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Oxidative Modification of Fibrinogen Is Associated With Altered Function and Structure in the Subacute Phase of Myocardial Infarction

Matteo Becatti, Rossella Marcucci, Giulia Bruschi, Niccolò Taddei, Daniele Bani, Anna Maria Gori, Betti Giusti, Gian Franco Gensini, Rosanna Abbate, Claudia Fiorillo

Objective—Among plasma proteins, fibrinogen represents a major target of oxidative modifications. In patients with post-acute myocardial infarction (6 months after the acute event), fibrinogen oxidation-induced carbonyls and fibrinogen function were estimated using in vitro and ex vivo approaches. Fibrinogen structural features and clot architecture were also explored.

Approach and Results—In 39 patients with post-acute myocardial infarction and 28 age-, sex-, and risk factor-matched controls, oxidative stress markers (in plasma and in purified fibrinogen fractions), thrombin-catalyzed fibrin polymerization, and plasmin-induced fibrin lysis were estimated. Circular dichroism spectra of purified fibrinogen extracts, electron microscopy, and differential interference contrast microscopy analyses of fibrin clots were also performed. Marked signs of oxidative stress in plasma ($P<0.01$ versus controls) and, correspondingly, an increased extent of fibrinogen carbonylation (3.5-fold over control values; $P<0.01$ versus controls) were observed in patients. Furthermore, fibrinogen fractions purified from patients exhibited significantly reduced clotting ability and decreased susceptibility to plasmin-induced lysis ($P<0.01$ versus controls). Alterations in fibrinogen secondary structure, as suggested by circular dichroism spectroscopy, and in fibrin clot architecture, as analyzed by electron and differential interference contrast microscopy, were also identified.

Conclusions—Here, we report for the first time that patients with post-acute myocardial infarction present with an overall imbalance in redox status and marked fibrinogen carbonylation associated with altered fibrinogen function, thus suggesting a role for carbonylation as a direct mechanism of fibrinogen function. The observed features occur along with modifications in protein structure and in clot architecture. (*Arterioscler Thromb Vasc Biol.* 2014;34:1355-1361.)

Key Words: fibrinogen ■ myocardial infarction ■ oxidative stress

The imbalance between reactive oxygen species production and antioxidant defenses leads to the condition known as oxidative stress. Disturbances in the normal redox status of tissues can cause toxic effects through the production of peroxides and free radicals that damage all cellular components, including proteins, lipids, and DNA. Proteins are the main targets for reactive oxygen species that may alter every level of their structure from primary to quaternary, causing physical changes.¹ Increased reactive oxygen species generation by vascular and inflammatory cells occurs in cardiovascular disease, and there is widespread evidence that oxidative injury contributes to vascular damage and cardiac dysfunction.² Elevated levels of oxidative biomarkers, such as protein carbonyls (PC) and lipid peroxidation markers (thiobarbituric acid reactive substances, 8-epi-prostaglandin F), have been found in atherosclerotic lesions and in the circulation

of patients with coronary artery disease (CAD).³⁻⁶ In plasma of patients with acute myocardial infarction (AMI) assayed within 24 to 96 hours after the acute event, an impaired fibrin lysis and an enhanced fibrinogen carbonylation has been recently observed.^{4,7}

No evidence for a possible role of carbonylation on fibrinogen function in patients with post-AMI is available. Hence, for the first time, we investigated by an in vitro and an ex vivo approach, whether fibrinogen function—assessed by thrombin-catalyzed fibrin polymerization and fibrin susceptibility to plasmin-induced lysis—is associated with alterations in its oxidative status. Additional data on modifications in fibrinogen structure and in clot architecture are also reported.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

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Nonstandard Abbreviations and Acronyms

AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
AMI	acute myocardial infarction
CAD	coronary artery disease
PC	protein carbonyls

Results

Subjects

Demographic and clinical characteristics of the population studied are summarized in Table 1.

Oxidative Stress Markers in Plasma and in Fibrinogen

As reported in Table 2, patient plasma displayed significantly higher total PC and thiobarbituric acid reactive substances levels, and lower total antioxidant capacity, than healthy controls ($P<0.05$ versus controls). Plasma total PC was positively and significantly correlated with fibrinogen PC ($P<0.05$; $R=0.555$; Figure 1A) and with thiobarbituric acid reactive substances levels ($P<0.05$; $R=0.444$; Figure 1B).

