Clinical haemorheology and microcirculation

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Summary. Hyperviscosity, due to alterations of blood cells and plasma components, can induce microvascular damage. Nitric oxide (NO) is released by endothelium and plays a crucial role in flow-mediated vasodilation. An impaired availability of NO, due to polymorphisms of endothelial NO synthase (eNOS), may influence erythrocyte deformability thus increasing blood viscosity. We investigated haemorheological variables in patients with idiopathic sudden sensorineural hearing loss (ISSHL), retinal vein occlusion (RVO) and systemic sclerosis (SSc), as possible models of microvascular damage, and their relationship with eNOS gene T-786C, G894T and 4a/4b polymorphisms. Whole blood viscosity and plasma viscosity were assessed with a rotational viscosimeter and erythrocyte deformability index (DI) with Myrenne filtrometer. eNOS polymorphisms were analyzed in ISSHL and SSc patients. At multivariate analysis alterations of some haemorheological variables resulted significantly associated with ISSHL, RVO and SSc. A significantly higher prevalence of eNOS -786C and 894T was found in both ISSHL and SSc patients than in controls; at multivariate analysis these two polymorphisms significantly affected DI in both groups of patients. These results suggest that hyperviscosity, either determined by genetic susceptibility or not, can be involved in the pathophysiology of these clinical disorders and can be the target of new therapeutic strategies.

Key words: hyperviscosity, erythrocyte deformability, eNOS polymorphisms, idiopathic sudden sensorineural hearing loss, retinal vein occlusion, systemic sclerosis.

Riassunto (Emoreologia clinica e microcircolo). L'iperviscosità, dovuta ad alterazioni delle componenti cellulari e plasmatiche del sangue, può indurre un danno microvascolare. L'ossido nitrico (ON) è rilasciato dall'endotelio e svolge un ruolo cruciale nella vasodilatazione flusso-dipendente. Una ridotta disponibilità di ON, dovuta ai polimorfismi della nitrossido sintetasi endoteliale (NOSe), può alterare la deformabilità eritrocitaria incrementando la viscosità ematica. Abbiamo valutato le variabili emoreologiche in pazienti con sordità improvvisa (SI), occlusione venosa centrale della retina (OVCR) e sclerosi sistemica (ScS), come possibili modelli di patologia del microcircolo, e la loro relazione con i polimorfismi della NOSe T-786C, G894T e 4a/4b. Le viscosità del sangue totale e plasmatica sono state misurate con un viscosimetro rotazionale e l'indice di deformabilità eritrocitaria (ID) con il filtrometro di Myrenne. I polimorfismi della NOSe sono stati analizzati nei pazienti con SI e ScS. All'analisi multivariata le alterazioni di alcune variabili emoreologiche sono risultate significativamente associate con la SI, l'OVCR e la ScS. Una prevalenza significativamente più alta degli alleli della NOSe -786C e 894T è stata osservata nei pazienti con SI e ScS rispetto al gruppo di controllo; all'analisi multivariata questi due polimorfismi sono risultati significativamente associati con un'alterazione dell'ID in entrambi i gruppi di pazienti. Questi risultati suggeriscono che l'iperviscosità, che sia o meno determinata da una suscettibilità genetica, può essere implicata nella fisiopatologia di queste malattie e può essere l'obiettivo di nuove strategie terapeutiche.

Parole chiave: iperviscosità, deformabilità eritrocitaria, polimorfismi dell'eNOS, sordità improvvisa, occlusione venosa retinica, sclerosi sistemica.

INTRODUCTION

Blood viscosity and erythrocyte deformability play a key role in maintaining and regulating microcirculation. Haemorheological variations due to alterations of blood cells and plasma components lead to hyperviscosity, which may slow blood flow and facilitate occlusive events through erythrocyte rouleaux formation and platelet aggregation. Erythrocytes have unique flow-affecting properties namely aggregability, deformability and adherence to endothelial cells, which play a major role in blood flow. Low blood viscosity leads to an

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improvement in microcirculatory flow, which enables the interactions between rheologic factors and the surrounding tissue [1]. Erythrocyte aggregation causes rheologic obstruction of the microcirculation, while damaged microcirculatory vessels in turn have a rheologic, functional and structural impact on erythrocytes. The alteration of erythrocyte membrane properties might be due to an oxidative injury, which occurs in microcirculatory disorders, mainly through lipid peroxidation.

Nitric oxide (NO) is released by the endothelium in response to shear stress and plays a crucial role in flow-mediated vasodilation [2, 3]; pharmacologic inhibition or a genetic deficiency of endothelial NO synthase (eNOS) impairs endothelium-dependent vasodilation and increases vascular resistance [4, 5]. NO, which is synthesized from L-arginine by at least 3 isoforms of NO synthase (NOS) (inducible, neuronal and endothelial) [6] contributes to vascular tone regulation [7] and maintains the functional and structural integrity of the vessel wall [8]. Finally in vitro and in vivo studies [9-12] suggested a role for NO in modulating erythrocyte deformability. An impaired availability of NO, due to nonsynonimous single-nucleotide polymorphisms (SNPs) in the coding region of NOS3, the gene for eNOS, may reduce shear stress, thus increasing blood viscosity and influencing erythrocyte deformability.

Since an impaired microvascular blood flow is at the basis of several clinical disorders, we sought to explore the role of hyperviscosity in patients with idiopathic sudden sensorineural hearing loss (ISSHL) [13], retinal vein occlusion (RVO) and systemic sclerosis (SSc) [14] as three possible models of microvascular damage. Moreover, to evaluate the role of eNOS polymorphisms in modulating haemorheological profile, the relationship between haemorheological variables and eNOS gene T-786C, G894T and 4a/4b polymorphisms was investigated in ISSHL [15] and SSc [14] patients.

