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Neutrophil Activation Promotes Fibrinogen Oxidation and Thrombus Formation in Behçet's Disease

Running title: *Becatti et al.; Role of neutrophils in thrombus formation*

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Abstract

Background—Behçet disease (BD) is a systemic vasculitis with a broad range of organ involvement, characterized by a multisystemic, immune-inflammatory disorder involving vessels of all sizes and often complicated by thrombosis. Systemic redox imbalance and circulating neutrophil hyperactivation have been observed in BD patients and are thought to be responsible for impaired coagulation. We here focused on the pathogenetic mechanisms potentially linking immune cell activation and thrombosis, and specifically examined whether neutrophil activation can affect fibrinogen modifications and consequently elicit thrombosis.

Methods and Results—Blood samples were collected from 98 consecutive BD patients attending our dedicated Center and from 70 age and sex-matched healthy controls; in all patients fibrinogen function and structure, fibrin susceptibility to plasmin-lysis, plasma redox status, leucocyte oxidative stress markers and possible ROS sources were examined.

Thrombin-catalyzed fibrin formation and fibrin susceptibility to plasmin-induced lysis were significantly impaired in BD patients ($p < 0.001$). These findings were associated to increased plasma oxidative stress markers ($p < 0.001$) and to a marked carbonylation of fibrinogen ($p < 0.001$) whose secondary structure appeared deeply modified. Neutrophils displayed an enhanced NADPH oxidase activity and increased ROS production ($p < 0.001$), which significantly correlated with fibrinogen carbonylation level ($r^2 = 0.33$ $p < 0.0001$), residual β -band intensity ($r^2 = 0.07$ $p < 0.01$) and fibrinogen clotting ability ($r^2 = 0.073$ $p < 0.01$).

Conclusions—In BD patients, altered fibrinogen structure and impaired fibrinogen function are associated with neutrophil activation and enhanced ROS production whose primary source is represented by neutrophil NADPH oxidase.

Key words: thrombosis; oxidative stress; fibrinogen; inflammation; Behçet's disease

Background

Chronic inflammation is a well-known risk factor for the development of thrombosis.¹ However, the details of the complex crosstalk between inflammation and hemostasis are far to be elucidated.²⁻⁸ Some interesting animal models of inflammation-induced thrombosis exist,^{9,10} but human models have not been yet proposed.

Among the systemic inflammatory diseases characterized by thrombotic tendency, Behçet's disease (BD) is a peculiar systemic vasculitis¹¹ with frequent muco-cutaneous, ocular, gastrointestinal and cerebral lesions; recurrent thrombosis occurs more frequently in male patients with active disease, and represents an important cause of morbidity and mortality.¹² Cardiovascular events affect up to 45% of BD patients involving both arterial and venous vessels of all sizes, but deep and superficial vein thrombosis of the lower extremities are the most common vascular manifestations of the disease.¹³

An overall imbalance in blood redox status (assessed by ischemia-modified albumin, IMA, advanced oxidation protein products, AOPP, and pro-oxidant/antioxidant balance, PAB) has been reported in BD.¹⁴ Moreover, systemic inflammation more than usual thrombophilic factors is thought to be the main trigger of thrombosis in this condition and seems to be mainly mediated by T lymphocytes, monocytes, neutrophils and pro-inflammatory cytokines along with endothelial cell dysfunction.¹⁵ In particular, neutrophils can mediate tissue injury in different ways: actually, neutrophils in BD display an intrinsic hyperactivation, probably HLAB*51-related, and usually participate in perivascular infiltration in BD lesions.¹⁶ Based on these pathogenetic concepts and clinical experience, the European League Against Rheumatism (EULAR) recommendations for the management of BD suggest that thrombosis should be treated with immunosuppression rather than anticoagulation,¹⁷ as an inflammation-induced thrombosis.

Recent studies have provided evidence that fibrinogen plays a multifaceted role in inflammatory responses and autoimmunity.^{18,19} The ability of fibrinogen to participate in the inflammatory response depends on its specific interaction with integrins, the leukocyte cell surface adhesion receptors. M2 (CD11b/CD18, Mac-1) and X2 (CD11c/CD18, p150,95) are the main fibrinogen receptors and are expressed on neutrophils, monocytes, macrophages and several subsets of lymphocytes.²⁰ In BD, endothelial cell dysfunction, increased reactive oxygen species (ROS) production²¹ and neutrophil hyperfunction have been reported,¹⁵ together with an impaired fibrinolysis.²²

In this study, in order to highlight the mechanisms of inflammation-induced thrombosis, we investigated fibrinogen modifications, fibrin susceptibility to plasmin-lysis, plasma redox status, leucocyte oxidative stress and possible ROS sources in a population of BD patients. Moreover, we explored fibrinogen structure and its possible relationship with neutrophil-dependent ROS production.

Methods

Patients enrollment

From November 2009 until June 2014 ninety-eight patients with BD who attended the Florence Behçet Centre (47 males and 51 females) and 70 age-matched healthy control subjects, were included in the study (**Table 1**). All the patients were diagnosed as having Behçet's disease according to International Study Group criteria.²³ Patients with other autoimmune diseases, active infections or neoplastic conditions were excluded. Blood samples were collected from patients without immunosuppressive therapy and only prednisone assumption under 10 mg/day were allowed. In colchicine-treated patients (21 males and 27 females), therapy was suspended at

least 7 days before blood sample collection. The study protocol was approved by local Ethical Committee and informed consent was obtained from all subject enrolled. Demographic and clinical characteristics of the population studied are summarized in **Table 1**.

Sample collection

Blood samples were collected in Vacutainer tubes containing 0.109 mol/L buffered trisodium citrate (1:10) or EDTA (0.17 mol/L). After centrifugation (1500 x g for 15 minutes at 4°C), aliquots of plasma were used for experiments or stored at 80°C for further analysis. Another aliquot of sodium citrate plasma was used for fibrinogen purification.

Fibrinogen purification

Fibrinogen was purified using the previously described ethanol precipitation method.²⁴ After the purification procedure, fibrinogen concentration was determined by ultraviolet spectroscopy at a wave length of 280 nm, assuming an extinction coefficient of 1.51 mg/ml. The yield of purified fibrinogen was not statistically different between patients and controls (9.7±1.4 vs 8.9±1.9, mg/10 ml of plasma, respectively). The purity of the fibrinogen preparations (from 10 ml of citrated plasma) was assessed by densitometry of Coomassie-stained polyacrylamide gels after electrophoresis under reducing conditions. In our purification procedures, the amount of fibrinogen, expressed as a percentage of total protein content, yielded 97.1±1.4% of total protein content in controls and 94.0%±1.1% in Behçet's patients. No significant statistical difference was observed in the purification yield between controls and patients.