Purified fibrinogen from patients displayed significantly increased carbonylation (3.5-fold) in comparison with healthy controls ($P<0.01$; Figure 1C). PC amounts obtained in human, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-challenged purified fibrinogen (SIGMA, Milan, Italy) were comparable with those observed in fibrinogen from patients with post-AMI and resulted significantly increased compared with untreated fibrinogen (Table 3).

Circular Dichroism Spectra: Analysis of Secondary Structure

Secondary protein structure was analyzed by far-UV circular dichroism spectroscopy (Figure 2). In control subjects, the observed spectrum for fibrinogen suggested a typically α -helical structure with minima at 208 and at 222 nm.

Table 1. Clinical Characteristics of Post-Acute Myocardial Infarction Patients and Control Subjects

	Patients (n=39)	Controls (n=28)	P Value
Men/Women (n, %)	27/12 (69.2)	20/8 (67.9)	0.999
Age, y; median and interquartile range	71.0 (59–77)	69.8 (58–78)	0.845
Hypertension (n, %)	26 (66.7)	18 (59.2)	0.999
Smoking habit (n, %)	9 (23.1)	6 (21.4)	0.777
Dyslipidemia (n, %)	18 (46.2)	12 (33.9)	0.809
Diabetes mellitus (n, %)	11 (28.2)	5 (17.9)	0.393
Family history of CAD (n, %)	19 (48.7)	4 (14.2)	0.004
Overweight (n, %)	8 (20.5)	6 (18)	0.759
ACE-inhibitors (n, %)	27 (69.2)	10 (35.7)	0.012
Statins (n, %)	24 (61.5)	11 (39.3)	0.087
Proton pump inhibitors (n, %)	37 (94.9)	1 (3.5)	<0.001
β -Blockers (n, %)	34 (87.2)	10 (35.7)	<0.001

ACE indicates angiotensin-converting enzyme; and CAD, coronary artery disease.

Table 2. Plasma Redox Status and Fibrinogen Carbonyl Content in Patients With Post-AMI and Control Subjects

	Control Subjects	Patients With Post-AMI
TBARS, nmol/mL	11.2 \pm 3.3	30.5 \pm 13.6* (28.90)
TAC, nmol/mL	470.2 \pm 45.3	306.9 \pm 98.7* (292.0)
Total PC plasma, nmol/mg	1.15 \pm 0.31	2.87 \pm 1.02* (2.73)
Fibrinogen carbonyl content, nmol/mg	0.19 \pm 0.05	0.66 \pm 0.32* (0.69)

All data are represented as mean \pm SD. PC indicates protein carbonyls; post-AMI, post-acute myocardial infarction; TAC, total antioxidant capacity; and TBARS, thiobarbituric acid reactive substance.

*Statistically significant difference vs Control at the $P<0.05$ level, as assessed using the ANOVA-Bonferroni test.

Fibrinogen from patients with post-AMI displayed an altered circular dichroism spectrum consisting mainly of a decrease in the negative peak in the 215 to 225 nm region, therefore, suggesting a decrease in α -helical content (Figure 2).

Fibrinogen Polymerization and Fibrin Formation

Representative curves of thrombin-catalyzed fibrinogen polymerization are shown in Figure 3. In an in vitro assay, increasing concentrations of AAPH affected thrombin-induced polymerization of pure fibrinogen (Figure 3A). In particular, in the presence of increasing AAPH concentrations, V_{max} and Max absorbance progressively and significantly decreased, whereas lag time increased in a dose-dependent manner ($P<0.01$ versus controls).

To evaluate whether an antioxidant treatment could prevent the observed alterations, 0.05 mmol/L Trolox was added to the AAPH incubation reactions and thrombin-catalyzed polymerization of fibrinogen was performed. As Figure 3A reports, the simultaneous incubation of AAPH with Trolox was able to prevent the observed changes.

Similarly, in patients, the ability of fibrinogen to undergo clotting was diminished: significant differences in lag time, V_{max} , and Max absorbance versus controls were found ($P<0.01$; Figure 3B). In Figure 3C, the correlation analysis between fibrinogen carbonyl content and Max absorbance was reported: the relationship seems inverse and significant ($P<0.05$ $R=0.419$).

