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Human blood plasma advanced oxidation protein products (AOPP) correlates with fibrinogen levels

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Abstract

In 1996 a novel oxidative stress biomarker, referred to as advanced oxidation protein products (AOPP) was detected in the plasma of chronic uremic patients. The aim of the present studies was to find out that which plasma fraction(s) is responsible for AOPP reactivity. Thermal treatment of pooled samples of human citrate-plasma or EDTA-plasma at 50°C resulted in a rapid and parallel loss of fibrinogen concentration and AOPP reactivity. On the basis of time course and $t_{1/2}$ values following thermal treatment, AOPP was indistinguishable from fibrinogen. There was a statistically significant ($p < 0.0001$) correlation between levels of blood plasma fibrinogen and AOPP in patients ($n = 61$) with various peripheral vascular or cardiovascular diseases. There was also a significant ($p < 0.0001$) relationship between plasma levels of fibrinogen and molar AOPP/fibrinogen ratio indicating that higher fibrinogen concentrations were associated with more oxidatively transformed groups on the molecule. Results of the present studies suggest that post-translationally modified fibrinogen is a key molecule responsible for human plasma AOPP reactivity. It remains to be elucidated what is the pathophysiological significance of the post-translationally modified fibrinogen in the inflammation-associated events of atherosclerosis, in platelet aggregation, and as a cardiovascular risk biomarker.

Keywords: *Advanced oxidation protein products (AOPP), oxidant stress, fibrinogen, cardiovascular risk*

Introduction

In 1996 a novel oxidative stress biomarker, referred to as advanced oxidation protein products (AOPP) was detected in the plasma of chronic uremic patients [1]. AOPP levels correlated with plasma concentrations of dityrosine and advanced glycation end-products (AGE)-pentosidine as indices of oxygen-mediated protein damage. It was suggested that AOPP measure highly oxidized proteins, especially albumin. The authors used a semi-automated microplate-based spectrophotometric technique at 340 nm where AOPP concentrations were expressed in chloramine-T equivalents (μmol/L) serving as standard [2]. It has recently been demonstrated that the spectral characteristics of AOPP correspond to several chromophores, which include dityrosine, carbonyls and pentosidine and that AOPP result from myeloperoxidase-derived oxidative stress, but not exclusively [3]. In addition to uremia, plasma levels of AOPP were found elevated in patients with coronary artery disease [4], in preterm neonates [5,6], in diabetes mellitus [7,8], in systemic sclerosis [9], in ankylosing spondylitis [10] and in critically ill patients [11]. AOPP were also found a strong prognostic markers in patients with IgA nephropathy [12].

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The aim of the present *in vitro* studies was to find out that which plasma fraction(s) is responsible for AOPP reactivity.

Materials and methods

Chemicals

Chloramine-T (N-chloro-p-toluenesulfonamide sodium salt), potassium iodide (KI) and purified fibrinogen from human plasma were purchased from Sigma-Aldrich (Budapest, Hungary), acetic acid (96%, v/v) was from REANAL (Budapest, Hungary).

Blood samples

Peripheral venous blood samples were collected in K_3EDTA (lavender top) or sodium citrate (0.109 mol/L, 3.2%) anticoagulated (light blue top) or red top serum vacuette tubes (Greiner Bio-One, Kremsmünster, Austria) from patients admitted to our Department with various peripheral vascular or cardiovascular diseases. Blood samples were centrifuged at 4000 rpm (3000 g) for 10 min (swing-out rotor, Jouan CR3i laboratory centrifuge, Saint-Herblain, France) and the supernatant plasma or serum were promptly used either separately or pooled for studies.

Thermal treatment of plasma

Plasma samples (0.4 ml aliquots) were pipetted in Eppendorf tubes and the tubes were accommodated in an electrically heated water bath adjusted to a temperature of 50°C (±1°C). After termination of the heat treatment period, tubes were sequentially removed and centrifuged in an angle rotor for Eppendorf tubes at 4000 rpm (1200 g) for 10 min (same centrifuge).

Laboratory tests

Determination of AOPP. AOPP was determined on the principle of published technique [1], modified and adapted by us to Cobas Mira Plus (Roche, Basel, Switzerland) clinical chemistry analyzer and described in details elsewhere [11]. Each assay was performed in duplicates. AOPP concentrations are expressed in chloramine-T equivalents (μmol/L) as described [1]. Albumin was assayed by bromocresol green dye binding (Cobas Mira Plus).