Protein Concentration assay

Protein concentration in the samples was determined using the Bradford assay.²⁵ A standard curve of bovine serum albumin (0–15 µg protein/200 µl volume) was used.

Protein Carbonyl (PC) determination

Oxidative modification on plasma proteins and on purified fibrinogen fractions was assessed based on carbonyl content using 2,4-dinitrophenylhydrazine (DNPH), as previously reported.²⁶

DNPH reacts with PC, forming a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically. Briefly, plasma (100 μ l), after incubation with DNPH (400 μ l), was precipitated with Trichloroacetic acid (TCA) and the pellet washed several times with a 1:1 mixture of ethanol/ethylacetate. Finally, the pellet was resuspended in 500 μ l of guanidine hydrochloride and measured at 370 nm. PC content was calculated by using a molar extinction coefficient of 22000 $M^{-1}cm^{-1}$. The results, expressed in terms of nmol/ml of PC, were then normalized for protein concentration.

TBARS (Thiobarbituric Acid Reactive Substances) estimation



Plasma TBARS levels were measured using a TBARS assay kit (OXI-TEK, ENZO, USA) in accordance with the manufacturer's instructions. Briefly, the adduct generated by reacting malondialdehyde with Thiobarbituric acid after 1h at 95°C was measured spectrofluorimetrically, with excitation at 530 nm and emission at 550 nm. TBARS were expressed in terms of malondialdehyde equivalent (nmol/ml) and then normalized for protein concentration.

Circular Dichroism (CD) spectra of purified fibrinogen extracts

CD spectra were recorded at 25°C in 0.2 cm quartz cells from 250 to 195 nm (farUV), using a protein concentration of 1mg/ml. Samples were filtered through 0.22 μ M filters and five spectra recorded for each sample. Molar ellipticity values $[\theta]$ were calculated according to the equation: $[\theta](deg\text{-}cm^2dmol^{-1}) = [\theta(MRW)]/[10(l)(c)]$, where θ is the displacement from the baseline value X to the full range in degrees; MRW is the mean residue weight of the aminoacids; (l) is the path length of the cell (cm); and (c) is protein concentration (g/ml).²⁷

Thrombin-catalyzed fibrinogen and polymerization assays

For functional analysis, purified fibrinogen fractions stored at -80°C and not previously thawed were used. Fibrin polymerization was monitored at 595 nm in a 96-well micro titer plate reader (model550, Bio-Rad Milan, Italy) at 25°C , as previously described.²⁸ Prior to the polymerization assay, control and patient fibrinogen samples were extensively dialyzed against 100 mM Tris/HCl buffer, pH 7.4, and diluted to a final concentration of 1 mg/ml. To each reaction (in triplicate), 240 μl of fibrinogen (1 mg/ml) in 100 mM Tris/HCl, 5 mM CaCl_2 , pH7.4 was added. The polymerization reaction was started by adding 60 μl thrombin (at a final concentration of 0.25 U/ml). Absorbance was monitored for 90 min at 25°C .

Absorbance curves were characterized using the following parameters: 1) the maximum slope (V_{max}), calculated as the slope of the steepest part of the polymerization curve (using 15 time points), which represents the rate of lateral protofibril association; 2) the lag phase, measured as the time elapsed until an increase in absorbance was seen, which reflects the time to the start of lateral fibril aggregation; 3) maximum absorbance (MaxAbs) of the growing clot, recorded 60 min after polymerization was initiated, which reflects an average fibrin fiber size and the number of protofibrils per fiber.²⁹

Determination of fibrin structure based on label-free differential interference contrast (DIC) microscopy

Fibrin clots prepared as described above were analysed by DIC microscopy. This is a label-free microscopy technique with a high sensitivity to thin cellular material, even when it is located within thick tissue.³⁰ DIC microscopy is superb for observing transparent objects and very thin filaments or sharp interfaces, which produce good contrast even when their diameter falls below the resolution limit of the optical system. DIC microscopy causes one side of an object to appear bright while the other side appears darker. This shadow effect gives a pseudo-three-dimensional

appearance to the specimen, at excellent resolution.

Purified fibrinogen fractions stored at -80°C and not previously thawed were used. Before polymerization, control and patient fibrinogen samples were extensively dialyzed against 100 mM Tris/HCl buffer, pH 7.4, and diluted to a final concentration of 1 mg/ml. To each reaction (in duplicate) 240 μl of fibrinogen (1 mg/ml) in 100 mM Tris/HCl, 5 mM CaCl_2 , pH 7.4 was seeded on glass coverslips. The polymerization reaction was started by adding 60 μl of thrombin (at a final concentration of 0.25 U/ml) at 25°C . After 90 min the sample was analyzed using a Leica TCS SP5 microscope (Mannheim, Germany), using DIC. The observations for each examined sample were obtained using a Leica Plan Apo 63 \times oil immersion objective.

Fibrin digestion with plasmin and electrophoretic analysis of plasmin digests



Fibrin clots were prepared in microcentrifuge tubes by incubating bovine thrombin (12 units/mL final concentration) with fibrinogen (2 mg/mL final concentration) in 20 μL of 100 mM Tris/HCl, 5 mM CaCl_2 , pH 7.4, for 1 h at 25°C . Plasmin was then added (5 μL of 100 $\mu\text{g}/\text{mL}$), and the fibrin clots were digested over a period of 6 h at 37°C , as previously described.¹ The digestion reaction was terminated by adding 10 μL of lithium dodecylsulfate (LDS) gel electrophoresis sample buffer. The same lot of thrombin and of plasmin were used for all experiments. Samples were heated at 70°C for 10 min under reducing conditions (50 mM dithiothreitol). Then, aliquots from each digest (equivalent to 10 μg of fibrin) were loaded onto 4–12% Bis-Tris gels. After electrophoresis, gels were stained with Coomassie blue. Band intensities of stained gels were quantified by densitometry using the Chemi-Doc system and Quantity-One software (Bio-Rad, Milan, Italy). Data were expressed as the ratio between the densitometric reading of the purified protein at a given digestion time and that of the undigested protein (time 0 for incubation with plasmin).

