

# Serum Elastin-Derived Peptides in Age-Related Macular Degeneration

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**PURPOSE.** Dysregulation of the extracellular matrix (ECM) plays an important role in the pathogenesis of age-related macular degeneration (AMD). Elastin is a fibrous protein constituent of the ECM, degradation of which may be detected by the presence of serum elastin-derived peptides (S-EDPs) in circulation. This study was undertaken to estimate levels of S-EDPs in patients with AMD compared with age-matched control subjects.

**METHODS.** Fifty-six patients with AMD were classified into two groups: early age-related maculopathy (ARM) and neovascular AMD. The control group consisted of 15 age-matched subjects with no AMD. S-EDP levels in the serum of these subjects was estimated by competitive ELISA, using solubilized  $\alpha$ -elastin from human aorta and polyclonal antibodies to this antigen.

**RESULTS.** S-EDPs were significantly higher in patients with AMD than in control subjects. In addition, subjects with neovascular AMD had higher levels of S-EDPs than did those with early disease.

**CONCLUSIONS.** The cause of this association between S-EDPs and AMD is unknown, but it suggests that systemic elastin degradation may increase the risk of conversion from early ARM to neovascular AMD. Further studies are needed to confirm whether the serum level of S-EDPs is a useful predictor of conversion from early ARM to neovascular AMD. (*Invest Ophthalmol Vis Sci.* 2005;46:3046-3051) DOI:10.1167/iops.04-1277

Age-related macular degeneration (AMD) is a leading cause of blindness in the elderly in the Western world.<sup>1-3</sup> AMD is classified according to the International Classification and Grading System.<sup>4</sup> Early age-related maculopathy (ARM) is defined as the presence of intermediate-sized, soft, distinct drusen ( $>63 \mu\text{m}$ ) with retinal pigment epithelial depigmentation or hyperpigmentation; soft, indistinct drusen ( $>125 \mu\text{m}$ ); or reticular drusen. "Wet," or neovascular, AMD is characterized by the presence of choroidal neovascularization (CNV).

The pathogenesis of AMD is undoubtedly complex and multifactorial.<sup>5</sup> Several investigators have shown that changes in matrix biology play an important role in the pathogenesis of AMD.<sup>6,7</sup> Elastin is an extremely insoluble fibrous protein constituent of the extracellular matrix (ECM).<sup>8</sup> It is the main

protein of the elastic fiber and contributes to the elastic property of several tissues such as the vascular wall, lungs, and skin. In the eyes, elastin is present in Bruch's membrane and the choroidal vessels.<sup>9</sup> Partial proteolysis of elastin by activated proteinases results in the release of soluble elastin-derived peptides (S-EDPs) into the circulation. Therefore, S-EDP measurement is indicative of systemic elastin turnover.<sup>10</sup>

Large amounts of S-EDPs are produced during several pathologic processes.<sup>11,12</sup> The measurement of S-EDPs has been proposed as a method of monitoring disease processes such as emphysema and as a predictor of expansion of small abdominal aortic aneurysms.<sup>13,14</sup> Accelerated elastolysis has also been reported in patients with various manifestations of atherosclerosis.<sup>15</sup> Several clinical and epidemiologic studies have concurrently illuminated established cardiovascular risk factors and markers of atherosclerosis with AMD.<sup>16</sup> Therefore, we postulated that both the vascular wall matrix and Bruch's membrane may share several common changes, including degradation of elastin and release of S-EDPs into the circulation.

In this study, we analyzed S-EDP levels in patients with AMD compared with S-EDPs in age-matched control subjects.

## MATERIALS AND METHODS

### Subjects

Fifty-six subjects with a clinical diagnosis of AMD were included in the study. The control group consisted of 15 healthy subjects without AMD (defined as the absence of drusen, pigmentary abnormalities, and neovascular AMD). All enrolled subjects underwent a complete ophthalmic examination by the recruiting retinal specialist: visual acuity testing, slit lamp examination, and retinal examination after pupil dilation were performed. Each subject also had 30° color stereo fundus photographs of both eyes taken. Fluorescein angiography was also performed if there was a clinical suspicion of CNV. Subjects with coexistent fundus disease and subjects with ungradable photographs were excluded from the study.