Plasma fibrinogen concentrations were determined using the micro version of the Clauss method (Stago Compact, Asnières sur Seine, France) in thermal treatment study of pooled citrate-plasma, and normal version in assaying individual patient's plasma, or by immuno-nephelometric technique (Turbox Fibrinogen Orion Diagnostica, Espoo, Finland) in thermal treatment study of pooled EDTA-plasma and in the chromatographic studies to determine fibrinogen in the effluent fractions. D-dimer concentrations were determined in the post-chromatography samples using immuno-turbidimetric assay (STA Liatest, STAGO).

Chromatographic studies. Size exclusion chromatography of purified human fibrinogen (340 kDa) of Sigma-Aldrich origin, pooled human plasma and pooled human serum was performed essentially as described (3). Column used: Superose 12 HR 10/30 adapted to a FPLC system (Amersham Pharmacia). Column was equilibrated with phosphate buffer (50 mM, pH 7.4) and calibrated with calibrators (BIO-RAD) as follows, thyroglobulin (670 kDa), aldolasae (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B-12 (1.35 kDa). Purified human fibrinogen was dissolved in the buffer at a concentration of 5 mg/mL (AOPP: 49.9 μmol/L) and 0.2 ml of this solution was injected onto the column. Pooled human plasma (AOPP: 82.9 μmol/L) or pooled serum (AOPP: 16.8 μmol/L) collected from six identical patients was diluted 5-fold in the buffer and an aliquot of 0.3–0.3 ml were injected onto the column. Flow rate was 0.3 ml/min in each run. Detection was performed at 280 nm. Collected fractions (1.2 ml each) were analyzed for absorbance at 280 nm, AOPP reactivity at 340 nm (1) in a plate reader (Chamelon, Hidex Oy, Turku, Finland), for fibrinogen immunoreactivity and D-dimer concentrations as described above.

The Local Scientific Ethics Committee approved this study.

Statistical analyses

Data were statistically evaluated by using the SPSS (12.0.1 for Windows) package. Values exhibited normal distribution on the basis of Kolmogorov-Smirnoff's test. Linear correlation was calculated and Pearson's correlation coefficients are given in comparisons of plasma vs. serum values, and in comparison of plasma fibrinogen and AOPP concentrations. Intergroup comparisons were made using two-tailed t test. A p value less than 0.05 was considered statistically significant.

Results

Comparison of AOPP concentrations in EDTA-plasma, citrate-plasma and serum

We compared AOPP concentrations in EDTA-plasma, citrate-plasma and serum of the same group of patients (Table I). It appears that AOPP values measured in EDTA-plasma and citrate-plasma were comparable and there was a statistically significant correlation ($r = 0.897$, $p < 0.01$) between values measured in the two groups. Serum levels of AOPP were, however, much lower and did not

Table I. Comparison of AOPP concentrations in EDTA-plasma, citrate-plasma and serum of the same group of patients.

Type of sample	AOPP (μmol/L)	AOPP %
EDTA-plasma ($n = 20$)	44.1 ± 13.3	100
Citrate-plasma ($n = 20$)	37.6 ± 11.7	86.1 ± 11.1
Serum ($n = 20$)	14.6 ± 9.0‡	32.5 ± 19.3

AOPP concentrations were promptly determined in the respective plasma and serum samples of the same patients (11M/9F, age range: 30–79 y) in duplicates as described in Materials and methods section.
Values are mean ± SD. Linear correlations (Pearson's) were calculated for EDTA plasma vs. citrate-plasma ($r = 0.897$, $p < 0.01$), EDTA-plasma vs. serum ($r = 0.324$ ns) and citrate-plasma vs. serum ($r = 0.361$ ns) using the values of each individual patient (ns: not significant).‡ $p < 0.001$ (intergroup comparisons: serum vs. EDTA-plasma or serum vs. citrate-plasma (two-tailed t test)).

show relationship with values measured either in EDTA-plasma ($r = 0.324$ ns) or citrate-plasma ($r = 0.361$ ns).