Assessment of ROS generation by flow cytometry

After collection, 100 µl EDTA-anticoagulated blood samples was resuspended in 2 ml of BD FACS Lysing Solution (Becton Dickinson Biosciences, San Jose, CA, USA), gently mixed, and incubated at RT in the dark for 10 min, following manufacturer's protocol. Next, the cells were centrifuged, the supernatant discarded and cells washed twice in PBS. To determine the level of intracellular ROS generation, cells were incubated with H₂DCF-DA (2.5 µM) (Invitrogen, CA, USA) in RPMI without serum and phenol red for 15 min at 37 °C. After labelling, cells were washed and resuspended in PBS and analysed immediately using a FACSCanto flow cytometer (Becton-Dickinson, San Jose, CA). The sample flow rate was adjusted to about 1000 cells/s. For a single analysis, the fluorescence properties of 20000 leukocytes were collected. The respective gates were defined using the distinctive forward-scatter and side-scatter properties of the individual cell populations. Moreover, the viability of the cells was controlled by flow cytometry with propidium iodide staining and was found to exceed 95%. Data was analysed using BD *FACSDiva software* (Becton-Dickinson, San Jose, CA, USA).

Leukocyte subpopulation cell sorting

After collection, 5 ml EDTA-anticoagulated blood samples was resuspended in 100 ml of BD FACS Lysing Solution (Becton Dickinson Biosciences, San Jose, CA, USA), gently mixed, and incubated at RT in the dark for 10 min, following manufacturer's protocol. Next, cells were centrifuged, supernatant discarded and cells washed twice in PBS. For cell sorting, cells were resuspended in 2.5 ml 1640 RPMI (containing glutamine, supplemented with 10% heat-inactivated FCS, 1% Penicillin/Streptomycin, Cellegro, Washington, DC) and flow-sorted with a FACSaria (BD Bioscience, San Jose, CA). The sample flow rate was adjusted to about 10000 cells/s. Forward and side scatter were used for identification of leukocyte subpopulations

(lymphocytes, monocytes and neutrophils). In our experimental design, the use of label-free leukocyte subpopulations is mandatory for all planned experiments (i.e. incubation of leukocyte subpopulations with fibrinogen and leukocyte subpopulations NADPH oxidase activity).

Forward and side scatter were also used to exclude debris and dead cells. Purity of each leukocyte subpopulation was >95% as confirmed by post-sorting FACS analysis with specific antibodies. Moreover, the viability of the cells was controlled by flow cytometry with propidium iodide staining and was found to exceed 95%. Data was analysed using BD FACSDiva software (Becton-Dickinson, San Jose, CA, USA). The obtained leukocyte subpopulation were used for NADPH oxidase activity assay and for *in-vitro* leukocyte subpopulation-induced fibrinogen carbonylation assay.



NADPH oxidase activity assay

NADPH oxidase activity assay was performed on each leukocyte subset using a Lumat LB 9507 single-tube luminometer (Berthold Technologies, GmbH & Co, Bad Wildbad, Germany). After washing with phosphate-buffered saline, 7.5×10^5 cells were resuspended in 150 μ l Krebs-HEPES buffer (99 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1 mM KH₂PO₄, 1.9 mM CaCl₂, 25 mM NaHCO₃, 20 mM HEPES, 11.1 mM glucose, pH 7.44) and incubated for 10 minutes at 37°C. Then, lucigenin was added to the sample at a final concentration of 25 μ M. Luminescence was recorded continuously for 30 min. Each sample was also assayed in the presence of the NADPH inhibitor diphenyleneiodonium chloride, DPI, (20 μ M) to obtain basal luminescence values. NADPH oxidase activity was expressed as relative units of luminescence (RLU)/s.

***In-vitro* fibrinogen carbonylation induced by leukocyte subsets**

For *in-vitro* leukocyte-induced fibrinogen carbonylation assay, each FACS-sorted leukocyte subset (lymphocytes, monocytes and neutrophils) (7.5×10^5 cells) was incubated with 0.4 mg of

fibrinogen in 200 μ l 1640 RPMI (in presence or absence of the NADPH oxidase inhibitor apocynin 1.5 mM) for 3h at 37 °C. After centrifugation (1500xg for 10 min), the supernatant was collected and used for the estimation of carbonyl content.

Statistical Analysis

To assess the quality of analyses, all the experiments were performed three times on the same sample, each one with three replicates. After assessing the low intra-experiment and inter-experiment variability and the reproducibility of measures (Repeated measures ANOVA, data not shown), each value per subject was calculated as the overall mean of the means of the three experiments. Therefore, group means and SD were calculated by using the overall mean for each subject as single value in the calculations.



All data presented a normal distribution. Statistical significance was evaluated with a standard unpaired Student t test (two-tailed; $P < 0.05$) when appropriate. For multiple-comparison analysis, 1-way ANOVA followed by the Bonferroni post hoc test was applied when appropriate. Correlation analysis was performed using the Pearson's test. All statistical operations data were processed using the Graph Pad Prism 5 software. A value of $P < 0.05$ was considered to be statistically significant.

Results

Oxidative stress markers in plasma, lymphocyte, monocyte and neutrophil fractions and in purified fibrinogen

The main plasma oxidative stress markers- namely protein carbonyls (PC), accounting for protein oxidative damage, and thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation- were significantly increased in BD patients compared to healthy controls

($p < 0.001$). Purified fibrinogen obtained from patients showed a significantly increased extent of carbonylation as compared to that obtained from healthy controls ($p < 0.01$) (**Table 2**). ROS production in BD patients in lymphocyte and monocyte fractions was almost two-fold and in neutrophils was three-fold compared to healthy controls (**Fig.1A**).

Interestingly, only neutrophil ROS production positively and significantly correlated with fibrinogen PC content ($p < 0.0001$, $r^2 = 0.3327$), while monocyte/lymphocyte ROS production did not (**Fig. 1B**). ROS production in granulocyte/monocyte/lymphocyte populations did not show any significant correlation with total plasma PC content (data not shown).

Circular Dichroism Spectra: analysis of secondary structure

The secondary protein structure, mainly represented by alpha-helices and beta-pleated sheets, deeply influences protein function. In our study, secondary protein structure was analyzed by far-UV Circular Dichroism (CD) spectroscopy (**Fig. 2**). In control subjects the observed spectrum for fibrinogen suggested a typical alpha-helical structure with minima at 208 nm and at 222 nm. Fibrinogen from BD patients displayed an altered CD spectrum mainly consisting in a decrease in the negative peak in the 215-225 nm region, therefore suggesting a decrease in alpha-helical content (**Fig. 2**), which can account for functional protein modifications.

Fibrinogen polymerization and fibrin formation

Fibrinogen functioning was assessed by evaluating the kinetics of fibrin polymerization.