A detailed medical history of each patient was taken. Particular care was taken to note history of hypertension (defined as being prescribed antihypertensive drugs), angina pectoris, myocardial infarction, intermittent claudication, chronic obstructive airway disease (COAD), abdominal aortic aneurysm (AAA), and hypercholesterolemia (defined as being prescribed statins). History of smoking was classified as never smoked, ex-smoker, or current smoker. All current smokers were excluded.

This research adhered to the tenets of the Declaration of Helsinki. Institutional ethics committee approval was obtained, and all patients gave their full informed consent. The patients in this study were recruited as part of a Medical Research Council-funded project on AMD.

### Grading of AMD

Color fundus photographs of the subjects were graded by two graders using the nomenclature and classification recommended by The International ARM Epidemiologic Study Group.<sup>4</sup> Fundus fluorescein angiography was available, to exclude CNV in patients classified as having early ARM by fundus appearance. The graders were masked to the age and clinical history of the participants. Double grading for intra- and

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interobserver variability was performed. Discrepancies were resolved by discussion. The subjects were classified as either early ARM or neovascular AMD. Early ARM was defined as the presence of intermediate, soft, distinct drusen ( $>63 \mu\text{m}$ ) with retinal pigment epithelial depigmentation or hyperpigmentation; soft, indistinct drusen ( $>125 \mu\text{m}$ ); or reticular drusen. Neovascular AMD consisted of subjects with CNV. If the grades in the two eyes were different, the subject was categorized according to the severity of changes in the worse eye.

### Blood Samples

Venous blood was collected from all subjects in a serum separator tube (SST-BD Vacutainer; BD Diagnostics, Oxford, UK), allowed to clot for 30 minutes, and centrifuged for 10 minutes at 1500g at room temperature. Serum was aliquoted and stored at  $-70^\circ\text{C}$  within an hour of collection and then thawed when required. The samples were randomized so that the scientist who analyzed the samples was blinded to the clinical history of the subjects.

### Estimation of S-EDPs

**Confirmation of Antibody Specificity by Dot-Blot.** This procedure was performed to check the suitability of capture antibodies to use in enzyme-linked immunosorbent assay (ELISA). On a membrane (Hybond C; GE Healthcare, Buckinghamshire, UK), 10- $\mu\text{L}$  aliquots of 1.25  $\mu\text{g}/\text{mL}$   $\alpha$ -elastin from human aorta (Sigma-Aldrich, Poole, UK) in 0.1 M sodium carbonate (pH 9) were placed in dots in grid formation and allowed to dry. The membrane was blocked with blocking solution consisting of 5% (wt/vol) low-fat milk powder (Marvel; Premier Foods, St. Albans, UK) in tris-buffered saline (TBS) for 1 hour on an orbital shaker. The capture antibodies tested included a mouse monoclonal antibody to  $\alpha$ -elastin (Abcam Ltd., Cambridge, UK) and a rabbit polyclonal antibody to  $\alpha$ -elastin (Elastin Products Co., Pacific, MO). Each capture antibody was added in a range of dilutions to the blocking solution described earlier, and a strip of membrane was dotted with an  $\alpha$ -elastin for 1 hour at room temperature. The membrane was then washed with TBS containing 0.05% (vol/vol) Tween 20 (TBST) five times, for 5 minutes each. The strips were then incubated with appropriate detection antibody (both horseradish peroxidase [HRP]-conjugated antibodies purchased from Santa Cruz Technology/Autogen Bioclear, Wiltshire, UK). Donkey anti-mouse detection antibody was used for the monoclonal capture antibody (1:1000 in 5% wt/vol milk in TBS) and goat anti-rabbit detection antibody for the polyclonal capture antibody (1:1000 in 5% wt/vol low-fat milk powder in TBS), both incubated for 1 hour at room temperature. Membranes were then washed again in TBST for 5 minutes five times. Chemiluminescence detection was then performed with a kit (Enhanced Chemiluminescence; GE Healthcare) and the membrane strips were placed in a cassette with a high-performance chemiluminescence hyperfilm (GE Healthcare) before the film was developed for analysis.

