4.93 (3.22–7.55)	< 0.001	6.90 (3.95–12.03)	< 0.001
Erythrocyte lipid peroxidation*	1.42 (1.60)	< 0.001	1.53 (1.32–1.76)	< 0.001
DPH*	5.21 (3.10–8.77)	< 0.001	7.69 (3.61–16.40)	< 0.001
TMA–DPH*	3.11 (2.31–4.18)	< 0.001	3.60 (2.51–5.16)	< 0.001

CI, confidence interval; DPH, 1,6-diphenyl-1,3,5-hexatriene; F, female; M, male; OR, odds ratio; ROS, reactive oxygen species; TMA–DPH, 1-[4′-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene.
*OR for each 100-Unit increase.

Table 2 Partial correlation coefficients between erythrocyte oxidative stress-related parameters (reactive oxygen species [ROS] production and lipid peroxidation) and erythrocyte membrane rigidity (1,6-diphenyl-1,3,5-hexatriene [DPH] and 1-[4′-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene [TMA–DPH]), whole blood viscosity (WBV) and erythrocyte deformability (erythrocyte elongation index [EI]) after controlling for age, sex, cardiovascular risk factors, medications, leukocyte number, and mean corpuscular volume

	Erythrocyte ROS (RFU)	Erythrocyte lipid peroxidation (RFU)
DPH (RFU)	$r = 0.652$ $P < 0.001$	$r = 0.609$ $P < 0.001$
TMA–DPH (RFU)	$r = 0.605$ $P < 0.001$	$r = 0.593$ $P < 0.001$
WBV 0.512 s^{-1} (mPa*s)	$r = 0.593$ $P < 0.001$	$r = 0.508$ $P < 0.001$
WBV 94.5 s^{-1} (mPa*s)	$r = 0.462$ $P < 0.001$	$r = 0.405$ $P < 0.001$
Erythrocyte EI	$r = -0.455$ $P < 0.001$	$r = -0.570$ $P < 0.001$

RFU, relative fluorescence units.

passage through the microcirculation, thus contributing to microvascular complications. The major determinants of erythrocyte deformability are cell geometry, intracellular fluid viscosity, and the viscoelastic properties of the cell membrane [34,35]. Several articles have described a decrease in membrane fluidity in different cell membranes as a consequence of lipid peroxidation [36–38]. However, the exact mechanisms by which oxidative stress affects membrane functions remain unclear.

Two main reasons have been suggested for the loss of membrane fluidity in oxidative stress condition. First, the polyunsaturated/saturated fatty acid ratio in the membrane could be reduced [39], because ROS have a particular affinity for electron-rich unsaturated covalent bonds, which are found in polyunsaturated fatty acids [40]. Second, the formation of cross-links among the membrane lipid moieties may limit motion within the membrane, contributing to rigidity [41]. In fact, the existence of a direct relationship between lipid peroxidation and membrane leakiness has been suggested [42–45]. Moreover, lipid peroxidation can alter lipid membrane permeability by increasing the dielectric constant of the membrane interior and by increasing the microviscosity, possibly through cross-linking of lipid radicals [46]. The effect of peroxidation on lipid dynamics and membrane order is still a matter of debate. According to some researchers, peroxidation does not affect the fluidity of the membrane [47] or the reorientational dynamics of the lipids [48]. According to others, membrane fluidity is decreased [49–52], and the decrease is more marked near the double bonds of the bilayer than in other regions [49]. Our results indicate that erythrocyte membrane fluidity is significantly altered in RVO patients as compared with controls, suggesting that cell membranes are stiffer and less fluid in RVO erythrocytes.

Erythrocyte deformability is critical to microvascular perfusion and oxygen delivery [53]. Alterations in this contribute to the severe vascular pathology of RVO. Furthermore, because the size, shape and diffusion capacity of an erythrocyte depend on the structure of its membrane, alterations in membrane structure could lead to a

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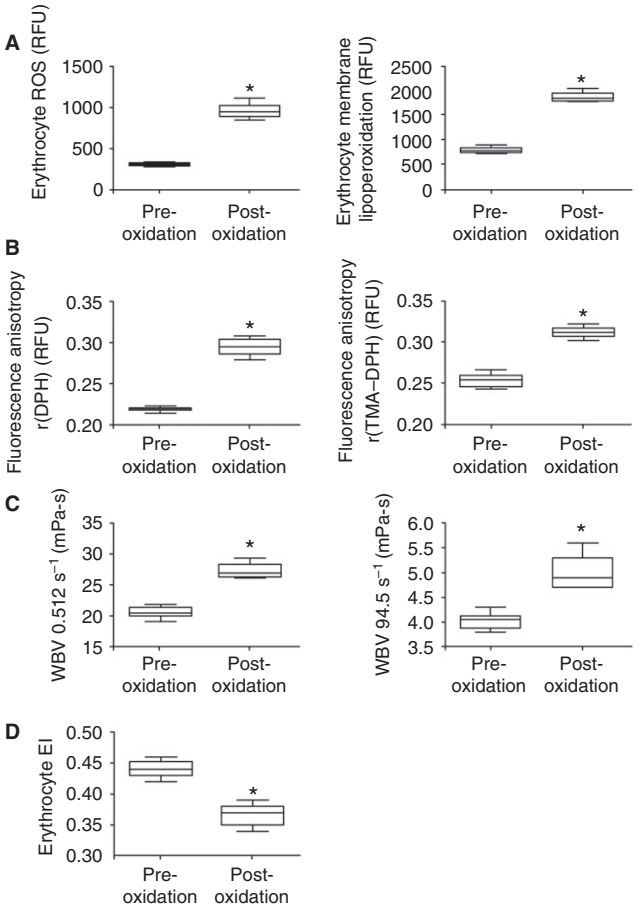