STUDY POPULATIONS AND EXPERIMENTAL PROCEDURES Idiopathic sudden sensorineural hearing loss, hyperviscosity and eNOS gene polymorphisms

Sixty-three consecutive ISSHL patients (30 males and 33 females), with a median age of 54 years (range 19-78 years), who referred to Florence Thrombosis Centre from January to July 2003 less than one week after the acute event, were enrolled. All patients underwent complete audiologic examination, complete history taking and general physical examination. The diagnosis of ISSHL was made by experienced audiologists of Audiological Clinic (University of Florence) by excluding other causes of sudden deafness such as viral, congenital, inflammatory, degenerative or traumatic. Sixty-seven healthy subjects matched for age and sex were also studied. Exclusion criteria for controls were a history of cardiovascular disease or other chronic diseases. Patients and controls were not on antithrombotic therapy at the time of the study.

A second study was performed in 80 ISSHL patients to evaluate the role of three eNOS gene polymorphisms (T-786C, G894T, 4a/4b) in affecting erythrocyte deformability.

Retinal vein occlusion and hyperviscosity

This study was performed to evaluate haemorheological variables in 180 consecutive patients affected by RVO, which was diagnosed by ophtalmoscopic fundus examination revealing disc swelling, venous dilation or tortuosity, retinal hemorrhages and cotton-wool spots and by fluorescein angiography demonstrating extensive areas of capillary closure, venous filling defects and increased venous transit time. The control population consisted in 180 healthy subjects matched for age and sex. Patients and healthy subjects with a personal history of glaucoma or cardiovascular disease were excluded from the study.

Systemic sclerosis, hyperviscosity and eNOS gene polymorphisms

Finally, we explored the role of haemorheological variables and eNOS gene polymorphisms also in 113 consecutive SSc patients who referred to the Division of Medicine I and Rheumatology of the University of Florence. Patients with symptoms overlapping with those of other connective tissue diseases were excluded from the study. Patients underwent complete clinical examinations and were also examined for nailfold capillaroscopy changes and tested for circulating autoantibodies characteristic of SSc (anti-topoisomerase I [anti-Scl-70], anticentromere and antinuclear with a nucleolar pattern); 108 of 113 patients presented with Raynaud's phenomenon. A detailed interview addressing personal and family history was performed in the context of a physical examination by expert physicians in order to identify symptom-free subjects and exclude those who were suspected of having any form of vascular disease (i.e. cardiovascular and cerebrovascular disease, venous thromboembolism or other chronic diseases). In order to evaluate the possible influence of calcium channel blockers (CCBs) on haemorheological parameters, we analyzed the rheologic profile in 20 SSc patients not receiving CCB therapy. One hundred-thirteen healthy subjects matched for age and sex were also studied as controls; exclusion criteria for controls were a positive history of cardiovascular disease, venous thromboembolism or other chronic diseases. All subjects in the patient and control groups were Caucasian, unrelated to each other and residing in the same area.

An extensive clinical profile was established for each SSc patient. Patients were classified as having limited cutaneous SSc (lcSSc) or diffuse cutaneous SSc (dcSSc) according to the criteria proposed by LeRoy *et al.* [16]. Skin involvement was evaluated with the modified Rodnan skin score, which was assessed by an experienced rheumatologist [17]. Nailfold videocapillaroscopy for analysis of microvascular abnormalities was performed as previously reported [14]. According to this analysis, patients were grouped as having capillaroscopy changes with an early, active or late pattern [18].

Two-dimensional echocardiogram and standard electrocardiogram were performed to assess cardiovascular involvement. Lung involvement was evaluated by forced vital capacity, diffusing capacity for carbon monoxide and high resolution computed tomography, and kidney involvement was evaluated by renal function tests (including 24hour creatinine clearance) [19]. We determined the presence of antinuclear antibodies by indirect immunofluorescence on rat liver, anticentromere antibodies by indirect immunofluorescence on Hep-2 cells and by enzyme-linked immunosorbent assay (ELISA) for CENP antigen, anti-topoisomerase I antibodies by immunoblot analysis and rheumatoid factor by ELISA.

Exclusion criteria were as follows: age <18 years, pregnancy, stroke in the 4 months preceding the study, myocardial ischemia, heart failure, systemic arterial hypertension not pharmacologically controlled, thrombocytopenia (platelet count < 100 $\times 10^{9}$ /liter), thrombocytosis (platelet count > 500 ×10⁹/liter), renal failure, chronic hepatitis, diabetes mellitus and malignancy. SSc patients treated with drugs potentially able to modify the evolution of the disease (corticosteroids, methotrexate, cyclophosphamide) were excluded, as were patients whose conditions did not allow a complete pharmacologic washout (patients with severe ulcers, severe pulmonary arterial hypertension, severe respiratory failure, congestive heart failure (NYHA class III-IV), creatinine values ≥ 1.5 mg/dL, megaesophagus and/or malabsorption).

The presence of traditional cardiovascular risk factors was assessed on the basis of patient's interview and hospital records. Dyslipidemia was defined according to the Third Report of the National Cholesterol Education Program; hypertension in the presence of blood pressure above 130/80 mmHg and/or an antihypertensive treatment and diabetes according to American Diabetes Association criteria. Coronary artery disease was defined on the basis of a history of myocardial infarction or stable and unstable angina. Patients were considered overweight when their body mass index (BMI) was above 25 kg/m². All subjects gave their informed consent for the experimental study which was approved by the Institutional Review Board.

Venous blood samples were obtained from overnight fasting subjects in a resting condition in the morning (from 8 to 10 am) by venepuncture of the antecubital vein with minimal stasis. To assess haemorheological profile 20 mL of blood were anticoagulated with EDTA. To determine PAI-1 plasma levels an aliquot of 4.5 mL blood was collected into an ice-cold polypropylene tube containing 0.13 mol/L sodium citrate (0.5 mL; 1:10, V/V) and kept in melting ice. Finally, another aliquot of citrated blood was maintained at room temperature for Sonoclot analysis and measurement of fibrinogen plasma levels and factor VIII:C.