**Competitive ELISA.** The concentration of S-EDPs was determined with a competitive ELISA adapted from a protocol described by Peterson et al.<sup>15</sup> Soluble  $\alpha$ -elastin was obtained from human aorta (Sigma-Aldrich, Poole, UK). The rabbit anti-human elastin IgG (Elastin Products Co.) was raised against  $\alpha$ -elastin prepared from human aorta.

The wells of a microtiter plate (Nunc; Fischer Scientific, Loughborough, UK) were coated with 150  $\mu\text{L}$   $\alpha$ -elastin (1.25  $\mu\text{g}/\text{mL}$ ) in 0.1 M sodium carbonate (pH 9) by incubating at  $4^\circ\text{C}$  for 12 hours. The plate was then washed four times with 150  $\mu\text{L}$  phosphate-buffered saline (PBS) containing 0.05% (vol/vol) Tween 20 (PBST). A standard curve was generated using dilutions of human aortic  $\alpha$ -elastin (0–100 ng/mL). Anti- $\alpha$ -elastin antibodies (1:1000) in PBST and 7% (wt/vol) bovine serum albumin (PBST-7% BSA) were preincubated with variable dilutions of  $\alpha$ -elastin in PBST-7% BSA overnight at  $4^\circ\text{C}$ . These solutions were then transferred to the coated plate and incubated at room temperature for 1 hour. The preincubation period before addition to the  $\alpha$ -elastin-coated microtiter plate creates the competitive aspect of the ELISA. Before addition of the detection antibody, the microtiter plate wells were washed with PBST four times. Then, 150  $\mu\text{L}$  HRP-

conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology/Autogen Bioclear) was added to each well (1:2000 dilution in PBST-7% BSA). The plate was incubated for 1 hour at room temperature and again washed with PBST four times. Thereafter, 100  $\mu\text{L}$  of ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Roche Diagnostics, Lewes, UK) buffer was added, and the plate was incubated for a further hour at room temperature. Absorbance was read at 495 nm with an automated plate reader (Dynex Technologies, West Sussex, UK). A standard curve was generated from the absorbance readings obtained from the  $\alpha$ -elastin dilutions.

During the assay optimization process, pooled human sera from healthy volunteers were used as a diluent for the standard curve instead of the assay buffer (PBST-7% BSA), to examine the potential effect of serum components on the ELISA. The standard curves generated with standards in pooled human sera and assay buffer were correlated and the level of significance tested by sign test. Because the standard curves were well correlated and there was no significant difference between them, subsequent standard curves were based on  $\alpha$ -elastin in assay buffer.

Standards and serum samples were analyzed in duplicate. The S-EDP concentrations were calculated from the standard curve and expressed as nanograms per milliliter.

The following controls of the reaction were used: (1) substrate control: only assay buffer, wash solutions and ABTS substrate added to the polystyrene wells coated with  $\alpha$ -elastin; (2) no-capture-antibody control: detection antibody added directly to the wells coated with  $\alpha$ -elastin and the wells incubated with the ABTS substrate solution; (3) negative control to assess the specificity of the reaction: tested samples replaced with human albumin solution and serum samples from healthy volunteers and assayed by using the standard protocol; and (4) positive control: the tested sample replaced with human aortic  $\alpha$ -elastin at 1 g/mL in assay buffer.