Effects of thermal treatment on fibrinogen concentrations and AOPP reactivity of blood plasma

Thermal treatment of citrate- or EDTA-plasma at 50°C resulted in a rapid and parallel loss of fibrinogen concentrations and AOPP reactivity, whereas albumin concentrations remained unaltered over the whole observation period (Figure 1). On the basis of time-course or $t_{1/2}$ values, fibrinogen and AOPP were indistinguishable from each other in the citrate-plasma ($t_{1/2}$ for fibrinogen: 184 s, for AOPP: 180 s). In EDTA-plasma $t_{1/2}$ for fibrinogen was 129 s, for AOPP 100 s.

Correlation between fibrinogen and advanced oxidation protein products (AOPP) concentrations in the blood plasma of patients

We simultaneously determined fibrinogen and AOPP concentrations in the citrate-plasma of 61 patients at our Department with various cardiovascular or peripheral vascular diseases. Results showed a statistically strong correlation ($r = 0.9199$, $p < 0.0001$) between fibrinogen and AOPP concentrations (Figure 2). With increase of plasma concentrations of fibrinogen, AOPP/fibrinogen molar ratio (mol chloramine-T equivalents/mol fibrinogen) increased significantly ($r = 0.5705$, $p < 0.0001$) indicating that higher fibrinogen concentrations were associated with more oxidatively transformed groups on the molecule (Figure 3).

Gel exclusion chromatographic separation of samples

Chromatographic separation of purified human plasma fibrinogen resulted in one peak at 280 nm

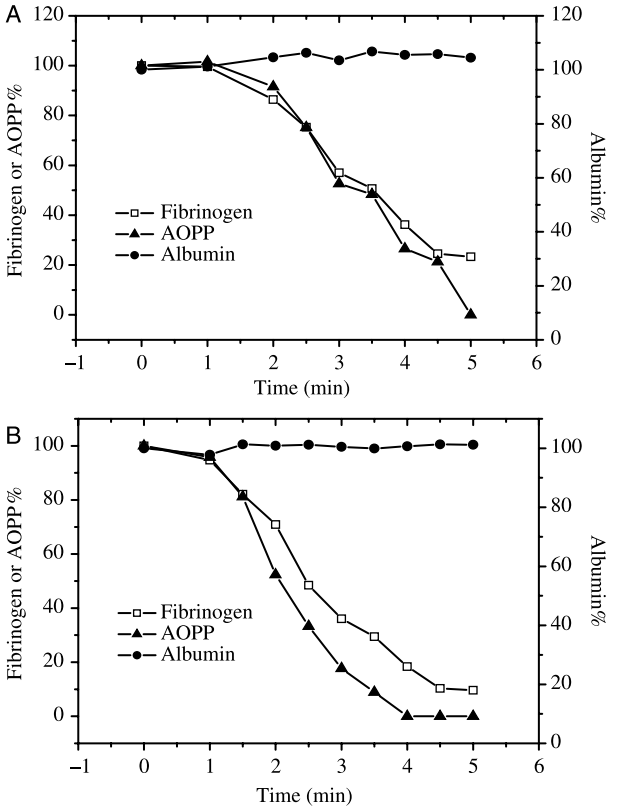

Figure 1. Loss of fibrinogen concentration and AOPP reactivity in blood plasma subjected to thermal treatment. Pooled citrate (panel A) or EDTA-plasma (panel B) samples from six to eight patients were subjected to thermal treatment at 50°C as described in Materials and Methods section. After centrifugation (1200 g, 10 min), fibrinogen, AOPP and albumin concentrations were promptly determined in the supernatant. Each point represents the mean of three independent series of investigations. Hundred percent corresponds to values measured in samples with no thermal treatment. Values for $t_{1/2}$ were calculated using a semi-log plot (incubation time vs. log % concentration).

