*Free Radical Research*, October 2011; 45(10): 1115–1123

informa
healthcare

**REVIEW ARTICLE**

# Advanced oxidation protein products (AOPP): novel uremic toxins, or components of the non-enzymatic antioxidant system of the plasma proteome?

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*(Received: 05 May 2011; Accepted: 23 June 2011)*



**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. It was suggested that AOPP measure highly oxidized proteins, especially albumin. Recent data in turn appear to indicate that oxidized fibrinogen is the key molecule responsible for the AOPP reaction in the human plasma. Since fibrinogen is an acute-phase reactant, it is evident that during each episode of inflammatory response, the antioxidant capacity of the plasma is enhanced. In this context, fibrinogen can be regarded as a component of the antioxidant system of the plasma proteome. It was also demonstrated that oxidized fibrinogen is bound to apolipoprotein(a) of lipoprotein(a) via lysine binding sites. Thus, apo(a) could compete with plasminogen (and/or tissue plasminogen activator) for its binding sites of fibrin(ogen), causing inhibition of fibrinolysis, and thereby promote atherosclerosis and cardiovascular disease.

**Keywords:** *Antioxidant defence, apolipoprotein(a), oxidized albumin, oxidized fibrinogen, reactive oxygen species (ROS)*

**Abbreviations:** *AGE, advanced glycation end-products; AOPP, advanced oxidation protein products; apo(a), apolipoprotein(a); CE, cholesterol ester; CRF, chronic renal failure; CAPD, continuous ambulatory peritoneal dialysis; CV, cardiovascular; Cys, cysteine; DM, diabetes mellitus; DNA, deoxyribonucleic acid; EACA, ε-aminocaproic acid; EDTA, ethylenediamine-tetraacetic acid; FF, fibrinogen fragments; HD, hemodialysis; HDL, high-density lipoprotein; HK-2 cells, human proximal tubular cells; $H_2O_2$, hydrogen peroxide; HOCl, hypochlorous acid; HSA, human serum albumin; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); Lys, lysine; Met, methionine; MPO, myeloperoxidase; OFR, oxidized fibrinogen reactivity; PD, peritoneal dialysis; SR-BI, scavenger receptor class B type I; tPa, tissue plasminogen activator; TRA, tranexamic acid*

## The discovery of advanced oxidation protein products (AOPP)

In 1996, a novel oxidative stress biomarker, referred to as advanced oxidation protein products (AOPP), was detected in the plasma of chronic uremic patients. Plasma levels of AOPP were the highest in patients on hemodialysis (HD), followed by those on peritoneal dialysis (PD). Undialyzed patients with advanced chronic renal failure (CRF) had lower levels than those on dialysis, but markedly higher than healthy controls [1]. The authors used a semi-automated microplate-based spectrophotometric technique at 340 nm, where AOPP concentrations were expressed in chloramine-T equivalents (μmol/L) [2]. 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. By size-exclusion chromatography, AOPP were retrieved in two distinct

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10715762.2011.602074

peaks at 600 and below 80 kDa in uremic plasma, while no such peaks were found in control plasma. The high molecular weight AOPP peak was mostly due to albumin that appeared to form aggregates likely resulting from disulphide bridges and/or dityrosine cross-linking. Likewise, the low molecular weight AOPP also contained albumin in its monomeric form [1]. The AOPP assay did not react with non-oxidized native serum albumin, as evidenced by studies using purified human serum albumin (HSA), or control plasma, or by fractionation showing that the AOPP peak did not exactly match the albumin peak. Two interpretations are possible: either the low molecular weight AOPP may contain structurally and oxidatively modified albumin that gives positive reaction in the AOPP assay; or alternatively, low molecular weight AOPP are derived from other plasma proteins [1]. Later it was demonstrated that the spectral characteristics of AOPP correspond to several chromophores, which include dityrosine, carbonyls and pentosidine, and that AOPP result from myeloperoxidase (MPO)-derived oxidative stress, but not exclusively [3]. AOPP remained stable during sample storage both at −20°C and −80°C for 6 months [4].