Intra- and interplate reproducibility tests were also performed. For the former, a pair of positive and negative controls was each tested 30 times in the same ELISA plate (intraplate). In the latter test, the positive and the negative serum controls were tested 30 times in different plates from the same lot on different dates (interplate). The mean, standard deviation (SD), and coefficient of variation (CV) were calculated for both the inter- and intraplate optical densities (ODs). The ELISA was considered reproducible if the variations of each of the 30 positive and 30 negative control ODs was within  $\pm 2$  SD of the mean of the individual runs. Sample results were discounted if the duplicates disagreed by more than 5%.

### Statistical Methods

Analysis was performed on computer (Statistical Package of the Social Sciences; ver. 11.0; SPSS, Chicago, IL). Results were reported as the mean  $\pm$  SEM. The normal distribution of S-EDPs was assessed with the Shapiro-Wilk test. The standard curves generated with standards in pooled human sera and assay buffer were correlated and the level of significance tested by sign test. Multiple regression analysis was performed with S-EDPs as the dependent variable and the disease status of the subjects as the independent variable. The relationship of aging to S-EDP levels was analyzed independently and in the three groups, by using the correlation coefficient. Student's *t*-test and one-way ANOVA were used for analysis of results.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Characteristics of Subjects

Serum was analyzed from 71 subjects, including 30 with ARM, 26 with neovascular AMD, and 15 normal control. Table 1 presents the demographic details and baseline clinical data of the study group. Age and gender distribution were similar in the AMD, ARM, and control groups. No statistically significant

TABLE 1. Characteristics of the Subjects

	ARM ( <i>n</i> = 30)	Neovascular AMD ( <i>n</i> = 26)	Controls ( <i>n</i> = 15)	<i>P</i>
Mean age (y)	77	79	75.2	0.7
(range)	(65-88)	(62-92)	(61-91)	
Gender (M:F)	12:18	10:16	6:9	0.5
Exsmoker (%)	3.3	3.8	0	0.9
Hypertension (%)	16.6	15.4	13.3	0.8
Myocardial infarction (%)	3.3	3.8	0	0.9
Angina pectoris (%)	0	0	6.6	0.2
Intermittent claudication (%)	0	3.8	0	0.4
Stroke (%)	3.3	0	0	0.5
COAD (%)	3.3	0	0	0.5
AAA (%)	0	0	0	1.0
Hypercholesterolemia (%)	3.3	3.8	0	0.9

differences in clinical data were observed between the subjects in the three groups.

### Antibody Specificity

The results of dot-blot analysis demonstrated that only the polyclonal antibody tested would consistently detect human  $\alpha$ -elastin. The mouse monoclonal antibody tested failed to indicate binding to the antigen using either a dot-blot or ELISA protocol (results not shown).

### Assay Reproducibility

The assay was found to be sensitive from 0.5 to 100 ng/mL of  $\alpha$ -elastin. For the intra- and interplate reproducibility studies, ODs of the individual positive and negative control were all within  $\pm 2$  SD of the mean ODs, as shown in Table 2. Figure 1 shows a typical standard curve. There was an excellent correlation between the standard curve created with  $\alpha$ -elastin in PBS containing BSA and a standard curve created with  $\alpha$ -elastin in pooled serum from healthy volunteers. The detected levels of  $\alpha$ -elastin were slightly higher in pooled sera than in PBS containing BSA, but the difference was not statistically significant ( $P = 0.13$ , sign test).

### Estimation of S-EDPs in AMD and Controls

The mean  $\pm$  SEM of S-EDPs in the AMD and ARM groups combined ( $n = 56$ ) was  $29.6 \pm 3.95$  ng/mL compared with  $15.4 \pm 2.71$  ng/mL in the control group ( $n = 15$ ;  $P < 0.05$ , Student's *t*-test). The mean  $\pm$  SEM of S-EDPs in ARM ( $n = 30$ ) and neovascular AMD ( $n = 26$ ) was  $23.9 \pm 1.75$  ng/mL and  $36.2 \pm 4.52$  ng/mL, respectively.

