Associations of Plasma-Soluble Fas Ligand with Aging and Age-Related Macular Degeneration

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PURPOSE. To evaluate the associations between plasma-soluble Fas ligand (sFasL) and age-related macular degeneration (AMD).

METHODS. Plasma samples were obtained from 230 individuals (age range, 45–85), with or without AMD. The concentrations of sFasL were determined by an enzyme-linked immunosorbent assay (ELISA). The measured sFasL levels were transformed into cubic roots and were fitted into linear regression models against AMD status, with adjustment for age and sex.

RESULTS. Plasma sFasL increased with age and AMD. There was a linear correlation between age and the cubic roots of sFasL. The plasma sFasL concentrations in non-AMD subjects ranged from 0 to 1.65 ng/mL (median, 0.69 ng/mL), whereas in patients with AMD, sFasL ranged from 0 to 2.43 ng/mL (median, 0.18 ng/mL). Between the ages of 61 and 84, the subjects with AMD had significantly higher sFasL than did the non-AMD subjects. There was a sexual dimorphism of the plasma sFasL levels. In non-AMD subjects, sFasL was lower in the females. In patients with AMD, sFasL was higher in the females.

CONCLUSIONS. An elevation of plasma sFasL with aging may play a role in the development of AMD and is a potential peripheral marker for monitoring disease progression. (Invest Ophthalmol Vis Sci. 2008;49:1345–1349) DOI:10.1167/iovs.07-0308

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gle-related macular degeneration (AMD) is the most common cause of legal blindness among people older than 60 years.1 Aging, smoking, and oxidative stress are some of the well-characterized risk factors for AMD.2,3 Inflammation and, in some cases, infection have been suggested to play key roles in the pathogenesis of AMD.4–10 For instance, infection with either Chlamydia pneumoniae or cytomegalovirus has been associated with both dry and wet AMD.11 In addition, recent genetic studies have identified that polymorphisms of the complement factor genes, including complement factor H (CFH), factor B (BF), and component 2 (C2), are associated with wet AMD.12–14

The recognition of infection and inflammation as risk factors is particularly important in understanding the etiology of AMD, because both provide mechanistic links with the other risk factors—that is, aging, smoking, and oxidative stress. Plasma levels of CFH are altered with both aging and smoking.10 In addition, results from an AREDS ancillary study demonstrated that the levels of C-reactive protein (CRP), a systemic inflammatory biomarker, are significantly higher in patients with advanced AMD.11 Oxidative stress is a central mechanism of host response to infection and reactive oxygen species are generated in cell signaling mechanisms of inflammation.12,13 Consequently, a more detailed understanding of how the components of the immune system change with aging, as well as which components are associated with AMD, will provide molecular targets for intervention to prevent disease development and progression.

Soluble Fas ligand (sFasL) is an important cytokine involved in inflammation,14,15 functioning through binding to the death receptor Fas and activating apoptosis. It is a soluble form of Fas ligand (FasL), a 40-kDa type II transmembrane protein that is a member of the tumor necrosis factor family of cytokines, generated in cell signaling mechanisms of inflammation.12,13 As one of the most potent inducers of receptor-mediated apoptosis, FasL is predominantly expressed in activated T cells and natural killer cells, but is also expressed in immune-privileged sites such as the testis and placenta, as well as the anterior and posterior chambers of the eye.16,17 The FasL-induced apoptosis is implicated in the regulation of immune responses, killing of tumor cells, and maintenance of immune-privileged sites.17–19

Malfunctions of the FasL/Fas system can result in either survival of cells that should be eliminated (e.g., cancer) or inappropriate death of functioning cells (e.g., AIDS). In a chronic disease such as AMD, metalloproteinase activation or a decline in clearance mechanisms of generated sFasL could result in elevated circulating sFasL concentrations. As a result of elevated sFasL, critical cells, such as those of the retinal pigment epithelium, would be vulnerable to elimination by apoptosis. On the other hand, changes in plasma sFasL can be one of the consequences of gene–environment interactions that are associated with various chronic diseases. Blood sFasL levels are elevated in many pathologic conditions, including cancer, AIDS, acute graft-versus-host disease, and autoimmune diseases,20–24 and previous studies have also identified associations between sFasL and rheumatoid arthritis, atherosclerosis, and infection.25–27

The potential involvement of the FasL/Fas system in AMD and other age-related eye diseases has not been well characterized. In the present study, we measured the plasma sFasL levels in a cohort of subjects with AMD and compared the values to the measurements from non-AMD subjects after adjusting for age and sex. Our results showed that there is an increase of plasma sFasL in association with age and with AMD.