Representative curves of thrombin-catalyzed fibrinogen polymerization are shown in **Fig. 3A**. In BD patients, the ability of fibrinogen to undergo clotting was diminished: significant differences in BD patients vs. controls were found in the main parameters which characterize the polymerization curves (as described in the Methods section) i.e. Lag time, V_{max} and Max Abs ($p < 0.01$), suggesting a different clot structure. In **Fig.3B** the significant correlation between

fibrinogen PC and Max Abs is reported ($P < 0.0001$, $r^2 = 0.2894$). When polymerization kinetics was assayed in patients' plasma, the obtained Max Abs was significantly reduced as compared with that of controls ($p < 0.01$, data not shown).

ROS production in the leucocyte subpopulations correlated with Max Abs, which resulted from fibrinogen polymerization curves. No significant correlation was found between Lymphocyte/Monocyte ROS production and Max Abs (**Fig. 3C, 3D**). Conversely, Max Abs significantly correlated ($p < 0.01$) with neutrophil ROS production (**Fig. 3E**).

Differential Interference Contrast (DIC) microscopy

In order to evaluate the structure of the fibrin network in BD patients and controls, fibrin samples obtained from BD patients and controls were analyzed by DIC microscopy. Representative images are shown in **Fig. 4**. After 90 minutes a tight fibrin network is still evident after plasmin-induced lysis in fibrin obtained from BD patients compared to controls.

Fibrin susceptibility to plasmin-induced lysis

To evaluate fibrin susceptibility to plasmin-induced lysis, we focused on the degradation rate of the fibrin β chain; the degradation before and 3h and 6h of plasmin digestion in BD patients and controls is reported in **Fig. 5A**. In BD patients the relative band intensity at each plasmin incubation time was significantly increased with respect to controls ($p < 0.01$) (**Fig. 5B**). Moreover, in BD patients, the correlation between fibrinogen carbonyl content and the relative fibrin β chain intensity after 6 h of plasmin digestion was shown to be significant ($p < 0.001$, $r^2 = 0.1254$, **Fig. 5C**). The correlation between ROS production in leucocyte fractions and residual fibrin β chain after 6 h of plasmin digestion is shown in **figures 5D, 5E, 5F**. No significant correlation was found between lymphocyte or monocyte ROS production and residual fibrin β chain (**Fig. 5D, 5E**). Conversely, a significant correlation ($p < 0.01$, $r^2 = 0.073$) was found when

neutrophils were analyzed (**Fig. 5F**).

NADPH oxidase activity in lymphocyte, monocyte and neutrophil fractions

The activity of NADPH oxidase, one of the major physiological ROS source, was measured in leukocytes obtained from BD patients and controls. NADPH oxidase activity measured in lymphocyte, monocyte and neutrophil fractions, in the presence or absence of a specific NADPH oxidase inhibitor, revealed only in BD neutrophils a significantly increased (about five-fold) enzyme activity as compared to that of neutrophils obtained from controls (**Fig. 6**).

Neutrophils are responsible for fibrinogen carbonylation

In the *in vitro* experiments equal amounts of lymphocytes, monocytes and neutrophils from BD patients were incubated with human fibrinogen for 3h incubation at 37 °C. Interestingly, a significantly increased PC content ($p < 0.01$) was observed only in fibrinogen samples incubated with patients' neutrophils (**Fig. 7A**). Simultaneously, fibrinogen samples incubated with the different leucocyte fractions were tested for susceptibility to plasmin-induced lysis. Only fibrinogen incubated with patients' neutrophils showed a marked resistance to plasmin-induced lysis as evidenced by increased residual β chain intensity compared to controls after 6 h of incubation with plasmin (**Fig. 7B**).

Discussion

Although inflammation-induced thrombosis is a well-known process, its pathogenic mechanisms still remain a matter of debate.² BD may be considered a prototype of systemic inflammatory disease causing thrombosis. In fact, thrombosis, which is the most common vascular manifestation in this condition, responds to immunosuppressive treatment rather than anticoagulation.

The association among oxidative stress, inflammation and endothelial dysfunction has been repeatedly suggested^{2,31} and blood oxidative stress markers (lipid peroxidation markers, protein carbonyls) have been indicated as prognostic tools in vasculitis and particularly in BD.³² Only few studies until now have explored the possible blood ROS sources in BD,²¹ whereas it is unknown whether oxidative stress may affect the structure and function of fibrinogen, a plasma protein involved both in inflammation and coagulation and particularly susceptible to oxidation.

Inflammation-induced endothelial injury emerges as a key factor connecting chronic inflammation and thrombosis.³³ However, the mechanisms underlying this pathogenetic correlation remain to be elucidated. Our results indicate that leucocyte ROS production, particularly by neutrophils, is significantly enhanced in BD patients. This finding is associated with increased plasma levels of oxidative stress markers and in particular with a greater extent of fibrinogen carbonylation. In the blood of BD patients only neutrophil-derived ROS (but not lymphocyte- or monocyte-derived ROS) showed a significant correlation with fibrinogen carbonyl content, so suggesting that neutrophil activation drives fibrinogen oxidation.

Neutrophils play a critical role in host defense by the engagement of specific surface receptors, with a multitude of ligands including fibrinogen.³⁴ It has been reported that neutrophils and monocytes promote vascular injury through the generation of inflammatory mediators, tissue infiltration, and oxidation of lipoproteins and other proteins. Neutrophils release serine proteases promoting efficient thrombus formation in mice by stabilizing fibrin deposition, likely by proteolytic inactivation of endogenous anticoagulants.³⁵ Moreover, in neutrophils, soluble fibrinogen is able to trigger, through an integrin-dependent mechanism, an activating signal that promotes degranulation, phagocytosis enhancement, and apoptosis delay. These events may deeply affect the fate of the inflammatory response.³⁶ In the reported *in vitro* experiments,

purified fibrinogen resulted markedly carbonylated when incubated with neutrophils derived from BD patients, but not with monocytes or lymphocytes from the same patients.

