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Non-Enzymatic Post-Translational Modifications in the Development of Age-Related Macular Degeneration

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1. Introduction

Age-related macular degeneration (AMD) is a leading cause of blindness among Caucasians in various countries.1-3 In addition, cases of AMD are increasing among non-Caucasians, and AMD has become one of the major causes of blindness worldwide. To address this problem, several etiological, pathological, and basic science studies are being conducted. Etiological studies have revealed that the risk factors of AMD include smoking,4 increasing age, and the presence of cardiovascular disorders.5 However, the molecular mechanisms linking these risk factors to AMD are still unclear.

Pathoclinical studies have revealed macular drusen, which are small lumps of abnormally accumulated proteins beneath the retinal pigment of epithelial cells, to be a sign of AMD (Figure 1).6 Proteomics analysis of drusen has revealed that it is composed of various proteins, including clusterin, albumin, TIMP3, vitronectin, complement components, and crystallin. However, the pathological role of drusen in the development of AMD is still unclear.

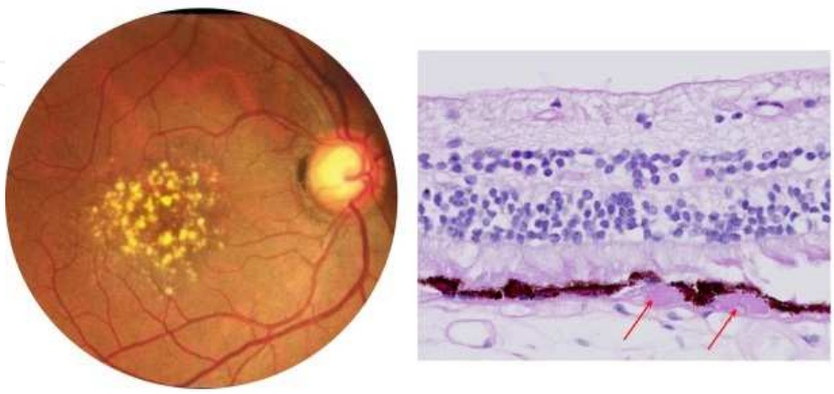

Fig. 1. **Drusen as an early sign of age-related macular degeneration**
Drusen are seen as yellow to white materials especially in the macular area (Left). Histologically, drusen are recognized as extracellular deposits that form between the retinal pigmented epithelium (RPE) and Bruch's membrane (arrows in the right Fig.).

AMD and other aging-related changes in the body have certain common characteristics, including the aggregation of abnormal proteins, seen in the lens and brain of patients with cataract and Alzheimer's disease, respectively. Post-translational modifications of proteins are also aging-related changes commonly seen in the target organ. For example, advanced glycation of proteins and racemization of amino acids with resultant D-amino acid formation in proteins are well-documented changes related to aging. Post-translational modification of proteins occurs because of aging, and it contributes to the aging changes of organs.

In order to elucidate the molecular mechanism of AMD, we evaluated the role of post-translational modifications of proteins in the development of AMD, particularly the formation of advanced glycation end products (AGEs) and D-amino acids in the development of drusen.

2. Post-translational modifications in age-related disorders

Post-translational modification of proteins is a molecular characteristic of the aging process. This is of 2 types as follows: enzymatic post-translational modification, which includes phosphorylation and glycosylation and is essential for protein function; and non-enzymatic post-translational modification that includes advanced glycation, racemization (and the resultant D-amino acid formation), and truncation, which impairs protein function, contributing to the aging process at the molecular level in various organs (Figure 2).