METHODS

Study Populations

From 2000 to 2002, we enrolled 161 subjects without and 69 patients with AMD from both the Emory Eye Center and the Veterans Affairs
Hospital (Atlanta, GA), with ages ranging from 45 to 85 years. The study was reviewed and approved by the Institutional Review Board of Emory University and was performed in accordance with the ethical standards outlined in the 1975 Declaration of Helsinki, as revised in 1983 and 1996. Each participant gave informed consent before inclusion in the study. Subjects were given a short questionnaire to obtain information on sex, race, smoking, alcohol consumption, exercise, medications, and nutritional supplementations. The patients had AMD diagnosed based on the definitions outlined in the AREDS (categories 2, 3, and 4). The number of patients in disease stages 2, 3, and 4 was 4, 9, and 56, respectively. The diagnosis was made on clinical retinal examination and confirmed with fundus photographs and/or fluorescein angiography.

Sample Collection
Blood was collected from an antecubital vein with a heparinized 23-gauge butterfly needle and syringe. Plasma was immediately prepared and stored at −80°C in aliquots until further analysis.

Measurements
The plasma soluble FasL levels were measured by ELISA (Oncogene Research Products, Boston, MA) according to the standard protocol provided by the manufacturer. This ELISA system measures sFasL by a sandwich method using anti-FasL monoclonal antibodies against two different epitopes. Briefly, 50 μL biotinylated detector antibodies were added to wells of 96-well plates precoated with another anti-FasL antibody. Plasma samples were diluted 1:3 with the sample diluents and 100 μL was added to the wells and incubated simultaneously with detector antibodies for 3 hours at room temperature. Subsequently, the wells were washed three times with ELISA wash buffer (PBS containing surfactant and 2% chloroacetamide) and incubated with 100 μL horse-radish peroxide–conjugated streptavidin for 30 minutes in room temperature. After three washes with ELISA wash buffer and one wash with distilled water, the substrate tetramethylbenzidine (100 μL) was added, and the plates were incubated in the dark for 30 minutes. The reaction was stopped by adding 1.25 M sulfuric acid. Peroxidase activity was determined using absorbance at 450 nm. Purified human recombinant FasL was used as a standard. Standards were assayed as duplicate and performed on the same plates and at the same time as the samples. The concentration of sFasL was calibrated from the standard curve and had a detection limit of 0.02 ng/mL. Levels below the detection limit were coded 0 and were included in statistical analysis.

Statistical Analysis
Linear regression models were used to analyze the effects of age, sex, and AMD status on plasma sFasL. Because the levels of the measured plasma sFasL were heavily skewed (Fig 1), we used cubic root transformation of sFasL to provide an acceptable distribution, which was fit versus age and sex in linear regression models.

Because of limitations in patient recruitment, the age distributions were different for the AMD patients than for those without. To maximize inclusion of both groups, therefore, we focused on subjects older than 61 but younger than 84 (inclusive).

RESULTS
sFasL in Human Plasma Samples
We measured the plasma sFasL concentrations in 230 subjects, including 69 patients with AMD. The sex distribution was comparable between the two groups; however, the median age of non-AMD subjects was younger than that of those with AMD (P < 0.0001, Table 1). In non-AMD subjects, the levels of plasma sFasL ranged from 0 to 1.65 ng/mL. In patients with AMD, sFasL ranged from 0 to 2.43 ng/mL. The median values and the interquartile range (IQR) are presented in Table 1. The IQR was taken from data located in the interval between the first quartile (25% position) and the third quartile (75% position).

There was a significant difference in the sFasL level between subjects with and without AMD (P < 0.0001, Table 1).

sFasL with Aging
To address possible roles of sFasL in aging and age-related diseases, plasma sFasL levels were analyzed as a function of age. The measured plasma sFasL levels were heavily skewed (Fig 1). We systematically examined statistical transformations (e.g., ln, square root) that would achieve approximately normal distribution and found that the cubic root transformation provided an acceptable distribution (Fig 1).