To determine homocysteine, whole venous blood was collected in tubes containing ethylenediaminotetracetate (EDTA) 0.17 mol/L, immediately put in ice and centrifuged within 30 minutes at 4 °C ($1500 \times g$ for 15 min).

Haemorheological studies were performed by assessing whole blood viscosity (WBV), plasma viscosity (PLV) and erythrocyte deformability index (DI) as already reported [13]. WBV and PLV were measured at 37 °C using the Rotational Viscosimeter Low Shear 30 (Contraves, Zürich, Switzerland). WBV was analyzed at shear rates of 0.512 s⁻¹ and 94.5 s⁻¹ and was determined at native hematocrit. PLV test was only performed at 94.5 s⁻¹ shear rate. Erythrocyte filtration was measured by a microcomputer-assisted filtrometer, model MF4 (Myrenne GmbH, Roetgen, Germany). Erythrocyte deformability was estimated by a curve indicating erythrocyte filtration through a 10 min recording in order to determine rheologic properties of erythrocytes, passing them through polycarbonate filters with 5 µm micropores (Nucleopore[®], Pleasanton, CA). The initial flow rate from the microcomputer generated curves was taken for assessing DI. Blood count was performed by a Coulter counter (Coulter Corporation, Miami, Florida).

Sonoclot analysis, a global test to assess whole blood coagulation, was performed by a special device (Sonoclot Analyzer, Sienco Inc, Morrison, CO, USA) [20]. The Sonoclot device consists of an open-ended disposable plastic probe, mounted on an ultrasonic transducer, which is immersed in a cuvette containing 1.5 mg of celite, which is used as clotting activator, and 360 µL of whole blood. The viscous force of the forming clot creates impedance to the vibrating probe, which is converted to an output signal [20]. The changes in the viscoelastic properties of blood clot are recorded in the form of a graph ("Sonoclot Signature"). Sonoclot variables taken into consideration were: 1) the SonACT (the time until onset of initial fibrin formation) expressed in seconds (s); 2) the clot rate value (used to evaluate the acceleration of clotting formation) expressed as % slope/min; 3) the time to peak (the time till fibrin formation is completed) expressed in min.

Fibrinogen was assayed according to the Clauss method. Factor VIII was measured by a clotting method using factor VIII:C-deficient plasma (DADE Behring, Marburg, Germany) and an ELISA was used to determine PAI-1 antigen (ag) plasma levels (Asserachrom® PAI-1, Stago, Asnieres-sur-Seine, France) in ISSHL patients. Plasma levels of total homocysteine (free and protein bound) were determined by fluorescence polarization immunoassay (IMX Abbott Laboratories, Oslo, Norway) in RVO patients.

Polymorphisms of *NOS3* were analyzed in ISSHL and SSc patients after genomic DNA extraction from peripheral blood leukocytes using a QIAmp Blood kit (Quiagen, Hilden, Germany), as previously described [15].

Statistical analysis was performed using the STATA 7.0 program (Stata Corporation, College Station, Texas, USA) for the first study and Statistical Package for the Social Sciences software for Windows, version 11.5 (SPSS, Chicago, IL) for the others. All odds ratios (OR) are given with their 95% confidence intervals. A value of p < 0.05 was chosen as the cut-off level for statistical significance.

RESULTS

Idiopathic sudden sensorineural hearing loss, reduced erythrocyte deformability, hypercoagulability and eNOS gene polymorphisms

Among the variables studied, hematocrit, WBV at both 0.512 s⁻¹ and 94.5 s⁻¹, PLV, DI, SonACT, clot rate, time to peak, factor VIII:C and PAI-lag resulted significantly altered in ISSHL patients than in controls. In Table 1 data obtained from both univariate and multivariate analysis are reported. At multivariate analysis WBV at 94.5 s⁻¹, DI, SonACT, clot rate, factor VIII:C and PAIlag plasma levels remained significantly and independently associated with ISSHL after adjustment for sex, age, dyslipidemia, smoking habitus, hypertension, hematocrit, fibrinogen, haemorheological and haemostatic variables. Significant correlations (Spearman's rank correlation coefficient) were found between haemostatic (clot rate, factor VIII:C and PAI-1ag) and haemorheological variables (WBV at both 0.512 s⁻¹ and 94.5 s⁻¹, DI). Significant correlations were also found between clot rate values and both factor VIII:C (r = 0.32, p < 0.001) and PAI-1ag (r = 0.23, p < 0.01) plasma levels.

eNOS gene genotype distribution and allele frequency were in agreement with those predicted by Hardy-Weinberg equilibrium in patients and controls. A significant higher prevalence of eNOS -786C and 894T, but not of 4a allele was observed in patients in comparison to controls.

When we assumed a dominant model of inheritance (*i.e.* eNOS -786CC+TC vs -786TT) a significant association between the eNOS gene -786C and 894T rare variants and the disease was found. The multivariate analysis, adjusted for gender, age and traditional cardiovascular risk factors (hypertension, dyslipidemia and smoking habit) revealed that eNOS 894T rare variant was an independent predisposing factor to ISSHL. When the eNOS -786C and 894T rare variants were present (-786CC+TC and 894TT+GT combined genotype), the OR for the predisposition to the disease was 2.99 (95%CI 1.43-6.20; p = 0.003) and in subjects carrying all three eNOS rare alleles (-786CC+TC and 894TT+GT and 4a4a+4a4b combined genotype), the OR was 3.6 (95%CI 1.83-7.18; p = 0.0002).

