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Article

Sex and Age-Related Differences in Complement Factors Among Patients With Intermediate Age-Related Macular Degeneration

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Purpose: Age-related macular degeneration (AMD) is an acquired degenerative disease of the retina classified into early, intermediate, and advanced AMD. A key factor in the pathogenesis of AMD is the complement system. The interaction of age and sex with the complement system may affect the risk of developing AMD. The purpose of this study was to determine if there were sex-specific differences in levels of complement factors among patients with the intermediate phenotype of AMD (iAMD) and explore the correlation between age and complement proteins.

Methods: We studied complement factors in patients with iAMD and controls without AMD. Nonparametric, rank-based linear regressions including a sex by AMD interaction were used to compare levels for each analyte. Correlations between age and complement proteins were evaluated using the Spearman rank correlation coefficient.

Results: We found significantly higher levels of factor B and factor I in females compared with males with iAMD, whereas no differences were seen in complement levels in male and female controls. The ratios of Ba/factor B, C3a/C3, C4b/C4, and C5a/C5 were not different in males and females with iAMD.

Conclusions: We demonstrate disparities in a subset of systemic complement factors between females and males with iAMD, but apparent complement turnover as measured by ratios of activation fragments to intact molecules was not different between these groups. The results suggest that complement system levels, including complement regulator factor I, exhibits sex-related differences in patients with iAMD and highlights that stratification by sex might be helpful in the interpretation of clinical trials of anticomplement therapy.

Introduction

Age-related macular degeneration (AMD) is an acquired degenerative disease of the retina that leads to progressive central vision loss. It is the third leading cause of blindness worldwide and it is estimated that by 2040, 288 million individuals will be affected by the disease, representing approximately a 50% increase from 2020.[1] The hallmark finding of AMD is the presence of drusen within the macula between the basal surface of the retinal pigment epithelium and Bruch's membrane.[2] As described by the Beckman Initiative for Macular Research Classification Committee,

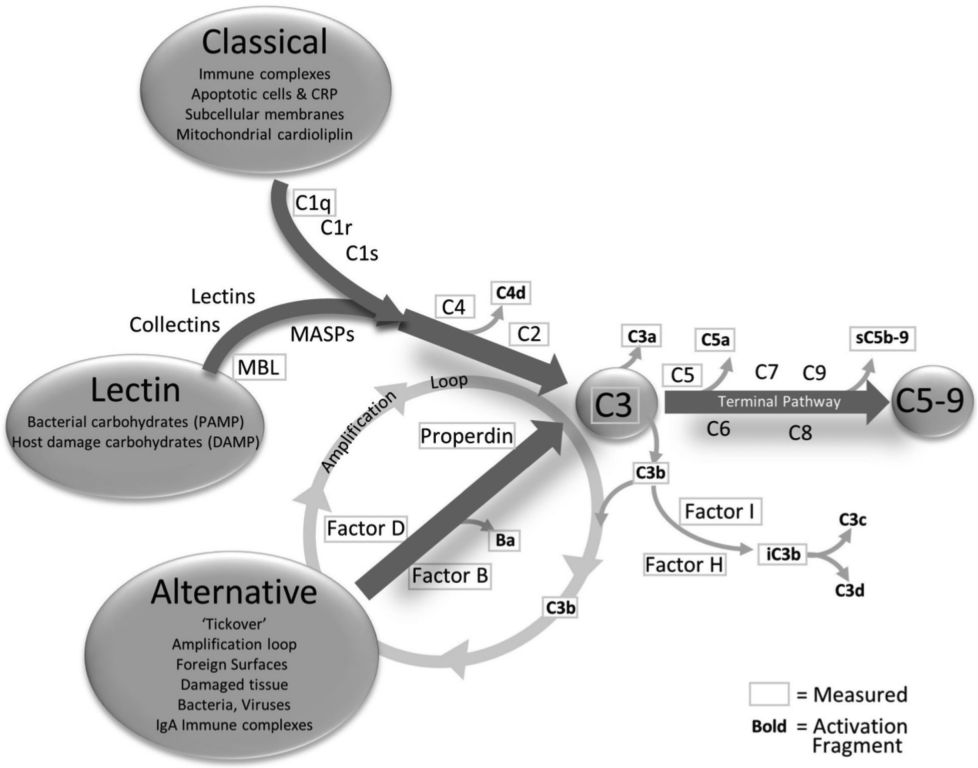

Figure 1. The classic, lectin, and alternative complement pathways. These three pathways are activated differently but all connect at the central point C3. The three pathways are interrelated by the action of AP as an amplification loop for the classical and lectin pathways. After C3, the complement system enters the terminal pathway, culminating in the membrane attached complex (also known as C5b-9). As activation flows through the cascade, the components are cleaved yielding activation fragments that are released into circulation. *Note.* Reprinted with permission from BMJ Open Ophth. Lynch AM, et al 2020;5:e000361 page 2. License date Nov 13,2021.