Transmission Electron Micrographs and Differential Interference Contrast Microscopy

Using electron microscopy, we found that fibrin filaments from patients and controls examined preplasmin digestion displayed differences in size and morphology: in particular, control fibrin filaments seemed as bundles of densely packed, longitudinally arranged fibrin molecules (Figure 4A, top), whereas post-AMI fibrin filaments consisted of thinner fibrin fibers (mean diameter, 120 \pm 13.2 nm versus control, 151 \pm 13.6 nm; Figure 4B). After plasmin digestion, samples from control subjects showed a complete disarrangement of fibrin filaments, with short fibrin monomers forming a loose 3-dimensional network (Figure 4A, lower left). Samples from patients with post-AMI after plasmin digestion still showed a filamentous pattern although with slightly decreased average fiber size (mean diameter, 89 \pm 10.9 nm versus control 40 \pm 3.7

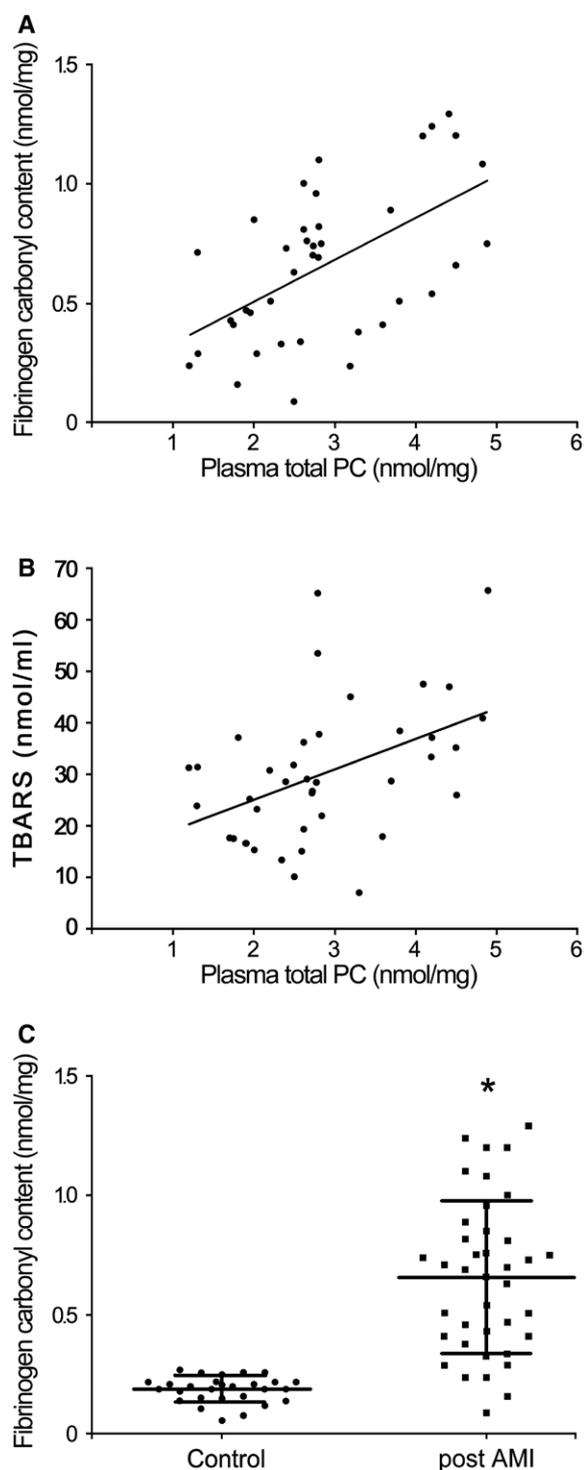


Figure 1. Pearson rank correlation analysis in patients with post-acute myocardial infarction (AMI) comparing plasma total protein carbonyls (PC) and fibrinogen carbonyl content ($P<0.05$; $R=0.555$; **A**) and comparing plasma total PC and thiobarbituric acid reactive substance (TBARS) levels ($P<0.05$; $R=0.444$; **B**). Fibrinogen carbonyl content in purified fibrinogen fractions from patients with post-AMI ($n=39$) and controls ($n=28$; **C**). Protein carbonyl content was measured as described in the Materials and Methods in the online-only Data Supplement. All experiments were performed by running each sample in triplicate. Values are represented as mean \pm SD. *Significant difference vs control at the $P<0.01$ level (ANOVA-Bonferroni test).

nm; Figure 4B) and evident fibrin monomers (Figure 4A, lower right).