### *AOPP as oxidative stress biomarkers*

In addition to chronic uremia, levels of AOPP were also found elevated in preterm hypoxic babies [5], in patients with coronary artery disease [6], in diabetes mellitus (DM) types 1 and 2 [7–9], in systemic sclerosis [10], in ankylosing spondylitis [11], in Behçet's disease [12], in critically ill patients [13], in chronic rheumatic valve disease [14], in the first and second trimesters of pregnancy [15], in colorectal cancer [16], in the relapse of acute myeloid leukemia [17] and also in several other diseases and pathological conditions. In harmony with the original description, all of the studies published so far appear to provide convincing evidence that AOPP are oxidative stress biomarkers. The statement that the measurement of oxidative stress lacks standardized methods [18] also holds true for AOPP. This is a major cause of the fact that AOPP control values published by various groups vary considerably (Table I). It was recently recognized that the high triglyceride concentration in uremic plasma due to interference leads to over-estimation of AOPP values [19], and a modified AOPP assay by precipitation of triglycerides before analysis can overcome this problem [20]. This was a substantial contribution to the standardization issue. Another obstacle in the proper comparison of the results of the various studies is that some authors use serum for AOPP assay instead of plasma, as it was originally published. These authors fail to give any justification or explanation as to why do they prefer serum over plasma. Actually, this is a crucial point in AOPP-related studies, as it will be demonstrated later.

Table I. Advanced oxidation protein products (AOPP) adult healthy control values.

| AOPP (μmol/L) | Sample | Reference |
|---|---|---|
| 29.4 ± 4.9 (n = 10) | Plasma | [1] |
| 32.0 ± 1.4 (n = 140) | Plasma | [6] |
| 79.8 ± 23.7 (n = 24) | Serum | [7] |
| 60−65 (n = 40) | Plasma | [8] |
| 47.0 ± 7.0 (n = 23) | Plasma | [10] |
| 36.0 ± 13.0 (n = 30) | Plasma | [11] |
| 126.97 ± 27.74 (n = 46) | Plasma | [14] |
| 106.4 ± 37.8 (n = 8) | Serum | [19] |
| 170.9 ± 101.9 (n = 8) | Plasma | [19] |
| 121 (14−414) n = 25 | Plasma | [44] |
| 89.2 (median) (n = 14) | Serum | [48] |

### *Oxidative damage to proteins*

Oxidative damage to proteins is caused by the action of free radicals and various other oxidizing compounds [21]. A common term of these compounds is reactive oxygen species (ROS). ROS are formed during normal or pathological processes in the organism. ROS-induced pathological changes in protein structure, their mechanisms and consequences are described in detail elsewhere [22–25]. A kinetic model predicted that plasma proteins consume the majority of hypochlorous acid (HOCl), a potent oxidant with limited damage to other materials [26].

### *Albumin*

HSA with a molecular mass of approximately 66 kDa is the most abundant protein in plasma, representing 50–60% of total proteins. It is synthesized in the liver, secreted into the circulation, where its half-life is about 20 days. Albumin is a multi-functional protein: it maintains appropriate oncotic pressure and transports bilirubin, cholesterol, fatty and amino acids, metal ions, hormones and various other ligands and drugs. It is a negative acute-phase protein. Albumin is also a very important extra-cellular antioxidant [27–29]. In general, albumin represents the major and predominant antioxidant in plasma, a body compartment known to be exposed to continuous oxidative stress [30]. More than 70% of the free radical trapping activity of serum was attributed to HSA in the free radical-induced hemolysis test [31]. Methionine (Met) and cysteine (Cys) accounted for 40–80% of total antioxidant activity of HSA, Cys chiefly worked as a free radical scavenger, whereas Met acted as a metal chelator [32].

Since AOPP were discovered in the plasma of uremic patients, the majority of AOPP-related scientific

papers published so far are concerned with the study of the potential inter-relationship between the accumulation of the new biomarker and chronic kidney disease. Even so, AOPP were suspected as being novel uremic toxins [33].