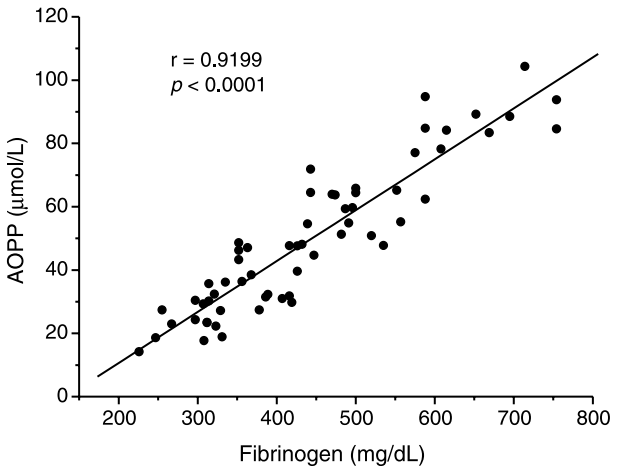

Figure 2. Correlation between blood plasma concentrations of fibrinogen and AOPP in patients. Fibrinogen and AOPP concentrations were simultaneously determined in the citrate-plasma of 61 inpatients (M/F, 32/29, mean age: 61.6 y, range 22–82) at our Department with various cardiovascular or peripheral vascular diseases. Pearson's correlation coefficient was calculated.

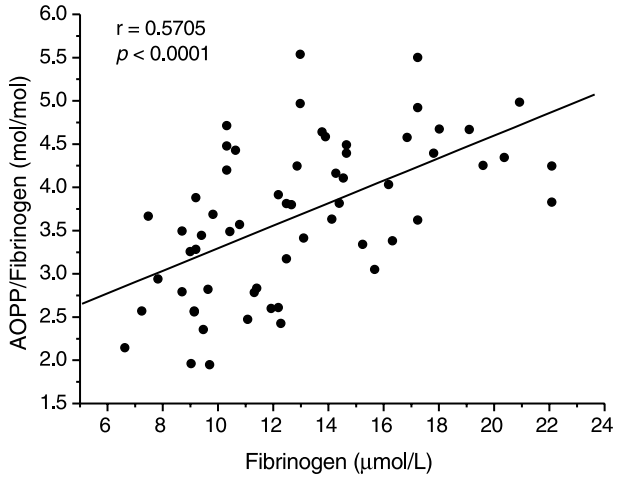

Figure 3. Relationship between plasma concentrations of fibrinogen and AOPP/fibrinogen molar ratio in patients. AOPP/fibrinogen molar ratio is given as mol chloramine-T equivalents/mol fibrinogen. Data belong to the same patients as in Figure 2. Pearson's correlation coefficient was calculated.

which completely overlapped with immunoreactivity of the molecule. Part of AOPP reactivity of fibrinogen molecule could be recovered in fractions under the peak (Figure 4A).

After chromatographic separation of pooled human plasma, AOPP reactivity was detected in two peaks. The first one co-eluted with fibrinogen immunoreactivity (fractions 7–11) whereas the second peak eluted in fractions from 11 to 15 (Figure 4B). D-dimer concentrations in fractions (6–17) tested were under detection threshold (0.22 μg/mL).

The elution pattern of pooled human serum revealed a smaller AOPP peak in the fibrinogen zone (fractions 7–11) followed by a second one, peaked in fraction 15 in the very low molecular mass range (Figure 4C). Fibrinogen immunoreactivity resulted in a flat curve in the fibrinogen zone (not shown). In fractions 14, 15 and 16 D-dimer concentrations were 0.31, 0.25 and 0.29 μg/mL, respectively. In earlier fractions D-dimer was under detection threshold.

Discussion

Fibrinogen is a dimeric glycoprotein with a molecular mass of 340 kDa, synthesized primarily in the liver. It represents after albumin and globulins the third abundant protein in the blood plasma with an average concentration of 1.5–4.0 g/L (4.4–11.7 μmol/L). Its half-life in humans is 3–5 days [13]. Fibrinogen plays an essential role in blood clotting, platelet aggregation and is also involved in inflammatory processes and atherogenesis. Oxidant stress leads to covalent oxidative modifications of several plasma proteins, chief among which is fibrinogen. *In vitro* exposure of fibrinogen to