AMD can be classified into early, intermediate and advanced AMD.[2] There is important prognostic and therapeutic value in studying patients with intermediate AMD (iAMD), given that many patients in this group progress to the advanced and visually threatening forms of AMD. Established risk factors for AMD include age, cigarette smoking, race, genetics, and a family history of AMD.[1,3,4] A key factor in the pathogenesis of AMD is the complement system.[5,6] This finding is supported by the fact that complement components are found within drusen[7–9] and in the aqueous humor of patients with AMD.[10] In addition, multiple genetic variants associated with AMD are in genes encoding for complement proteins.[3] In fact, common genetic variants near key regulatory complement genes together may explain up to 60% of the heritability of AMD.[11]

The complement system is composed of three activation pathways and an amplification loop: the classical pathway, alternative pathway (AP), and lectin pathway.[12] Each pathway (Fig. 1) is triggered by distinct activators, but they all converge at the central step through the generation of C3-convertases, which cleave C3 into C3a and C3b. C3a is an anaphylatoxin, and C3b binds to the C3-convertase yielding the C5-convertase, which subsequently cleaves C5. The cleavage of C5 leads to the formation of C5a (another anaphylatoxin and potent chemoattractant) and C5b, which ultimately forms the membrane–attack complex (C5b-9), facilitating the generation of a pore in targeted cells leading to lysis. The classical pathway can be initiated by binding of C1q to antigen–antibody complexes after the activation of C2 and C4. The activation of the AP involves factor B and factor D, which are known to interact with C3b to form a C3 convertase. In addition to acting as its own activation pathway, the AP also has an important role as the amplification loop of complement activation, regardless of initiating pathway. In summary, the amplification loop is a balance between the C3b feedback cycle and the C3b breakdown cycle.[13] Lastly, the lectin pathway is activated by Mannose-binding lectin, ficolins, or collectines by binding sugars or acetylated surfaces.[12] To prevent unwanted complement activation, humans have a variety of plasma and membrane-bound inhibitory proteins to regulate the location and activity of complement. Examples of plasma regulators include complement factor I and factor H, both key players of the AP complement

regulation.[14] It is established that complement activation is altered in patients with AMD compared with controls.[10,15,16] In fact, evidence suggests that complement activation levels vary significantly among AMD disease stages, with higher levels of C3d/C3 ratios in patients with more advanced disease.[18]

Regarding sex differences in the prevalence of AMD, reports from the National Eye Institute of the National Institute of Health suggest that women represent two-thirds of prevalent cases of AMD.[17] Several investigators have also shown that sex plays a role in the prevalence and presentation of other ocular diseases.[18] It is suggested that genetic differences and fluctuation in sex hormones (such as estrogen) may account for some of these differences.[19–21] In fact, our group recently described the relationship between hormone replacement therapy and AMD, with findings suggesting that hormone replacement therapy is a protective factor for AMD,[22] further highlighting the relationship of sex-related differences and AMD. Moreover, within the last few decades there has been an increased recognition of sex-driven differences in the immune response, with a much higher frequency of autoimmune conditions observed in females compared with males.[23–25] The overall consensus is that sex chromosome genes, environmental factors, and hormones contribute to the differential regulation of immune responses between sexes.[19,20,26] Indeed, as discussed by Miller et al.,[27] the failure to present data stratified by sex may obscure important sex-related differences in health-related outcomes.

Our group has recently described changes in systemic levels of complement factors in patients with AMD[15] and specifically in patients with iAMD.[28] The research described herein was prompted by our prior research, the important emerging role of incorporating sex as a biological variable in clinical research and specifically by the paucity of research in sex differences in activation of the complement system in AMD.[27,29,30] Thus, the purposes of our study were to (a) determine if there were any sex-specific differences in levels of complement factors among patients with iAMD, and (b) explore the correlation between age and complement proteins.