Fig. 6. *In vitro* oxidative stress-induced erythrocyte membrane alterations. To demonstrate the key role of oxidative stress in erythrocyte alterations, blood samples from 10 control subjects were incubated (or not) at 37 °C for 2 h in the presence of the free radical generator 2,2′-azobis(2-amidinopropane) dihydrochloride. Then, all experiments were performed in treated and untreated samples. (A) Erythrocyte lipid peroxidation and erythrocyte reactive oxygen species (ROS) production were significantly increased as compared with unoxidized samples ($P < 0.001$). (B) Fluorescence anisotropy (1,6-diphenyl-1,3,5-hexatriene [DPH] and 1-[4′-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene [TMA–DPH]) was significantly increased in oxidized samples ($P < 0.001$), demonstrating the key role of oxidative stress in altering erythrocyte membrane rigidity. (C) A significant difference in whole blood viscosity (WBV) at shear rates of 94.5 s^{-1} and at 0.512 s^{-1} was observed between treated and untreated blood samples ($P < 0.001$). (D) Erythrocyte deformability (elongation index [EI]) was significantly reduced in oxidized samples ($P < 0.001$). RFU, relative fluorescence units.

decrease in tissue oxygenation [54] and retinal ischemia. Erythrocyte deformability plays a major role in determining blood viscosity in the central retinal vein at the level of the lamina cribrosa, where slow venous flow and high vascular resistance are present. Interestingly, our results show an inverse correlation between erythrocyte membrane lipid peroxidation and deformability, demonstrating that membrane peroxidation can perturb membrane properties. This evidence is also confirmed by the significant and inverse correlation between erythrocyte ROS production and the EI. There are several possible reasons for the marked heterogeneity found in the literature, including the use of different methodologies for peroxide generation, leading to different (and usually not well defined) membrane lipid compositions [54]. However, despite the numerous studies on the effects of oxidation on the structure and dynamics of lipid membranes, the relationship between increased membrane permeability and modifications in the structure and dynamics of lipid bilayers is not clear.

A number of studies have demonstrated that patients with arteriosclerosis, or high levels of plasma glucose and high arterial blood pressure, show an increased risk of developing RVO [55,56]. Several hemodynamic changes in the retinal microvasculature (such as increased vascular permeability, vasodilatation and adhesion of inflammatory cells to the blood vessel wall) are associated with hyperglycemia-induced inflammation [57,58]. Furthermore, strong evidence suggests that oxidative stress induced by hyperglycemia is involved in the development of vascular alterations in the retina [59]. Increased internal membrane viscosity resulting from glycosylation leads to decreased erythrocyte deformability in patients with diabetes [60]. In addition, it has been demonstrated that the severity of hypertension is associated with erythrocyte aggregation and deformability [61], and that erythrocytes contribute to the high incidence of atherosclerotic diseases in patients with hypertension, partly because of the association of erythrocyte hemorheologic alterations with increased oxidative stress [62]. However, our statistical analysis demonstrated that, after adjustment for several potential confounders (including hypertension and diabetes), erythrocyte-derived ROS and erythrocyte lipid peroxidation remained significantly associated with RVO and positively correlated with erythrocyte membrane viscosity and deformability.

Some limitations of this study should be pointed out. The control group consisted of healthy volunteers, which may have led to higher ORs in this study. First, RVO patients are likely to have originated from a different source population than the healthy controls; therefore, the variables studied may have followed a different distribution than that of the population from which the patients originated. Moreover, the size of the study groups is limited, and these results need further confirmation in a larger population. Finally, all laboratory parameters were evaluated on a single occasion after RVO, and, at present, these results do not allow us to establish a definite role of these alterations in the onset of RVO, owing to the retrospective design of the study. In spite of these limitations, our results show significant erythrocyte membrane alterations in RVO patients, at the structural and functional levels, and an increase in membrane lipid peroxidation and intracellular ROS production. The main findings here reported suggest that erythrocyte oxidative stress plays a key role in the pathogenetic mechanism of

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RVO disease, and pave the way to possible therapeutic interventions with antioxidant supplementation.

Addendum

M. Becatti, R. Marcucci, A. M. Gori, L. Mannini, and E. Grifoni were responsible for data collection and analysis, and performed experiments. A. Alessandrello Liotta, A. Sodi, and R. Tartaro monitored patient inclusion. R. Abbate and C. Fiorillo were responsible for protocol development and study funding, and supervised the study. N. Taddei, S. Rizzo, and D. Prisco gave critical guidance during the project. M. Becatti and C. Fiorillo designed the experiments and wrote the manuscript. R. Abbate critically revised the manuscript. All authors contributed substantially to the critical revision of the manuscript, and gave approval of the final draft.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical characteristics of study population.

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