At the same time, a marked increase in NADPH oxidase activity was observed only in the neutrophil fraction. NADPH oxidase-derived ROS are key players in oxidative stress and inflammation, and the NOX complex (the major isoforms of NADPH oxidase) is considered to be a major source of ROS in phagocytic polymorphonuclear neutrophils and monocytes.^{37,38}

In the present study, both plasma oxidative markers and fibrinogen PC were markedly and significantly increased in BD patients when compared with control subjects. In BD, fibrinogen PC significantly correlated with neutrophil-derived ROS, but not with lymphocyte- or monocyte-derived ROS. In this context, it has been reported that fibrinogen is more prone to oxidation than albumin, and upon oxidation, clot formation rate has been observed to be decreased.^{39,40}

However, other investigators reported that exposure of fibrinogen to oxidizing conditions (i.e. Fe³⁺ ascorbate) promoted fibrin formation, enhanced platelet aggregation, and supported less efficient plasminogen activation by tissue plasminogen activator (tPA).⁴¹

In our study, the relationship between oxidized fibrinogen and fibrinogen function has been explored by measuring the clotting ability of purified fibrinogen, using an *in vitro* thrombin-catalyzed polymerization assay. In this system, the magnitude of the turbidity increase relates to the architecture of the formed clot and the altered maximum absorbance of fibrin polymerization reflects the formation of thinner and more compact fibers. When we assessed, in BD patients, thrombin-catalyzed fibrin polymerization, a slower rate and turbidity was observed compared with healthy controls, as indicated by decreased V_{max} and Max abs values. In particular, this latter parameter was significantly and inversely correlated with fibrinogen carbonyl content suggesting a direct influence of carbonylation on fibrin polymerization.

Moreover, in BD patients, only neutrophil (but not lymphocyte or monocyte) ROS production inversely and significantly correlated with turbidity/Max Abs parameters in clotting assays.

When fibrinogen secondary structure was investigated by far-UV circular dichroism spectroscopy, a reduction in α -helical content was detected in BD patients. Considering that protein functionality largely depends on its secondary structure, this finding suggests that carbonylation promotes the formation of a species less rich in α -helix, which may specifically affect the biological activity of fibrinogen. Our observation concerning the modified fibrinogen function is in keeping with our and previous reports, showing that fibrinogen oxidation impairs the capacity of isolated fibrinogen to form a fibrin clot under the effect of thrombin.^{24,39,42} To study another important feature of fibrinogen function in relation to carbonylation, we determined, both in BD patients and controls, fibrin resistance to plasmin-induced lysis. In BD patients, fibrin showed a marked resistance to lysis and its degradation was significantly decreased with respect to healthy controls. Moreover, fibrin resistance to lysis significantly correlated with fibrinogen PC and with neutrophil ROS production (but not with lymphocyte-or monocyte-derived ROS).

Interestingly, one of our major findings is a strong and positive correlation between fibrinogen PC and residual β -chain intensity after plasmin-induced lysis. Moreover, a significant correlation between residual β -chain intensity after plasmin-induced lysis and neutrophil ROS production was found.

The reported findings are consistent with the observation, in patients with acute coronary syndromes, that clots composed of dense networks are more resistant to lysis and these features correlate with inflammation and oxidative stress.⁴³ Hence, clot structure, analyzed by electron and differential interference contrast microscopy, revealed, in BD patients, an altered clot

architecture mainly characterized by a tight fibrin network composed of filaments with slightly decreased average fiber size that are resistant to plasmin-induced lysis when compared with control subjects. Clots composed of thin fibers and small pores have been suggested to be more thrombogenic^{44,45} but the mechanisms underlying the formation of these abnormal fibrin clots have not yet been established. The post-translational oxidative modifications of fibrinogen could play an important role in this context.⁴⁶

In conclusion, our data highlight that neutrophil activation promotes fibrinogen oxidation and thrombus formation in BD. In particular, our results suggest that an altered fibrinogen structure and an impaired fibrinogen function are associated with neutrophil activation and enhanced ROS production whose primary source is represented by neutrophil NADPH oxidase. BD is a systemic vasculitis¹¹ frequently complicated by recurrent thrombosis, especially in male patients with active disease.¹⁶ Our results were observed in BD patients regardless of the presence of vascular involvement. Furthermore, all patients had inactive disease, thus suggesting that BD is *per se* a model of inflammation-induced thrombosis. Altogether, these data may improve our understanding of the pathogenesis of inflammation-induced thrombosis and may suggest potential targets for innovative therapeutic approaches.

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Conflict of Interest Disclosures: None.

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Clinical Perspective

Behçet disease (BD) is a systemic inflammatory disease which frequently affects medium-sized and large vessels, histologically characterised by neutrophil-rich inflammatory infiltrates. BD is often complicated by both arterial and venous thrombosis, which is often resistant to common anti-coagulation therapy and rarely causes embolism. The pathogenesis of BD-related thrombosis is still unclear. In this study, we demonstrate that in BD patients neutrophils have a significantly increased reactive oxygen species (ROS) production due to abnormal NADPH-oxidase activity; ROS promote fibrinogen carbonylation and secondary structure modification which ultimately cause altered fibrinogen clotting ability and reduced fibrin susceptibility to plasmin-induced lysis. These data offer a new model of inflammation-induced thrombosis and support current therapeutic concepts regarding the use of immunosuppressive (rather than anticoagulation) therapy in BD-related thrombosis. The model of inflammation-induced thrombosis warrants further investigation in BD and in other autoimmune diseases.

Table 1. International Study Group criteria and non-criteria characteristics of the patients with Behçet's disease: demographic and clinical features.

International Study Group criteria for Behçet's disease		
Major criteria		
Recurrent oral ulcerations: minor aphthous, major aphtous or herpetiform ulceration observed by physician or patient, which recurred at least 3 times in one 12 month period n (%) patients = 98/98 (100%)		
+		
Minor criteria		
Recurrent genital ulceration: aphthous ulceration or scarring observed by physician or patient	2 minor criteria n (%) patients = 73/98 (74%) Genital ulcers + Eye lesions 14 (19%) Genital ulcers + Skin lesions 25 (34%)	3 minor criteria n (%) patients = 25/98 (26%) Genital ulcers + Eye lesions + Skin lesions 16 (64%)
Eye lesion: anterior uveitis, posterior uveitis, or cells in vitreous on slit lamp examination or retinal vasculitis observed by ophthalmologist	Eye lesions + Skin lesions 25 (34%) Skin lesions + Pathergy test 8 (11%) Eye lesions + Pathergy test 1 (1%)	Skin lesions + Eye lesions + Pathergy test 7 (28%) Genital ulcers + Skin lesions + Pathergy test 2 (8%)
Skin lesions: erythema nodosum observed by physician or patient, pseudofolliculitis or papulopustular lesions, or acneform nodules observed by physician in post-adolescent patients not on corticosteroid treatment		
Positive pathergy test read by physician 24-48 hours		
Other non-criteria demographic and clinical features of the patients with Behçet's disease		
Males		47/98 (48%)
Females		51/98 (52%)
HLAB*51		53/98 (54%)
Vascular involvement		31/98 (32%)
Articular involvement		55/98 (56%)
Gastrointestinal involvement		36/98 (37%)
Central nervous system involvement		18/98 (18%)

Table 2. Oxidative stress markers. Group mean \pm SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. *indicates significant difference vs control at the $p<0.01$ level (Student t test).