We observed a higher percentage of altered erythrocyte deformability in subjects carrying the eNOS rare variants in comparison to those carrying the wild-type allele: DI was altered in 73.2%, 70% and 74% of patients carrying the eNOS -786C, 894T and 4a rare variants, respectively, and in 41.7%, 53.3% and 56.6% of patients carrying the -786T, 894G and 4b wild-type alleles. The same pattern was observed in control subjects: erythrocyte deformability was altered in 9.3%, 8.6% and 8.3% of controls carrying the eNOS -786C, 894T and 4a rare variants, respectively, and in 2.7%, 4.4% and 5.4% of controls carrying the -786T, 894G and 4b wild-type alleles. To evaluate the influence of eNOS polymorphisms on erythrocyte deformability apart from the disease we used a logistic model in which erythrocyte deformability was considered the dependent variable; from the univariate analysis, eNOS -786C and 894T rare alleles significantly affected the altered erythrocyte deformability, and the influence on this parameter was higher in subjects carrying these two variants. After adjustment for traditional cardiovascular risk factors, the eNOS rare variants remained associated with altered erythrocyte deformability, and the presence of -786C and 894T alleles significantly modified the influence on erythrocyte deformability.

 Table 1 | Univariate and multivariate analysis of clinical and laboratory characteristics in ISSHL patients and controls

	Univariate analysis OR (95% CI)	Multivariate analysis° OR (95%Cl)			
Hematocrit (%)	1.18 (1.05-1.32)**	1.06 (0.86-1.32)			
WBV 0.512 s ⁻¹ (mPa·s)	6.97 (3.12-15.55)**	1.43 (0.34-5.95)			
WBV 94.5 s ⁻¹ (mPa·s)	17.08 (6.85-42.59)**	5.62 (1.28-24.77)*			
PLV (mPa·s)	4.73 (1.75-12.76)**	3.92 (0.90-17.06)			
DI	12.20 (5.32-27.97)**	5.96 (1.61-22.03)**			
SonACT (s)	4.93 (1.93-12.57)**	4.79 (1.02-22.48)*			
Clot rate (%/min)	7.63 (2.87-20.23)**	5.41 (1.30-22.59)*			
Time to peak (min)	1.07 (0.33-3.51)	1.31 (0.18-9.70)			
Fibrinogen (mg/dL)	1.66 (0.45-6.17)	0.76 (0.08-7.32)			
Factor VIII:C (%)	2.95 (1.18-7.39)*	5.02 (1.02-24.69)*			
PAI-1ag (ng/mL)	2.60 (1.21-5.56)*	3.74 (1.01-13.87)*			

* p < 0.05; ** p < 0.01

° Adjusting for sex, age, hypertension, dyslipidemia, smoking habitus, haemorheological and Sonocot variables, factor VIII: C, PAI-1ag, hematocrit, fibrinogen.

WBV = whole blood viscosity; PLV = plasma viscosity; DI = deformability index; SonACT = activated clotting time; PAI-1ag = plasminogen activator inhibitor 1 antigen.

	Model 1	*	Model 2*	**	Model 3***					
	OR (95%CI)	р	OR (95%CI)	р	OR (95%CI)	р				
Age	0.99 (0.98-1.02)	0.9	0.99 (0.97-1.02)	0.8	0.99 (0.96-1.02)	0.7				
Gender (males vs females)	1.08 (0.68-1.69)	0.7	1.27 (0.78-2.07)	0.3	1.23 (0.68-2.23)	0.5				
Hypertension	3.15 (1.94-5.12)	< 0.0001	3.19 (1.91-5.23)	< 0.0001	3.20 (1.74-5.88)	< 0.0001				
Diabetes	2.09 (0.93-4.73)	0.07	2.29 (0.97-5.4)	0.06	2.91 (1.11-7.68)	0.03				
Smoking habit	2.34 (1.35-4.07)	0.002	2.24 (1.24-4.03)	0.007	2.05 (1.02-4.12)	0.04				
WBV at 94.5 s⁻¹ shear rate, mPa⋅s										
1 st tertile	Reference		Reference		Reference					
2 nd tertile	1.6 (0.82-3.16)	0.2	1.68 (0.82-3.45)	0.2	1.90 (0.72-5.06)	0.2				
3 rd tertile	4.29 (2.52-7.31)	< 0.0001	4.08 (2.30-7.21)	0.006	8.97 (3.72-21.63)	< 0.0001				
WBV at 0.512 s ⁻¹ shear rate, mPa·s										
1 st tertile	Reference		Reference		Reference					
2 nd tertile	0.55 (0.29-1.04)	0.06	0.57 (0.29-1.09)	0.9	1.17 (0.48-2.84)	0.7				
3 rd tertile	2.78 (1.15-6.72)	0.005	1.73 (1.00-3.00)	0.05	2.78 (1.15-6.72)	0.02				
Deformability Index										
1 st tertile	4.99 (2.76-9.05)	< 0.0001	4.75 (2.53-8.94)	< 0.0001	4.84 (2.05-11.44)	< 0.0001				
2 nd tertile	1.20 (0.61-2.36)	0.6	1.13 (0.54-2.34)	0.7	1.02 (0.37-2.79)	0.9				
3 rd tertile	Reference		Reference		Reference					

Table 2 | Multivariate logistic regression analysis of clinical and laboratory characteristics in RVO patients and controls

*Adjusted for age, gender, hypertension, diabetes, smoking habit.

** Adjusted for age, gender, hypertension, diabetes, smoking habit and white blood cells.

*** Adjusted for age, gender, hypertension, diabetes, smoking habit, white blood cells, hematocrit, fibrinogen and homocysteine.

