Sickle cell anemia: haemorheological aspects

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Summary. The maintenance of erythrocyte shape and membrane integrity is bound to the modification of deformability and/or permeability. Usually, this features are not investigated with normal laboratory tests. The membrane stiffness, the cell geometry, and the viscoelasticity components are influencing factors on survival and functionality of the erythrocytes. Only few studies have analyzed the viscoelastic characteristics of red blood cells, even less are the studies on patients affected by sickle cell disease (SCD), a pathology characterized by acute and chronic impairment of cell flexibility due to the formation of intracellular sickle haemoglobin (Hb S) polymers. A critical point of SCD is represented by the rheologic alterations of sickle cells determined by the transition from sol to gel of haemoglobin producing a dramatic change in cell viscosity and viscoelastic properties. We have investigated the behaviour of the blood in SCD, from an original rheological point of view, by evaluating the viscoelastic properties of sickle cells in oscillating harmonic sinusoidal mode. A comparison between patients with different severity of the disease, with transfusion dependence (TD) or without transfusion dependence (NTD), has been carried out. This study has confirmed the rheologic impairment of SC blood. The TD patients showed a minor heterogeneity of rheologic behaviour in comparison with NTD patients, because of the normalizing effect of transfusion. The analysis of viscoelastic properties might be an additional useful tool for monitoring transfusional and pharmacological treatments.

Key words: sickle cell disease, blood viscosity, erythrocyte deformability, blood visco-elastic properties.

Riassunto *(Anemia falciforme: aspetti emoreologici)*. Le alterazioni morfologiche, la modificazione in deformabilità e/o permeabilità dell'eritrocita a cui seguono danni di tipo emoreologico, normalmente non son evidenziabili con i normali test di laboratorio, e innescano una serie di reazioni a catena che possono determinare un danno irreversibile con conseguente sequestro da parte degli organi emocateretici. Non molti sono gli studi sulle caratteristiche viscoelastiche degli eritrociti, ancora meno sono quelli su pazienti portatori di anemia falciforme (AF), una patologia caratterizzata da un danno acuto e cronico della flessibilità cellulare dovuta alla formazione di polimeri intracellulari di emoglobina S. Un aspetto critico della AF è rappresentato dalle alterazioni reologiche delle cellule falcemiche determinate dalla transizione da sol a gel dell'emoglobina accompagnata da un drammatico cambiamento della viscosità e delle proprietà viscoelastiche del globulo rosso. In questo lavoro sono stati studiati pazienti sia omozigoti per AF che portatori di doppia eterozigosi S/α o β - talassemia, trasfusione dipendenti (TD) e non trasfusione dipendenti (NTD), valutando le proprietà viscoelastiche tramite regime oscillatorio. Questo studio ha confermato la presenza di alterazioni reologiche nella AF. I pazienti TD, essendo normalizzati dall'effetto della trasfusione, hanno mostrato una minore eterogeneità rispetto ai pazienti NTD. La valutazione in regime oscillatorio delle proprietà viscoelastiche potrebbe essere un ulteriore utile strumento che permette di monitorare trattamenti trasfusionali e farmacologici.

Parole chiave: anemia falciforme, emoreologia, deformabilità dei globuli rossi, proprietà viscoelastiche del sangue.

INTRODUCTION

"The history of our understanding of sickle cell disease can be likened to the opening of the proverbial Russian egg: at once another egg appears within, and inside that another and yet another and so on. Thus, in the study of sickle cell disease, first we see the ailing and anemic patient, then his deoxygenated, sickling red cell, then its abnormal haemoglob-

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in, and finally, hidden away inside the beta chain of the molecule, a single displaced amino acid" [1].

Oral history passed down through African generations told of an inherited illness with episodes of sudden onset bone pains and of many infantile deaths. The earliest known medical paper with the original name of "sickle cell disease" was published in 1874 by Africanus Horton [2].

The scientific pathway from man to molecule begins with the recognition of the clinical aspects of sickle cell disease (SCD) at the beginning of the past century. Herrick's initial description in 1910 of elongated crescent-shaped cells led to a variety of *in vitro* experiments which attempted to explain this phenomenon.

Many later studies showed that the formation of sickle polymer is complex and involves multiple types of chemical interactions. A large body of precise and coherent informations has been gathered on the mechanisms underlying the polymerization of S hemoglobin (Hb S). They form the cornerstone of our understanding of the pathogenesis of SCD [3-6].