Methods

Study Design and Population

This study was conducted on patients with iAMD whose records and samples were part of the University of Colorado AMD research registry and repository.[15,28,31,32] The registry includes patients with AMD who attend the retina clinics of the UCHealth Sue Anschutz-Rodgers Eye Center and control cataract patients with no AMD. This registry conforms with the Declarations of Helsinki and is approved by the Colorado Multiple Institutional Review Board. The informed consent, recruitment, and exclusion and inclusion criteria are described in detail elsewhere.[31,33] In brief, each patient is consented for review of their medical history, collection of blood for biomarker studies, and classification of the AMD disease phenotype[34] after an assessment of multimodal imaging (spectral domain optical coherence tomography, color fundus photography, and autofluorescence).[32,33] Patients who are between 55 and 99 years of age, have AMD in one or both eyes, and have the capacity to provide consent are eligible for inclusion in the registry. Exclusion criteria for the registry included terminal illness, active ocular inflammatory disease, prior treatment with anti–vascular endothelial growth factor injections, panretinal photocoagulation, and branch and central retinal vein occlusion. Further exclusion criteria are proliferative diabetic retinopathy, nonproliferative diabetic retinopathy, diabetic macular edema, cystoid macular edema, macula-off retinal detachment, central serous retinopathy, full-thickness macular hole, ocular melanoma, pattern or occult macular dystrophy, macular telangiectasia, corneal transplant, drusen not caused by AMD, and current systemic treatment for cancer or any serious mental health or advanced dementia issues. Control patients included in the registry are enrolled 1 month after cataract surgery with no evidence of AMD by review of multimodal imaging.

AMD Classification

As discussed elsewhere in this article, the diagnosis of AMD was confirmed using multimodal imaging. Two reviewers masked to the clinical diagnosis classified the images into early AMD, intermediate AMD, or advanced AMD, using the classification described by the Beckman Initiative for Macular Research Classification Committee.[2] Reviewers also examined the retinal images of controls to confirm no evidence of AMD using the same criteria.[2] For this study we concentrated on patients with iAMD. Intermediate AMD was defined as pigmentary abnormalities with at least medium drusen or large drusen (>125 μm) in either eye with no indication of advanced AMD in either eye as evaluated by multimodal imaging.

Complement Measurements

We collected plasma samples from each patient. The plasma ethylenediaminetetra-acetic acid tube for each patient was spun at 3000 rpm in a chilled (4°C) centrifuge for 10 minutes to isolate plasma. The plasma was then pipetted into aliquots and immediately stored in a –80°C freezer. Aliquots of plasma were transferred to the laboratory for measurement of complement factors. Measured proteins included complement components and activation fragments of the lectin pathway, classical pathway, and AP, more specifically, mannose-binding lectin, C1q, C2, C4, C4b, C3, C3a, factor D, factor B, Ba, factor H, factor I, C5, C5a, and soluble C5b-9 (sC5b-9). The units for each measurement are specified in the respective tables. Ratios of Ba/factor B, C3a/C3, C4b/C4, and C5a/C5 were also determined. The measurement of proteins described elsewhere in this article was performed by two methods. Ba and C3a levels were measured by ELISA (Quidel, San Diego, CA). The remaining measurements were performed by multiplex Luminex immunoassays (MilliporeSigma, Burlington, MA). Measurements of analytes was performed in duplicate wells and the average value was reported. Standard curves and a four-parameter parametric curve fit were used to calculate the absolute quality in nanograms per milliliter or micrograms per milliliter. For all testing runs, three quality controls were included on the assays, two from the manufacturer and one internally developed. Assays were considered acceptable if controls were within specifications. Samples were run in two batches (102 samples in batch 1 and 172 samples in batch 2); all controls were run in batch 1, and there was no difference between representation of sex between batches.

Statistical Analysis

Patient characteristics were compared between groups using a two-sample *t*-test for continuous variables and a χ^2 test or Fisher's exact test, when indicated, for categorical variables. A nonparametric (rank-based) regression model was fit to each analyte with a sex by AMD category interaction. Linear contrasts of regression coefficients were used to evaluate the pairwise comparisons that included iAMD cases versus control, male versus female controls, male versus female cases, male controls versus male cases, and female controls versus female cases. Correlations between age and complement proteins were evaluated using the Spearman rank correlation coefficient. *P* values are presented as both raw values and after adjustment for multiple comparisons using the false discovery rate.[35] All analyses adjusted for batch effect and were performed using SAS version 9.4 (The SAS Institute, Cary, NC).

