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Inflammasome Proteins as Inflammatory Biomarkers of Age-Related Macular Degeneration

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Purpose: Age-related macular degeneration (AMD) can result in severe vision loss and blurriness in the older population. The early and intermediate stages of AMD typically start without noticeable symptoms and can only be detected with a comprehensive eye exam. Because of the quiet onset of the disease, it is necessary to identify potential biomarkers to aid in the diagnosis, staging, and association with disease onset. Inflammasome signaling proteins are prominent biomarkers in the central nervous system, and the inflammasome has been shown to play a role in the innate inflammatory response in aging and AMD.

Methods: Serum from healthy controls and AMD patients were analyzed for the protein levels of Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), interleukin (IL)-18 and C-reactive protein (CRP) to determine cutoff points, positive and negative predictive values, and receiver operator characteristic curves, as well as univariate and multivariate linear and logistic regression models.

Results: ASC, IL-18, and CRP were elevated in the serum of AMD patients when compared to healthy controls. The area under the curve (AUC) for ASC was 0.98 with a cutoff point of 365.6 pg/mL, whereas IL-18 had an AUC of 0.73 and a cutoff point of 242.4 pg/mL, and the AUC for CRP was 0.67 with a cutoff point of 8,684,152 pg/mL. Levels of IL-18 had a statistically significant linear correlation with that of ASC with an adjusted R^2 of 0.1906, indicating that 19% of IL-18 could be explained by ASC protein levels in serum. Moreover, a logistic regression model for the diagnosis of AMD consists of ASC and having a diagnosis of hypertension, indicating that these two factors (elevated levels of ASC and a diagnosis of hypertension [HTN]) are associated with the diagnosis of AMD.

Conclusions: ASC, IL-18, and CRP are elevated in patients with AMD, and the protein levels of IL-18 are partially the result of ASC protein expression. Moreover, elevated protein levels of ASC in serum and a diagnosis of HTN increase the odds of patients having a diagnosis of AMD.

Translational Relevance: Biomarkers of AMD may be used to monitor disease risk, response to treatment and disease progression.

Introduction

Age-related macular degeneration (AMD) is a leading cause of blindness in the older population and affects more than 11 million people in the United States alone[1] and more than 170 million people worldwide.[2] AMD is a progressive degenerative disease that can result in irreversible vision loss.[3] Patients in the early stages of AMD often experience no symptoms, and the disease is typically not detected until later, when vision loss begins to occur. Because there is currently no cure

for this disease, it is imperative to identify biomarkers to help screen for the disease to diagnose the early stages of AMD, monitor response to treatment, slow disease progression, and identify novel therapeutic strategies.[4,5]

The inflammatory response is a common influence in several aging-related diseases such as Parkinson's disease and Alzheimer's disease (AD).[6] Previous research has studied the inflammatory response due to aging, a process known as inflammaging.[7] Accordingly, the inflammasome is involved in inflammaging, which is associated with the early stages of neurodegeneration.[7–10] The inflammasome is a multiprotein complex of the innate immune response,[1] and the activation of the inflammasome protein caspase-1, in association with apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC), triggers a cascade of molecular events that promote the release of the proinflammatory cytokines interleukin (IL)-1β and IL-18.[11] The most definitive risk factor for AMD is aging, with smoking and hypertension (HTN) also possible contributors, among others.[12] Recent evidence suggests that AMD is an ocular manifestation of a systemic disease.[13] Thus research is needed to identify blood biomarkers that can provide systemic data associated with the diagnosis of AMD.

We have previously shown that inflammasome signaling proteins are promising biomarkers of inflammation in a variety of conditions.[14–16] Thus inflammasome proteins may be also promising biomarkers of the inflammatory components associated with AMD that may also underlie the aging and systemic components of the disease. Therefore in the following study we investigated the potential for the inflammasome signaling proteins ASC and IL-18 as biomarkers of the inflammatory response associated with AMD. In addition, we measure the protein levels of C-reactive protein (CRP) as a general marker of systemic inflammation because CRP has been shown to be a promising measure of systemic inflammation in AMD patients. [17,18] Hence, here we determined cutoff points, receiver operator characteristic (ROC) curves with associated sensitivity and specificity calculations, as well as negative and positive predictive values, accuracy, and likelihood ratios for ASC, IL-18, and CRP. In addition, we fit linear regression models to explain the protein levels of the proinflammatory cytokine IL-18 and a logistic regression model to determine the odd of patients having a diagnosis of AMD taking into consideration the protein levels of ASC, CRP in serum, as well as having a diagnosis of HTN.