Patients with uremia are exposed to increased oxidative stress, and HSA is the major plasma protein target of oxidative stress in CRF and HD patients [34–43]. In these pathological conditions, circulating AOPP concentrations are also increased [44–52]. In vitro oxidized (chlorinated) HSA containing carbonyls and dityrosine was capable of triggering the oxidative burst of human monocytes in cultures. This finding first demonstrated that AOPP act as a mediator of oxidative stress and monocyte respiratory burst, which points to monocytes as both target and actor in the immune dysregulation associated with chronic uremia [18,53,54]. HOCl generated via MPO activity could represent one of the pathways for AOPP production in plasma proteins exposed to activated phagocytes [3]. Plasma AOPP correlated with plasma MPO protein concentration in HD patients, but not in control subjects or pre-dialysis patients, suggesting that in the latter AOPP did not predominantly result from MPO activity [55]. To determine the pharmacokinetic properties of AOPP, oxidized HSA was prepared in vitro using chloramine-T (a hypochlorite analogue). The AOPP and dityrosine contents of oxidized-HSA were similar to those of uremic patients. In pharmacokinetic analysis, oxidized HSA after injecting to mice, left the circulation rapidly. Data for organ distribution showed that the liver and spleen play important roles in the elimination of AOPP [42]. In vitro preparation of chloramine-modified HSA was significantly endocytosed in a dose-dependent manner at a higher level than HSA by human proximal tubular cells (HK-2 cells) in vitro. AOPP-HSA up-regulated the expression of CD36 pathway. These results suggest that AOPP-HSA may cause renal tubular injury via the CD36 pathway [56]. In vitro generated AOPP-albumin binds with high affinity to the high-density lipoprotein (HDL) receptor scavenger receptor class B type I (SR-BI). Already an equimolar concentration of AOPP-albumin to HDL blocked HDL association to SR-BI and effectively inhibited SR-BI-mediated cholesterol ester (CE) uptake. Moreover, albumin isolated from HD patients, but not albumin isolated from healthy controls, markedly inhibited SR-BI-mediated HDL-CE transfer in vitro dependent on the AOPP content of albumin. Thus, depressed plasma clearance of HDL-cholesterol may contribute to the abnormal composition of HDL and the high cardiovascular (CV) risk observed in patients with CRF [57]. In the studies concerned with the in vivo or in vitro actions of AOPP, the authors used in vitro oxidized (transformed) albumin, which may not be identical with AOPP spontaneously generated in vivo. This statement is supported by the finding that the inhibitory activity of in vitro generated AOPP-albumin was stronger compared to albumin isolated from HD patients, despite similar AOPP content of albumin [57]. Although, in the literature, AOPP reactivity is linked to albumin, this hypothesis is not supported by any convincing evidence.

### *Fibrinogen*

Fibrinogen is a soluble glycoprotein found in the plasma, with a molecular weight of 340 kDa, which is synthesized primarily in the liver. After albumin and globulins, it represents the third most abundant protein in plasma, with an average concentration of 1.5–4.0 g/L (4.4–11.7 μmol/L). Its biological half-life is 3–5 days [58]. It comprises three pairs of non-identical polypeptide chains (Aα, Bβ, $\gamma)_2$ linked to each other by disulphide bonds [59]. The molecule consists of the central nodule connected to the distal β- and γ-nodules by two coiled-coil connectors. Fibrinogen plays an essential role in blood clotting and platelet aggregation. It is also involved in inflammatory processes and atherogenesis. Fibrinogen is also known as an acute-phase reactant. In addition to its free form, fibrin(ogen) can exist in the human blood plasma in forms associated with proteins [60] and lipoproteins [61–63].

Oxidative stress leads to covalent modification of several plasma proteins. By using this approach, it was found that plasma fibrinogen was much more susceptible to oxidative modification compared to the other major plasma proteins, albumins, immunoglobulins and transferrin [64]. Thus, protein oxidation serves as a useful marker for assessing oxidative stress in vivo [65]. When human fibrinogen was modified with $H_2O_2$ inter- and intra-molecular cross-links of fibrinogen were formed, accompanied with oxidation of tryptophan, Met and tyrosine residues [66]. Other authors also published data on the antioxidant effects of fibrinogen [67,68]. In a cell-free system, fibrinogen was a much more powerful antioxidant for protecting lipoproteins than albumin, since on a molar basis albumin was present in about a 100 times greater concentration [69].