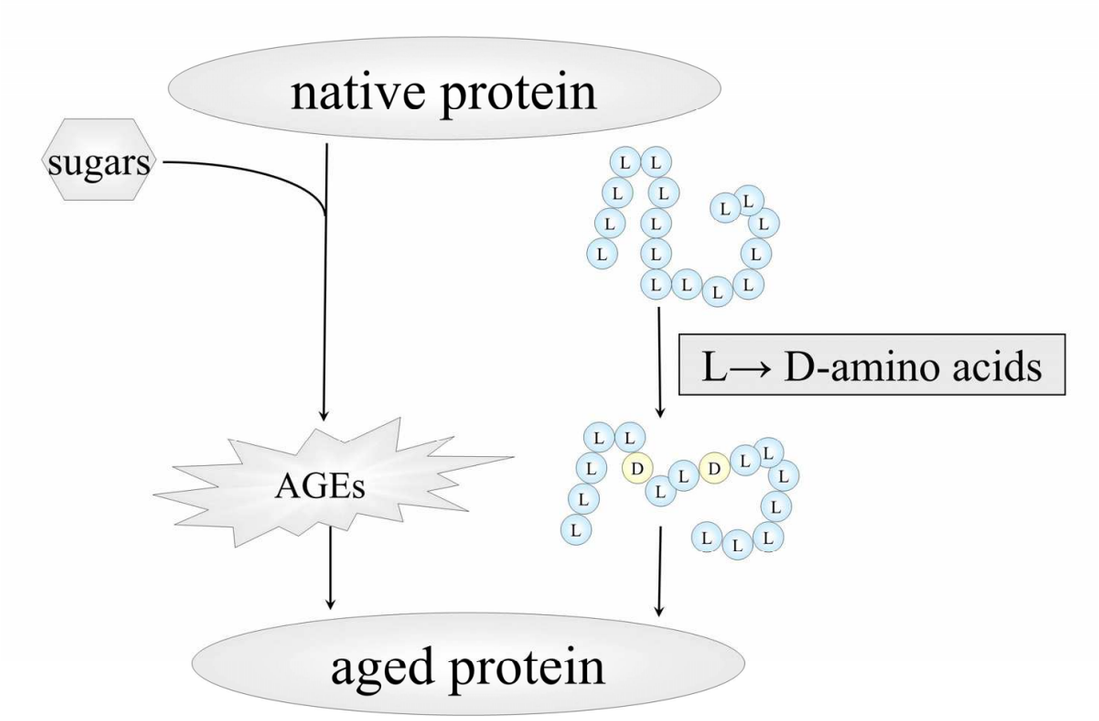

Fig. 2. **Aging of proteins at the molecular level**
Non-enzymatic post-translational modifications of proteins, including formation of AGEs and D-amino acids, are recognized as an aging process of proteins at the molecular level.

2.1 Advanced glycation end products

Advanced glycation end products (AGEs) are the final reaction products of proteins and reducing sugars. The reaction of reducing sugars (glucose and fructose) with Lys and Arg residues in proteins leads to the formation of Schiff base and Amadori products, which slowly undergo oxidation, dehydration, and condensation to form AGEs (Figure 3). The final products vary depending on the proteins, sugars, and the reactions involved. However, common structures called AGE motifs are seen in the products, irrespective of the proteins and sugars involved. Recently, a number of AGE motifs, including N^{ε}-(carboxy) methyl-L-lysine, imidazoline, pyrraline, and pentosidine, have been revealed.

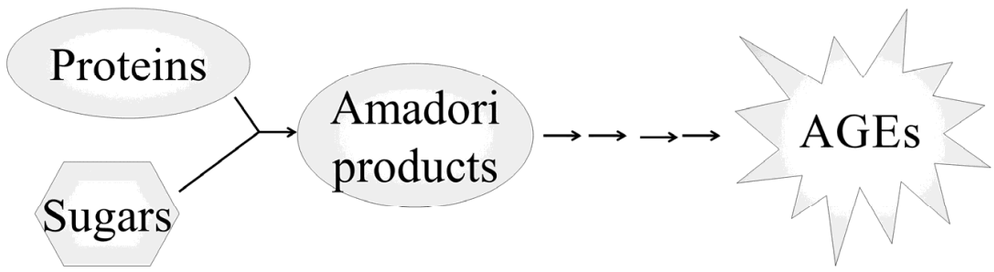

Fig. 3. **Multistep reactions of AGE formation**
Proteins and reducing sugars react with each other to form Amadori products. After multistep reactions, including oxidation, dehydration, and condensation, AGEs are generated.

Our body is like an incubator containing sugars and proteins, in which AGEs are naturally formed, particularly in the tissues with a low turnover rate such as bone, teeth, dura mater, and lens. AGEs tend to accumulate with age, and they are known to do so in the target organ of age-related disorders such as Alzheimer's disease and atherosclerosis. Thus, accumulation of AGEs in tissues is thought to be a biomarker of the aging process.