In Figure 4A, fibrin samples obtained from patients with post-AMI and controls analyzed by differential interference contrast microscopy are shown. A tight fibrin network is still evident after plasmin-induced lysis in fibrin obtained from patients with post-AMI when compared with that obtained from controls.

Fibrin Susceptibility to Plasmin-Induced Lysis

Fibrin β -chain degradation at 0, 3, and 6 hours after plasmin digestion in vitro experiments consisting of human purified fibrinogen treated with AAPH (in the absence or presence of Trolox) and quantification of residual β -chain intensity after 6 hours plasmin digestion is reported in Figure 5A. Fibrin clots obtained with pure fibrinogen after incubation with increasing AAPH concentrations showed reduced susceptibility to plasmin-induced lysis, at each considered time of plasmin digestion. To evaluate whether an antioxidant treatment could prevent the observed alterations, 0.05 mmol/L Trolox was added to AAPH incubation reactions and fibrin susceptibility to plasmin-induced lysis was then assessed. As shown in Figure 5A, the simultaneous incubation of AAPH and Trolox was able to prevent the observed changes in fibrin digestion by plasmin.

Fibrin β -chain degradation after 0, 3, and 6 hours of plasmin digestion in patients with post-AMI and controls is reported in Figure 5B. In patients with post-AMI, the relative band intensity at each considered time of plasmin digestion was significantly higher with respect to controls.

In patients, the correlation between fibrinogen carbonyl content and the relative fibrin β -chain intensity after 6 hours of plasmin digestion was shown to be positive and significant ($P<0.05$; Figure 5C). In patients with post-AMI, fibrin β -chain intensity after 6 hours of plasmin digestion was also significantly correlated with plasma PC ($P<0.01$; $R=0.540$) and thiobarbituric acid reactive substances levels ($P<0.01$; $R=0.616$, data not shown).

Discussion

The results of the present study show, for the first time, that in patients with post-AMI (1) a systemic redox imbalance is coupled with an increased level of fibrinogen carbonylation; (2) changes in the secondary protein structure of fibrinogen are present; (3) the increased extent of fibrinogen carbonylation is associated with altered polymerization and susceptibility to plasmin-induced lysis; and (4) marked modifications in the global clot architecture are evident.

Atherosclerosis is characterized by lipid and protein oxidation in the vascular wall that contributes to important clinical manifestations of CAD represented by plaque disruption and endothelial dysfunction.^{2,8}

Increased levels of oxidative biomarkers and decreased antioxidant activity in the circulating blood of patients with CAD have been reported,^{9,10} whereas raised PC values have also been found in patients with myocardial infarction, providing further evidence for enhanced oxidative stress in these subjects.¹¹

Table 3. In vitro Assay: Protein Carbonyl Content in AAPH-Treated Pure Fibrinogen

	Untreated	0.01 mmol/L AAPH	0.05 mmol/L AAPH	0.1 mmol/L AAPH	1 mmol/L AAPH
Pure fibrinogen carbonyl content, nmol/mg	0.1±0.02	0.41±0.12*	1.22±0.22*	2.09±0.41*	9.19±1.32*

All data are represented as mean±SD. AAPH indicates 2'-azobis(2-amidinopropane) dihydrochloride.

*Statistically significant difference vs untreated at the $P<0.05$ level, as assessed using the ANOVA-Bonferroni test.

In the present study, both plasma PC and fibrinogen PC were markedly and significantly increased in patients with post-AMI when compared with control subjects and were significantly correlated. In this context, previous reports indicate that fibrinogen is more susceptible to oxidation than most other plasma proteins;¹² in particular, fibrinogen is 20× more susceptible to oxidation than albumin,¹³ as also reported in patients with MI, where it was found that total plasma carbonyls were formed preferentially on fibrinogen.⁷

When we investigated, using far-UV circular dichroism spectroscopy, whether the alterations observed in fibrinogen functions could be related to secondary structure modifications, we found a shift in the mean residual ellipticity and a reduction in α -helical content in patients with post-AMI in comparison with controls, suggesting a change in secondary structure content. Taking into consideration that secondary structure of proteins plays a major role in determining their functionality in specific physiological processes, this preliminary result needs further investigation and leads to speculation that carbonylation promotes the formation of a less α -helix-rich species.¹⁴

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; the altered maximum absorbance of fibrin polymerization reflects the formation of thinner and more compact fibers.¹⁵