Results

The baseline demographic and clinical characteristics for the participants are shown in Table 1. In total, 274 patients were included, of which 211 were patients with iAMD and 63 were controls. There were 129 females (61%) in the iAMD group and 41 (65%) in the control group ($P = 0.57$). The mean age of participants was higher in the iAMD cases compared with controls without AMD (77 years old vs75 years old, respectively; $P = 0.03$). Furthermore, we found a significantly higher frequency of a family history of AMD in cases versus controls (35% vs 19%; $P = 0.02$). We also examined these demographic and clinical characteristics by sex within the iAMD cohort. The only significant risk factor was a higher frequency of treated hypertension (HTN) in females compared with males in the iAMD group (60% vs 44%; $P = 0.03$; Supplementary Table S1).

In Supplementary Table S2, we demonstrate the levels of systemic complement factors in cases with iAMD and in controls without AMD. As previously reported in a smaller group of patients from this cohort,[28] we found differences in select complement factors between cases and controls. Levels of C1q and C4b were significantly lower in iAMD cases compared with controls. In contrast, C2, C3, C3a, and C5 levels were significantly higher in iAMD cases compared with controls. Factors B, H, and I were all significantly lower in iAMD cases compared with controls. Regarding the ratio measurements, the Ba/factor B and the C5a/C5 ratios were significantly different in cases versus controls.

We next evaluated differences in complement levels between males and females in the iAMD cohort. As shown in Table 2, we found significantly higher levels of factor B and factor I in females with iAMD compared with males. However, ratios of Ba/factor B, C3a/C3, C4b/C4, and C5a/C5 were not statistically different between males and females with iAMD. Given our cohort's higher history of HTN in females in the iAMD group, a subgroup analysis of the significant markers was performed within patients with iAMD using an HTN by sex interaction model. Using this model, factor B and factor I were found to also be higher in females with HTN compared with males with HTN ($P < 0.01$ and $P = 0,01$ respectively). No other pairwise

Table 1. Clinical Characteristics of Patients With Intermediate AMD and Controls Without AMD

	Control ($n = 63$)	iAMD ($n = 211$)	*P* Value
Sex, female	41 (65%)	129 (61%)	0.57
Family history of AMD			<0.01
None	48 (76%)	109 (52%)	
Yes	12 (19%)	73 (35%)	
Uncertain	3 (5%)	29 (14%)	
Age, mean (SD)	74.5 (3.8)	76.6 (7.2)	0.03
Body mass index, mean (SD)	27.0 (5.6)$n = 61$	26.9 (5.3)$n = 202$	0.84
Smoking			0.33[†]
Never	34 (54%)	97 (46%)	
Current	0	6 (3%)	
Former	29 (46%)	108 (51%)	
History of			
Treated HTN	37 (59%)	113 (54%)	0.47
Kidney disease	8 (13%)	26 (12%)	0.94
Peripheral vascular disease	14 (22%)	34 (16%)	0.26
Cardiac disease	21 (33%)	74 (35%)	0.80

P values obtained from χ^2 test for categorical variables and *t*-test for continuous variables unless noted otherwise.
[†]*P* value calculated from Fisher's exact test.

Table 2. Complement Levels for Males and Females Within the Intermediate AMD Cohort

Complement FactorsMedian (IQR)	FemalesiAMD ($n = 129$)	MalesiAMD ($n = 82$)	*P* value	FDR *P* value
C1q µg/mL	81 (71–95)	84 (74–90)	0.75	0.87
C4 µg/mL	117 (104–131)	116 (103–137)	0.47	0.70
C2 µg/mL	4.4 (3.4–5.4)	4.0 (3.2–5.0)	0.47	0.70
C4b µg/mL	8.0 (5.3–10.5)	6.1 (5.0–9.7)	0.87	0.92
Mannose-binding lectin ng/mL	714 (241–1183)	826 (361–1629)	0.13	0.42
C3 µg/mL	78.4 (64.8–96.8)	78.1 (58.0–93.6)	0.26	0.60
C3a ng/mL	58.0 (44.6–71.0)	52.1 (40.0–69.0)	0.14	0.42
Ba ng/mL	658 (533–816)	627 (530–808)	0.77	0.87
Factor B µg/mL	116.6 (100.8–130.2)	105.4 (95.1–121.4)	<.01	0.03
Factor D µg/mL	1.6 (1.3–2.1)	1.5 (1.3–2.0)	0.67	0.86
Factor H µg/mL	187.5 (169.5–219.9)	177.2 (160.1–198.2)	0.02	0.10
Factor I µg/mL	27 (23–32)	25 (22–28)	<.01	<.01
C5 µg/mL	51 (38–82)	71 (39–93)	0.34	0.68
C5a ng/mL	574 (418–724)	518 (315–684)	0.61	0.85
Ba/factor B	0.006 (0.004–0.007)	0.006 (0.005–0.007)	0.06	0.29
C3a/C3	0.001 (0.001–0.001)	0.001 (0.000–0.001)	0.98	0.98
C4b/C4	0.079 (0.040–0.095)	0.048 (0.041–0.093)	0.26	0.60
C5a/C5	0.013 (0.005–0.018)	0.008 (0.003–0.017)	0.43	0.70