Concentrations of plasma fibrinogen are increased in chronic uremic and hemodialyzed patients [70–72]. Plasma AOPP concentrations were also enhanced in such patients [45–55]. Moreover, there was a statistically significant correlation between plasma AOPP and fibrinogen concentrations in patients with CRF [44] and in non-diabetic pre-dialysis patients [73].

In 1995, the existence of increased quantities of a macromolecular protein complex (MPC) was reported in the plasma of patients with type I DM [60], which reacted with a specific antibody to human fibrinogen [74]. The molecular properties of MPC were similar, if not identical to AOPP [75]. In

non-uremic patients with various peripheral vascular or CV diseases, plasma AOPP were studied with the aim to clarify the potential relationship between fibrinogen and AOPP. Thermal treatment of pooled 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 post-treatment, AOPP was indistinguishable from fibrinogen. There was also a statistically significant correlation between the plasma levels of fibrinogen and molar AOPP/fibrinogen ratio, indicating that higher fibrinogen concentrations were associated with more oxidatively transformed groups of the molecule. These results appear to indicate that post-translationally modified fibrinogen is a key molecule responsible for human plasma AOPP reactivity. A considerable relative difference was found among plasma and serum AOPP values collected from the same patients: EDTA plasma: 100%, citrate plasma: 86.1%, serum: 32.5% [76]. In another study, significant differences were also detected among plasma and serum AOPP values in control, HD and continuous ambulatory peritoneal dialysis (CAPD) patients. The authors suggest that among others, coagulation factors might be responsible for this difference [19]. Since fibrinogen is practically absent in serum, the question of what molecules are responsible for the still high serum AOPP concentration in uremic patients arises. There is accumulating evidence that fibrinogen fragments (FF) concentrations are increased in uremia [44,77–80]. The mean FF concentration in uremic plasma was eight times greater than in normal plasma. The uremic plasma showed several bands of high intensity and diffuse staining in a broad range of molecular mass, in contrast to normal plasma, which exhibited only faint bands with MW below 200 kDa. The mean FF levels decreased by 48.25% following HD [80]. Since FF are not clottable, they are present not only in plasma, but also in serum. Thus, FF, when enhanced, can considerably contribute to both plasma and serum AOPP concentrations. FF already present in vivo in the blood plasma and/or generated in the non-anticoagulated vacuette tubes during the pre-analytical phase may serve as source of serum AOPP. The latter mechanism seems to operate, since concentrations of fibrin monomer were more than twofold in the serum than in the plasma of the same patients [81]. The outcome of such reactions is, however, rather erratic and may strongly depend on the length of the pre-analytical phase, ambient temperature, patient's pathology and probably on many other unknown factors beyond control. All these facts may offer explanation as to why serum AOPP concentrations are low and fail to show the relationship with respective values of plasma containing fibrinogen.

The ancient fibrinogen molecule was composed of all identical chains [82]. Fibrinogen-like domains originated early in evolution, and it is likely that their specific and tightly controlled inter-molecular interactions were involved in immune functions [83] and inflammation [84]. Evidence has also been provided for the common ancestor for innate immunity and blood coagulation system [85]. Since fibrinogen also represents a target for oxidative stress, it seems feasible that the primitive fibrinogen molecule participated in the first-line defence of the host against the deleterious effects of ROS. In addition to its key role played in hemostasis and thrombosis, through conservation of the respective chemical groups during several hundred million years of evolution of the molecule, fibrinogen still retained this function until now. In healthy individuals, fibrinogen occurs in more than 1 million different forms because of the many possible combinations of biosynthetically or post-biosynthetically modified or genetically polymorphic sites [86].