SICKLE CELL DISEASE: A FOUNDER MUTATION

SCD, marked by misshapen red blood cells, is usually caused by a founder mutation. It fits in the germ-line mutations category and it is passed down intact over generations [7].

The SC mutation today can be found in five different haplotypes (Senegal, Benin, Bantu, Arab-India, Cameroon) leading to the conclusion that the mutation appeared independently five times in five different founders in human history. Their highest frequency occurs in tropical areas but the population migrations have ensured that they are encountered in most different countries [8].

The frequency of a founder mutation in the population is governed by two competing forces in which the beneficial effects drive the frequency of the mutant gene up, while the harmful effects damp down the frequency. The SCD is a well-known example of the so called balancing selection: it apparently arose repeatedly in regions ridden by malaria, in Africa and the Middle East. A single copy of SC gene helps the carrier to survive malarial infection (protective effect) while two copies produce a homozygous sick state with lower survival rates [7].

As a result of heterozygote advantage against malaria, the inherited haemoglobin disorders are the commonest monogenic disease [8]. The inherited disorders are classified into two groups: the structural variants and the thalassaemias (thal). The first group results from single-amino-acid substitutions in α or β chains; the second group is characterized by abnormal globin gene function resulting in total absence or in quantitative reduction of α or β globin chain synthesis. The α- and β-thalassaemias are a heterogeneous group of haematological disorders with a high incidence in a wide areas extending from Mediterranean and African regions, the Middle East, the Indian sub-continent, South-East Asia, Melanesia and Pacific Islands [8].

The distribution of the β^s gene in the Old Word corresponds to that of falciparum malaria [9-11]. In the various populations of equatorial Africa, the gene frequency ranges from 5% to more than 14%; it is about 4% among Caribbeans, Europeans, and North and South Americans of African descendent. The sickle gene is also indigenous to Sicily, Greece, India, Saudi Arabia, Israel, Turkey and Iran [9-14].

SICKLING DISORDERS

The sickle cell picture shows the propensity of the red blood cells to assume a sickled configuration when blood is deoxygenated, accompanied by the loss of potassium and water which it provides an inhospitable environment for the parasite of *Plasmodium Falciparum*.

SCD pathophysiology is mediated by acute and chronic impairment of cell flexibility due to the formation of intracellular Hb S polymers as cells are partially deoxygenated in the microcirculation.

The vicious circle of the erythrocyte membrane damage of SCD is well-known. The sickling phenomenon depends on the formation of deoxyhaemoglobin S and the transition from a sol to a gel is accompanied by a dramatic increase in viscosity. The consequent increase in intracellular haemoglobin concentration accelerates and potentiates the rate of deoxygenation of the erythrocytes at which further polymerization can occur. This is the beginning of the numerous structural and functional abnormalities of sickle red blood cell [3-6, 13-16].

The clinical manifestations of SCD vary enormously between and among the major genotypes ranging from asymptomatic subjects to patients disabled by recurrent pain and chronic complications. Typically, patients are anaemic but lead a relatively normal life with painful episodes. Virtually every organ system in the body is subject to vaso-occlusion, which accounts for the characteristic acute and chronic multisystem failure of this disease.

In the homozygous state (Hb SS) and in double heterozygous conditions (Hb S/β thalassemia), the presence of Hb S induces heterogeneous morbidity, that can be grouped in four major categories: chronic haemolytic anaemia, systemic manifestations with increased susceptibility to infections, vaso-occlusive episodes or painful "crises" of varying severity and frequency, organ damage as consequence of multiple vaso-occlusive events [15].

SICKLE BLOOD CELL RHEOLOGY

The whole blood viscosity is a function of both the number of erythrocytes and their deformability and of the plasma proteins. It is known that the blood is a non-Newtonian fluid and its viscosity is markedly dependent on shear rate. The plasma has a high

protein-mediated effects and exhibits RBC-RBC adhesive interactions at lower shear rates that are mediated by large plasma proteins (*i.e.* fibrinogen) [4-8, 17-19].