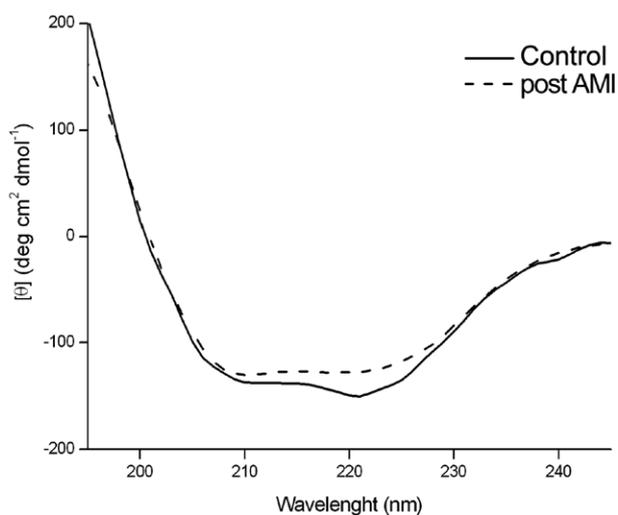


Figure 2. Spectroscopy analysis of fibrinogen secondary structure. Representative far-UV circular dichroism spectra of fibrinogen purified from a patient with post-acute myocardial infarction (AMI) and a control subject. Spectroscopic analyses were performed as described in the Materials and Methods in the online-only Data Supplement.

Interestingly, the functional alterations that we observed when fibrinogen was subjected to oxidation could easily be reversed by simultaneous treatment with a water soluble analogue of α -tocopherol (Trolox), emphasizing the role of oxidative modification of fibrinogen on its function.¹⁶

In patients with post-AMI, we observed a slower rate of thrombin-catalyzed fibrinogen polymerization; this alteration in fibrinogen function was found to be directly and significantly correlated to fibrinogen carbonyl content, suggesting an influence of carbonylation on thrombin-induced polymerization of fibrinogen.

The introduction of carbonyl groups into proteins can be triggered by different reactive oxygen species or secondary by-products of oxidative stress and can arise at different sites and by different mechanisms.¹⁷ Carbonylation can result in several different protein modifications, which may specifically affect the biological activity of proteins.¹⁸ Among the functional effects that have been described, modified binding activities, enzyme inactivation, and altered susceptibility to proteolytic degradation are the most represented.¹⁹ It has been shown that the introduction of carbonyl derivatives (aldehydes and ketones) may alter the conformation of the polypeptide chain, thus determining functional modification of proteins.²⁰ The 2 amino acids that are perhaps the most prone to oxidative attack are cysteine and methionine, both of which contain susceptible sulfur atoms. Other amino acyl moieties, especially lysine, arginine, proline, and threonine, incur formation of carbonyl groups on the side chains.²¹ The presence of oxidizable amino acids (Pro and Arg) in the cleavage site of thrombin, or the cleavage of peptide bonds by oxidation of glutamyl residues, may explain the altered thrombin-induced fibrin polymerization observed in patients with post-AMI.

In a recent in-depth study of the thrombin cleavage site, the authors show that mutating both Pro and Arg results in a 200- to 400-fold drop in cleavage, which highlights the importance of these 2 positions for maximal substrate cleavage.²²

In our experimental model, carbonylation of purified human fibrinogen was responsible for an oxidant-dependent decrease in thrombin-induced clot formation likely determined by a covalent protein modification. In line with this, the observed alteration in clotting ability of purified human fibrinogen was completely prevented when it was incubated with both the oxidant generator AAPH and the antioxidant Trolox.

Our findings showing alteration of fibrinogen function strengthen other reports, showing that fibrinogen oxidation impairs the capacity of isolated fibrinogen to form a fibrin clot under the effect of thrombin¹⁶; these results seem to contrast with those reported by Paton et al,⁷ obtained in 12 patients with MI, which were performed in the acute phase of myocardial infarction (ie, within 24–96 hours of the acute event). This discrepancy could be because of the different clinical setting: the