Retinal vein occlusion and hyperviscosity

Among the traditional cardiovascular risk factors, hypertension, smoking habit and diabetes, but not dyslipidemia, were significantly more frequent in patients than in healthy subjects. With regard to laboratory parameters, a significant difference in WBV at both 0.512 s⁻¹ and 94.5 s⁻¹, white blood cells, DI and homocysteine, but not PLV, hematocrit and fibrinogen, was observed between patients and controls. In order to investigate the possible association between RVO and haemorheological parameters the study population was divided into tertiles of WBV at 0.512 s⁻¹ and 94.5 s⁻¹ and DI and a logistic regression analysis was performed which has shown, at univariate analysis, a significant association of the highest tertiles of WBV at 0.512 s⁻¹ (OR: 2.31, 95%CI 1.42-3.77; p < 0.0001), WBV at 94.5 s⁻¹ (OR: 4.91, 95%CI 2.95-8.17; p < 0.0001), and the lowest tertile of DI (OR: 5.53, 95%CI 3.13-9.75; p < 0.0001) with the disease.

After adjustment for age, sex, hypertension, smoking habit and diabetes (model 1 analysis), white blood cells (model 2) as well as for hematocrit, fibrinogen and homocysteine (model 3) the highest tertiles of WBV at both shear rates and the lowest tertile of DI were found to be significantly associated with the disease (*Table 2*).

Systemic sclerosis, haemorheologic profile and eNOS polymorphisms

A marked alteration of all haemorheological parameters in SSc was found. Levels of fibrino-

gen and C-reactive protein (CRP), but not hematocrit, were significantly higher in SSc patients than in controls. Patients with either lcSSc (n = 75) or dcSSc (n = 38) showed altered haemorheological variables compared with controls (p < 0.0001). A significant difference in CRP levels was observed between the lcSSc and dcSSc groups. No relationship was found between high CRP levels and rheologic parameters (p > 0.05 for all variables); no correlation was detected between the haemorheologic profile and ulcers (n = 54 patients) (p > 0.05 for all rheologic parameters).

On videocapillaroscopy, 57 patients had an early pattern, 41 had an active pattern and 15 had a late pattern of changes. The haemorheologic profile was not different among the three groups (data not shown). In Table 3, data obtained from univariate and multivariate analyses are reported. In multivariate analysis, WBV at 94.5 s⁻¹, DI and PLV remained significant and independent risk factors for SSc after adjustment for age, sex, hypertension, hematocrit, fibrinogen, NOS3 polymorphisms and haemorheological parameters. In 20 SSc patients not receiving CCB therapy, we analyzed the rheologic profile. No difference was observed between SSc patients receiving CCB therapy and those not receiving CCB therapy (p = 0.3 for WBV at 0.512 s⁻¹, p = 0.5 for WBV at 94.5 s⁻¹, p = 0.8 for PLV and p = 0.4 for DI, by Mann-Whitney test for unpaired data).

No deviation from the expected population genotype proportions predicted by Hardy-Weinberg equilibrium was detected at NOS3 polymorphisms site. A significant difference in genotype distribution for the NOS3 894G > T polymorphism, but not for the -786T > C and 4a/4b polymorphisms, was observed between SSc patients and controls; with regard to the NOS3 allele frequency, we found a significantly higher prevalence of the NOS3 -786C (p = 0.04) and 894T (p = 0.007) rare alleles, but not of the 4a rare allele, in patients than in controls. Fifty-nine of 113 patients (52.2%) carried the -786C/894T haplotype. At univariate analysis, by using a dominant model of inheritance, NOS3 -786C and 894T alleles influenced the predisposition to SSc (p = 0.02 and p = 0.003, respectively). After adjustment for age, sex and hypertension, only the simultaneous presence of the -786C and 894T alleles represented a susceptibility factor for SSc (p = 0.004). Nevertheless, after adjustment for haemorheological parameters as well, the simultaneous presence of these 2 alleles did not remain a factor predisposing to the disease.

In order to evaluate the influence of NOS3 polymorphisms on haemorheological parameters, we used a logistic model in which the single haemorheological parameter was considered the dependent variable apart from the disease; in the univariate analysis, the NOS3 -786C and 894T alleles, but not the 4a allele, significantly influenced all rheologic parameters; nevertheless, after adjustment for age, sex, hypertension, hematocrit and fibrinogen, these NOS3 rare variants significantly influenced the DI (Table 4). In subjects carrying the NOS3 -786C and 894T alleles we observed that NOS3 -786C/894T haplotype significantly influenced the DI, but not the other rheologic parameters, at both univariate and multivariate analyses (OR 4.26, 95%CI 2.37-7.65, p < 0.0001 and OR 2.65, 95%CI 1.24-5.67, p = 0.01, respectively).

The role of *NOS3* polymorphisms in influencing the haemorheologic profile was also tested in the control population. We observed that in subjects carrying the *NOS3* 894T allele, the percentages of altered WBV and DI were higher than those in subjects carrying the 894G wild-type allele (WBV at 0.512 s⁻¹, 17.6 % vs 11.3 %; WBV at 94.5 s⁻¹, 17.6 % vs 6.5 %; DI, 17.6 % vs 4.8 %). Interestingly, the *NOS3* 894T allele influenced the DI, but not the other rheologic parameters, at both univariate and multivariate analyses (OR 4.21, 95%CI 1.08-16.50, p = 0.04 and OR 3.97, 95%CI 1.00-17.58, p = 0.05, respectively).