When the plasma sFasL levels were examined as a function of age, a significant correlation was identified between the sFasL1/3 and age in the non-AMD subjects (Fig 2; Spearman’s rank correlation coefficient: 0.36; P < 0.0001). In addition, sex appeared as an important factor. The sFasL levels in non-AMD females were lower than those in non-AMD males. However, the linear regression lines had very similar slopes (P = 0.94), indicating that the effect of age on sFasL1/3 was similar for both sexes. We fitted a linear regression of sFasL1/3 against both age and sex. The fitted model was sFasL1/3 = −0.23 + 0.01 × age + 0.15 × I[sex], where I[sex] is an indicator function for sex (female = 0, male = 1). The results suggest that for every year older, sFasL1/3 increases by 0.01. Both the age and sex effects were significant, with P = 6.2 × 10⁻⁶ and 1.6 × 10⁻³, respectively. Moreover, the variation in sFasL1/3 increased slightly as age increased.

sFasL in AMD
To investigate whether increased sFasL levels with aging was also associated with AMD, we compared the sFasL levels between non-AMD subjects and those with AMD. However, in the data set, the age distributions were different between the subjects without and those with AMD. Because age influenced sFasL level significantly and the age distributions differed, in-
The age-matched control subjects (Table 3). In patients with AMD was significantly higher than in those without. When analyzed by linear regression model, subjects with AMD tend to have higher sFasL levels than do ages. As shown in Table, 2, between the ages of 61 and 84, the concentration decreased in association with age in the AMD group. Consequently, the difference between the AMD and non-AMD group, the sFasL concentration decreased in association with age and are higher in patients with AMD after adjustment for age. To our knowledge, this is the first report of an association between plasma sFasL and AMD and suggests sFasL to be a possible biomarker for AMD. The FasL/Fas system plays essential roles in inflammation and immune responses. Altered cytokine production, along with lymphopenia and abnormal immune response, is frequently observed in aging and age-related diseases. Oxidative stress, infection, and inflammation can upregulate FasL expression and stimulate sFasL release from T cells, monocytes, macrophages, endothelial cells, and other cell types. An increased expression of Fas and FasL in T-cell subsets is associated with greater susceptibility of T cells to apoptosis in aging humans. The increased sFasL with aging, as demonstrated in this study, may be a consequence of increased oxidative stress, infection, or inflammation, and it may contribute to other age-related disease events.

Although the plasma sFasL level could be indicative of systemic inflammation and immune response, AMD is a focal disease in which the expression profile in the lesion tissue is highly relevant. Using cultured human RPE cells, we showed that ligation of Fas on the RPE cells by recombinant soluble FasL or agonistic anti-Fas antibody induced apoptosis. A study by Lambooij et al. showed that there is high FasL expression in choroidal neovascular membranes. Although the

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933446/ on 11/25/2018)

**Figure 2.** Association between plasma sFasL and age in control subjects. The amount of measured plasma sFasL was plotted as a function of age and sex in non-AMD subjects. The data were transformed to obtain normal distribution before statistical analyses.

### Table 1. Distribution of Sex, Age, and sFasL in Non-AMD Subjects and AMD Patients

<table>
<thead>
<tr>
<th></th>
<th>Non-AMD (n = 161)</th>
<th>AMD Cases (n = 69)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of females (%)</td>
<td>86 (53.4%)</td>
<td>41 (59.4%)</td>
<td>0.40</td>
</tr>
<tr>
<td>Age (mean ± SD)</td>
<td>66 (57.74)</td>
<td>78 (73.82)</td>
<td>3.6 × 10⁻³⁵</td>
</tr>
<tr>
<td>sFasL (ng/mL, median (IQR))</td>
<td>0.18 (0.04, 0.50)</td>
<td>0.69 (0.41, 0.86)</td>
<td>2.3 × 10⁻¹¹</td>
</tr>
<tr>
<td>sFasL₁/³, median (IQR)</td>
<td>0.56 (0.34, 0.79)</td>
<td>0.88 (0.74, 0.95)</td>
<td>2.3 × 10⁻¹¹</td>
</tr>
</tbody>
</table>

Results are given as median and interquartile range (IQR). * Wilcoxon rank test.