Formation of AGEs increases in diabetes partly because the concentration of reducing sugars increases in the blood. An accumulation of AGEs is seen in the thickened basement membrane and sclerotic lesions of the glomerulus in diabetic nephropathy. Furthermore, AGEs in the vitreous body and cornea are involved in the development of diabetic retinopathy and keratopathy, respectively.11-14

AGEs, along with being the accessory products of the aging process and diabetes, also possibly contribute to the aging process and diabetic complications. AGEs are known to alter the structure of proteins by intra- and inter-molecular crosslinking, consequently disrupting their function. For example, naïve laminin has a figure of the cross, but AGE-modified laminin is deformed, and thus, loses its adhesion property to epithelial cells.

2.2 D-amino acids

Proteins of all living organism on earth are composed of 20 types of amino acids. Among them, 19 amino acids other than glycine have chiral carbon atoms in the molecule.

Depending on the configuration of the side chains around the chiral carbon atoms, amino acids are either l- or d-amino acids. If amino acids were synthesized chemically, the quantity of l- and d-amino acids would be equal. However, proteins in all living organisms are composed exclusively of l-amino acids.

L-amino acids are converted to D-amino acids with a half-life of several thousand years, thus creating an equal amount of L- and D-amino acids over a particular period. This amount of D-amino acids is used for the age determination of fossils. Thus, D-amino acids are regarded as the products of post-mortal change and are irrelevant to living organisms. However, biologically uncommon D-amino acids that are enantiomers of L-amino acids have been found in the lenses16-19, teeth20, bones, brains21, skin22, aortas23, erythrocytes24, lungs25, and ligaments of elderly donors.26 The presence of D-amino acids in aged tissues of the living body is considered to be a result of racemization of L-amino acids in proteins in metabolically inert tissues during one's lifetime (Figure 4).

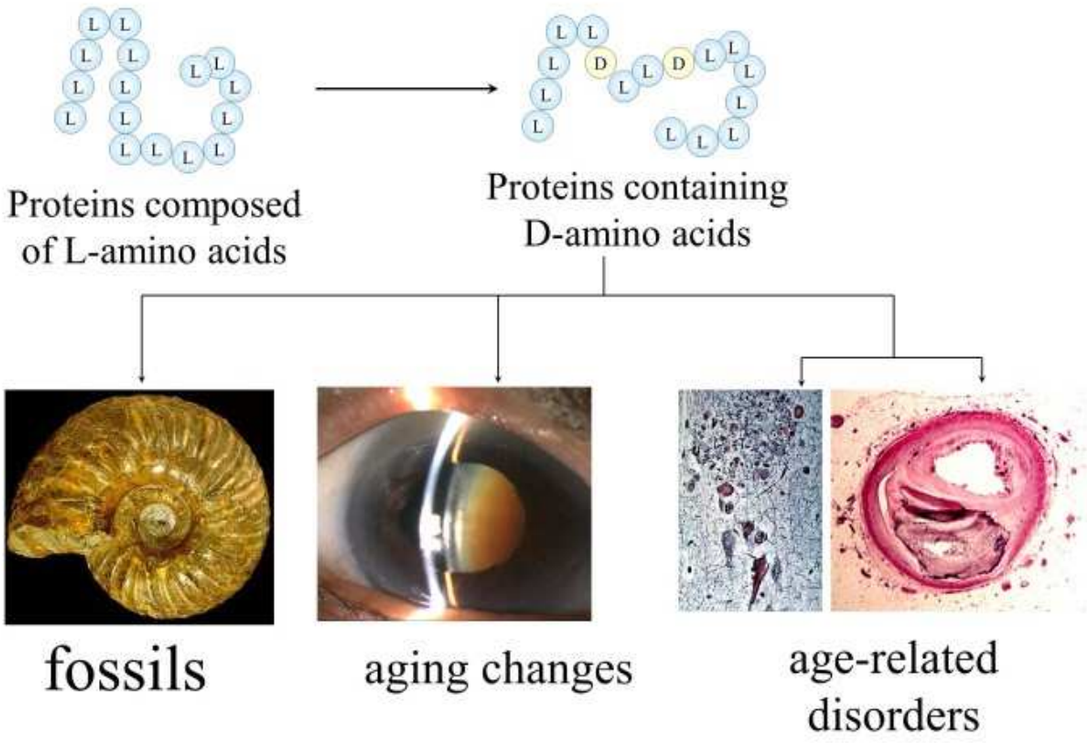

Fig. 4. **Significance of D-amino acids in proteins**
D-amino acids in proteins are seen in fossils as well as in the target organs of aging changes and age related disorders such as cataract, Alzheimer's disease, and atherosclerosis.

