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Morphological changes and oxidative damage in Rett Syndrome erythrocytes

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ABSTRACT

Background: Hypoxemia and increased oxidative stress (OS) have been reported in Rett Syndrome (RTT), a genetical neurodevelopmental disorder. Although OS and hypoxemia can lead to red blood cells (RBCs) shape abnormalities, no information on RBCs morphology in RTT exists. Here, RBCs shape was evaluated in RTT patients and healthy subjects as a function of OS markers, blood oxygenation, pulmonary gas exchange, and cardio-respiratory parameters.

Methods: RBCs morphology was evaluated by Scanning Electron Microscopy. Intraerythrocyte and plasma non protein-bound iron (NPBI), esterified F_2-Isoprostanes (F_2-IsoPs), 4-HNE protein adducts (4-HNE PAs) were measured. Pulmonary oxygen gradients and PaO_2 were evaluated by gas analyzers and cardiopulmonary variables by pulse oximetry. In RTT patients these parameters were assessed before and after ω-3 polyunsaturated fatty acids (ω-3 PUFAs) administration.

Results: Altered RBCs shapes (leptocytes) and increased NPBI were present in RTT, together with increased erythrocyte membrane esterified F_2-IsoPs and 4-HNE PAs. Abnormal erythrocyte shapes were related to OS markers levels, pulmonary gas exchange, PaO_2 and cardio-respiratory variables. After ω-3 PUFAs, a decrease of leptocytes was accompanied by a progressive increase in reversible forms of RBCs. This partial RBCs morphology rescue was related to decreased OS damage markers, improved pulmonary oxygen exchange, and cardiopulmonary physiology.

Conclusions: These findings indicate that in RTT 1) RBCs shape is altered; 2) the OS-hypoxia diad is critical in generating altered RBCs shape and membrane damage; 3) ω-3 PUFAs are able to partially rescue RBCs morphology and the OS-derived damage.

General significance: RBCs morphology is an important biosensor for OS imbalance and chronic hypoxemia.

1. Introduction

Rett Syndrome [OMIM ID: 312750] (RTT) [1] is a severe and complex neurodevelopmental disorder included in the autism spectrum disorders (ASDs). Although it is a relatively rare condition, affecting almost exclusively females with a frequency of approximately 1:10,000, RTT represents the second most common cause of mental retardation in the female gender, and is caused by mutations in X-linked methyl-CpG binding protein 2 gene (*MeCP2*) in up to 95% of cases [2]. A wide genetical and phenotypical heterogeneity is well established in RTT, with about 80% of RTT clinical cases showing the typical clinical picture, characterized by an early neurological regression, followed by loss of acquired cognitive, social, and motor skills in a typical 4-stage neurological regression, together with development of autistic behavior [3,4]. To date, no effective treatment exists. One of

Abbreviations: OS, oxidative stress; RTT, Rett syndrome; RBCs, red blood cells; NPBI, non protein-bound iron; F_2-IsoPs, F_2-Isoprostanes; 4-HNE PAs, 4-HNE protein adducts; PaO_2, partial arterial pressure of oxygen; ω-6 PUFAs, ω-6 polyunsaturated fatty acids; ω-3 PUFAs, ω-3 polyunsaturated fatty acids; ASDs, Autism spectrum disorders; MeCP2, methyl-CpG-binding protein 2; respiratory bronchiolitis-associated interstitial lung disease, RB-ILD; COPD, chronic obstructive pulmonary disease; SEM, Scanning electron microscopy; Met-Hb, methemoglobin; AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SpO_2, oxyhemoglobin saturation; PI, Perfusion Index; PVI, pleth variability index; CO-Hb, carboxy-hemoglobin; (A-a)PO_2, O_2 alveolar-arterial gradient; NAC, N-acetylcysteine; CBZ, carbamazepine.

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the main reasons for a lack of an effective therapy in RTT may reside in an incomplete understanding of the disease pathogenesis.

In previous studies we have reported chronic hypoxia, impaired pulmonary gas exchange, and increased oxidative stress (OS) in typical RTT [5]; pulmonary lesions, respiratory bronchiolitis-associated interstitial lung disease (RB-ILD)-like lesions, were observed on imaging studies in about half of typical patients with RTT [6].

Although emerging evidence indicates that OS imbalance and hypoxemia can lead to red blood cells (RBCs) shape abnormalities in adult patients with chronic obstructive pulmonary disease (COPD) [7–10], to date no information regarding erythrocyte morphologic characteristics in RTT is available.

RBCs, owing to high levels of cellular iron and the unique property of hemoglobin to autooxidize, are a constant source of superoxide production, and have been extensively used for investigating mechanisms of OS [11,12]. Erythrocytes show a characteristic biconcave shape, of key importance for all their functions (i.e. deformability, O_2 exchange), but also very susceptible to morphological changes with consequent lack of functionality. Among the factors causing morphological modifications, changes in osmolarity, pH conditions and presence of oxidants are described [13,14]. Oxidants, in fact, produce alterations in erythrocyte membranes, as manifested by a decreased cytoskeletal protein content and production of high-molecular-weight proteins, which can lead to abnormalities in erythrocyte shape and rheological properties [15–18]. Experiments with erythrocytes incubated with H_2O_2 and ascorbate/Fe^{2+} have shown morphological changes characterized by a dose dependent increase in echinocyte formation, indicating the role of oxidative damage in compromising the rheological behavior of the erythrocytes [19].

Our previous studies have demonstrated that OS induces in RBC iron release in a free redox active form (NPBI, i.e. non protein-bound iron), the release is accompanied by methemoglobin (Met-Hb) formation and by oxidative alterations of membrane lipids (lipid peroxidation) and proteins [20,21]. Lipid peroxidation is a major consequence of oxidative damage to a variety of tissues in vivo [22,23]. A series of prostaglandin F_2-like compounds, i.e. F_2-isoprostanes (F_2-IsoPs), are formed by free radical-catalyzed peroxidation of esterified arachidonic acid (AA) in phospholipids, a pathway that is independent of the cyclooxygenase pathway. Once formed, F_2-IsoPs are released into the circulation and due to their relatively low reactivity, they can be easily measured in biological samples. Therefore, F_2-IsoPs are considered specific and reliable OS markers [23–25].

Furthermore, one OS-related molecule is 4-hydroxynonenal (4-HNE), a major and toxic aldehyde generated by free radical attack on ω-6 polyunsaturated fatty acids (ω-6 PUFAs) (arachidonic, linoleic, and linolenic acids) and considered a second toxic messenger of oxygen free radicals [26]. 4-HNE is highly reactive toward proteins, forming stable covalent adducts and this process introduces carbonyl groups into proteins [26].

Our previous morphological [18] scanning electron microscopy (SEM) study on mouse erythrocytes after incubation with oxidant agents showed a number of alterations in shape, including echinocyte transformation and the appearance of codocytes, stomatocytes, and knizocyte-like forms. These alterations were more prominent with increasing of iron release and lipid peroxidation appearance and were prevented by the addition of an iron-chelating antioxidant agent.