	Controls n=70	BD patients n=98	
Plasma PC (nmol/mg)	11.57 \pm 3.42	18.91 \pm 5.73	p<0.01
Plasma TBARS (nmol/ml)	0.610 \pm 0.070	1.905 \pm 0.961	p<0.01
Fibrinogen PC (nmol/mg)	0.450 \pm 0.050	1.941 \pm 0.817	p<0.01

Figure Legends

Figure 1. A) Lymphocytes/monocytes/neutrophils ROS production in controls and BD patients. Group mean \pm SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. B) Pearson rank correlation analysis in BD patients, comparing fibrinogen carbonyl content and lymphocytes/monocytes/neutrophils ROS. ROS production and fibrinogen carbonyl content was measured as described in the Materials and Methods section. Each point represents the overall mean of different experiments/replicates for each subject. *indicates significant difference vs control at the $p<0.01$ level (Student t test).

Figure 2. Spectroscopy analysis of fibrinogen secondary structure. Far-UV Circular Dichroism (CD) spectra of fibrinogen purified from 98 BD patients and 70 healthy controls. Spectroscopic analyses were performed as described in the Materials and Methods section. Group mean \pm SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported.

Figure 3. A) Thrombin-catalyzed fibrinogen polymerization and corresponding Lag time, Vmax and Max Abs in fibrinogen purified from BD patients and controls are reported.

Thrombin-catalyzed fibrin polymerization was performed as described in the Materials and Methods section. Group mean \pm SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. *indicates significant difference vs control at the $p<0.01$ level (Student t test). B) In BD patients, Pearson rank correlation analysis comparing BD fibrinogen carbonyl content and Max Abs showed a statistical significance ($C, p<0.0001, r^2=0.2894$). Each point represents the overall mean of different experiments/replicates for each BD patient. C-D-E) Pearson rank correlation analysis comparing lymphocytes/monocytes/neutrophils ROS and Max Abs in BD patients. Each point represents the overall mean of different experiments/replicates for each BD patient.



Figure 4. Representative Differential Interference Contrast (DIC) Microscopy images of fibrin from a BD patient and a control subject. The same fibrinogen concentration was used in these subjects, who were matched for age and sex. DIC microscopy analyses were performed as described in the Materials and Methods section.

Figure 5. A) Representative gel of fibrin degradation after 0h, 3h and 6h of plasmin digestion, using fibrinogen purified from BD patients and controls. B) Residual fibrin β chain intensity after 6h of plasmin digestion in fibrinogen purified from controls and BD patients. Group mean \pm SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. * indicates significant difference vs control ($p<0.01$, Student t test). Plasmin-induced fibrin digestion, and electrophoretic analyses of plasmin-digests were performed as described in the Materials and Methods section. C) In BD patients, Pearson rank correlation analysis comparing BD fibrinogen carbonyl content and residual beta chain intensity after 6h of plasmin digestion

showed a statistical significance (C, $p < 0.001$ $r^2 = 0.1254$). Each point represents the overall mean of different experiments/replicates for each BD patient. D-E-F) Pearson rank correlation analysis comparing lymphocytes/monocytes/neutrophils ROS and Max Abs in BD patients. Each point represents the overall mean of different experiments/replicates for each BD patient.

Figure 6. NADPH oxidase activity was assessed by luminometric assay (as described in the Materials and Methods section) in lymphocytes/monocytes/neutrophils from BD patients and controls. All assays were performed in the absence and in the presence of a specific NADPH oxidase inhibitor (apocynin). Group mean \pm SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. * indicates significant difference vs control ($p < 0.01$, ANOVA-Bonferroni test).

Figure 7. A) Leukocyte- dependent fibrinogen carbonylation.

Lymphocytes/monocytes/neutrophils from BD patients were incubated with purified control fibrinogen and its carbonyl content was then estimated. All assays were performed in the absence or presence of a specific NADPH oxidase inhibitor (as reported in the Materials and Methods section). Group mean \pm SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. * indicates significant difference vs FG + lymphocytes and # indicates significance vs Fibrinogen + monocytes ($p < 0.01$, ANOVA-Bonferroni test). B) Fibrinogen susceptibility to plasmin-induced lysis. Representative gel of fibrin degradation after 0h, 3h and 6h of plasmin digestion, using control fibrinogen incubated with lymphocytes/monocytes/neutrophils from BD patients. Residual fibrin β chain intensity after 6h of plasmin digestion

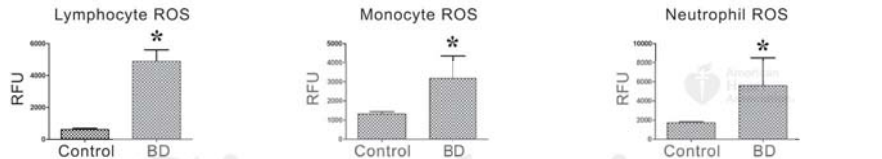
in control fibrinogen incubated with lymphocytes/monocytes/neutrophils from BD patients. Group mean \pm SD of 98 patients or 70 controls, calculated by considering the overall mean of different experiments/replicates as single value for each subject, are reported. * indicates significant difference vs control ($p < 0.01$, ANOVA-Bonferroni test).



Circulation

A

Leukocyte ROS production



B

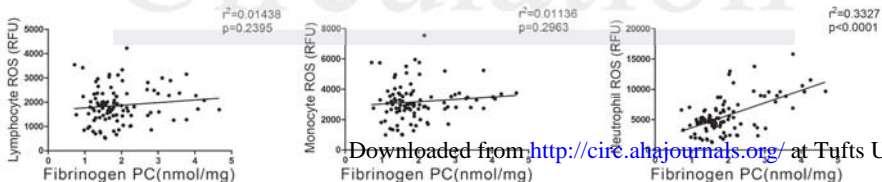
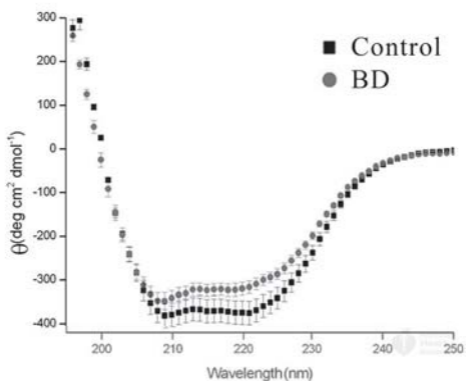
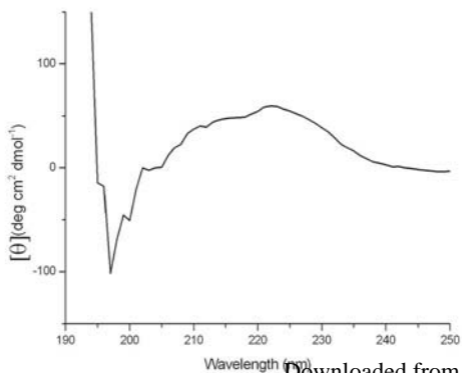