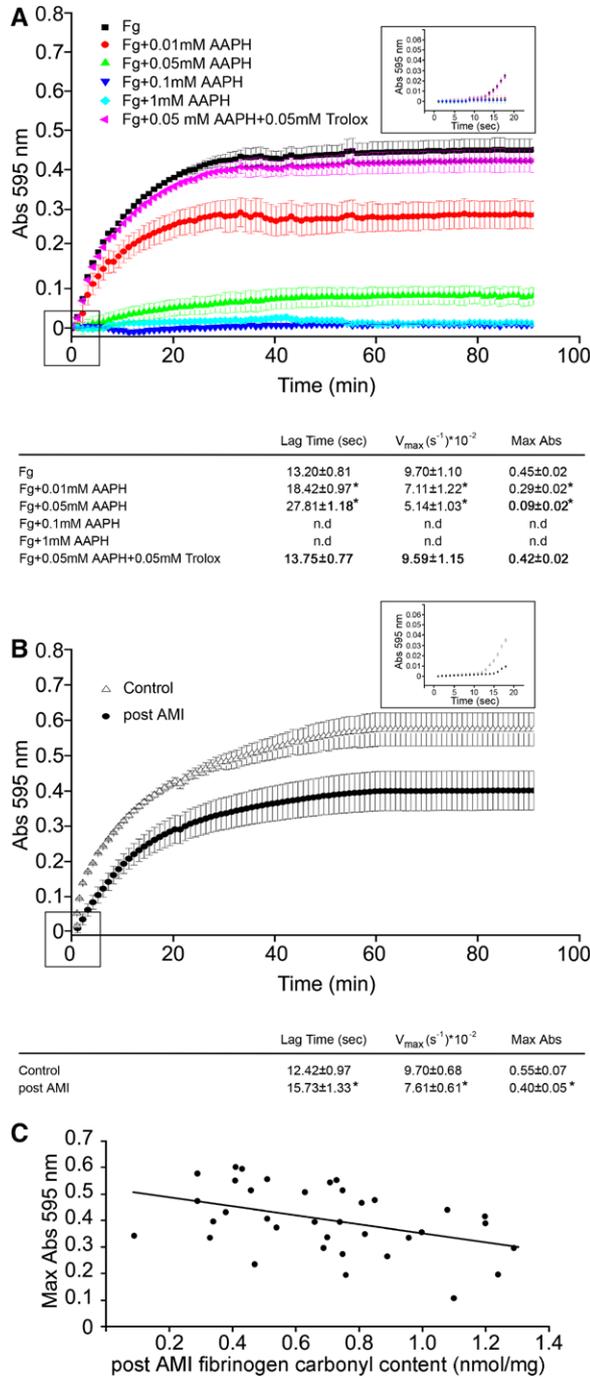


Figure 3. Representative curves of thrombin-catalyzed fibrinogen polymerization and corresponding lag time, V_{max} and Max absorbance in purified human fibrinogen (Fg; Sigma, Milan, Italy) incubated with increasing concentrations of the peroxy radical generator 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH; **A**) and in Fg purified from patients with post-acute myocardial infarction (AMI) and controls (**B**). AAPH treatment protocol (in the absence or presence of the antioxidant Trolox) and thrombin-catalyzed fibrin polymerization were performed as described in the Materials and Methods in the online-only Data Supplement. Values are reported as mean±SD of 3 independent experiments performed in triplicate (ie, each sample run in triplicate; **A**) and of 3 independent experiments performed on 39 patients with post-AMI and 28 controls (**B**). *Significant difference vs control at the $P<0.01$ level (ANOVA-Bonferroni test). In patients with post-AMI, Pearson rank correlation analysis comparing post-AMI Fg carbonyl content and Max absorbance showed a statistical significance (**C**; $P<0.05$; $R=0.419$).