Lipoprotein(a) [Lp(a)] is an LDL-like lipoprotein whose concentration in plasma is correlated with atherosclerosis. The characteristic protein component of Lp(a) is apolipoprotein(a) [apo(a)], which is disulphide-linked to apolipoprotein B-100. Sequencing of cloned human apo(a) complementary DNA shows that it is very similar to human plasminogen [87–88]. DNA sequence comparisons and phylogenetic analysis indicate that the human type of apo(a) evolved from a duplicated plasminogen gene during recent primate evolution [89]. Lp(a) inhibits the binding of plasminogen to plasmin-modified immobilized fibrinogen, indicating that both molecules compete for similar lysine (Lys)-binding sites [90]. On the basis of these findings, it was attempted to determine whether the inclusion of the Lys analogue plasmin inhibitors, tranexamic acid (TRA) or ε-aminocaproic acid (EACA) in the assay medium could influence plasma AOPP reactivity. To this aim, a recently published kinetic photometric assay was applied [91] and the results were expressed directly as oxidized fibrinogen reactivity (OFR) instead of AOPP concentration expressed in chloramines-T equivalents. The results of the study with the involvement of 65 non-uremic patients with various peripheral vascular diseases indicated that OFR resides in two compartments in the plasma; a free form, and bound to apo(a) component of Lp(a). Although, the latter is about 50% greater, an equilibrium appears to exist between the two compartments, since there was a highly significant correlation between the free and bound OFR. The fact that the actions of the Lys analogues TRA and EACA were dose-dependent unequivocally demonstrates that the interaction between OFR and apo(a) is accomplished through Lys-binding sites [92]. Since fibrinogen is an acute-phase reactant, it is evident that during each episode of inflammatory response, the antioxidant capacity of the plasma proteome is increased. In this context,

fibrinogen can be regarded as a part of the antioxidant system of the plasma proteome. On the other hand, after oxidation of fibrinogen, the transformed molecule is bound to apo(a) of Lp(a) and thereby gets sequestered from the plasma. The schematic representation of the key steps of the function of this newly recognized antioxidant system is presented in Figure 1 (Figure 1).

Lampreys were the first vertebrates in which fibrinogen appeared. It was about 450 million years ago. The duplication of plasminogen gene occurred in primates about 80 million years ago [89]; thus, several hundred million years should have been elapsed until the protein-protein interaction between fibrinogen and apo(a) molecules could occur. It means that this event took place relatively recently on the time scale of evolution.

The Lys-binding site in kringle IV type 10 of apo(a) is the major fibrin(ogen) binding site, but Lys-independent interactions have also been demonstrated [93]. A novel Lys-dependent high-affinity apo(a) binding site was demonstrated in the γ-chain of fibrinogen [94]. The apo(a) and plasminogen binding sites in intact fibrinogen are cryptic [95–97], but some cryptic sites are exposed upon conformational changes [98]. The apo(a) binding sites are located in the COOH-terminal half of the αC-domain, the same region that also binds plasminogen and tissue plasminogen activator (tPa) [99]. Thus, apo(a) could compete with plasminogen (and/or tPa) for its binding sites in the αC-domain of fibrin(ogen), causing inhibition of fibrinolysis, and it could also promote atherothrombosis. Elevated plasma levels of Lp(a) [100] and fibrinogen are independent risk factors for atherosclerotic CV diseases [101,102]. On the other hand, fibrinogen deficiency in apo(a) transgenic mice reduced the accumulation of apo(a) in the vessel walls and lesion development, suggesting that fibrin(ogen) may provide one of the major sites to which apo(a) binds to the vessel wall and participates in the generation of atherosclerosis [103,104]. Competition between apo(a) and plasminogen for Lys-dependent binding sites of fibrin associated with atherosclerotic lesions resulted in inhibition of fibrinolysis and is regarded as the major mechanism by which apo(a) and fibrin(ogen) promote atherosclerosis [105] . Protein damage occurs at susceptible hot spots in oxidative stress and when occurring at functional sites of physiological fragility, minor extents of protein damage can likely have profound pathophysiological effects [106]. It remains to be elucidated whether fibrinogen oxidation during oxidative stress, such as in uremia, occurs at such hot spots of the fibrinogen molecule, which may expose cryptic Lys-binding sites and thereby enhance affinity of the molecule to apo(a). The notion that the increased oxidative stress contributes to the excessive increase of CV risk in uremic patients [107,108] can readily be reconciled with the novel interpretation of the AOPP system outlined in the present review.