Table 1. Patients With AMD

AMD Patients	32
Sex	
Males	13 (41%)
Females	19 (59%)
Race	
Caucasian	31 (97%)
African	1 (3%)
Age Range	
Range	65–93
Median	78
Mean	78.84
Comorbidities	
HTN	20 (63%)
Hypercholesterolemia	12 (38%)
Diabetes	7 (22%)
HLD	5 (16%)
Wet AMD	19 (59%)
Dry AMD	13 (41%)

Methods

Participants

Samples were purchased from BioIVT (Hicksville, NY, USA). Informed consent was obtained from donors enrolled in the study Prospective Collection of Samples for Research sponsored by SeraTrials, LLC. with IRB number 20170439. The age range of donors was from 55 to 93 years old, with 61 samples in the control no-AMD group and 32 in the AMD group (Table 1). Age-matched healthy controls (no-AMD) presented with no known diagnosis of any disease.

Simple Plex Assay

Concentrations of inflammasome proteins (ASC and IL-18) and CRP in serum samples from AMD and age-matched controls were analyzed using the Ella System (Protein System).[14,19] In short, 50 µL of diluted serum sample was loaded to each well of the cartridge, and 1 mL of washing buffer was loaded into specified wells. The assay was analyzed by Simple Plex Runner Software. Results shown are the mean of each sample run in triplicate for all three analytes.

Biomarker Analyses

Data obtained from the Simple Plex assay was analyzed using Prism 8 software (GraphPad). Initially,

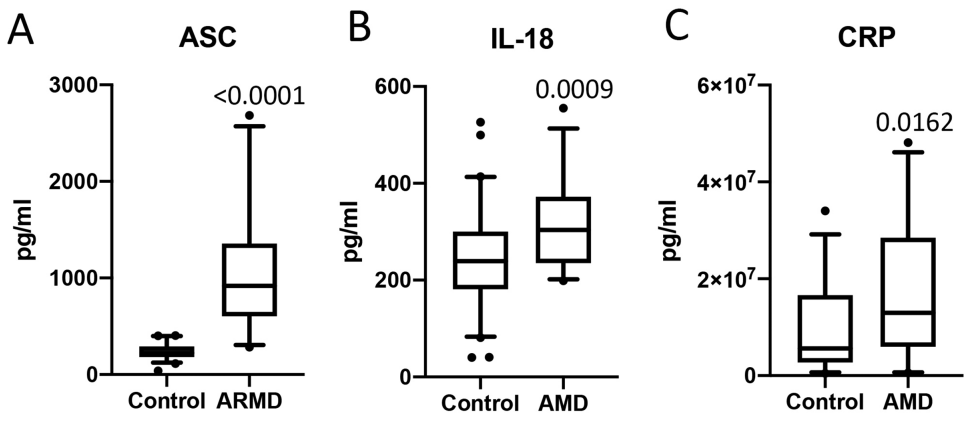

Figure 1. ASC, IL-18 and CRP are elevated in the serum of patients with AMD. Protein levels in pg/ml of ASC (**A**), IL-18 (**B**) and CRP (**C**)in serum samples from patients with AMD and healthy donors (controls). ASC: N = 46 control, 32AMD.IL-18: N = 61 control, 27 AMD.CRP: N = 35 control, N = 29 AMD.Box and whiskers are shown for the 5th and 95th percentile.

outliers were removed, followed by the calculation of descriptive statistics and ROC to obtain the area under curve (AUC), which provided the specificity, sensitivity and likelihood ratio, as well as the 95% confidence interval, standard deviation, and *P* value. A cutoff point was identified for the different ranges of specificities and sensitivities taking into consideration the likelihood ratio. Positive and negative predictive values were calculated in addition to the accuracy of each analyte.