2.3 Co-localization of advanced glycation end products and D-amino acids in age-related macular degeneration

To reveal the role of AGEs and D-amino acids in the development of AMD, we analyzed the immunohistochemical localization of AGE and D-amino acid-containing proteins in human ocular samples of various ages.

Eye samples: Nine eyes from 9 donors of 18 to 88 years of age were obtained at the time of necropsy and used as samples for this study. Among them, 4 eyes from donors older than 68 years had drusen.

Antibodies: N^{ε}-(carboxy) methyl-L-lysine (CML) is the major component of AGEs in the body. A monoclonal antibody to CML was purchased (Transgenic Co. Ltd, Kumamoto, Japan).

The preparation and characterization of the primary antibody of D-β-Asp containing proteins was as described previously.27 The polyclonal antibody to the synthetic peptide called peptide 3R(Gly-Leu-D-β-Asp-Ala-Thy-Gly-Leu-D-β-Asp-Ala-Thy-Gly-Leu-D-β-Asp-Ala-Thy) corresponding to 3 repeats of positions 149–153 of the human α-A-crystallin was prepared and purified.

Immunohistochemistry: Immunohistochemical localization of CML and D-β-Asp containing proteins was investigated using the antibodies mentioned above. After fixation with 10% formalin solution, 4-μm-thick sections of the paraffin-embedded ocular samples were prepared. After deparaffinization, the sections were treated with 2 mg/mL of monoclonal antibody to CML or 1:500 diluted polyclonal antibody to D-β-Asp-containing proteins. After washing with phosphate-buffered saline, the sections were treated with secondary antibodies labeled with a polymer of horse radish peroxidase (Hitofine, Max-PO kit, Nichirei Co. Ltd, Tokyo, Japan). The final products were visualized using diaminobenzidine solution dissolved in phosphate-buffered saline.

No immunoreactivity to CML was seen in the retinas, choroids, or scleras of 5 eyes of donors younger than 18 years of age. In contrast, moderate immunoreactivity was seen in the retinal nerve fiber layers, and strong immunoreactivity was seen in the drusen and the thickened Bruch's membrane of the 5 eyes of donors older than 68 years (Figure 5).

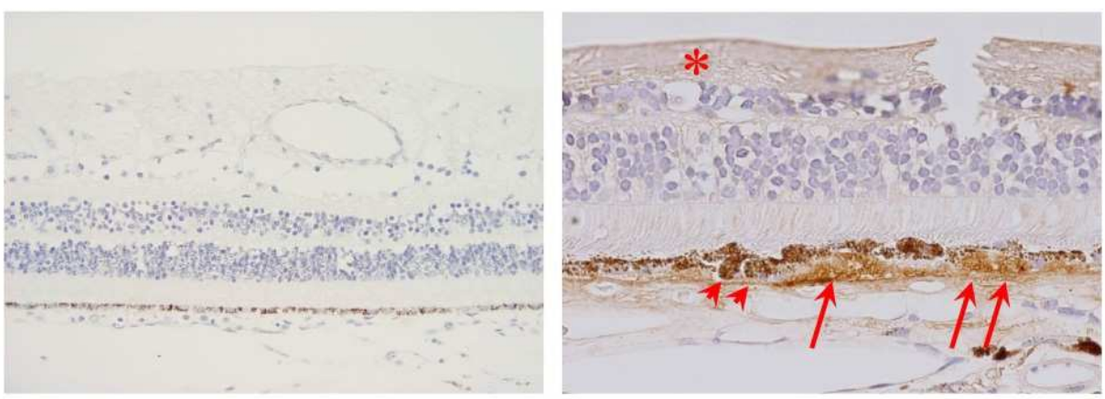

Fig. 5. **Immunohistochemical localization of AGE in human retina and choroid**
No immunoreactivity to CML, a major component of AGEs, is noted in young donor eyes. In contrast, immunoreactivity to CML is seen in the retinal nerve fiber layer (*), drusen (arrows), and thickened Bruch's membrane (arrowheads).