The aim of this study was to investigate whether OS, identified by our group as a potential key pathogenetic factor of RTT and to which erythrocytes are likely exposed, could induce morphological changes and oxidative damage in the erythrocytes of typical RTT patients. NPBI, esterified F_2-IsoPs and 4-HNE membrane protein adducts (4-HNE PAs) were determined in erythrocytes; NPBI was also measured in plasma; hypoxia and impaired gas exchange were evaluated by gas analyzer and pulse oximetry monitoring. The RBCs morphological changes and oxidative damage markers were evaluated before and after ω-3 polyunsaturated fatty acids (ω-3 PUFAs) supplementation, known antioxidants with multiple actions [27].

2. Materials and methods

2.1. Subjects

The study included 12 female patients with clinical diagnosis of typical RTT (mean age: 9.5 ± 8.9 years, range 2–34) with demonstrated *MeCP2* gene mutation and 12 age-matched healthy controls (mean age: 9.8 ± 8.08 years, range 3–32). RTT diagnosis and inclusion/exclusion criteria were based on the recently revised RTT nomenclature consensus [3]. All the patients were consecutively admitted to the Rett Syndrome National Reference Centre of the University Hospital of the Azienda Ospedaliera Universitaria Senese (AOUS). The subjects examined in this study were on a typical Mediterranean diet. RTT clinical severity was assessed using either the total clinical severity score (CSS), a validated clinical rating specifically designed for RTT, or the "compressed" clinical severity score (CCSS) both based on 13 individual ordinal categories measuring clinical features common in RTT [28]. Clinical and genetic characteristics of the examined RTT patients are reported in Table 1. RTT patients with menstrual cycles (N = 6) were sampled in the mid-follicular phase. Blood samplings in the control group were carried out during routine health checks, sports, or blood donations, while blood samplings in patients were obtained during the periodic clinical checks. The healthy control subjects were sex- (given that over 98% of RTT patients are females, we selected a female control group) and age-matched.

The study was conducted with the approval by the Institutional Review Board and all informed consents were obtained from either the parents or the legal tutors of the enrolled patients.

2.2. ω-3 PUFAs (DHA plus EPA) oral supplementation

A cohort of 12 patients (mean age 12.7 ± 7.6 years; range 2.5–34) with a clinical diagnosis of typical RTT were supplemented with ω-3 PUFAs for 6 and 12 months. An average dose of 20–40 mg/kg b.w./day of commercially available ω-3 PUFAs, with docosahexaenoic acid (DHA) plus eicosapentaenoic acid (EPA) as ethyl esters preparation, with a EPA to DHA ratio of about 1.5 (Eskim, Sigma-tau, Pomezia, Roma, Italy) was administered orally. Use of ω-3 PUFAs in RTT was previously obtained by the AOUS Ethical Committee.

All data obtained in typical RTT patients after ω-3 PUFAs supplementation were compared to those obtained in unsupplemented typical RTT patients, a comparable group for age (differences between the two groups, i.e., unsupplemented vs. supplemented, P = 0.3538), disease stage, and clinical severity.

2.3. Blood sampling

Blood was collected in heparinized tubes and all manipulations were carried out within 2 h after sample collection. The blood samples were centrifuged at 2400 × *g* for 15 min at 4 °C; the platelet poor plasma was saved and the buffy coat was removed by aspiration.

RBCs were washed twice with physiologic solution (150 mM NaCl). An aliquot of packed erythrocytes was resuspended in Ringer solution [125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 mM glucose, 1 mM $CaCl_2$], pH 7.4 as a 50% (vol/vol) suspension for the determination of intraerythrocyte NPBI, and GSH. The remaining volume of packed erythrocytes was used for erythrocyte membranes preparations (i.e., hemoglobin-free ghosts) for esterified F_2-IsoPs and 4-HNE PAs determinations. Plasma was used for the NPBI assay.

Table 1
Clinical and genetic characteristics of untreated RTT patients.

Item	Patient number											
	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	#11	#12
MeCP2 mutation type	Other	ETMs	Other	R270X	T158M	ETMs	R294X	C TER DEL	Other	R133C	R133C	T158M
Neurological regression	+	+	+	+	+	+	+	+	+	+	+	+
Somatic growth deficit	−	−	+	−	−	−	−	+	−	−	−	+
Microcephaly	+	+	+	+	+	+	+	+	+	+	+	+
Motor/independent sitting	−	+	−	−	−	+	+	−	+	−	−	−
Ambulation	−	+	−	−	−	+	+	−	+	−	−	−
Hand use	−	−	−	−	−	−	−	−	−	−	−	−
Scoliosis	+	+	+	+	−	+	−	+	−	−	+	+
Language	−	−	−	−	−	−	−	−	−	−	−	−
Nonverbal communication	+	+	+	+	+	+	+	+	+	+	+	+
Respiratory dysfunction	+	+	+	+	+	+	+	+	+	+	+	+
Autonomic symptoms	+	+	+	+	+	+	+	+	+	+	+	+
Stereotypies	+	+	+	+	+	+	+	+	+	+	+	+
Epilepsy/seizures	−	−	+	−	+	−	−	−	+	+	+	−
Compressed clinical severity score (CCSS)[28]	6	9	9	7	7	9	8	7	9	6	7	8

Legend: +, item present; −, lacking item.
MeCP2 mutation type legend: C TER DEL: C-terminal deletions; ETMs: early truncating mutations; Other: other pathogenetic mutations.

An aliquot of each blood sample (1 ml) was centrifuged at 800×*g* for 10 min at 4 °C for Scanning Electron Microscopy (SEM) analysis of erythrocytes.

2.4. Intraerythrocyte and plasma NPBI

Intraerythrocyte NPBI (nmol/ml erythrocyte suspension) was determined as a desferrioxamine (DFO)-iron complex (ferrioxamine) as previously reported [5]. Briefly, 25 μM DFO was added to the samples (1 ml erythrocyte suspension), the erythrocytes were then lysed by adding water (1 vol) and freezing (−70 °C) and thawing. The hemolysate was ultrafiltered at 3373×*g* for 30 min in centrifugal filters with a 30-kDa molecular weight cut-off (VIVASPIN 4, Sartorius Stedim Biotech GmbH, Goettingen Germany) and the ultrafiltrate was stored at −20 °C until analysis. The DFO excess was removed by silica (Silicagel 25–40 μm; Merck, Darmstadt, Germany) column chromatography (Varian Inc., CA, USA). The DFO-iron complex was determined by HPLC and the detection wavelength was 229 nm.

Plasma NPBI (nmol/ml) was determined as reported for intraerythrocyte NPBI. DFO (25 μM) was added to plasma diluted (1:1; vol:vol) with Ringer solution, then the samples were ultrafiltered and stored at −20 °C until analysis as above.

The calibration curve correlation for intraerythrocyte NPBI and plasma NPBI was adequate ($r^2 = 0.994009$), the minimum detection limit was 0.1 nmol/ml, and mean intra- and inter-observer coefficients of variation of ≤2.5% and ≤5%, respectively.

2.5. Intraerythrocyte glutathione (GSH)

Glutathione (GSH) content determinations in RBCs were carried out according to the spectrophotometric method of Beutler et al. [29]. After RBC lysis and subsequent metaphosphoric acid protein precipitation, the method involved GSH oxidation by sulfhydryl reagent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the derivative 5′-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm.