Figure 2 depicts the progressive increase in S-EDPs with the increase in severity of AMD ( $P < 0.05$ , ANOVA). The multiple regression analysis showed that the various clinical conditions did not significantly influence the levels of S-EDPs in this cohort (Table 3).

### Correlation of S-EDPs with Aging

The levels of S-EDPs of control and pathologic sera did not correlate with that age of the subjects. There was a strong

dispersion of results ( $r = -0.01$ ). The separate evaluation of the retinopathy groups did not show a significant age-dependent trend for the levels of S-EDPs ( $r = 0.02$ ).

### DISCUSSION

Elastin is the main protein of elastic tissue, contributing to the elastic properties of many tissues such as vascular wall, skin, and lung.<sup>8</sup> In the eye, elastin is found in many structures, including the trabecular meshwork, uvea, and optic nerve. The major sites of elastin deposition in the uvea include the choroidal blood vessels and Bruch's membrane. The elastic lamina of Bruch's membrane is thinner and less abundant in the macula than in the periphery and it is also thinner and less abundant in the macular region of AMD eyes.<sup>17</sup> Age-related changes in Bruch's membrane include lipid accumulation, calcification, and fragmentation.<sup>18</sup> In a study by van der Schaft et al.,<sup>19</sup> age-related calcification of Bruch's membrane was noted to commence as early as the third decade of life. Calcification and fragmentation of Bruch's membrane facilitates ingrowth of choroidal neovascular membranes with consecutive development of neovascular AMD.<sup>20</sup> Moreover, elastin complexed with lipids and calcium favors its degradation compared with native elastin.<sup>21</sup> In a case-control study, Blumenkranz et al.<sup>22</sup> noted elastotic degeneration in non-sun-exposed skin in patients with AMD, suggesting that photic insults are not primarily responsible for this change but that there are inherent, systemic differences in elastin synthesis and degradation. This change in elastic fibers is an important risk factor for CNV.

Elastolytic enzymes mediate the degradation of elastic fibers. These enzymes include: elastase from neutrophils and platelets, cathepsin G and metalloproteinases (MMPs) such as gelatinase A (MMP-2), gelatinase B (MMP-9), matrilysin (MMP-7), and the macrophage metalloelastase (MMP-12).<sup>23</sup> Degradation of the elastic fibers by these enzymes results in the release of S-EDPs into the circulation. Therefore, the quantification of increased S-EDPs may reflect the increased production or activity of these enzymes.

Several immunologic methods have been used to estimate S-EDPs.<sup>24</sup> Because elastin is insoluble, it cannot be used directly

TABLE 2. Reproducibility of S-EDP ELISA

S-EDP	Intraplate Precision			Interplate Precision		
	<i>n</i>	OD (Mean $\pm$ SD)	CV (%)	<i>n</i>	OD (Mean $\pm$ SD)	CV (%)
0 ng/mL	30	1.125 $\pm$ 1.67	31.0	30	1.089 $\pm$ 0.53	12.5
75 ng/mL	30	0.645 $\pm$ 0.42	11.3	30	0.625 $\pm$ 0.18	6.7