Similarly, no immunoreactivity to the D-β-Asp containing proteins was seen in the retinas, choroids, or scleras of donors younger than 18 years. In contrast, strong immunoreactivity was seen in the drusen seen in donors older than 68 years. In addition, moderate immunoreactivity to D-β-Asp containing proteins was seen in the sclera, the internal limiting membrane of retinal vessels, and Bruch membranes (Figure 6).

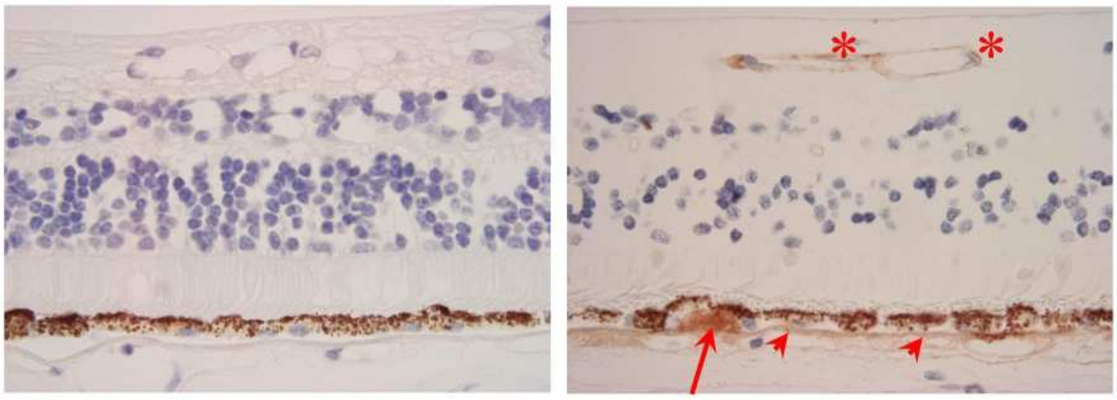

Fig. 6. **Immunohistochemical localization of D-amino acid-containing proteins in human retina and choroid**
No immunoreactivity to D-β-Asp-containing proteins, one of the major components of D-amino acids, is noted in young donor eyes. In contrast, the immunoreactivity to D-β-Asp-containing proteins is seen in the vessel walls (*), drusen (arrows), and thickened Bruch's membrane (arrowheads).

3. Possible mechanism of age-related macular degeneration

Drusen, an early sign of AMD, are small lumps of aggregated proteins rich in AGEs and D-amino acid containing proteins, which, in addition to being accessory products of the aging process, also possibly accelerate the aging process, suggesting that AGEs and D-amino acids in drusen play a central role in the development of AMD via various mechanisms.

One possible mechanism involves the interaction of AGEs and AGE receptors, which increases inflammation and accelerates neovascularization. AGE-modified proteins are recognized by the receptor for AGE (RAGE),30 galectin-3,31 macrophage scavenger receptors, and CD36.32 Particularly, the interaction of AGEs with RAGE induces inflammatory cytokines such as TNF-α and VEGF.33 RAGEs are expressed on the surface of retinal pigment epithelial cells. Furthermore, the interaction of AGE and RAGE increases the expression of RAGE, which serves as a positive feedback for the reaction. Thus, the constant interaction of AGEs in drusen with RAGE on retinal pigment epithelial cells would increase the expression of VEGF and induce neovascularization, resulting in AMD (Figure 7).