Figure 1

Fibrinogen CD spectra



Fibrinogen CD difference spectrum

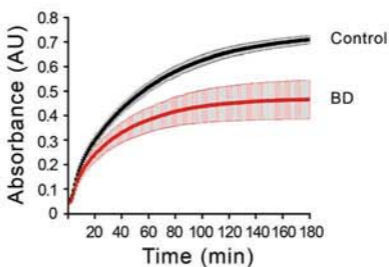


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Figure 2

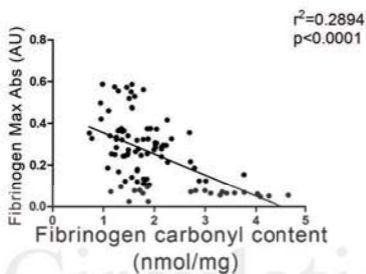
A

Thrombin-catalyzed fibrin formation

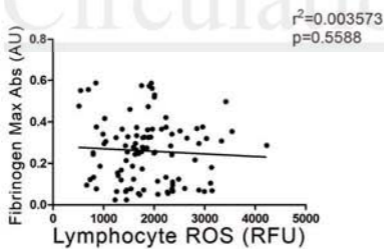


	Control n=70	BD patients n=98
Max Abs(AU)	0.550±0.07	0.256±0.15*
Vmax(sec ⁻¹ ×10 ⁻²)	5.39±0.70	3.09±1.69*
Lag Time(min)	11.03±0.63	20.31±1.13*

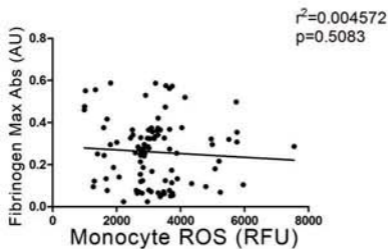
B



C



D



E

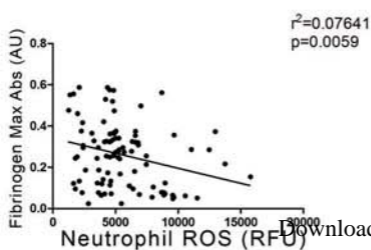
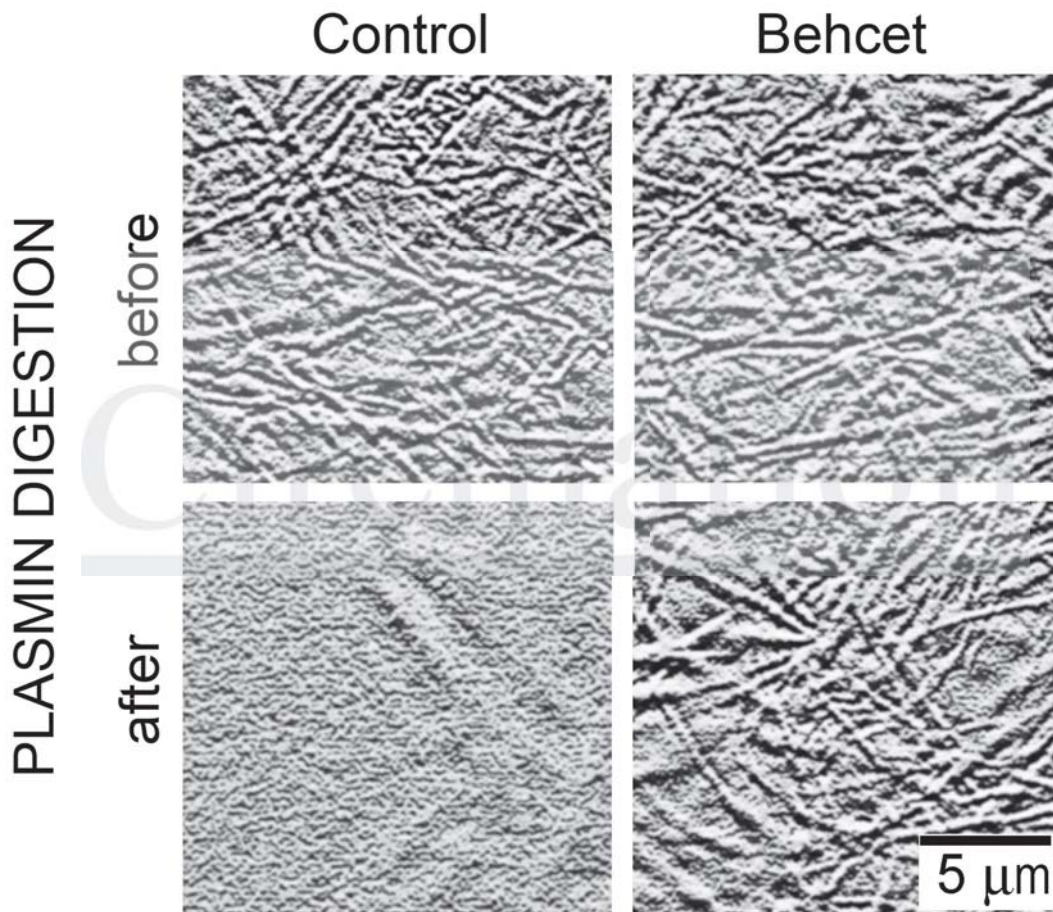
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Figure 3