In SCD the viscosity is dominated by HbS gelation and by the presence of dense sickle cells. As reported by Ballas and Mohandas [18], a wide number of interrelated factors influence the micro – and macro – rheology of the sickle blood: plasma components, haematocrit (Hct), the unsickling-sickling red cells cycles, the cellular dehydration, the erythrocyte deformability and mechanical fragility,vascular factors, α-globin genotypes and β globin aplotypes, the white cell populations, the haemostatic factors, the epistatic genes, and the environment. All the above-mentioned determinants together with the alterations of the Virchow triad on the haemostatic system and genetic factors [19-21] are responsible for aberrant interaction of sickle RBC with vascular endotelium and can modulate the SCD severity.

Upon deoxygenation the viscosity of sickle blood rises sharply, because the polymerization of deoxy-HbS, therefore in SCD the presence of anaemia with a decrease in Hct can be considered protective of microcirculatory flow. The increase in Hct by transfusion therapy, used in severe cases of SCD, can rise the viscosity and upset this balance resulting in hyperviscosity and clinical deterioration. The transfusion guidelines for SCD have defined the post-transfusional Hct value is less than 0. 35 (or Hb concentration of <11-12 g/dL) and sickle red blood cells (SS-RBCs) between 30 and 50 % [22, 23]. Until today the management of SCD is of supportive care and the red cell membrane deformability and the hypoxia are the cardinal points of the pharmacodynamic research. The use of hydroxyurea (HU), a rheological drug, has been proposed for its ability to rise the level of fetal haemoglobin (Hb F), increase mean cell volume and reduce neutrophil count. Therefore, HU increases the whole cell deformability with a consequent better oxygen supply to the tissues [2, 22].

The viscoelastic properties of the sickle gel depend on both the density and rigidity of the component fibers. Thus, rheological measurements on sickle gels have not only provided new insights into the structure of the gel but also have clear-cut implications regarding the deformability of sikle cells *in vivo* [15].

The effects of the rheological properties of the gel and the intrinsic behaviour of haemoglobin S, with or without other genetically determined abnormalities of the α or β chain, can be regarded as the proximate and most immediate cause of the vaso-occlusive manifestations in these patients. In fact, a hallmark of these patients is the vaso-occlusion of small and sometimes large vessels that allow a higher morbidity and mortality, because of the involvements of the vaso-occlusive formations.

Decreased deformability of sickle cells has been documented using a variety of techniques, including viscosimetry, filtration, ektacytometry, and micropipete aspiration of individual cells [18-21] that have pointed out, respectively, an increased viscosity of blood, decreased filtration rate of diluted cell suspension through narrow pores, decreased ability of cells to undergo deformation in shear fields, and increased aspiration pressures needed to induce entry of cells into micropipets. These studies have demonstrated a marked heterogeneity in the extent of erythrocytes rheologic abnormalities in different SCD subjects and often in the same individual blood sample [18-21].

In order to study the rheological behaviour of sickle blood cells in SCD, a comparison between transfusion dependent (TD) or no-transfusion dependent (NTD) patients with different severity of the disease was carried out by evaluating the viscoelastic properties of sickle cells in oscillating harmonic sinusoidal mode.

MATERIALS AND METHODS

Blood samples

The blood samples were collected by venepuncture using plastic tubes containing 4.7 mM K_3 ethylenediaminetetra-acetic acid (EDTA K_3) and processed within 2 hours. The samples from SCD patients (Day Hospital Talassemici, S. Eugenio Hospital, Rome, Italy) were analyzed in triplicates and in comparison with samples from healthy blood donors as controls (periodic healthy blood donors of Donors Group – Italian Red Cross, Centro Aziendale Produzione Emocomponenti – Azienda Ospedaliera San Camillo – Forlanini).

Patients

Ten SCD patients (mean age 33 years) were analyzed: n. 5 subjects TD and n. 5 subjects NTD. Both groups included two HbSS and three HbS/β thalassaemia patients. Moreover, two TD patients and one NTD patient carried a combined 3.7 alfa globin gene mutation. All TD patients showed a rate of HbS less than 35% whereas the rate of NTD ranged from 66 to 68%.

The regular transfusional terapy of TD patients was justified by clinical severity of the disease: bone infarction, hepatobiliary injury, priapism, skin ulcers and cardiac complications. The NTD group showed a milder clinical picture with the exception of the patient n.8 that had manifested the avascular necrosis of the femoral head.

Haemorheological assays

For the haemorheological assays a rotational viscosimeter RV20 was used with the couette measuring system CV100. The coaxial cylinder sensor systems are known as ME 31. Shear rate and test temperature were controlled through the rheocontroller RC-20 (HAAKE - Karsruhe, Germany). Blood samples were analyzed according to the Recommendation of the International Committee for Standardization in

Haematology (ICSH) [24]. Each blood sample was measured at 37 °C in oscillation harmonic sinusoidal mode to determine its viscoelastic properties. The oscillating movement allows us to investigate the structure of the fluid and to study its dynamic and mechanical behaviour.