### Table 2. Association between Plasma sFasL and AMD, after Adjustment for Age and Sex

<table>
<thead>
<tr>
<th>Age Range</th>
<th>Non-AMD Male (n = 48)</th>
<th>Non-AMD Female (n = 54)</th>
<th>AMD Male (n = 25)</th>
<th>AMD Female (n = 33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>61–65</td>
<td>0.29</td>
<td>0.21</td>
<td>0.44</td>
<td>NA</td>
</tr>
<tr>
<td>66–70</td>
<td>0.42</td>
<td>0.31</td>
<td>0.65</td>
<td>1.18</td>
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<tr>
<td>71–75</td>
<td>0.67</td>
<td>0.27</td>
<td>0.92</td>
<td>0.64</td>
</tr>
<tr>
<td>76–80</td>
<td>0.91</td>
<td>0.55</td>
<td>0.54</td>
<td>0.90</td>
</tr>
<tr>
<td>81–84</td>
<td>0.46</td>
<td>0.42</td>
<td>0.44</td>
<td>0.48</td>
</tr>
</tbody>
</table>

The transformed levels of plasma sFasL were averaged and presented as functions of age, sex, and AMD status. The data were determined by measurements performed in 58 AMD and 102 control subjects aged between 61 and 84 years.
immunohistochemical staining did not identify significant age-or AMD-dependent changes in membrane-bound FasL, in cases of wet AMD, the integrity of the blood-retina barrier is compromised. Consequently, sFasL may reach the subretinal space through circulation, bind to the Fas receptor on RPE cells and photoreceptors, and cause apoptosis. The combination of these findings (i.e., that sFasL is increased in plasma with age, that increased plasma concentration of sFasL is associated with AMD, that RPE cells are preferentially lost during AMD, and that RPE cells are sensitive to sFasL), suggests that this could be a major mechanism in the development and progression of AMD.

Unexpected findings from the present study involve the differences in sFasL levels for non-AMD males and females. It is well established that sexual dimorphism exists within the neuroendocrine and immune systems. Females have higher levels of immunoglobulin, greater antibody responses, higher incidence of autoimmune diseases, higher corticosterone levels, and higher corticosteroidogenesis. Consequently, the observed difference of sFasL between the females and males may be a result of the sexual dimorphism of the neuroendocrine and immune systems. In patients with AMD, however, the females showed higher plasma sFasL than did the males. This effect could be a statistical aberration due to the number of subjects studied, or could reflect underlying sex differences in the sensitivity of the Fas/L/Fas system.

Because of the age differences in AMD and non-AMD subjects in the overall population, we analyzed only the association between sFasL and AMD in subjects between 61 and 84 years of age. To test the hypothesis of the present study further, studies are needed in which more age-matched non-AMD subjects are enrolled. Because of the limited number of subjects, the present study did not address smoking status, which is known to be an important risk factor for AMD, as well as one that may affect inflammatory status. Nonetheless, smoking causes oxidative stress, oxidative stress causes increased sFasL, sFasL triggers apoptosis in the RPE, and RPE death occurs early in the development of AMD. Thus, the overall conclusion appears valid, even if smoking is an upstream factor contributing to elevated sFasL levels.

AMD is likely to be a multifactorial disease. Genetic and environment factors can affect complement factors, sFasL, and other proinflammatory cytokines that may contribute to conditions of chronic inflammations. Nonetheless, before the molecular mechanisms can be elucidated, it should be noted that results from such cross-sectional studies of peripheral biomarker measurements do not discern initiating and later events and sometimes are influenced by many confounding factors, especially other preexisting disease conditions. For example, CRP was initially reported as a potential marker of AMD, but several of the subsequent studies did not confirm association between CRP and the AMD progression. Therefore, large-scale prospective studies involving carefully phenotyped cohorts of subjects are always necessary, to confirm any disease-related peripheral biomarker. In addition to the clinical studies, animal models can often reveal important mechanistic information. Previous studies have shown that the Fas/FasL system correlates negatively with laser-induced neovascularization but is positively related to tissue damages induced by acute inflammation. It remains to be determined how the Fas/FasL system functions in some of the newly developed AMD models with progressive and chronic retinal degeneration.

In summary, in the present study, plasma sFasL increased with aging in humans and there was a significantly higher level of plasma sFasL in AMD. Sex played an important role in determining the sFasL level, with low sFasL in non-AMD females but high sFasL in females with AMD. The results of this pilot study suggest that sFasL may be a promising biomarker for assessment of risk for AMD and that Fas/L/Fas may play an central role in the pathogenesis of AMD. Thus, development of therapeutic measures for systemic and localized modulation of FasL/Fas may provide a new strategy in the prevention and treatment of AMD.

### References