Statistical Analyses

Power analysis was carried out for ASC with a power of 0.8, a delta of 807 and a standard deviation of 624 for significance levels of 0.05, with a two-sided alternative resulting in a sample size of 10.44 per group. For IL-18 the power was also set to 0.8, a delta of 75.5 and a standard deviation of 96.53 for significance levels of 0.05 with a two-sided alternative resulting in a sample size of 26.66 per group. Finally, for CRP the power was also set to 0.8, a delta of 8,353,147 and a standard deviation of 9,329,045 for significance levels of 0.05 with a two-sided alternative resulting in a sample size of 20.59 per group. Normality was tested using the D'Agostino & Pearson omnibus and Shapiro-Wilk normality tests. Differences between groups were determined using the Mann-Whitney two-tailed test for non-normally distributed data and a two-tailed *t*-test for data that were normally distributed. The *P* value of significance was set at $P < 0.05$.

Regression Analyses

Linear regression analysis to explain the protein levels of IL-18 using ASC and CRP as the variables were run using RStudio/RMarkdown. After identifying the best lambda by a BoxCox transformation, data sets were transformed using a logarithmic transformation for ASC and IL-18 to normalize the distribution of the data. CRP was not logarithmically transformed since upon testing, after such transformation the adjusted R^2 was lower for the transformed CRP data. Univariate linear regression models were fit for IL-18 ~ ASC and IL-18 ~ CRP. An adjusted r^2 value was obtained to determine the approximate contribution of ASC and/or CRP to IL-18 protein levels. The Durbin Watson (DW) statistic was used to test for autocorrelation. The analytes that had a DW statistic *P* value > 0.05 were then used to fit a forward stepwise multivariate logistic regression model based on the lowest AIC. The final model was then further evaluated by residual analysis.

In addition, a binomial logistic regression analyses of the probability of a patient having a diagnosis of AMD as determined by the protein levels of ASC, IL-18 and CRP, as well as a comorbidity of HTN, diabetes, hyperlipidemia (HLD), hypercholesterolemia and diabetes were run using RStudio/RMarkdown. Discrimination and goodness of fit was tested through the AUC, the Akaike information criterion (AIC), the Bayesian information criterion (BIC), the McFadden-pseudoR^2 and the Hosmer–Lemeshow goodness-of-fit test. A multivariate binomial logistic model was chosen for variables with variables that univariately showed an AUC > 0.65 and a Hosmer-Lemeshow *P* value > 0.05. A potential multivariate model was tested using a stepwise regression based on the lowest AIC.

The following *R* packages were used in these analyses: ggplot2, MASS, dplyr, broom, car, regclass, ROCit, blorr, pscl, ResourceSelection, caret, and pROC.

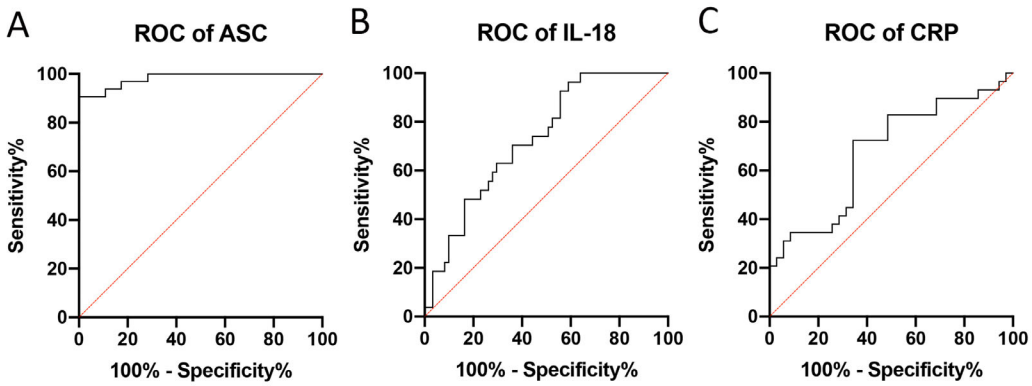

Figure 2. ROC curves for ASC (**A**), IL-18 (**B**) and CRP (**C**) from serum samples of AMD patients and healthy donors.ASC: N = 46 control, 32AMD.IL-18: N = 61 control, 27 AMD.CRP: N = 35 control, N = 29 AMD.