Viscoelastic properties were analyzed by elastic modulus G' and viscous modulus G". G' represents the elastically stored energy in a sample subjected to oscillating deformation, while G" marks the amount of energy which is dissipated into heat as the result of viscous flow.

We used two analytical procedures to evaluate the rheological behaviour: strain and frequency tests. Strain test was used to determine the deformability pattern of the sample in order to analyze the linear viscoelastic region. The test was conducted at constant ω (deformation velocity), varying the strain (amplitude of deformation). Frequency test was used to measure the dynamic and mechanical properties of the fluid which enables us to investigate its viscoelastic behavior under several stress conditions.

We studied the tangent of phase shift angle (tan delta = G"/ G') as a function of strain rate at a constant value of deformation amplitude, selected on the previously determined linear viscoelastic range by strain test. The frequency test was evaluated in the range from 0.1 to 10 Hz. ($f = \omega/2\pi$).

For the evaluation of G' and G" values we used the mathematical model known as "Power law":

$$X = K^* Y^n$$

where X is the independent variable and Y is the dependent variable.

It is possible to associate different variables to the X and Y value and to calculate the related constants. In this way we can evaluate all the behaviours of interest using only two constants instead of a large number of experimental data.

The meaning of each constant depends on the considered variable.

The function is represented by a curve, the constant "K" is associated with the position of the curve inside the graph plane, and the constant "n", with the slope of the curve. The calculated constants were compared with those obtained from healthy controls.

RESULTS

The distribution of the viscoelastic behaviours expressed as tan delta (tg δ), G', and G", are shown in *Figures 1-4*.

The tg δ curves obtained by TD patients (1-5), NTD patients (6-10) and normal control (C) have been evaluated in the linear viscoelastic range from 0.1 to 10 Hz. The tg δ curve of the normal control was independent from the applied strain rate, while the tg δ curves of both TD and NTD groups showed different trends. In the TD-patients 1 and 5 *(Figure 1)* tg δ values were higher than control ones at 0.1 Hz, while were comparable to control values at 10 Hz. In the TD-patients 2, 3, 4 the slope of the tg δ was comparable with that of control in the explored frequency region (0.1-10 Hz). As concerns TD-patient 2, a particular behaviour was observed being tg δ value comparable to control value at 0.1 Hz but increased at 10 Hz.

Similarly, the tg δ curves of the NTD patients *(Figure 2)* presented two different trends. In NTD-patients n. 6, 8 and 10, the tg δ values were higher than the control ones at 0.1 Hz, while the curves of the NTD-patients n. 7 and 9 ran together with the control one. It is notewhorty that the tg δ values of patients n. 6 and 8 were very low at 10 Hz as compared with the control ones.

Since tg δ curves represents the G"/ G' ratio, we have also analised G' and G" separately in order to evaluate the relationship between elastic G' and viscous G" modulus in TD and NTD patients.

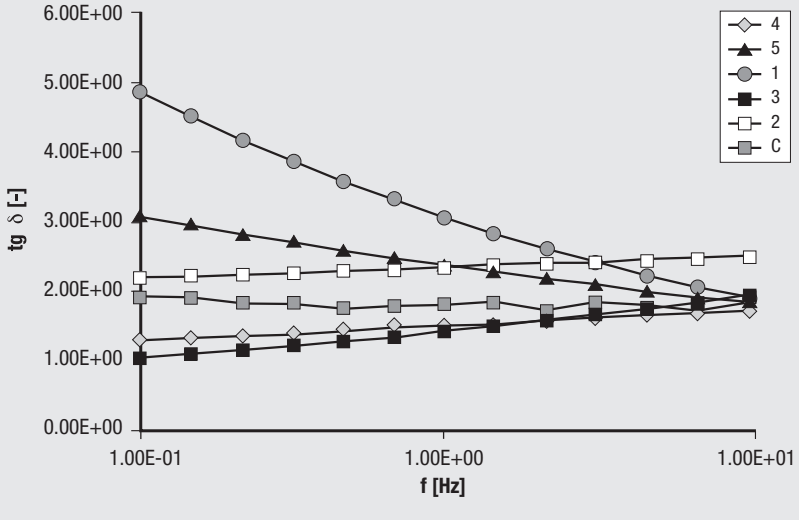

Fig. 1 | *Analysis of tangent δ (tg δ) in transfusion dependent SCD patients (1-5).*
C = control.