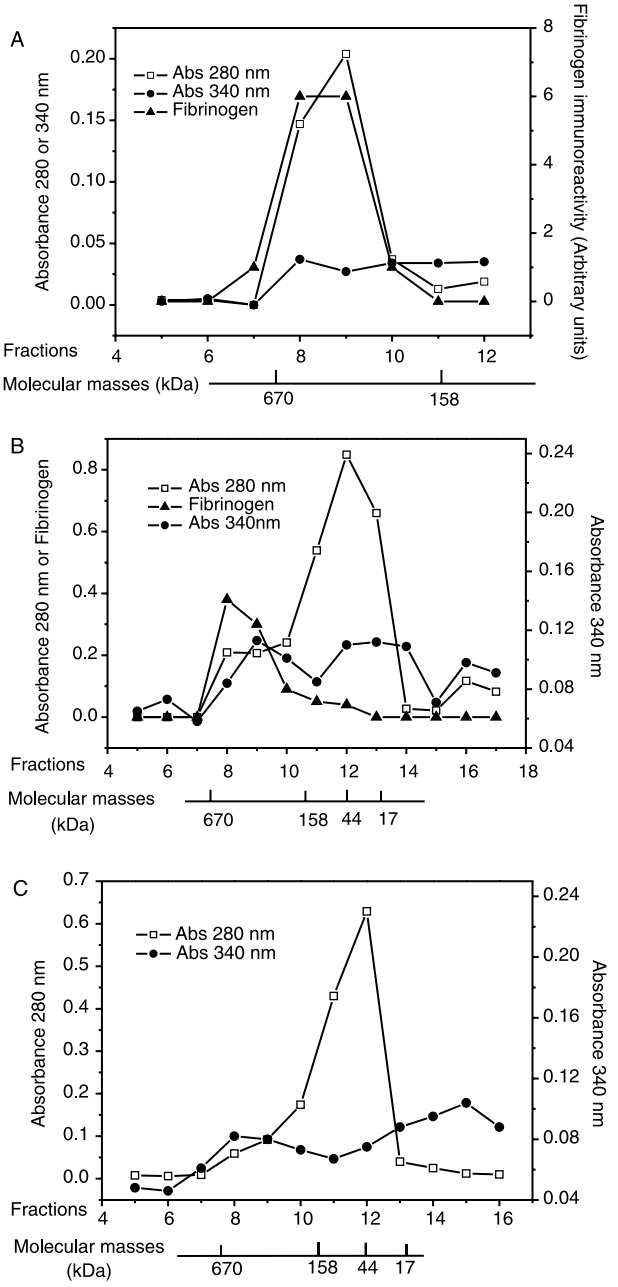

Figure 4. Elution profiles of various samples after gel exclusion chromatography on Superose 12 HR 10/30 column equilibrated with phosphate buffer (50 mM, pH 7.4). Flow rate was 0.3 mL/min, fraction volume 1.2 ml. Panel A: commercially purified human fibrinogen (Sigma-Aldrich) dissolved in phosphate buffer (5 mg/mL). AOPP concentration of the solution was 49.9 μmol/L. Injected volume: 0.2 ml. Panel B: Pooled human plasma collected from six patients in EDTA anticoagulated vacuette tubes. AOPP concentration was 82.9 μmol/L. Panel C. Pooled human serum collected from the same patients. AOPP concentration was 16.8 μmol/L. Both plasma and serum samples were diluted 5-fold with phosphate buffer and 0.3–0.3 ml aliquots were injected onto the column. Absorbance was continuously monitored at 280 nm. Eluted fractions were analyzed at 280 nm in a spectrophotometer, at 340 nm for AOPP absorbance in a microplate reader and for fibrinogen immunoreactivity (presented in arbitrary units) using immunonephelometric technique. In the eluted plasma and serum fractions D-dimer concentrations were also determined by immunoturbidimetric method.