Table 2. ROC Analysis

Biomarker	Area	Std. Error	95% C.I.	*P* Value
ASC	0.9823	0.01183	0.9592 to 1.000	<0.0001
IL-18	0.7286	0.05420	0.6224 to 0.8348	0.0007
CRP	0.6749	0.06864	0.5403 to 0.8094	0.0167

Results

ASC, IL-18 and CRP are Elevated in the Serum of Patients with AMD

Serum samples from patients with AMD and aged-matched healthy donors were analyzed for the protein expression levels of ASC (Fig. 1A), IL-18 (Fig. 1B), and CRP (Fig. 1C). ASC ($P < 0.0001$), IL-18 ($P = 0.0009$), and CRP ($P = 0.0162$) proteins were significantly higher in the AMD group when compared with the control group. These findings suggest that in human ASC and IL-18 do in fact play a role in the pathology of AMD, which corroborates previous findings showing elevated levels of CRP in patients with AMD.[17,18]

ASC as a Biomarker of AMD

To determine whether inflammasome signaling proteins are reliable biomarkers of AMD, the AUC was calculated for ASC (Fig. 2A), IL-18 (Fig. 2B), and CRP (Fig. 2C). Accordingly, ASC had the highest AUC of 0.9823 ($P < 0.0001$). IL-18 had an AUC of 0.7286 ($P = 0.0007$), and CRP had an AUC of 0.6749 ($P = 0.0167$) (Table 2). Moreover, ASC had a cutoff point of 365.6 pg/mL with 94% sensitivity and 89% specificity (Table 3). Comparatively, the cutoff point for IL-18 was 242.4 pg/mL, with a sensitivity of 74% and a specificity of 56%, and CRP had a cutoff point at 8,684,152 pg/mL with 72% sensitivity and 66% specificity (Table 3).

Linear Regression Analysis

A linear regression analysis was run to determine the relationship between ASC and CRP with the protein levels of IL-18. Two univariate models were fit using each analyte that showed statistically significant difference ($P < 0.05$) between controls and AMD patients (ASC and CRP) (Fig. 1) to explain the protein levels of IL-18. Both analytes presented Durbin Watson (DW) statistics with P values > 0.05 (Table 4). Then the analytes with DW statistics P values > 0.05 were used to fit a multivariate linear regression model using a stepwise forward approach in combination with HTN as another variable ($\log(\text{IL-18}) \sim \log(\text{ASC}) + \text{CRP} + \text{HTN}$). Accordingly, results from the stepwise regression resulted in the univariate model $\log(\text{IL-18}) \sim \log(\text{ASC})$ (Fig. 3, Supplementary Fig. 1) with an adjusted R^2 of 0.1906, indicating that approximately 19% of the protein levels of IL-18 are explained by the levels of ASC in serum, with the remaining being due to other proteins that were not included in this statistical model.

Logistic Regression Analysis

To predict the probability that protein levels of ASC, IL-18, CRP and the comorbidities (HTN, HLD, Hypercholesterolemia and Diabetes) contribute or not to the pathology of AMD, seven univariate models were tested for ASC, IL-18, CRP, and the comorbidities. ASC, IL-18, CRP, and HTN presented AUC values > 0.65 (ASC (0.98), IL-18 (0.70), CRP (0.67), and HTN (0.81)) and a Hosmer-Lemeshow P value > 0.05 (ASC (0.99), IL-18 (0.15), CRP (0.14) and

Table 3. Cutoff Point in Serum of AMD Patients

Biomarker	Cutoff Point (pg/mL)	Sensitivity (%)	Specificity (%)	LR	PPV (%)	NPV (%)	Accuracy (%)
ASC	>365.6	94	89	8.625	86	95	91
IL-18	>242.4	74	56	1.674	43	83	61
CRP	>8,684,152	72	66	2.112	64	74	69

Table 4. Univariate Analysis for Linear Regression

Analyte	Log(IL-18) ~ Log(ASC)	IL18 ~ CRP
Intercept (β_o)	3.79700	2.393e+02
SE	0.41625	4.022e+01
Significance	7.80e-14	1.35e-07
Coefficient (β_1)	0.30192	6.879e-06
SE	0.06902	2.159e-06
Significance	3.82e-05	0.00225
Multiple R^2	0.2011	0.1407
Adjusted R^2	0.1906	0.1269
Durbin-Watson test *P* value	0.506	0.156