2.6. Erythrocyte membrane preparation

Two aliquots (600 μl, each) of packed RBCs were lysed in Dodge buffer, and erythrocyte membranes were prepared according to Dodge [30]. Dodge buffer used to prepare erythrocyte membranes for F_2-IsoPs determination contained butylated hydroxytoluene (BHT) (90 μM) as an antioxidant. Samples were stored at −70 °C until analysis.

2.7. Erythrocyte membrane esterified F_2-IsoPs determination

A 100 μl aliquot of the erythrocyte membrane samples was resuspended with H_2O (0.9 ml) and 1 N KOH (500 μl) was added for the basic hydrolysis. After incubation at 45 °C for 45 min, the sample was acidified to pH 3 with HCl 1 N (500 μl), spiked with tetradeuterated Prostaglandin $F_2\alpha$ ($PGF_2\alpha$-d_4) (500 pg in 50 μl of ethanol; Cayman, Ann Arbor, MI, USA), as internal standard, and extracted with 10 ml of ethyl acetate. The upper organic layer, obtained after centrifugation at 1000×*g* for 5 min, was applied onto an aminopropyl (NH_2) cartridge (Waters, Milford, MA, U.S.A.) preconditioned with 10 ml of hexane. After derivatization the determination of F_2-IsoPs was accomplished by gas chromatography/negative ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) analysis [21]. Esterified F_2-IsoPs were normalized for membrane proteins, quantified by BioRad protein assay (BioRad, Hercules, CA) using 0.2% Triton X-100 to dissolve the membranes. The measured ions were the product ions at m/z 299 and m/z 303 derived from the $[M-181]^-$ precursor ions (m/z 569 and m/z 573) produced from 15-F_{2t}-IsoPs (the most represented F_2-IsoP isomer) and $PGF_2\alpha$-d_4, respectively [21].

2.8. Western blot for 4-HNE erythrocyte membrane protein adducts (4-HNE PAs)

For Western blot analysis, erythrocyte membrane samples (40 μg of membrane proteins, quantified by BioRad protein assay) (BioRad, Hercules, CA) were separated on 4–20% gradient SDS-PAGE gels (Lonza Group Ltd, Switzerland) and electro-transferred onto a hybond ECL nitrocellulose membrane (GE Healthcare Europe Gmbh, Milan, Italy). After blocking in 3% non-fat milk (BioRad, Hercules, CA, USA) in TBS-Tween20, membranes were incubated overnight at 4 °C with goat polyclonal anti 4-HNE adducts (cod.AB5605; Millipore Corporation, Billerica, MA, USA). Mouse anti-goat horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used as secondary antibody and bound antibodies were visualized by ECL reagents (BioRad, Hercules, CA, USA). Bands were visualized by autoradiography. Quantification of relevant bands was performed by digitally scanning the Amersham Hyperfilm™ ECL (GE Healthcare Europe Gmbh, Milan, Italy) and measuring immunoblotting image densities with imageJ software.

2.9. Scanning electron microscopy (SEM)

Erythrocytes were plated on poly-l-lysine coated slides and fixed in Karnowsky (2.5% glutaraldeyde — 4% paraformaldehyde in 0.1 M

sodium-cacodylate buffer, pH 7.2) for 2 h at 4 °C, rinsed twice for 10 min with 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide in 0.1 M sodium-cacodylate buffer for 1 h at 4 °C. Specimens were then dehydrated through a graded ethanol series, dried in a CO_2 critical point dryer (CPD010, Balzers Union, Liechtenstein), mounted on specimen stub, sputter coated with gold (Sputter Coater S150B, Edwards, England) and examined in a XL 20 SEM (Philips, Eindhoven, Netherlands).

Altered RBCs shapes were evaluated by counting ≥ 500 cells (50 erythrocytes for each different SEM field at a magnification of ×3000) from RTT patients (before and after ω-3 PUFAs supplementation) and control subjects. All countings were carried out in triplicate and averages were taken for data analysis.

2.10. Pulse oximetry

Continuous and noninvasive measurements of oxyhemoglobin saturation (SpO_2), perfusion index (PI), pleth variability index (PVI), carboxy-hemoglobin (CO-Hb) and Met-Hb were carried out using pulse oximetry motion artifact-free technology.

In particular, PI [31] is an objective indicator of skin perfusion (PI = AC/DC × 100%, where DC is the constant amount of light from the signal of the pulse oximeter absorbed by the skin, other tissues, and nonpulsatile blood, and AC is the variable amount of light absorbed by the pulsating arterial inflow) and the pleth variability index (PVI) [32] is a measure of the dynamic changes in the PI that occur during the respiratory cycle (PVI = PI maximum − PI minimum/PI maximum × 100%).

Each patient and control subject was equipped with an adequate pulse oximeter probe attached at the index of the left hand and wrapped to prevent outside light from interfering with the signal. The pulse oximeter was connected to a Masimo Radical 7 monitor (Masimo SET; Masimo Corp.), and plethysmographic waveforms were recorded on a personal computer using PhysioLog software (PhysioLog version 1.0.1.1; Protolink, Richardson, TX, USA) and analyzed by an observer who was blinded to clinical, biochemical, and respiratory function data. Randomly measured, 1 h-long data were obtained in triplicate, and averaged data were used for data analysis.

2.11. Blood gas analysis

To avoid a possible bias in SpO_2 measurements due to low peripheral perfusion (autonomic dysfunction), commonly observed in RTT patients, arterial blood was also obtained. Arterial blood for gas analyses was sampled from either the humeral or the radial artery. Partial arterial pressures of oxygen (PaO_2) and pH values were determined by a commercially available blood gas analyzer (ABL520 Radiometer; Radiometer Medical A/S; Copenhagen, Denmark).

2.12. Breathing and pulmonary oxygen exchange

Pulmonary gas exchange was assessed using a portable, commercially available gas analyzer (Hanky Hapy, version 1.2; Ambra Sistemi; Pianezza, Turin, Italy), as previously reported [5]. Briefly, the system works essentially on a multicompartment model based on the West function. This methodology has been proven to be sufficiently simple, non-invasive, accurate, and precise in determining alveolar-arterial gradient lung exchange for O_2 and ventilation/perfusion ratio inequalities. Air gas sampling was obtained by applying a facial mask of appropriate size connected to the gas analyzer. Respiratory rate, total ventilation, and expired gas composition were measured during a 60-s period. All measurements were carried out in duplicate, and the averages were used for data analysis. Arterial blood for gas analyses was sampled from either the humeral or the radial artery, and PaO_2 values were determined using a commercially available blood gas analyzer (see paragraph 2.11). Pulmonary gradients for O_2 [(A-a)PO_2] > 15 mm Hg were considered to be indicative for ventilation/perfusion mismatch.