### Standard curve of S-EDP

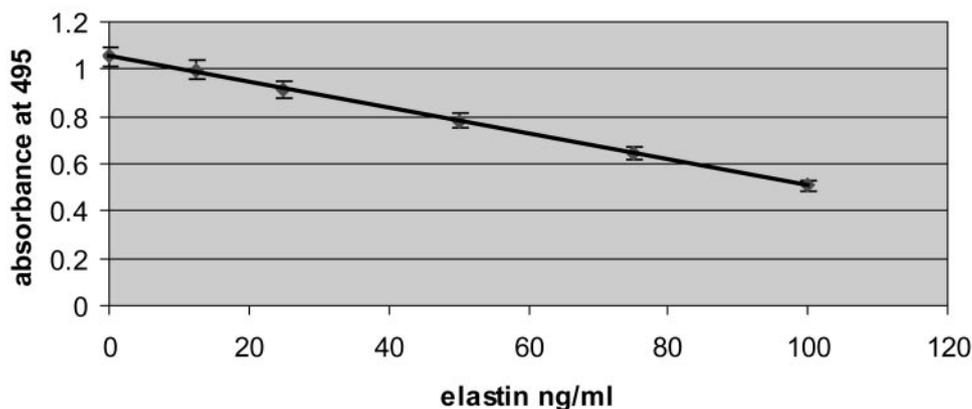


FIGURE 1. Typical standard curve of competitive ELISA for S-EDPs. Error bars,  $\pm 2$  SD.

in immunoassays. Therefore, elastin has to be solubilized before use. Insoluble elastin can be solubilized by treatment with 0.25 M oxalic acid, to obtain soluble  $\alpha$ -elastin, treatment with potassium hydroxide to obtain  $\kappa$ -elastin, or by enzymatic digestion of elastin with pancreatic or leukocyte elastase, or other enzymes such as pepsin, papain, and ficin.<sup>25</sup> However, a major problem in elastin histochemistry is that the purification and preparation of elastin and the reactivity of the antibodies influence the reported normal value of mean S-EDPs.<sup>26</sup> This phenomenon may explain, in part, the difference between the reported mean of control samples in our study versus that obtained by Petersen et al.<sup>15</sup> (15 ng/mL compared with 26 ng/mL, respectively), in that different capture antibodies were used. An improvement on our methodology would have been to include antibodies to peptides other than  $\alpha$ -elastin, but the present study was limited to use of those antibodies that were readily commercially available. Although the absolute S-EDP concentrations have been found to be variable, other authors suggest that it is the variation of S-EDP levels between individuals that is more significant than the absolute values.<sup>24,27</sup>

It is also important to consider factors that may influence the levels of S-EDPs that can be detected by an immunoassay. Circulating elastin autoantibodies may play a role in the interpretation of our results. Natural autoantibodies constitute a proportion of normal circulating immunoglobulins,<sup>28</sup> and S-EDPs have been shown to induce production of anti-elastin antibodies.<sup>29</sup> The levels of these antibodies in the serum or

Bruch's membrane may therefore indirectly influence the levels of S-EDPs. Moreover, our results could also be influenced by the presence of elastin-binding proteins and elastin-receptor interactions.

Despite the potential limitations of the immunoassay as described, the results of this study suggest that S-EDPs are significantly higher in subjects with AMD than in the control subjects, indicating increased elastin turnover in subjects with AMD. S-EDPs were also found to increase progressively from early disease to neovascular AMD.

The precise source of circulating S-EDPs is unclear, but there seems to be several possibilities. Although tissues such as lung parenchyma and skin could contribute to elastin degradation, most of the elastic fibers are present in the vascular wall, with the thoracic aorta containing 30% to 40% elastin and abdominal aorta approximately 20%,<sup>12</sup> indicating the most likely source of increased circulating S-EDPs. This may help explain the association of AMD with atherosclerosis<sup>30</sup> and emphysema.<sup>31</sup> The contribution of the Bruch's membrane and choroidal vessels to the circulating S-EDPs is presumably negligible, and so it is not thought likely that increased S-EDPs occurs as a result of AMD progression in the first instance. However, higher levels of MMP2 and -9 have been found in Bruch's membranes of patients with AMD than in age-matched control subjects,<sup>32,35</sup> and these enzymes may have a role in the degradation of elastic fibers of Bruch's membrane. This suggests that higher levels of EDPs at a local level in patients with