The median and interquartile range for each marker are presented, the *P* values compare the difference between sex after adjusting for batch effects.

comparisons were significantly different between those two groups. We found no significant differences in any of the complement factors between males and females in the control group (Table 3). These results are shown in Figures 2A, B.

Among patients with iAMD, age was positively correlated with factor D ($r = 0.33$; $P < 0.01$), Ba ($r = 0.20$; $P < 0.01$), C3a ($r = 0.17$; $P = 0.02$), and C2 ($r = 0.20$; $P < 0.01$) although the relationships were not strong (Table 4 and Supplementary Table S3).

Table 3. Complement Levels for Males and Females Within the Control Groups

Complement Factors Median (IQR)	Females ($n = 41$)	Males ($n = 22$)	*P* Value	FDR *P* Value
C1q µg/mL	91 (83–100)	88 (75–92)	0.12	0.55
C4 µg/mL	112 (100–122)	116 (96–129)	0.89	0.95
C2 µg/mL	3.3 (2.5–4.4)	3.7 (2.5–5.1)	0.32	0.76
C4b µg/mL	10.6 (8.9–11.4)	12.1 (9.0–13.1)	0.33	0.76
Mannose-binding lectin ng/mL	624 (309–1180)	462 (151–996)	0.62	0.86
C3 µg/mL	64.3 (51.4–80.9)	73.8 (58.8–83.9)	0.44	0.86
C3a ng/mL	50.8 (40.6–60.8)	48.1 (42.1–59.6)	0.83	0.95
Ba ng/mL	646 (525–709)	559 (527–749)	0.59	0.86
Factor B µg/mL	127.8 (109.2–141.1)	118.1 (105.1–125.3)	0.10	0.55
Factor D µg/mL	1.8 (1.5– 2.1)	1.8 (1.5–2.6)	0.55	0.86
Factor H µg/mL	235.1 (196.1–256.5)	214.9 (180.1–226.5)	0.07	0.55
Factor I µg/mL	32.0 (29.3–34.0)	29.8 (27.5–32.5)	0.15	0.56
C5 µg/mL	46.2 (40.1–49.8)	44.0 (38.8–47.4)	0.74	0.95
C5a ng/mL	556 (474–692)	596 (406–667)	0.96	0.96
Ba/factor B	0.005 (0.004–0.006)	0.005 (0.004–0.007)	0.34	0.76
C3a/C3	0.001 (0.001–0.001)	0.001 (0.001–0.001)	0.49	0.86
C4b/C4	0.091 (0.085–0.100)	0.099 (0.088–0.106)	0.03	0.55
C5a/C5	0.013 (0.010–0.015)	0.013 (0.011–0.015)	0.90	0.96

The median and interquartile range for each marker are presented, the *P* values compare the difference between sex after adjusting for batch effects.

Discussion

There were several key findings in our study analyzing sex-related differences in complement proteins in a cohort of patients with iAMD. First, we found higher levels of a key regulator of complement, factor I, and of AP component factor B in females compared with males with iAMD. Second, we found that apparent complement system turnover was not different between