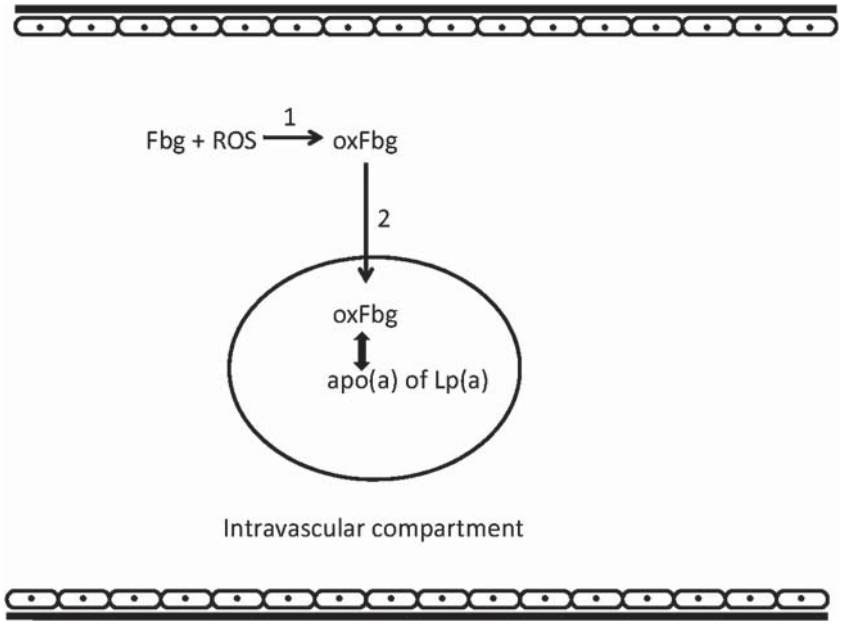


Figure 1. Schematic representation of the fibrinogen-linked antioxidant system of the human plasma proteome. Interaction between ROS and fibrinogen results in the formation of oxidized (transformed) fibrinogen (step 1), which can be detected in vitro as free advanced oxidation protein products (AOPP) or free oxidized fibrinogen reactivity (OFR), depending upon which method is used. The in vivo transformed molecule is bound to apolipoprotein(a) of lipoprotein(a) involving protein-protein interaction and thereby gets sequestered from the plasma (step 2). Under in vitro conditions, by the addition of lysine analogues to the plasma, oxidized fibrinogen can be released from apolipoprotein(a). After this manoeuvre, total (free + bound) AOPP or total OFR can be measured. Bound AOPP or bound OFR can be calculated as total and free.

## Conclusion

Oxidative stress is known to play an essential role in many human diseases and pathological conditions. There is a long list of laboratory methods available in the literature that are of help in establishing the presence of oxidative stress, but none of them proved to be unequivocally superior to the others hitherto. Sometimes they are used in combination with each other, but financial considerations strongly limit this practice in routine diagnostics. AOPP are just one of those techniques. The aim of this review is to give an account on the most important events that occurred since the discovery of AOPP until today and to outline a novel interpretation of the AOPP system. It appears that AOPP are integral parts of the non-enzymatic antioxidant system of plasma proteome. Oxidized fibrinogen is the key molecule responsible for the positivity of AOPP chemical reaction. Although, the involvement of other protein fractions in the AOPP reactivity cannot be excluded theoretically, but their contribution, if any, should not be essential. Oxidized fibrinogen is bound to apo(a) of Lp(a) due to the strong structural relationship between apo(a) and plasminogen. Thus, apo(a) could compete with plasminogen (and/or tissue plasminogen activator) for its binding sites on fibrin(ogen), causing inhibition of fibrinolysis and thereby promote atherosclerosis and CV disease. Therefore, it is suggested that the AOPP system functions as a double-edged weapon of the plasma proteome, but it by no means diminishes its diagnostic value as an oxidative stress biomarker.

## Declaration of interest

The author reports no conflict of interest. The author alone is responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 15 July 2011.