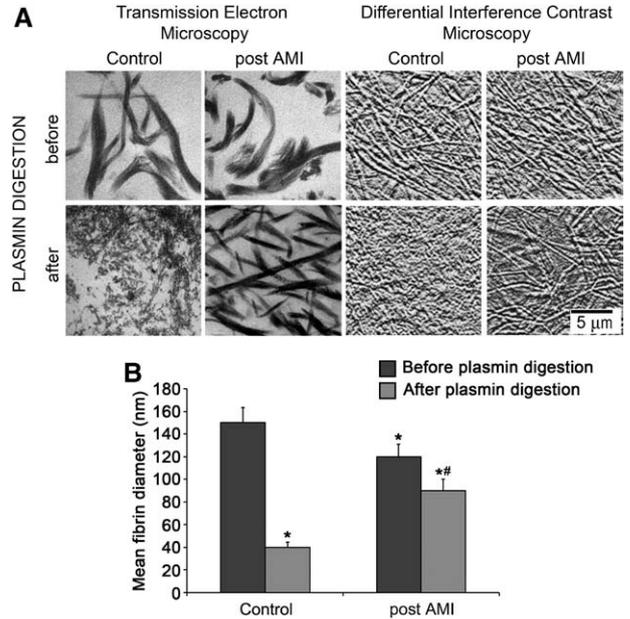


Figure 4. Representative transmission electron microscopy and differential interference contrast (DIC) microscopy images of fibrin from a patient with post-acute myocardial infarction (AMI) and a control subject (**A**). The same fibrinogen concentration was used in these subjects, who were matched for age and sex. Three different samples were analyzed, with 10 different clot areas sampled. Transmission Electron and DIC microscopy analyses were performed as described in the Materials and Methods in the online-only Data Supplement. The mean diameter of fibrin filaments from the different experimental groups was measured by computer-aided morphometry on $\times 50\,000$ transmission electron micrographs, using the freely available ImageJ 1.33 image analysis program (<http://rsb.info.nih.gov/ij>). For each sample, ≥ 50 filaments were measured and the values reported as mean±SD (**B**). *Significant difference vs control before plasmin digestion ($P<0.01$; ANOVA-Bonferroni test). #Significant difference vs post-AMI before plasmin digestion ($P<0.01$; ANOVA-Bonferroni test).

acute phase is characterized by an inflammatory and hypercoagulable state in which molecular alteration of fibrinogen because of fibrinopeptide A release was demonstrated.²³

Accordingly, a clear difference related to the clinical phase of CAD was documented by Undas et al,⁴ who reported significant differences in fibrinogen/fibrin alterations between patients with acute coronary syndrome and patients with stable angina.

To establish another important aspect of fibrinogen function in relation to carbonylation, we estimated fibrin resistance to plasmin-induced lysis.²⁴ We confirmed that in patients with post-AMI, fibrin is resistant to lysis and that its degradation is significantly lowered with respect to healthy controls.²⁵ Our results agree with previous investigations, demonstrating that patients with premature CAD produce abnormal plasma fibrin clots ex vivo that are resistant to fibrinolysis.²⁶

Interestingly, one of our main results is a strong and positive correlation between fibrinogen PC and residual β -chain intensity after plasmin-induced lysis. A similar correlation was found when correlating residual β -chain intensity after plasmin-induced lysis with plasma PC. The reported findings are consistent with the observation that F2-isoprostanes, which are stable markers of oxidative stress, are associated with reduced clot permeability and fibrinolysis in patients with cardiovascular.⁴