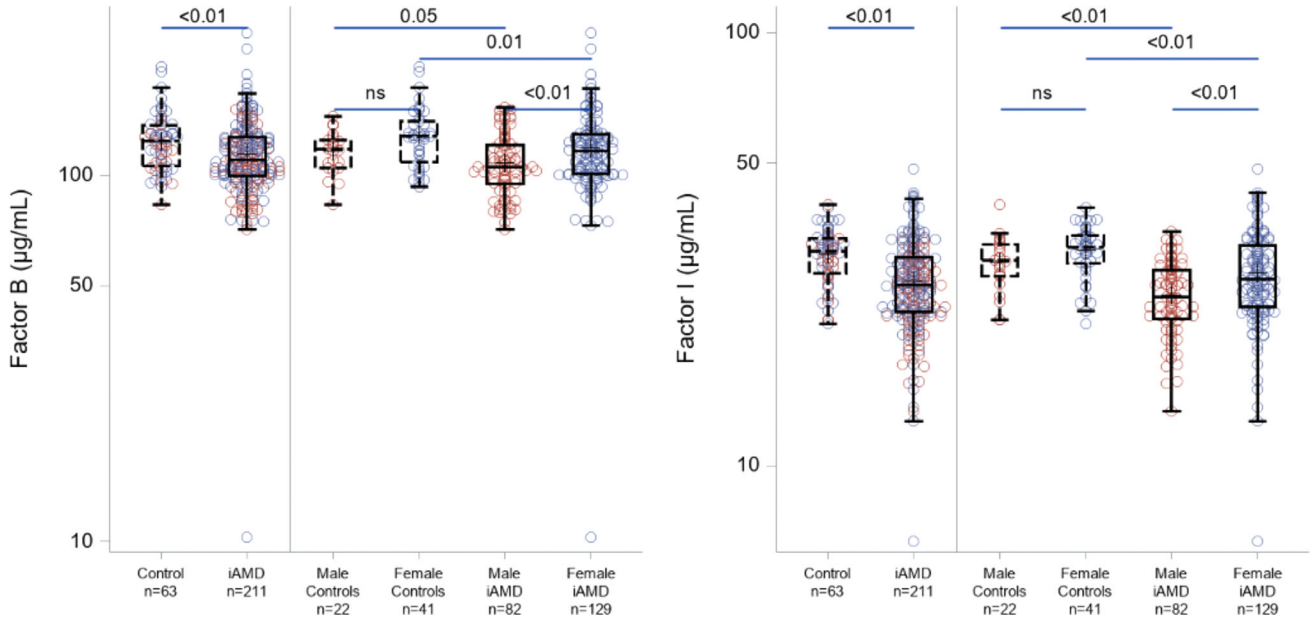

Figure 2. Boxplot showing difference among groups in complement components found to be significantly higher in females compared with males in patients with intermediate AMD. The boxplots extend to the 25th and 75th percentiles. The line represents the median and the large black pluses correspond to the mean values. Individual colored circles illustrate the raw values. Male patients are represented as blue circles and female patients are represented in red circles. "ns" represents no statistical difference between groups. All comparisons are adjusted for batch effects.

Table 4. Significant Spearman Correlation Coefficients Between Age and Complement Factors as Well as Complement Factors With Each Other Among Patients With iAMD*

Variable	By Variable	Spearman	*P* Value
Age	Factor D	0.33	<0.01
Age	Ba	0.20	<0.01
Age	C2	0.20	<0.01
Ba/factor B	C3a/C3	0.37	<0.01

*All correlations are presented in supplementary Tables 2 and 3.

males and females within the iAMD group as indicated by ratios of Ba/factor B, C3a/C3, C4b/C4, and C5a/C5. Third, in agreement with a previous study from our group,[28] we found that select complement factors were significantly different between cases with iAMD and controls with no AMD. Specifically, we demonstrated lower levels in iAMD for proteins involved in the AP including factor B as well as the complement regulators factor H and factor I. Fourth, we found no strong correlations between age and complement factors among patients with iAMD.

In this study, we found sex differences in iAMD, an area of AMD research that is understudied and underappreciated. Although some studies have shown that female sex is an independent risk factor for AMD disease and progression,[36–41] others have not.[42–44] We suggest that the variability by sex in the levels of complement factors demonstrated in our study could be used to better understand the pathogenesis of iAMD and to find targets for anticomplement therapy that could slow down progression to more advanced forms of AMD. Therefore, studies targeting differences in the innate and adaptive immune system in AMD may shed light on the controversy around sex as a potential risk factor for the development of AMD.