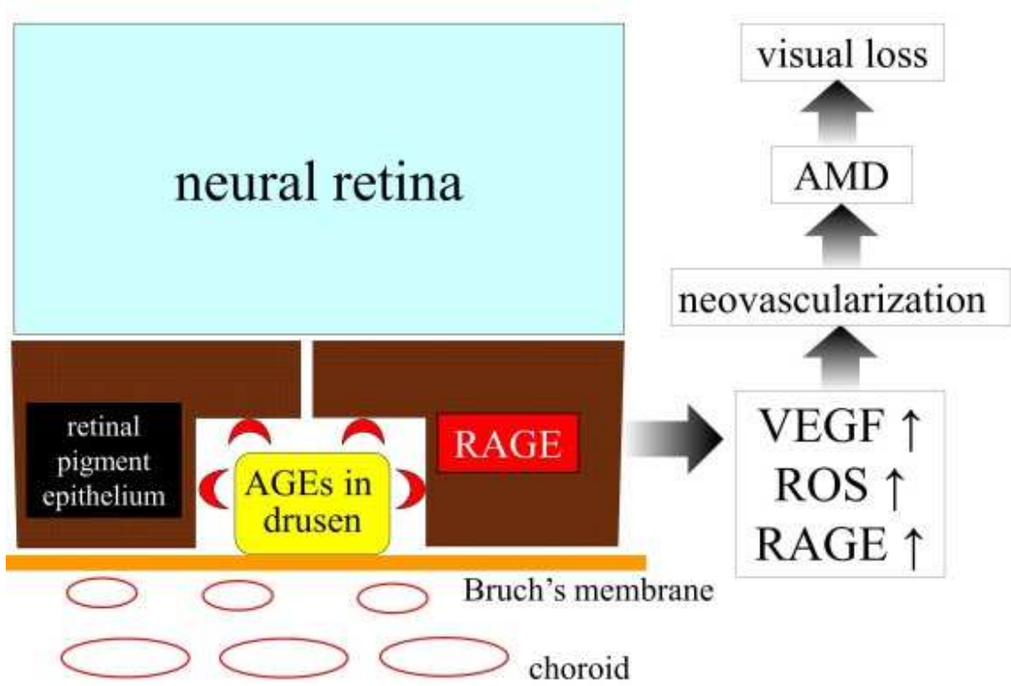

Fig. 7. **Possible mechanism of AMD in relation to AGEs in drusen**
The constant interaction of RAGE with AGEs in retinal pigment epithelial cells and drusen leads to the expression of VEGF, reactive oxygen species, and the increased expression of RAGE. This process then leads to neovascularization of the retina, which is a typical clinicopathological finding of AMD.

Another possible mechanism involves autoantibodies to AGE-modified proteins that have been detected in the elderly or in patients with rheumatic arthritis, which may induce inflammatory changes. Thus, autoimmune reactions may occur in tissues containing AGEs. In fact, proteomic analysis of drusen in human and animal models of AMD has revealed the deposition of IgG and complement factors. In addition, Becerra *et al.* have reported that the pathogenesis of AMD involves inflammatory changes.37 Based on these findings, intravitreal injection of corticosteroids to reduce the inflammation is clinically used to treat AMD.38 At present, the pathological role of D-amino acid containing proteins in the development of AMD is unknown. However, AGEs and D-amino acids need to be targeted for the prevention and treatment of AMD. For example, pyridoxamine inhibits the formation of AGEs in the body, and it has been used in clinical trials for the treatment of diabetic nephropathy. In addition, D-aspartyl endopeptidase has been shown to digest some D-amino acid containing proteins,41 suggesting that an increased expression of the intrinsic D-aspartyl endopeptidase would decrease the amount of D-amino acids-containing proteins in the target organ of the aging process. We suggest that the accumulating data on AGEs and D-amino acids will pave the way for new therapies for AMD in the near future.

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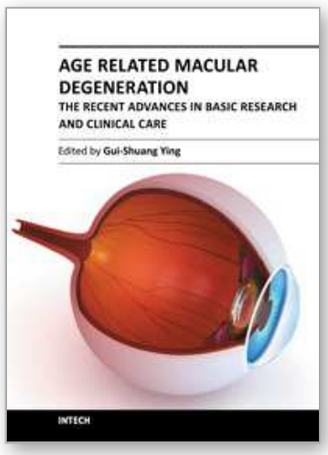

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Age-related Macular Degeneration (AMD) is the leading cause of vision loss and blindness in the developed countries. In the past decade, great progress has been made in understanding the pathobiology and genetics of this blinding disease, as well as in finding new therapies for its treatment. These include the discovery of several genes that are associated with the risk of AMD, new anti-VEGF treatments for wet AMD and new imaging techniques to diagnose and monitor the AMD. All chapters in this book were contributed by outstanding research scientists and clinicians in the area of AMD. I hope this timely book will provide the basic scientists and clinicians with an opportunity to learn about the recent advances in the field of AMD.

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