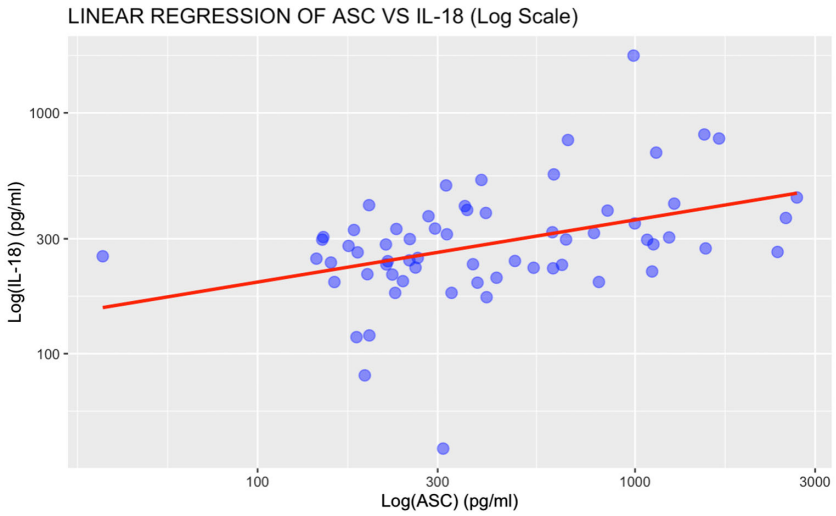

Figure 3. Linear regression plot of ASC vs IL-18.

HTN (0.99)) (Table 5). Moreover, the estimate for HTN ($P = 0.993$), HLD ($P = 0.993$), hypercholesterolemia ($P = 0.992$), and diabetes ($P = 0.990$) showed no significant values below 0.05. Therefore ASC, IL-18, and CRP were used to fit a multivariate logistic model using a forward stepwise regression, but the model obtained was the univariate model of AMD ~ ASC with an accuracy of 95%. Taken together, the odds of developing AMD increased with increased protein levels of ASC, IL-18, and CRP in serum. However, of the analytes studied, ASC is the best analyte associated with the AMD diagnosis.

Discussion

In this study, we aimed at studying inflammatory fluid biomarkers in the serum of patients with AMD. Our findings indicate that the inflammasome proteins ASC and IL-18 are reliable inflammatory biomarkers associated with the diagnosis of AMD. Accordingly, in comparison to age-matched healthy donors, ASC and IL-18 were significantly elevated in the serum of AMD patients. Similarly, the protein levels of CRP were also increased in the AMD cohort when compared

Table 5. Univariate Analysis

Analyte	ASC	IL-18	CRP	HTN
Intercept (β_o)	−8.436487	−2.022562	−9.624e-01	−1.1575
SE	2.576895	0.753904	4.012e-01	0.3457
Significance	0.00108	0.0073	0.0164	0.000812
Coefficient (β_1)	0.020634	0.005869	5.718e-08	20.7235
SE	0.006912	0.002372	2.315e-08	2534.7452
Significance	0.00283	0.0133	0.0135	0.993477
AUC	0.9793	0.6956	0.6749	0.8103
McFadden Psuedo-R^2	0.761832	0.1174	0.0814	0.4259
Hosmer-Lemeshow *P* value	0.9969	0.1542	0.1453	0.9999
AIC	24.997	81.809	84.976	54.607
BIC	29.314	86.127	89.294	58.925

to the control group. Importantly, the AUC for ASC (AUC = 0.982) provides argument for ASC being a strong biomarker in AMD. Previous studies have shown potential roles of cytokines in AMD, supporting the idea that the pathology of the disease is driven by inflammation. For instance, patients undergoing anti-VEGF therapy with elevated serum levels of IL-18, tumor necrosis factor (TNF) and IL-17 were found to have better long-term outcomes.[20,21] Thus supporting the view that inflammation is a principal contributor to the pathology of AMD. In addition, the acute phase protein CRP which has been associated with AMD pathology[22] was also elevated in the AMD group.