Autoimmune diseases are more prevalent in women compared with men.[45] For example, diseases such as systemic lupus erythematosus, autoimmune thyroid disease, and scleroderma have female to male ratios of between 7:1 and 10:1.[24,25,46] In addition, the symptoms, severity, disease course, and response to therapy may also be different between females and males with autoimmune-related disease.[46,47] Some of those disparities can partially be explained by the fact that sex differences occur in both innate and adaptive immune responses.[48] Yet, there is a paucity of data related to the influence of sex in the complement system. Kotimaa et al.[49] conducted research on a murine model and found that complement terminal pathway activity was weaker in females versus males across all pathways. Most recently, Gaya da Costa et al.[50] conducted a study in a healthy Caucasian population and found that females have lower levels of C3 and terminal pathway components compared with males. Our study in a select group of patients with iAMD was distinguished by the finding of higher systemic levels of factor B and factor I in females versus males. We suggest that an explanation for this finding is that the activation of all complement pathways ultimately results in a proinflammatory response that in turn leads to higher levels of factor B in females with iAMD. These changes could lead to a compensatory increase in factor I, further accelerating the decay of an AP components. Because of its higher levels, factor I can also inhibit further deposition of complement components and could then explain our results showing no differences in complement turnover across females and males with iAMD. Our study's novel findings emphasize the importance of stratification by sex in AMD research.[27]

Dysregulation of the above-mentioned complement components and regulators have already been implicated in the pathogenesis in AMD.[51–60] A genetic analyses by Gold et al.[61] identified a common risk haplotype (H1) and two protective haplotypes (L9H variant of BF and the E318D variant of C2) in patients with AMD. Moreover, higher levels of C3a have been seen in patients with neovascular AMD with subretinal fibrosis, and these patients had surprisingly a partial response to anti–vascular endothelial growth factor therapy.[62] Furthermore, there are promising data that the addition of antagonists to C3aR complexes can prevent the formation of basal deposits in the RPE.[63,64] In terms of AP regulation, one of the most replicated genetic variants associated with AMD is the tyrosine to histidine substitution at the amino acid 402 in complement factor H.[58] It has been demonstrated that higher circulating levels of splice variants of CFH may be a predisposing factor for AMD. In addition, rare genetic variants in CFI leading to low systemic levels of factor I have also recently been associated with AMD.[57]

We are aware that there are limitations to our study. The collection of data from single study site may not be representative of the entire population. A subgroup analysis within the iAMD groups and HTN yielded some attenuation in the magnitude differences when comparing males and females within the iAMD group. This finding, however, correlates with known reports associating HTN with higher levels of C3 complement fragments.[65,66] The rest of the markers found to be significantly different between females and males with iAMD were not different when stratified by HTN. Another limitation is that we did not assess sex-related risk factors such as the hormonal status of

the patients, which has been reported to play a role on aging and in the immune system.[48] Last, we did not examine the genetic polymorphisms in the complement pathways. Variations of CFH could potentially influence levels complement of components. However, assuming incidence of complement mutations does not differ by gender significantly, differences in complement levels found in our cohort remain a valid and interesting result. Last, we did not examine the interaction between complement proteins and sex with iAMD disease progression given the small sample size. To address this last point, recruitment into the registry is ongoing and we hope to address these limitations in the future with a larger sample size.

Patients with nonexudative forms of AMD have long been expecting an effective treatment that may reverse or slow progression of disease. With the advances in complement therapy, several therapeutic approaches have been investigated and are currently at different stages of clinical trials to reverse or slow progression of disease for patients with specifically the nonexudative forms of advanced AMD. Namely, a C3 inhibitor, pegcetacoplan (APL-2, Apellis Pharmaceuticals, Waltham, MA), was recently shown to significantly reduce geographic atrophy, a chronic progressive degeneration of the macula characteristic of late-stage AMD.[67,68] Avacincaptad pegol (Zimura, Iveric Bio, Cranbury, NJ), a novel complement C5 inhibitor was also shown to reduce the mean rate of geographic atrophy growth over 12 months compared with sham.[69] Other clinical trials with various complement inhibitors, however, have thus far been unsuccessful.[29,70] Our study highlights that stratification by sex should may add insight in the interpretation of clinical trials of anticomplement therapy for iAMD. Moreover, given the high rates of conversion from iAMD to the more advanced phenotypes of AMD, more attention should be placed on identifying markers that may predict and/or target disease progression. Moving forward, researchers should also focus on investigating sex-related differences in complement in patients with more advanced forms of AMD. More important, we believe that by combining systemic biomarkers, genetic profile, demographics, and imaging data it may be possible to discover individualized targets that could be of use for the treatment of intermediate and other forms of AMD.

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