DISCUSSION

The results of these studies suggest that hyperviscosity is associated with some clinical disorders in whom a microvascular damage is invoked as possible pathophysiological mechanism. Impairment of blood fluidity may significantly affect tissue perfusion and result in functional deterioration, especially if disease processes also disturb vascular properties. In particular, in the first study, we focused our attention on both haemorheological factors and parameters of haemostatic system. The findings obtained document, in fact, a high prevalence of alterations of blood rheology associated with haemostatic changes which suggest blood clotting activation in ISSHL patients. Interestingly, at multivariate analysis WBV at high shear rates and erythrocyte filtration represent risk factor for ISSHL with OR > 5. These data suggest a role for an altered erythrocyte deformability, hypercoagulability and hypofibrinolysis in the pathophysiology of blood flow reduction in cochlear microvascular district eventually leading to sudden hearing loss. Actually, at low shear rates, erythrocytes form rouleaux and WBV is predominantly a function of erythrocyte concentration and aggregation properties, whereas at high shear rates erythrocyte act as free particles, and WBV is primarily determined not only by erythrocyte concentration but also by PLV and erythrocyte deformability [21]. In the first study DI and WBV at high shear rate but not PLV and hematocrit, resulted independently associated with ISSHL, so suggesting that reduced erythrocyte deformability may be a relevant mechanism in determining ISSHL. Among the possible causes of decreased erythrocyte deformability, which is mainly determined by the physical properties of the membrane skeleton, we have analyzed the role

	Univariate analysis			Mult	ivariate anal	ysis*	Mul	Multivariate analysis**			
Variable	OR	95% CI	р	OR	95% CI	р	OR	95% CI	р		
WBV 0.512 s ⁻¹ (mPa.s) WBV 94.5 s ⁻¹ (mPa.s) PLV (mPa.s) Deformability index	12.4 15.8 8.0 10.6	6.4-24.1 7.9-31.8 4.4-14.5 5.2-21.4	< 0.0001 < 0.0001 < 0.0001 < 0.0001	1.4 5.4 2.8 3.7	0.4-5.02 1.4-20.5 1.2-6.5 1.4-9.9	0.6 0.01 0.01 0.01	1.5 5.4 2.8 3.9	0.4-5.5 1.4-19.9 1.2-6.5 1.4-10.8	0.5 0.01 0.01 0.007		

*Adjusted for age, gender, hypertension, hematocrit, and fibrinogen.

** Adjusted for age, gender, hypertension, hematocrit, fibrinogen, eNOS -786T > C and 894G > T polymorphisms, and for haemorheological variables.

		Uni	variate anal	ysis	Multivariate analysis*						
Dependent variable	Variable	OR	95% CI	р	OR 95% CI p						
Deformability index	<i>eNOS</i> -786CC+TC <i>eNOS</i> 894TT+GT <i>eNOS</i> aa+ab	3.9 3.1 1.3	2.1-7.4 1.6-5.9 0.7-2.4	< 0.0001 0.001 0.3	2.3 1.01-5.4 0.04 2.2 1.01-4.8 0.04						
WBV 0.512 s ⁻¹	<i>eNOS</i> -786CC+TC <i>eNOS</i> 894TT+GT <i>eNOS</i> aa+ab	2.2 3.6 1.1	1.2-3.8 2.04-6.4 0.6-2.03	0.008 < 0.0001 0.6	1.2 0.4-3.3 0.8 2.6 0.9-7.4 0.07						
WBV 94.5 s ⁻¹	<i>eNOS</i> -786CC+TC <i>eNOS</i> 894TT+GT <i>eNOS</i> aa+ab	2.2 3.3 1.5	1.2-3.9 1.8-5.8 0.8-2.6	0.008 < 0.0001 0.2	0.9 0.3-2.6 0.9 1.0 0.4-2.8 0.9						
PLV	<i>eNOS</i> -786CC+TC <i>eNOS</i> 894TT+GT <i>eNOS</i> aa+ab	1.9 1.7 1.7	1.1-3.3 1.02-2.9 0.9-3.1	0.02 0.04 0.06	1.2 0.6-2.4 0.6 0.8 0.4-1.7 0.6						
* 4 1 . 1 . 1 . 1	c1 · 1 · · · 1 · ·	11	1 1 . 1								

Table 4	Haemorheolo	gical profile	e and eNOS	polymor	phisms in	SSc	patients:	univariate	and mult	ivariate	anal	vsi
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Adjusted for age, gender, fibrinogen, hematocrit, hypertension, and haemorheological parameters.

of eNOS polymorphisms causing a reduced nitric oxide availability. In fact, experimental studies [9, 11, 12, 22] suggested that NO may modulate erythrocyte deformability and aggregation with a concentration-dependent effect and demonstrated that NOS inhibition resulted in an impairment of erythrocyte deformability, which could be restored by NO donors. Though, the mechanism responsible for the effect of NO on erythrocyte deformability has yet to be fully defined. A NO regulatory effect on red cell deformability by soluble guanylate cyclase, involved in the production of cGMP, has been hypothesized [23] and experimental studies showed a role of NO in modulating ion transport across the red cell membrane [24, 25]. To date, L-arginine, a precursor of NO, exhibits activity as a vasodilator, platelet aggregation inhibitor and modulator of immunologic processes and epithelial permeability [26]; so arginine supplementation might represent a therapeutic benefit in the presence of eNOS polymorphism related to reduced NO availability. The presence of eNOS gene rare variants, which are related to impaired NO availability, affect red blood cell deformability and blood viscosity, so possibly contributing to microcirculatory alterations, such as those found in ISSHL, which represent a suitable model. However, further studies are needed to address the molecular mechanism by which the eNOS gene is involved in the modulation of haemorheologic profile in patients suffering from other microvascular disorders.

Interestingly, we found a significant association between haemorheological alterations and changes in haemostatic system in ISSHL patients, suggesting that the occurrence of rheological modifications may be relevant in determining microvascular occlusion by triggering blood clotting activation and impaired fibrinolysis in these patients. Nevertheless in these patients factor VIII:C. coagulation parameters measured in whole blood test and PAI-1ag remained important risk factors for ISSHL after adjustment for haemorheological parameters. These results are in agreement with previous studies reporting both increased plasma levels of PAI-1ag and factor VIII:C as risk factors for arterial and venous thrombosis [27, 28]. A stimulated endothelium may account for the elevated levels of PAI-1 found in ISSHL patients which, in turn, contribute to the thrombophilic state. High wall shear stress, such as that occurring in stenosed vessels or due to blood hyperviscosity, may perturb vascular endothelium [29] and, thus, stimulate platelet adhesion and aggregation and the expression of procoagulant activities. At the same time high factor VIII levels may enhance thrombin generation and, thus, result in increased platelet activation and fibrin formation processes. In keeping with this finding are the results of the global evaluation of haemostasis by Sonoclot analysis that has shown significant alterations in SonACT and clot rate values in ISSHL patients with respect to controls. Clot rate is influenced by the rapid increase in blood viscosity, due to the conversion of fibrinogen to fibrin and reflects the speed at which the clot is being formed. In ISSHL patients hypercoagulability is suggested by shorter SonACT values and by higher clot rate values, indicating an enhanced rate of thrombin generation and fibrin formation, and by higher factor VIII:C plasma levels than in controls.