2.13. Data analysis

All variables were tested for normal distribution (D'Agostino-Pearson test) and data were presented as means with 95% confidence intervals (95% C.I.) for normally distributed variables or medians means with 95% C.I. for non-normally distributed data. Differences between groups were evaluated using independent-sample *t* test (continuous normally distributed data), Mann–Whitney rank sum test (continuous nonnormally distributed data), chi-square statistics (categorical variables with minimum number of cases per cell ≥ 5) of Fisher's exact test (categorical variables with minimum number of cases per cell <5), one-way analysis of variance (ANOVA), Student–Newman–Keuls post-hoc test, or Kruskal–Wallis test. Associations between variables were tested by univariate regression analysis (Pearson's coefficients or Spearman's rho, as appropriate). Two-tailed P values of less than 0.05 were considered significant if not otherwise specified. Correction for multiple comparisons was made (Bonferroni's correction). The MedCalc version 9.5.2.0 statistical software package (MedCalc Software, Mariakerke, Belgium) was used.

3. Results

3.1. Morphological erythrocytes alterations in untreated RTT

Scanning electron microscopy showed a variety of alterations in the shape (i.e., leptocyte, knizocyte, codocyte, leptocodocyte, stomatocyte) of erythrocytes from untreated RTT patients (Fig. 1; upper panel row: B–F, and lower panels: H and K). A majority of flat, thin forms (i.e., leptocytes) (means ± SE: 51.83 ± 3.5%) were accompanied by the appearance of knizocytes (21.99 ± 3.73%) (red cells with two or three concavities due to indentations in the cell membrane), codocytes (14.52 ± 3.36%) and leptocodocytes (5.25 ± 4.78%) (Fig. 2). Only 3.81 ± 1.67% of the cells showed their normal discoid shape (discocytes), but stomatocytes (2.45 ± 1.6%) (cells with a cup-shaped form with evagination of one surface and a deep invagination of the opposite face) were also observed (Fig. 2). Control erythrocytes showed a typical biconcave discoid shape and only few alterations were evident (Figs. 1; lower panels: G and J and 2).

3.2. OS and oxidative damage in RTT erythrocytes

Intraerythrocyte NPBI (1.6 ± 1.0 nmol/ml erythrocyte suspension) and plasma NPBI (0.9 ± 0.3 nmol/ml) were significantly increased in RTT patients as compared to controls (0.5 ± 0.2 nmol/ml; 0.3 ± 0.2 nmol/ml, respectively), thus confirming the occurrence of a systemic OS [5] (Fig. 3). Since free iron can induce oxidative alterations of lipid and protein cell membrane [20], we evaluated, as oxidative damage markers, esterified F_2-IsoPs formation (that is, the F_2-IsoPs still bound to membrane phospholipids) and the levels of 4-HNE PAs (Fig. 4). A significant increased formation of esterified F_2-IsoPs was observed in erythrocytes of RTT patients (P = 0.0001) in comparison of controls, as well as the levels of 4-HNE PAs were much higher (P<0.0001) in RTT erythrocytes than in controls (Fig. 4).

3.3. Correlation matrix for erythrocyte shape as a function of oxidative damage

The relationship between erythrocyte morphology in RTT and NPBI is reported in Table 2. Intraerythrocyte NPBI was found to be positively related to percentage of leptocytes (r = 0.6699, P = 0.0174) and inversely related to percentage of stomatocytes

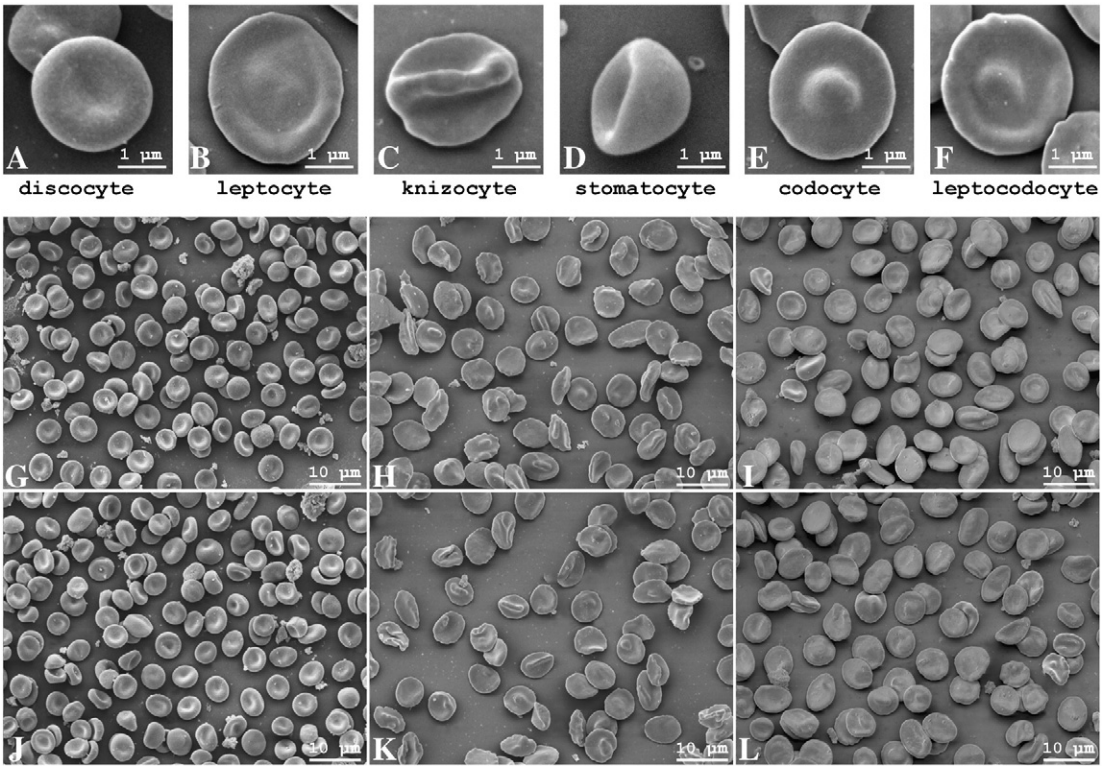

Fig. 1. Scanning Electronic Microscopy. Upper panel row: photograms of the main shape-altered RBCs from untreated RTT patients. (A: RBC normal shape; B–F: RBC altered shape). Lower panels: SEM of erythrocytes from RTT patients before and after ω-3 PUFAs supplementation (6 and 12 months) and healthy controls. (G, J) healthy controls. (H, K) typical RTT patients. (I) typical RTT patient after 6 months of ω-3 PUFAs supplementation. (L) typical RTT patient after 12 months of ω-3 PUFAs supplementation.

($r = -0.6075$, $P = 0.0362$). Plasma NPBI was positively related to leptocytes ($r = 0.6645$, $P = 0.0184$) and leptocodocytes ($r = 0.6454$, $P = 0.0234$) while was inversely related to the percentage of discocytes ($r = -0.5848$, $P = 0.0458$).

The relationship between erythrocyte morphology in RTT and membrane oxidative damage markers is also reported in Table 2. Esterified F_2-IsoPs were positively related to percentage of leptocytes ($r = 0.6281$, $P = 0.0287$) and inversely related to stomatocytes ($r = -0.8056$, $P = 0.0016$), reversible forms of erythrocytes. Likewise, 4-HNE PAs were positively related to leptocytes ($r = 0.7845$, $P = 0.0025$) and inversely related to codocytes ($r = 0.6180$, $P = 0.0322$). On the other hand, no significant correlations between the observed RBC morphological alterations and established symptoms of RTT ($P \geq 0.45$) were observed.