Differential Interference Contrast Microscopy



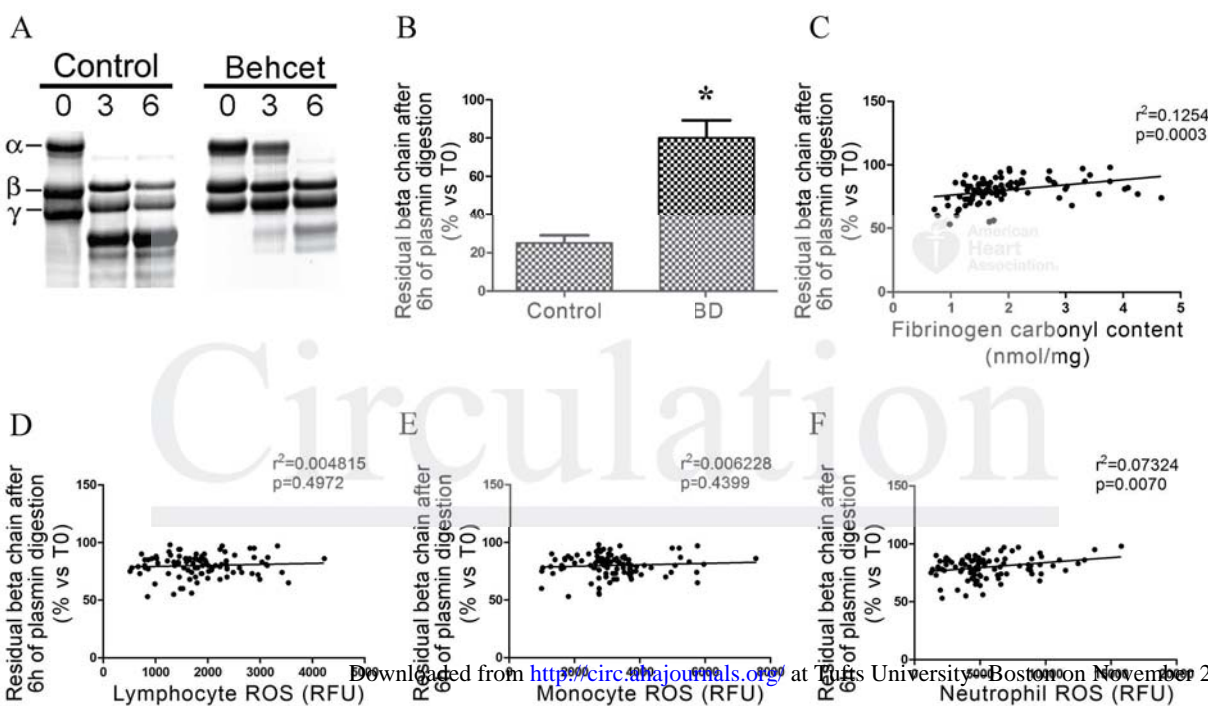


Figure 5

NADPH oxidase activity

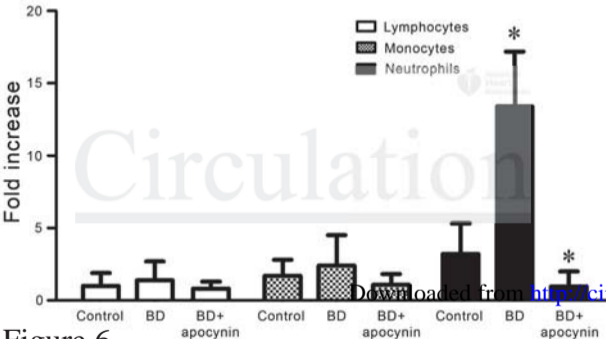
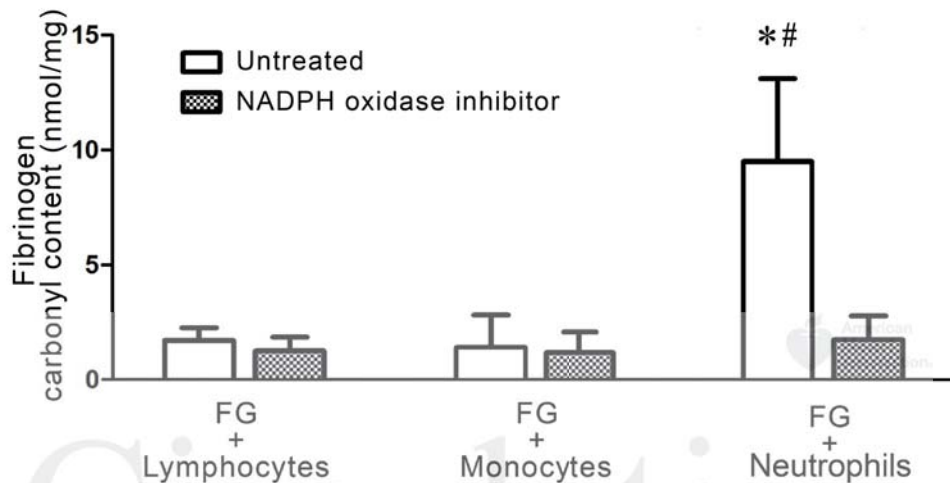


Figure 6

A

Leukocyte-dependent fibrinogen carbonylation



B

Fibrin susceptibility to plasmin-induced lysis

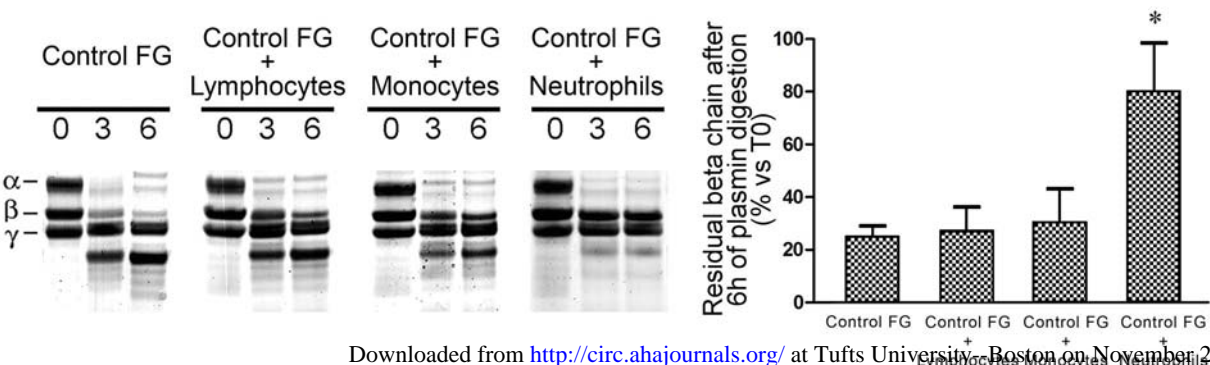
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Figure 7

Neutrophil Activation Promotes Fibrinogen Oxidation and Thrombus Formation in Behçet's Disease

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