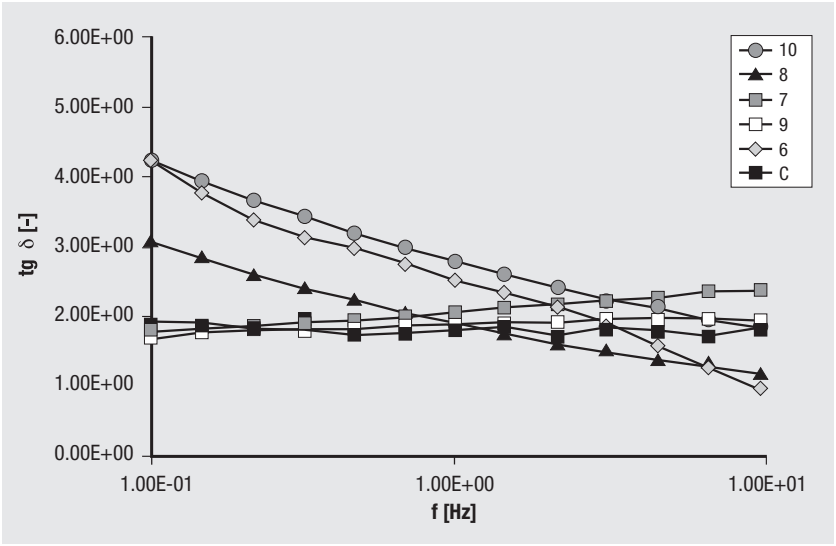

Fig. 2 | *Analysis of tangent δ (tg δ) in no-transfusion dependent SCD patients (6-10).*
C = control.

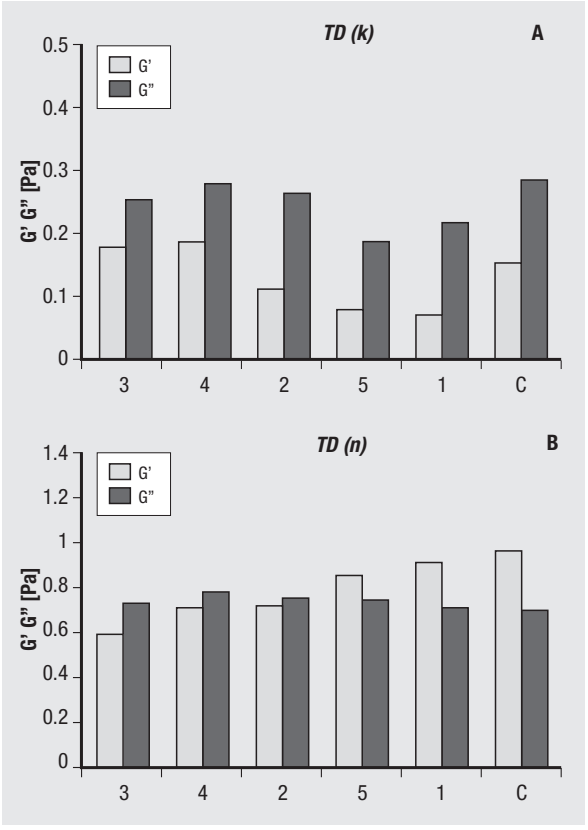

Fig. 3 A-B | *Analysis of G' elastic modulus and G" viscous modulus in transfusion dependent SCD patients (1-5).*
C = control; K = width constant; n = behaviour coefficient; TD = transfusion dependent.