3.4. Intraerythrocyte GSH content

Untreated RTT patients showed slightly higher GSH intraerythrocyte levels than healthy controls of comparable age (RTT, $n = 12$: 1098 ± 169.8 nmol/ml of erythrocyte suspension vs. 940 ± 89.9 nmol/ml of erythrocyte suspension, $P = 0.0093$). However, after ω-3 PUFAs supplementation, a non significant increase of GSH vs. untreated patients was observed (1149 ± 228.1 nmol/ml of erythrocyte suspension, $P = 0.5408$).

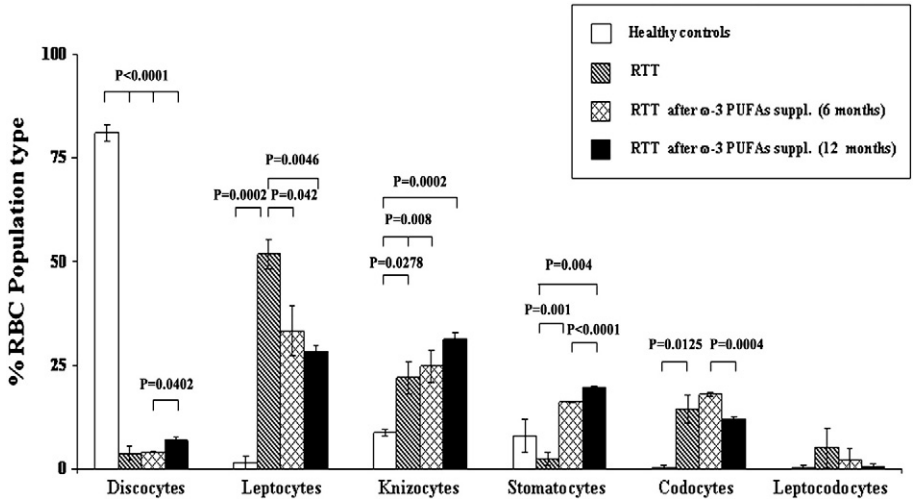

Fig. 2. RBC morphology distribution in RTT patients before and after ω-3 PUFAs supplementation (6 and 12 months) and healthy controls. All the statistical significant differences were reported.

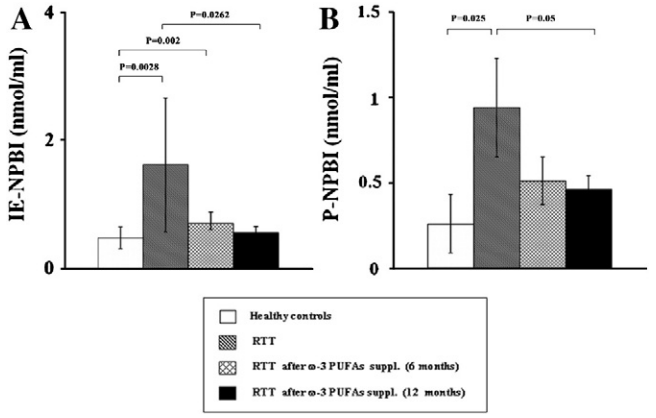

Fig. 3. NPBI in erythrocytes (A; IE-NPBI) and plasma (B; P-NPBI). The data were expressed as means ± SD. (IE-NPBI was reported as nmol/ml erythrocytes suspension). All the statistical significant differences were reported.

3.5. Erythrocyte morphology as a function of pulse-oximetry, pulmonary gradients for O_2, partial arterial pressure of oxygen and blood pH before and after ω-3 PUFAs supplementation

PaO_2 in untreated RTT patients was significantly lower than in controls, while levels returned to normal after ω-3 PUFAs supplementation (ANOVA, $P<0.001$) (see Supplementary Table). Peripheral oxygenation (SpO_2) was similarly decreased (data not shown). Likewise, untreated RTT patients significantly differ in PI, PVI, Met-Hb, CO-Hb and pulmonary gradients for O_2 from control subjects. After ω-3 PUFAs supplementation pulmonary gradients for O_2 [(A-a)PO_2: O_2 alveolar-arterial gradient] (at 6 and 12 months), CO-Hb (at 6 and 12 months), Met-Hb (at 12 months) returned to normal values. PI values increased while PVI decreased significantly (ANOVA, $P<0.0001$), albeit not returning within the range of controls at the end of 12 months of supplementation. On the other hand, pH was not different from controls and did not change after ω-3 PUFAs supplementation (ANOVA, $P=0.4440$).

Hypoxemia, compromised pulmonary oxygen exchange, Met-Hb and CO-Hb were significantly related to the occurrence of leptocytes, leptocodocytes, and partially codocytes (Table 3), whereas were inversely related to the presence of discocytes and stomatocytes (reversible forms to discocytes). A positive relation between knizocytes and PVI as well as a negative correlation between echinocytes and CO-Hb were observed.

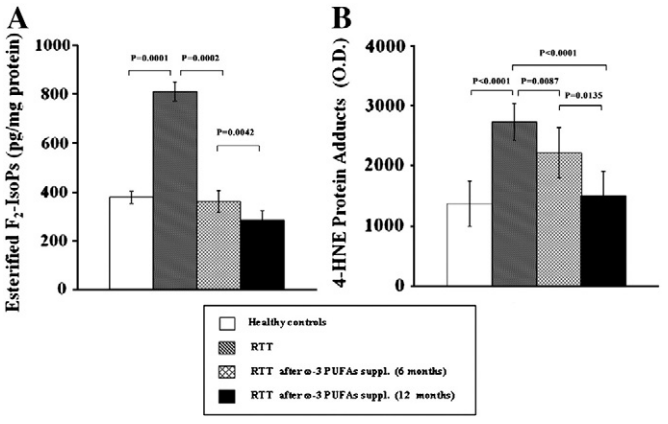

Fig. 4. RBC membrane oxidative damage. (A) Esterified F_2-IsoPs. (B) 4-HNE Protein Adducts. The data were expressed as means ± SE. All the statistical significant differences were reported.

3.6. RTT erythrocyte morphology after ω-3 PUFAs supplementation

ω-3 PUFAs supplementation induced several changes in erythrocyte morphology of RTT patients (Fig. 1; lower panels: I and L). After supplementation, a decrease of leptocytes (at 6 months, $P=0.042$; at 12 months $P=0.0046$) was observed and found to be accompanied by an progressive increase in reversible forms of erythrocytes, such as stomatocytes (at 6 months, $P=0.01$; at 12 months, $P=0.004$) [33]. In addition, a significant increase in knizocytes (at 6 months, $P=0.008$; at 12 months, $P=0.002$), and discocytes (i.e., normal RBCs shape) (at 12 months, $P=0.0402$) was recorded (Fig. 2).