### S-EDP in the three groups

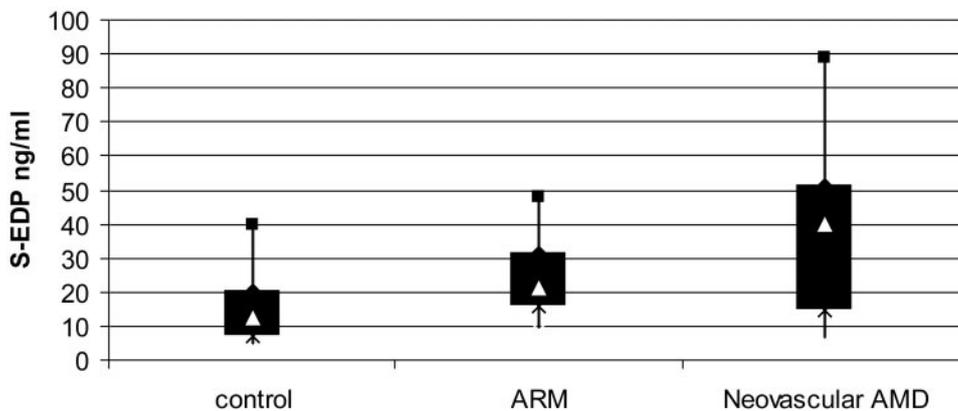


FIGURE 2. Box chart showing progressive increase of S-EDPs with increasing severity of ARM and AMD.

TABLE 3. Robustness Check of S-EDP

Independent Variable	Multiple Regression Analysis with S-EDP as the Dependent Variable
Mean age	0.53
Gender (females)	0.61
Exsmoker	0.68
Hypertension	0.31
Myocardial infarction	0.56
Angina pectoris	0.35
Intermittent claudication	0.62
Stroke	0.63
COAD	0.54
AAA	1.00
Hypercholesterolemia	0.54

neovascular AMD may be relevant. Fragmentation of Bruch's membrane is a prerequisite to the invasion of CNV into the subretinal space. Higher levels of S-EDPs in neovascular AMD suggest that S-EDPs may be directly involved in the pathogenesis of CNV.

S-EDPs interact with cell membrane receptors such as the elastin-laminin receptor and the integrins.<sup>34,35</sup> These interactions activate intracellular signaling pathways that lead to diverse cellular events.<sup>36</sup> Thus, S-EDPs are defined as matrikines as they originate from fragmentation of a matrix protein and have distinct cellular effects, such as increased elastase production, free radical release, induction of LDL oxidation, stimulation of endogenous cholesterol production, and chemotactic activity.<sup>37-39</sup> These peptides also act with various growth factors, cytokines, and vasoactive molecules released as a response to injury and stimulate endothelial cells to proliferate.<sup>40,41</sup> Thus, the disruption of elastin is not just an end product of elastin turnover. It may be an important contributor to the pathogenesis of neovascular AMD.

Several reports have indicated the role of inflammation in AMD. Vine and Powell<sup>42</sup> showed a correlation between elastolytic activity and inflammatory cell infiltrates in degenerative vascular disease. Likewise, Bruch's membrane is populated by macrophages and lymphocytes in AMD.<sup>43,44</sup> Macrophages elaborate an array of proteinases including metalloelastase (MMP-12). In addition, macrophages and lymphocytes are potential sources of cytokines that can activate other cells to produce degradative enzymes. Therefore, the release of leukocyte elastase can also lead to a collapse of the elastin network and fragmentation of Bruch's membrane.

Another mechanism that should be considered in light of our results is the possibility of altered elastin gene transcription in AMD. Indeed, many cytokines already studied in AMD have the ability to modulate elastin expression.<sup>45,46</sup> Further insight into transcriptional mechanisms accompanying tissue responses to the elastolytic events can lead to the design and testing of intervention strategies pertinent to AMD.

Although further studies are needed to clarify the influence of S-EDPs on AMD, this work demonstrates a potential role for elastin peptides in the pathogenesis of AMD. Larger studies are also needed to determine whether the serum level of S-EDPs can be used as a predictor for the conversion of early ARM to neovascular AMD.

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