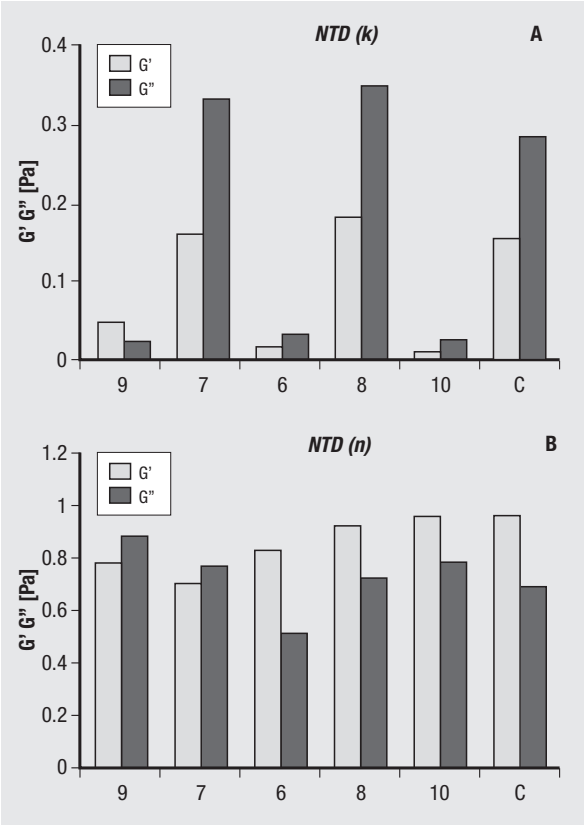

Fig. 4 A-B | *Analysis of G' elastic modulus and G" viscous modulus in no-transfusion dependent SCD patients (1-5).*
C = control; K = width constant; n = behaviour coefficient; NTD = no-transfusion dependent.

As regards the TD patients *(Figures 3A-B)*, the most relevant observations were a decrease in the constant K values in TD-patients 1, 2, and 5, while the TD-patients 3 and 4 reported K values similar to control ones.

The constant n values, that are associated with the slope of the G' and G" curves, presented viscous modulus greater than elastic one (G" > G') in the TD-patients 2, 3 and 4, while the trend of G' and G" was comparable to control in the TD-patients 1 and 5.

As regards the NTD patients *(Figures 4A-B)*, the analysis of G' and G" moduli demonstrated a wide heterogeneity of K values, and in particular a marked decrease in G' and G" was observed in NTD-patients 6, 9 and 10. About the constant n the viscous modulus was greater than the elastic one (G" > G') in NTD-patients 7 and 9 by showing a reversal of control trend.

DISCUSSION

Today, people affected by SCD have a "quality-adjusted life-years" and it is important to study this complicated disease with a multidisciplinary approach. It is not yet well understood the relationship between the physiological and rheological aspects and between the drug treatments and/or transfusional therapy and the rheological informations.

The red cell membrane viscosity and deformability in SCD has been showed to be different from those of normal erythrocytes, being sickled cells poorly deformable and this decrease in deformability leads to an increase in whole blood viscosity. Consequently, an increased impediment to flow induces an irregular erythrocyte aggregation, producing a reduction in oxygen saturation and resulting finally in further sickling of cells and vaso-occlusion in the microcirculation.

The measurement of viscoelasticity of the blood is a direct indicator of both the elastic and the viscous responses of the whole blood structure. The elastic (storage) modulus G' represents the assessment of the elastic storage of energy primarily due to kinetic deformability of the erythrocytes while the viscous (loss) modulus G" is the assessment of the rate of energy dissipation due to cell deformation.

We have investigated the behaviour of the blood in SCD, from an original rheological point of view, by evaluating the viscoelastic properties of sickle cells in oscillating harmonic sinusoidal mode. A comparison between patients with different severity of the disease, with TD or without NTD, has been carried out.

This study has confirmed the rheologic impairment of SC blood and has allowed to evidence an improvement of blood rheologic properties due to transfusion therapy. The TD patients showed a minor heterogenenity of rheologic behaviour in comparison with NTD patients, because of the normalizing effect of the transfusion. Furthermore, two different trends of tg δ were observed indipendently by the treatment: a control-like behaviour and a marked slope. The evaluation of G' and G" moduli has allowed to identify the different rheologic abnormalities of the patients, in particular the more solid-like behaviour of NTD patients with a decreased deformability. In spite of the undeniable damages of the transfusional therapy [23-25], up to today it seems to be a treatment strategy to prevent further complications of vasoocclusive events. Nevertheless, some silent subclinical processes can be masked for the existence of the chronic hypercoagulable state and in the presence of diminished flow and stasis.

The analysis of viscoelastic properties might be an additional useful tool in monitoring transfusional and pharmacological treatments and their effectiveness by providing particular insights into the deformability characteristics of sickle blood. Furthermore, the viscoelastic behaviour might give qualitative informations about the flow behaviour of the sickle blood and how the erythrocyte deformability affects the blood flow under different conditions.

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