3.7. OS and oxidative damage in typical RTT erythrocytes after ω-3 PUFAs

Following 12 months of ω-3 PUFAs supplementation, intraerythrocyte (0.55 ± 0.10 nmol/ml) and plasma (0.46 ± 0.08 nmol/ml) NPBI levels were significantly reduced as compared to values before supplementation (time zero) (−16.4%) and the values were similar to those of controls. Significant differences were already observed in the treated group at time 6 months in both intraerythrocyte (0.7 ± 0.17 nmol/ml) and plasma (0.51 ± 0.15 nmol/ml) NPBI (Fig. 3, Panels A and B).

A significant decrease was observed in oxidative damage markers levels: after 6 months of ω-3 supplementation esterified F_2-IsoPs were strongly decreased (−50%) ($P=0.0002$), and the value was similar to control; at 12 months of supplementation no further decrease was observed (Fig. 4, Panel A). 4-HNE- PAs levels were similarly decreased (−23.2%) after ω-3 supplementation, but values similar to controls were obtained after 12 months of treatment (Fig. 4, Panel B).

In ω-3 PUFAs supplemented group, clinical severity decreased of 25.5 and 30.1% at 6 and 12 months, respectively (CSS at time zero: 25.2 ± 10.9; CSS at time 6 months: 19.1 ± 9.5; CSS at time 12 months: 17.1 ± 8.9; ANOVA $P<0.005$; pairwise comparisons time 0 > time 6 months = time 12 months). Conversely, no significant differences in clinical severity were observed in the unsupplemented group of patients (CSS at time zero: 25.1 ± 11.5; CSS at time 12 months: 26.0 ± 10.8; $P=0.845$). Major improvements were observed in the areas of attention, breathing abnormalities, muscular tone, ambulation, autonomic dysfunction, and somatic growth.

4. Discussion

The study of erythrocyte morphology is of great importance in field of rheology, as the deformability of the circulating cells has a fundamental influence on the rheological properties of blood [34].

For the first time, we report that RBCs shape is abnormal in RTT and appears to be mainly modulated by OS. In particular, leptocytes were found to be the predominant altered erythrocyte shapes in untreated typical RTT.

We did not evidence a relationship between mutation type and RBCs morphological phenotype, as a quite homogenous RBCs shape phenotype correspond to a heterogeneous phenotypical clinical expression of the disease. Therefore, it is possible that the abnormal RBCs shape is linked to the *MeCP2* mutation by the presence of the mutation per se, with all the possible biochemical consequences, rather than the specific point mutations within *MeCP2* gene.

The percentages of abnormal RBCs shape were found to be related to increased (intraerythrocyte and plasma) NPBI and evidence of membrane oxidative damage, as demonstrated by the increased formation of esterified F_2-IsoPs and 4-HNE PAs.

We also showed that antioxidant substances such as ω-3 PUFAs, by inducing a decrease in the intraerythrocyte and plasma NPBI and in the membrane oxidative damage markers, can be able to partially rescue altered RBCs shape changes by increasing the percentage of the atypical forms reversible to discocytes.

Table 2
Correlation matrix for RBC shape as a function of OS markers.

OS markers	Erythrocyte shape category						
	Lepto-	Knizo-	Stomato-	Codo-	Leptocodo-	Disco-	Echino-
P-NPBI	**0.6645**	0.3266	−0.1999	0.2052	**0.6454**	**−0.5848**	−0.1522
	(0.0184)	(0.3001)	(0.5333)	(0.5222)	**(0.0234)**	**(0.0458)**	(0.6368)
IE-NPBI	**0.6690**	0.0927	**−0.6075**	0.4978	−0.1316	−0.3501	−0.2584
	(0.0174)	(0.7745)	**(0.0362)**	(0.0996)	(0.6835)	(0.2646)	(0.4173)
Membrane esterified F_2-IsoPs	**0.6281**	−0.1133	**−0.8056**	0.1859	0.5117	−0.2360	−0.4618
	(0.0287)	(0.7259)	**(0.0016)**	(0.5629)	(0.0890)	(0.4602)	(0.1307)
Membrane 4-HNE PAs	**0.7845**	0.2484	−0.4264	**0.6180**	0.2926	−0.5700	−0.0807
	(0.0025)	(0.4362)	(0.1669)	**(0.0322)**	(0.3560)	(0.0530)	(0.8031)

Data are Spearman's rho correlations coefficient with in brackets P-values (N = 12).
Legends: Lepto-, Leptocytes; Knizo-, Knizocytes; Stomato-, Stomatocytes; Codo-, Codocytes; Leptocodo-, Leptocodocytes; Disco-, Discocytes; Echino-, Echinocytes; IE-NPBI, intraerythrocyte NPBI; P-NPBI, plasma NPBI; F_2-IsoPs, F_2-Isoprostanes; 4-HNE PAs, 4-HNE protein adducts. Bold character: statistically significant correlations.

It is known since a long time that redox-active iron is one of the most active source of OS. In turn, in prior research, we have demonstrated that iron is released from hemoglobin in a free redox-active form when RBCs are challenged by OS [11]. Iron release is accompanied by Met-Hb formation and is higher under hypoxic condition that under normoxia [35]. In RTT patients, a hypoxic condition is evidenced (as reported in the Supplementary Table), thus confirming our previous data [5], and is associated with an increase in Met-Hb and CO-Hb concentrations in comparison to controls, as well as a decrease in PaO_2 and SpO_2. Furthermore, pulmonary gradient for O_2 [(A-a)PO_2: O_2 alveolar-arterial gradient] was increased in RTT patients as compared to controls, thus suggesting a compromised pulmonary gas exchange. The hypoxia-induced increase in iron release is consistent [35,36] with the observation [37] that during the hypoxic incubation of partially oxygenated hemoglobin superoxide radical is formed, the production of which coincides with autoxidation of hemoglobin. In addition, Nagababu et al. [38] have shown that iron release occurs after hemoglobin autoxidation, formation of reactive oxygen species and loss of normal tetragonal symmetry around heme iron.

In RTT erythrocytes, a similar condition likely occurs with a consequent free radical-catalyzed peroxidation of esterified AA in the membrane phospholipids and formation of esterified F_2-IsoPs. Likewise, 4-HNE, as generated by free radicals attack on ω-6 PUFAs [26], leads to protein damage (protein oxidation), as indicated by increased 4-HNE PAs here observed in RBCs membranes. While the presence of esterified F_2-IsoPs likely indicates an in situ damage occurring in the RBCs membranes, the source of 4-HNE is unclear, given its known role of toxic messenger of oxygen free radicals [26]. On the other hand, it is conceivable that 4-HNE may derive from lipid peroxidation either of the plasma or erythrocyte lipid components. Increased 4-HNE PAs in RTT patients, indicating a protein damage to the erythrocyte membranes, reinforce our prior findings of increased plasma 4-HNE PAs in patients with several clinical manifestations of RTT [39].

Erythrocytes in RTT are likely exposed to a highly pro-oxidant environment, both inside (increased intraerythrocyte NPBI) and outside the cell (increased plasma NPBI). In turn, high levels of NPBI can trigger the lipid peroxidation events and RBCs morphological changes. In contrast, two factors well known to potentially affect RBCs shape, such as blood pH and osmolality (data not shown), appear to be within the normal range in RTT.