We hypothesized that the altered erythrocyte deformability and the consequently increased blood viscosity may account for these haemostatic changes. Actually, the luminal surface of the blood vessel is constantly exposed to haemodynamic shear stress, that, according to Poiseuille's law, is proportional to blood flow viscosity and inversely proportional to the third power of internal radius. This condition may create a vicious circle between endothelial damage and erythrocyte deformability, which can influence each other, thus leading to microvascular occlusion.

As ISSHL patients were investigated during the first week after the acute event we cannot rule out that elevated PAI-lag and factor VIII:C plasma levels might be, at least in part, related to the acute phase reaction. However this seems unlikely because levels of fibrinogen were not different between patients and controls. A limitation of this study is that these parameters were measured after the event and there is no evidence here that those in the population who have such blood alterations will be at higher risk of ISSHL. However, this happens for all retrospective studies.

The observed changes in viscosity, blood clotting and fibrinolysis may contribute, at least in part, to the pathophysiological mechanism of ISSHL. Since the more recent studies performed in patients affected by ISSHL have shown inconsistent effects of haemodilution alone as a possible therapeutic option [30, 31], the clinical implications of these results are that not only hyperviscosity but overall hypercoagulability should be the target for therapeutic approaches in these patients.

Interestingly, hyperviscosity has been also found to be associated with the occurrence of retinal vein occlusive disease, in particular as regard the highest tertile of WBV at both shear rates as well as the lowest tertile of erythrocyte deformability index. Contrasting data has been previously reported by some studies concerning a possible association between alterations of haemorheological variables and the occurrence of RVO but no conclusive results have been obtained [32-37]. Such conflicting results can be explained by either the scanty number of patients studied in most of these studies or by the fact that only some of these studies analyzed the whole pattern of haemorheology in association with the disease. Our study, evaluating the entire haemorheological profile together with inflammatory parameters and hematocrit, in a relevant number of RVO patients and healthy subjects, confirms the significant role of haemorheological variables in the occurrence of this disease.

Our findings are in keeping with some previous studies evaluating haemorheological variables in RVO patients [33, 36]. In our study, WBV has been found to be associated with an increased risk of RVO at both shear rates investigated, after multiple statistical adjustment. This allows us to state that blood viscosity plays a relevant role in the pathogenesis of RVO. Indeed, haemorheology in RVO seems important particularly because altered blood fluidity could lead to further decreased flow, initiating and/or causing progression of vessel wall alterations.

Another relevant finding of our study is the observation that an increased erythrocyte deformability is significantly protective against RVO. Erythrocyte deformability plays an important role in determining blood viscosity in the presence of slow venous flow and high vascular resistance, conditions that are normally present in the central retinal vein at the level of lamina cribrosa. Hence, an altered pattern of deformability for red cells can lead to a venous stasis and then to a reduced blood flow, by determining occlusion of retinal vessels. In our study, abnormalities of DI have been found to be present in a high percentage of RVO patients as compared to age and gender-comparable healthy subjects and have been observed to be significantly associated with an increased risk of the disease. Previous studies investigated red cell deformability in RVO patients but inconclusive data were obtained [32-34]. This can be ascribed to the different methods used for the measurement of such parameter.

At variance with previous studies, conversely, we did not find a significant association between plasma viscosity and RVO [34, 35, 37]. This conflicting result can be explained by the differences among these studies in terms of study populations, methods of measurements as well as control populations and main cardiovascular risk factors.

Notably, however, the results obtained in our study can be helpful for the clinical management of RVO patients. Up to now, no established treatment for RVO is available. The increasing role of hypercoagulability in these patients supports the role of antithrombotic drugs, but medical management of patients with RVO consists primarily on the treatment of the underlying systemic diseases. The presence of an altered haemorheological profile, on the other hand, can give to physicians a further therapeutic option for the treatment of RVO patients. Currently, some reports indicating a possible beneficial role of haemodilution therapy in the management of RVO have been reported, but data are limited [32, 38]. Moreover, a beneficial effect of treatment with pentoxyfilline, a therapeutic agent able to determine a significant improvement of perfusion to occluded vessels, as well as of haemorheology, has been proposed in RVO patients by hypothesizing a relevant role for prohaemorheological agents in the clinical management of this occlusive disease, but data supporting this approach are lacking [39].

To date, statins and antiplatelet drugs have been reported to significantly influence haemorheological profile [40, 41] and, interestingly, a recent report showed a role for platelet activation in the pathogenesis of RVO [42]. Thus, the results of our study likely suggest a possible usefulness of the approach with these drugs to RVO patients. This is also in line with a recent meta-analysis of studies evaluating thrombophilia in RVO, which suggests that these patients have a risk profile of "arterial" more than of "venous" type [43].

In conclusion, recent literature and our findings point to a possible approach to RVO with haemodi-

lution, statins and antiplatelet drugs. Randomized controlled trials are needed to confirm usefulness of such treatment in these patients.