Fe^{3+}-ascorbate for 1 h the mol carbonyl/mol protein ratio increased significantly with an accompanying reduction in the alpha-helical content of the protein. Oxidized fibrinogen was more readily able to form fibrin, and acetylation prevented the enhancement of clot formation [14]. Overnight treatment of fibrinogen by oxidants caused a 20-fold increase of carbonyl content of the glycoprotein. Formation of dityrosine as well as loss of tryptophan following fibrinogen oxidation were observed along with altered function of the molecule [15]. Among plasma proteins fibrinogen is the most susceptible to site-specific metal–ion catalyzed oxidation [16]. *In vitro* carbonylation of fibrinogen resulted in inhibition of clotting [17]. During hemodialysis session, carbonyls on fibrinogen further increased when high dose iron gluconate was administered [18]. The unique susceptibility of fibrinogen to carbonylation *in vivo* is supported by the finding that in the blood plasma of smokers and lung cancer patients fibrinogen was the only carbonylated plasma protein [19]. Other authors, however, have published that albumin is the major target of oxidant stress and there were no differences in oxidation of plasma transferrin, fibrinogen and immunoglobulin in patients with chronic renal failure [20]. It is highly unlikely that AOPP reactivity would be linked to albumin, since AOPP concentrations were much greater in the plasma (both in EDTA or citrate anticoagulated) than in the serum. Moreover, thermal treatment of plasma at 50°C resulted in a rapid and parallel loss of fibrinogen concentration and AOPP reactivity without any appreciable alterations in albumin concentration over the whole observation period. On the basis of these and other results of the present study it appears that fibrinogen is the key molecule responsible for blood plasma AOPP reactivity. AOPP reactivity of fibrinogen becomes more enhanced via post-translational modifications (oxidation, nitration, glycation, carbonyl, dityrosine, pentosidine and eventual crosslinks formation) of the molecule under various pathological conditions. This notion is supported by our observation that higher fibrinogen concentrations were associated with an enhanced molar ratio of AOPP/fibrinogen (Figure 3). Hypochlorous acid generated via myeloperoxidase (MPO) activity could represent one of the pathways for oxidized protein [21] and AOPP production [3]. Fibrinogen split products, however, may also contribute to AOPP concentrations. We suggest that fibrin(ogen) derivatives already present *in vivo* in the blood plasma and/or generated in the non-anticoagulated vacuette tubes during the preanalytical phase may serve as source of serum AOPP. This latter mechanism seems to operate, since concentrations of fibrin monomer were more than twofold higher in the serum than in the blood plasma of the same patients [22]. The outcome of such reactions is, however, rather erratic and may strongly depend on the length of the preanalytical phase, ambient temperature, patient's pathology and many other unknown factors beyond control. All these facts may offer explanation why serum AOPP concentrations are low and fail to show relationship with respective plasma values.

Striking finding was that the commercially purified human fibrinogen preparation dissolved in phosphate buffer exhibited AOPP reactivity at a ratio of 3.4 mol/mol fibrinogen. This figure is well within the range calculated for plasma AOPP/fibrinogen ratio (Figure 3), and thus compatible with the notion that AOPP reactivity resides on fibrinogen.

The results of our chromatographic studies also appear to support the fibrinogen-AOPP concept since the first AOPP peak after separation of the pooled plasma co-eluted with fibrinogen immunoreactivity. Since it has been demonstrated that fibrinogen may undergo plasmic digestion during plasma protein chromatography [23], we presume that the second AOPP peak can be associated with fibrinogen fragment(s) or degradation products, but this needs further elucidation. After chromatographic separation of the pooled human serum, the first AOPP peak was also detected in the fibrinogen zone but it was less apparent than in the respective eluent fractions of pooled human plasma. Fractions under the second AOPP peak contained fibrinogen degradation products as detected by the D-dimer assay.

The ancient fibrinogen molecule was composed of all identical chains [24] and the ancestral function of fibrinogen was in innate immunity [25]. Since fibrinogen also represents a target for oxidative stress, it is also feasible that the primitive fibrinogen molecule participated in the first-line defense of the host against reactive oxygen species (ROS). Recently published reviews on the topic unequivocally agree that atherosclerosis is an inflammatory disease and elevated fibrinogen is a cardiovascular risk biomarker, however, the link is unclear [26–29]. In patients with coronary artery disease a selective increase in nitrated fibrinogen was reported, moreover, exposure of fibrinogen to nitrating oxidants significantly accelerated clot formation and factor XIII cross-linking [30]. MPO-catalyzed reactions have been attributed to potentially proatherogenic biological activities throughout the evolution of cardiovascular disease, including during initiation, propagation and acute complication phases of the atherosclerotic process [31]. Nonetheless, in patients with chronic kidney disease at predialysis stage, CRP, fibrinogen and AOPP independently predicted atherosclerotic cardiovascular events in a recently published prospective study [32]. Although, at present, F_2-isoprostanes are considered the most reliable oxidant stress biomarkers [33], we still believe that the recognition that AOPP reactivity resides on fibrinogen,

provides a tool to easily obtain information in relation to post-translationally modified fibrinogen in the blood plasma of patients. It is well established that oxidation of proteins have a wide range of consequences [34], thus it remains to be elucidated what is the pathophysiological significance of the post-translationally modified fibrinogen in the inflammation-associated events of atherosclerosis, platelet aggregation, and as a cardiovascular risk biomarker.

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