Interestingly, no pits and holes in the erythrocytes from RTT patients were observed in contrast with what was reported in vitro in mouse RBCs incubated with phenylhydrazine and consequent hemolysis [18]. However, while antioxidant levels were significantly reduced in those experimental models [18], GSH levels in RTT patients appear to be slightly increased, and correspondingly on the clinical side, no hemolytic anemia is usually observed in RTT patients. It is interesting to note that blood GSH is also increased in COPD patients [8,40], a further shared feature between COPD and RTT.

Overall, our in vivo data confirm the results of prior in vitro studies about the importance of a normal intra- and extra-cellular environmental redox status for a normal erythrocyte shape, and that exposure to oxidants lead to significant erythrocyte shape abnormalities [15–18,41].

OS and hypoxemia can lead to RBCs shape abnormalities in chronic pulmonary disease. To date, the oxygen transfer from the alveoli to the RBCs was considered as a constant. However, this is likely not to be the case whenever the erythrocyte or alveolar morphology changes.

All this is to be taken in mind when evaluating the physiopathology of O_2 lung exchanges in RTT, where chronic respiratory abnormalities are reported [5,6]. In addition, due to the hypocapnia very commonly detectable in RTT patients, the “real” hypoxemia could be more severe than the “nominal” hypoxemia. It can be hypothesized that at least part of the hypoxemia observed in RTT patients may be related to impaired oxygen gas exchange between RBCs and lung cells [5]. Some similarities and some differences between COPD and RTT regarding RBCs morphology appear to exist. In fact, different

Table 3
Correlation matrix for RBC shape as a function of pulse-oximetry parameters, pulmonary gradients for O_2 [(A-a)PO_2], and partial arterial pressure of oxygen (PaO_2).

Variables	Erythrocyte shape category						
	Lepto-	Knizo-	Stomato-	Codo-	Leptocodo-	Disco-	Echino-
PaO_2 (mmHg)	**−0.826****	−0.0018	**0.6420****	**−0.529****	**−0.3461***	**0.4670****	0.2610
(A-a)PO_2 (mm Hg)	**0.6566****	0.0575	**−0.4853****	0.2540	**0.7141****	**−0.3748***	−0.3125
Met-Hb (%)	**0.7173****	0.0782	**−0.5887****	**0.3550***	**0.7228****	**−0.457****	−0.1780
CO-Hb (%)	**0.7518****	−0.0703	**−0.6999****	**0.3687***	**0.4657****	**−0.3708***	**−0.3705***
PI	**−0.769****	−0.3029	0.3240	**−0.534****	**−0.5606****	**0.6140****	0.3178
PVI	**0.8678****	**0.3523***	**−0.4060***	**0.643****	**0.4944****	**−0.670****	−0.1361

Data are Pearson's coefficients from linear regression analyses (N = 12).
Legend: Lepto-, Leptocytes; Knizo-, Knizocytes; Stomato-, Stomatocytes; Codo-, Codocytes; Leptocodo-, Leptocodocytes; Disco-, Discocytes; Echino-, Echinocytes; PaO_2, partial arterial pressure of oxygen; (A-a)PO_2, O_2 alveolar-arterial gradient; Met-Hb, methemoglobin; CO-Hb, carboxy-hemoglobin; PI, Perfusion Index; PVI, pleth variability index. Bold character: statistically significant correlations. * 0.05 < P-value < 0.01; ** P < 0.01.

forms of altered RBCs, such as a majority of acanthocytes and a few leptocytes have previously been reported in COPD patients [7]. In the present study, we report a great majority of leptocytes without evidence of acanthocytes, thus suggesting only a partial similarity between RTT and COPD concerning RBCs shape abnormality. Interestingly, a short period (3 months) of supplementation with N-acetylcysteine (NAC) has been reported to be able to reestablish the normal shape in RBCs from COPD patients [7], while in the present study 6 to 12 months of ω-3 PUFAs supplementation partially rescued RBCs morphology in RTT patients. The concept that RBCs shape and integrity is to be considered as a biosensor of OS imbalance in chronic lung disease [7–10] is also likely to be applicable to the RTT. RTT and COPD, although quite different for multiple reasons, do share the diad hypoxia/oxidative stress (OS) which, in our opinion, per se justify an investigation of red cell morphology as an important biosensor for OS, independently from the presence or absence of anemia. Furthermore, it should be underlined that COPD patients rarely present anemia, whereas frequently do show polycythemia. RBC morphological alterations are reported in few other pathologies with proven OS enhancement, notably COPD [7] and, more recently, end-stage renal disease [42]. However, it is interesting to note that echinocytosis and stomatocytosis are the morphological hallmark features for these conditions. In line with these clinical observations, also prior in vitro experiments with known oxidants, such as phenylhydrazine [18], results mainly in echinocytosis. On the other hand, RTT patients show mainly a leptocytosis, an RBC morphological feature which is not specific to a particular pathology as it can be observed in a wide spectrum of conditions ranging from thalassemias [43–47] to iron deficiency (e.g., in dogs) [48], adverse effects to drugs (e.g. phenothiazine) [49], diseases of the liver and the biliary tract [50], or even as a possible familial trait [51,52]. However, in the examined RTT patients: 1) hemoglobin electrophoresis appears to be normal and there is no evidence of hemolysis (preliminary unpublished data); 2) plasma iron content was found to be normal (data not shown); and 3) none of the patients received phenothiazine or derived drug compounds. Thus, although likely nonspecific, we may conclude that the observed RBC shape changes in RTT appear to be distinct from those usually observed in other conditions with increased OS. Nevertheless, the correlations study, here reported, indicates an association between erythrocyte shape and the levels of biomarkers of OS in both plasma and RBCs.

Oxidative modifications in the pulmonary microenvironment are known to result in a number of functional changes in several tissues as well as in blood [8]. The erythrocyte seems to be one of the most important tools of antioxidant defenses in the vasculature and, likely for the whole organism [8]. If the oxidative insult of the microenvironment overcomes the RBCs' defenses, this cell undergoes oxidative alterations, such as change of rheologic/functional properties, that can still be stably detected at the periphery. This hypothesis is reasonable because this cell crosses the lungs once a minute, and it is conceivable that in the presence of intense and chronic OS, it is not possible to fully repair the damage [8]. RBCs deformability is a prerequisite for their journey through the blood vessels and is essentially due to (1) a peculiar microviscosity, (2) an extremely "flexible" cytoskeleton, and (3) the ability to adhere to each other without undergoing stable cluster formation. All in all, these features lead to a cell plasticity that allows RBCs movement in the blood flow. All these features are lost once the cell is exposed to an oxidative insult [53,54].

In the present study, pulse oximetry in RTT patients represents a faithful indicator of chronic hypoxemia as shown by decreased PI values (a marker of peripheral vasoconstriction), and increased PVI values (a marker of increased intrathoracic pressure, i.e. pulmonary obstruction).

In fact, in typical RTT the lung appears to be unexpectedly a key organ for the disease. To this regard, it is interesting to note that a chronic lung disease resembling RB-ILD, likely affecting terminal bronchioli, has been recently discovered in RTT patients [6]. Therefore, it is possible that chronic obstructive disease leads to RBCs shape changes through the complex mechanisms of hypoxia and enhanced OS.