Finally, we documented a marked alteration of haemorheological variables and the role of NOS3 gene -786T > C and 894G > T polymorphisms in influencing the haemorheologic profile in SSc patients. We found haemorheological alterations not only in dcSSc and in the presence of cutaneous ulcers, but also in lcSSc in the absence of cutaneous ulcers. This result may indicate that the haemorheologic status is involved in SSc "per se", independently of clinical manifestations. Our results confirm the effect of two NOS3 polymorphisms in modulating the rheologic phenotype and, in particular, the erythrocyte deformability also in SSc patients. Indeed, these results highlight a possible mechanism by which a potential reduced availability of NO, related to NOS3 -786T > C and 894G > T polymorphisms, might influence the predisposition to SSc. Actually, we demonstrated that an altered haemorheologic profile represents an independent risk factor for SSc, as described in ISSHL and RVO, two other clinical conditions in which a microcirculatory alteration is involved. Thus, it could be hypothesized that these polymorphisms might modulate susceptibility to the disease by influencing the haemorheologic profile in addition to their influence on vascular biology. The effect of the NOS3 polymorphisms on the haemorheologic environment, related to potentially reduced NO levels, might be supported by our observation of a higher percentage of altered haemorheologic parameters in healthy subjects carrying the NOS3 894T rare allele.

Previous studies involving a limited number of patients indicated rheologic disorders related to erythrocyte aggregation and PLV in SSc [44, 45]. This is the first study to evaluate all rheologic parameters, such as PLV, WBV and DI; their alteration may be involved in the modification of the microvascular district found in SSc.

The pathogenetic mechanism of SSc are not yet completely defined. However, we know that impaired endothelial production of NO is involved in the breakdown of vascular tone control and vessel patency.

To date, there is evidence for impaired NO production as a potential result of the 894G > T polymorphism in exon 7 and of the -786T > C polymorphism, which reduces the promoter activity by ~ 50%, thereby lending experimental support for the notion of a physiologic role of this SNP. Data from experimental studies demonstrated that homozygosity for the rare variant of the -786T > Cpolymorphism in the promoter region of the *NOS3* gene is associated with a deficit of eNOS expression in human endothelial cells exposed to laminar shear stress, as well as with a reduced NO-mediated vasomotor function [46]. Interestingly, a recent report by Sandrim *et al.* [47] suggested that there is a genetic contribution of *NOS3* haplotype to the development of endothelial dysfunction in hypertensive patients, and thus this contribution is obscured when specific *NOS3* genotypes alone are considered. Similarly, we demonstrated that the *NOS3* -786C/894T haplotype significantly affected the rheologic parameters in SSc, thus supporting the hypothesis that studies based on haplotypes may provide more reliable information than SNPbased studies.

Our results, which confirm that NOS3 polymorphisms represent a susceptibility factor for SSc [48], are concordant with those of Metzger et al. [49], who reported an association between NOS3 gene haplotype and lower circulating nitrate and nitrite concentrations. In contrast, the results of our study are not concordant with those of other studies that did not demonstrate the association between NOS3 polymorphisms and SSc [50, 51]. These conflicting findings might be a result of the interethnic differences in the distribution of genetic polymorphisms [52], thus explaining the different clinical significance in each ethnic group from a different genetic background. At present, the number of candidate genes analyzed is really limited for revealing the complex nature of this disease and its heterogeneous phenotype.

Nonetheless, the results of this study confirm that also in SSc patients a potential reduction in NO availability related to *NOS3* polymorphisms may modulate erythrocyte deformability, thus contributing to endothelial injury with the same pathophysiological mechanism above discussed for ISSHL patients.

In the present study, in addition to erythrocyte involvement, we observed an increased PLV in SSc patients. This datum is attributable not only to increased fibrinogen levels, but also to the increase of other plasma components, such as albumin and immunoglobulins [53]. Since CCB therapy has been reported to modulate rheologic parameters in hypertension, we investigated the rheologic profile in 20 additional SSc patients not receiving CCB therapy. Actually, an altered rheolgic profile has been observed in SSc patients independently of CCB therapy, possibly suggesting that the microcirculatory alteration is more relevant to rheologic parameters than the benefit derived from CCB therapy.

This study has two main limitations. First, apart from the -786T > C polymorphism, which is known to suppress NOS3 gene transcription, thus lending experimental support to its physiologic role, the effects of the 894G > T and 4a/4b polymorphisms remain at present a matter of debate. Actually, the 894G > T polymorphism in exon 7 results in a conservative amino acid substitution and could affect eNOS activity, but the initial suggestion that the 894T variant is more susceptible to proteolytic cleavage [54] might be

attributed to experimental conditions. Finally, a functional role for the 4a/4b polymorphism in intron 4 has not been demonstrated, even though it has been reported to be associated with altered plasma NO levels [55] and responsible for variations in the genetic control of plasma nitrite and nitrate levels [56].

The second limitation lies in the lack of studies about the possible benefit of therapy with NO donors in SSc patients. These experimental data might shed light on the potential link between the *NOS3* genotypes and the rheologic parameters, thus substantiating the relevance of our results.

Our findings document an altered rheologic profile in SSc patients and demonstrate that this alteration is modulated by *NOS3* polymorphisms. This seems to shed light on a novel mechanism that might influence the microcirculation in SSc and allows us to comprehend how disease genotypes associated with the basic biology become clinical phenotypes. These findings suggest further inves-

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tigation of the molecular mechanism by which the *NOS3* gene is involved in the modulation of the rheologic profile. Elucidation of additional interactions mediated by *NOS3* polymorphisms might offer exciting opportunities for future investigations and new therapeutic possibilities.

In conclusion, the results of our investigations suggest that alterations in haemorheological variables, either determined by genetic susceptibility or not, can be involved in the pathophysiological mechanism of some clinical disorders in which a disturb of the microvascular district is invoked and can be the target of new possible therapeutic strategies. However, prospective studies are needed to strengthen our findings in patients with microvascular disorders.

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