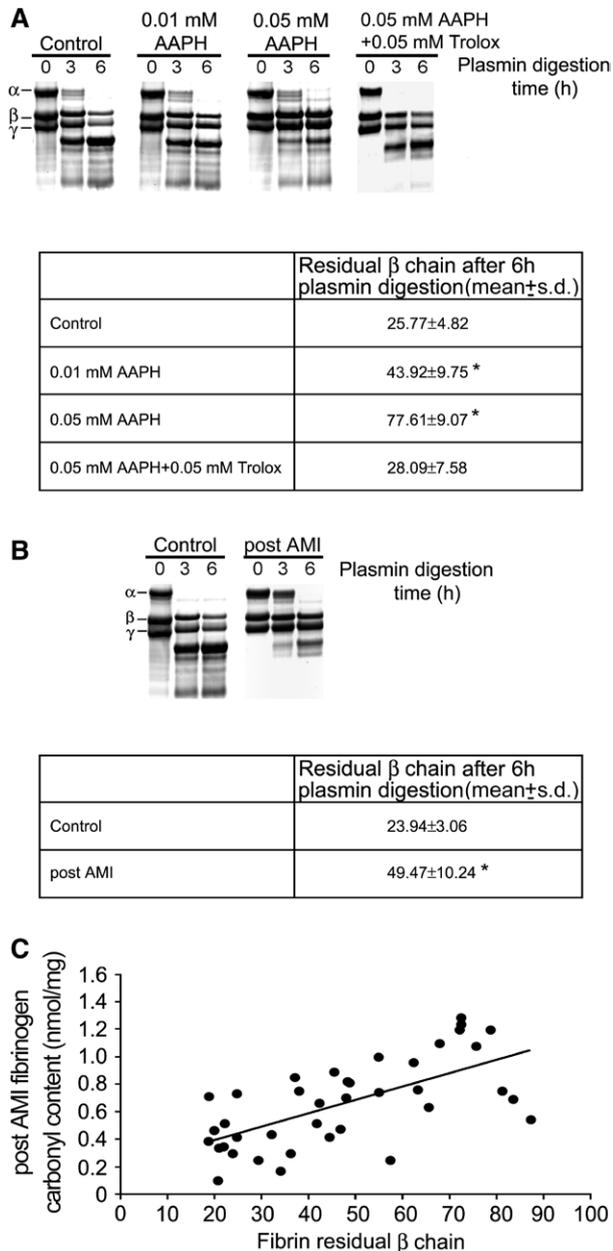


Figure 5. Representative gel of fibrin degradation after 0, 3, and 6 hours of plasmin digestion, using purified human fibrinogen (Sigma, Milan, Italy) incubated with increasing concentrations of the peroxyl radical generator 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH; **A**) and using fibrinogen purified from patients with post-acute myocardial infarction (AMI) and controls (**B**). AAPH treatment protocol (in the absence or presence of the antioxidant Trolox), fibrin digestion with plasmin, and electrophoretic analyses of plasmin-digests were performed as described in the Materials and Methods in the online-only Data Supplement. Residual fibrin β -chain intensity after 6 hours of plasmin digestion in fibrinogen purified from controls, patients with post-AMI, and in AAPH-treated purified human fibrinogen (Sigma, Milan, Italy) is reported. Values are represented as mean \pm SD of 3 independent experiments performed on 39 patients with post-AMI and 28 controls (or 3 independent experiments performed on AAPH-treated fibrinogen; ie, each sample run in triplicate). *Significant difference vs control ($P<0.01$; ANOVA-Bonferroni test). In patients with post-AMI, Pearson rank correlation analysis comparing post-AMI fibrinogen carbonyl content and fibrin β -chain intensity after 6 hours of plasmin digestion showed statistical significance (**C**; $P<0.01$; $R=0.540$).

Moving from the finding of altered thrombin-induced fibrin polymerization, possibly characterized by the presence of impaired lateral association of protofibrils and by alteration in fiber size resulting from fibrinogen carbonylation, we then performed an in-detail analysis of clot structure by electron and differential interference contrast microscopy. Our main finding relates to the presence, in post-AMI fibrin, of 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. Actually, clot structure is determined by several factors.^{27,28} In most purified systems, as fiber size increases, pore size increases too. Fibrin networks composed of thin fiber strands have small pores and are more rigid and less permeable. In contrast, clots formed by thick fiber strands have large liquid spaces, which imply higher permeability and accelerated fibrinolysis, likely because of a more efficient transport of fibrinolytic agents through a fibrin clot.²⁹ Clots composed of thin fibers and small pores are more thrombogenic and are associated with CAD.^{30–32} The mechanisms underlying formation of these abnormal fibrin clots have not yet been elucidated but could be related to post-translational modifications of fibrinogen.³³ The data presented here suggest a causative link between fibrinogen carbonylation and the structural alterations observed in patients with post-AMI.

An interesting aspect that must be taken into consideration is that all patients enrolled in the study were given aspirin as a secondary prevention treatment strategy, which could also have had an effect on clot architecture. Fibrinogen, in fact, is rich in lysine residues, which are the main target of acetylation by aspirin, treatment with which may disturb charge distribution on the protein surface. This action, however, has been shown to not affect the rate of fibrinogen gelation by thrombin.³⁴

In conclusion, our in vitro and ex vivo findings show that in patients with post-AMI, an overall imbalance in redox status and marked fibrinogen carbonylation are associated with altered clotting activity and susceptibility of plasmin to lysis. These features, observed far from the acute event, are accompanied by an alteration in protein structure and clot architecture.

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Disclosures

None.

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Significance

Fibrinogen represents an important target of oxidative modifications. In patients with post-myocardial infarction (6 months after the acute event), an overall imbalance in plasma redox status and marked fibrinogen carbonylation were associated with impaired clotting activity and reduced plasmin susceptibility to lysis. Fibrinogen protein secondary structure and clot architecture were also markedly altered. The features described here, observed far from the acute event, provide new insights into the mechanisms that control fibrin structure and function. These new findings might allow the development of pharmaceutical strategies to modulate fibrin structure *in vivo* and thereby might be useful for the primary and secondary prevention of coronary artery disease.