As prior studies on RBCs in patients with chronic pulmonary diseases have demonstrated microfilament network redistribution (actin and spectrin) [9] and a concomitant decrease in the band 3 phospho-tyrosine phosphatase activity, and altered function of anionic exchanger [10], further studies are needed in order to better characterize possible alterations of erythrocyte membrane proteins in RTT.

Several substances, such as primaquine [41], diclofenac [55], phenylpropanolamine [56] have been reported to induce structural RBCs changes, whereas antioxidants such as NAC [57], desferrioxamine [18], and *Rodhiola rosea* acqueous extract [58] can counteract erythrocyte alterations. Specifically, carbamazepine (CBZ), an antiepileptic drug commonly used in RTT, has been reported to produce echinocyte formation in vitro [59].

In our study, altered RBCs shapes were observed both in patients taking CBZ as well as in those not assuming this drug. In addition, the observed percentages of echinocytes were negligible (≤2%) and no significant changes were observed in the peripheral blood of typical RTT, either in untreated or ω-3 PUFAs supplemented patients. Although stomatocytes represent abnormally shaped RBCs, the reduction of the relative distribution of stomatocytes are seen as reflecting a hostile environment in that a reduction of these RBC shapes can also be considered equivalent to a reduction of those of erythrocyte shapes that are known to be reversible to normal discocytes. On the other hand, a wide family of drugs and substances are known to be "stomatogenic" [58,60,61]. It is therefore possible that ω-3 PUFAs, as known antioxidant agents, could induce stomatogenesis in the treated patients. Likewise, knizo- and codocytes are also detectable in conditions other than RTT. For instance, increased knizocytes, thought to be the results of mechanical deformation forces [33], are elevated also in diabetic vasculopathy [62]. In our study, knizocytes are found to be increased in the untreated RTT patients. Our unpublished data suggest that a systemic lupus erythematosus (SLE)-like microvasculopathy is present in the great majority of patients with the typical form of RTT when evaluated by nailfold capillaroscopy (Hayek et al., unpublished). Therefore, it is conceivable that this RTT-associated microangiopathy could, at least in part, be a cause for the increased percentage of knizocytes in the untreated patients, although further increase after ω-3 PUFAs supplementation here. Likewise, it is important to underline that the percentages of codocytes are already elevated in untreated RTT patients and they are further increased by ω-3 PUFAs supplementation. To date, no clear explanations for these phenomena exist. However, it has been reported that dietary fatty acids can affect erythrocyte shape in rodent models [63].

ω-3 PUFAs are known to influence the physical nature of cell membranes and membrane protein-mediated responses, lipid-mediator generation, cell signaling, and gene expression in many different cell types [27]. Here, we have shown that supplementation with ω-3 PUFAs is able to partially rescue RBCs shape changes by increasing the percentage of the atypical forms reversible to discocytes.

This morphological rescue is associated with a decrease in the intraerythrocyte and plasma NPBI levels as well as a reduced formation (up to normalization) of esterified F_2-IsoPs and 4-HNE PAs. Likewise, improved oxygen lung exchanges (decreased pulmonary gradients for O_2) and PaO_2 levels were observed, together with reduced production of Met-Hb and CO-Hb, decrease of PI and PVI values. It is interesting to note that no significant changes were observed between 6 and 12 months of ω-3 PUFAs supplementation. On the other hand, we had previously noticed that, following ω-3 PUFAs supplementation, clinical and biochemical changes are usually observed within the first six months [64,65], while the beneficial

effects appear to maintained with no further significant improvement. We have previously observed that if ω-3 PUFAs supplementation is suddenly interrupted for some reasons (lack of compliance, transient lack of availability of the pharmaceutical formulations, etc.…), then a rapid decrease of the beneficial effects follows [64].

In addition, cumulating evidence suggests that RTT could be considered as a "free radicals disease", somehow triggered by a *MeCP2* gene mutation or deletion, although the precise pathogenetic mechanisms leading from gene mutation to disease expression are still to be clarified. It is interesting to note, however, that we have a clear example of a genetic disease that benefits from the administration of particular fatty acids ("Lorenzo's oil," i.e., a 4:1 mixture of glyceryl trioleate and glyceryl trierucate) as it is the case for the X-linked adrenoleukodistrophy [66]. A positive effect from free radical scavengers in this same disease has been reported to reduce clinical symptoms in patients [67] and halting axonal degeneration in a mouse model [68]. Moreover, recent evidence indicates a beneficial action of propolis, a resinous substance collected from plants by bees, in the genetically determined disease of the RBC membrane hereditary spherocytosis [69]. Taken together, these data indicate that in certain genetically determined conditions, the action of administered fatty acids can lead to therapeutical results far more effective than simply providing symptomatic relief, and may account for the observed effects of ω-3 PUFAs on the relative distribution of knizocytes, stomatocytes and codocytes shown in our RTT patients population.

Although the mechanisms of action for ω-3 PUFAs are far from being fully understood, EPA and DHA have been shown to compete with AA for the conversion by cytochrome P450 enzymes, resulting in the formation of alternative, physiologically active, metabolites (17,18-epoxyeicosatetraenoic, and 19,20-epoxydocosapentaenoic acid, respectively) that could mediate some of the observed beneficial effects [70]. As a consequence, the partial rescue of chronic mild hypoxia and gas-exchange compromise observed in our RTT patients could be partially mediated by an effect on target respiratory chain components, ultimately leading to increased average PaO_2 values. Therefore, the reduction of leptocytosis after ω-3 PUFAs supplementation, here reported, may be seen as reflecting a partial rescue of hypoxia.

Furthermore, in considering the role of antioxidant activity of ω-3 PUFAs we have also to keep in mind the relationship between redox status and menstrual cycle [71,72]. As our previous studies have shown the importance of oestradiol (E_2) in the modulation of redox status in healthy women [73,74]. Therefore, RTT patients with menstrual cycles were sampled in the mid-follicular phase.

In conclusion, these findings indicate that in RTT patients 1) RBCs shape is specifically altered; 2) the OS-hypoxia diad is a key factor in generating the RBCs altered shape and membrane damage observed, and 3) ω-3 PUFAs are able to partially rescue altered RBCs morphology, the OS-derived damage and the cardio-respiratory dysfunction. Consequently, monitoring of RBCs morphology can be an important new diagnostic and prognostic tool in this particular form of ASDs in which the lung seems to represent an unexpectedly key organ for the disease pathogenesis. The prognostic significance of our findings may be related in particular to prediction and prevention of apnoeas, since erythrocyte shapes were found to be related to hypoxia and increased PVI. In the near future, it is conceivable that patients with significantly abnormal RBC shapes could be assigned to nasopharyngeal continuous positive airway pressure ventilation, alpha lipoic acid, theophylline or caffeine. Moreover, the presence of apnoeas can, in turn, trigger seizures. As a consequence, the study of RBC morphology can be potentially helpful in the prevention or early treatment of apnoeas in RTT.

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Conflicts of interest

All authors declare that they have no conflicts of interest related to the present study.

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