There are two forms of AMD. The most common form of AMD is dry macular degeneration, which is characterized by the presence of insoluble extracellular aggregates or drusen in the macula. Drusen affect the retinal pigmented epithelium (RPE) and the photoreceptor layer, and when advanced, these aggregates may lead to RPE atrophy, resulting in severe vision loss. The less-common form of AMD is wet macular degeneration, which is characterized by choroidal neovascular membranes and if left untreated may rapidly progress to blindness.[23] Importantly, although there is an overlap between both forms of AMD, it is important to differentiate the forms beyond the presence of choroidal neovascularization (CNV). Treatments effective for wet AMD, such as intravitreal anti-VEGF injections are not effective for the dry form of the disease; hence, highlighting the importance for a deeper understanding at the mechanistic, therapeutic, and diagnostic levels of AMD.

Several studies have shown a strong link between the inflammasome and AMD.[24–28] Accordingly, the inflammasome has been described to contribute to dysfunction in RPE cells.[25] In addition, inflammasome inhibition decreases the pathological effects of VEGF-induced AMD in mice.[27]

Recent evidence suggests that AMD may not be a disease limited to the eye. For instance, AMD has been associated with obesity, dyslipidemia, and HTN.[29] Moreover, patients with AMD seem to be at a higher risk of developing stroke, dementia, and kidney disease,[13] further highlighting the systemic involvement of AMD. Therefore measurement of inflammasome proteins as inflammatory markers in a cohort powered for the diagnosis of AMD offers the potential that ASC and IL-18 are capable of serving as useful biomarkers of the inflammatory response associated with AMD as determined by the high AUC values of 0.98 for ASC and 0.73 for IL-18. Consequently, future studies will aim to determine how the protein levels of ASC and IL-18 are affected in response to intravitreal anti-VEGF therapies in patients with wet AMD. Considering these findings, we hypothesize that increased levels of ASC and IL-18 are released[30] by cells in the retina such as retinal pigmented epithelial cells that are undergoing inflammasome activation as a result of oxidative stress, which is a known contributor to macular degeneration.[31] However, it is possible that a systemic inflammatory response is present and that a genetic predisposition or environmental susceptibility/stress such as smoking or diet[32] makes these patients prone to have ocular manifestations of a systemic disease that we refer to as AMD.

Furthermore, elevated protein levels of CRP in blood correlate with disease progression in AMD patients. Accordingly, CRP levels of less than 0.5 mg/L correspond to a low risk of disease progression, and CRP levels greater than 10 mg/L correspond to a higher risk of disease progression. [22,33–35]

We have recently shown that inflammasome proteins are good inflammatory biomarkers of traumatic brain

injury,[16,36] stroke,[15] psoriasis,[37] and multiple sclerosis,[14] as well as mild cognitive impairment (MCI) and AD.[38] Interestingly, it has been suggested that AMD and AD share a common pathologic mechanism.[39,40] Certainly, both conditions are associated with aging and protein aggregations like drusen and amyloid-β, respectively. Moreover, recently it has been shown that AMD and AD share nine molecular pathways and 63 biological processes, as well as 10 affected genes on chromosome 7.[41] However, histopathological findings have failed to show a positive correlation between AMD and AD.[42] Thus, although a stronger link is yet to be identified between AMD and AD, here we have found that the median serum levels of ASC in AMD patients was 918 pg/ml (range, 283 and 2684 pg/mL), whereas we have previously shown that the median serum levels of ASC in MCI was 681 pg/mL (range, 267 to 1616 pg/mL),[38] and for AD the median ASC levels in serum was 452 pg/mL (range, 157 to 1205 pg/mL).[38] Therefore serum levels of ASC are higher in AMD than in MCI patients, and the protein levels of ASC are higher in MCI than in AD patients. Thus suggesting that the levels of ASC start to decrease in serum with increased AD pathology (from MCI to AD) and yet whether AMD represents an early sign or comorbidity of AD pathogenesis before or associated with MCI remains a premature speculation. Certainly, there is evidence that decreased amyloid-β in tissue fluids, namely cerebrospinal fluid, is consistent with accumulation of amyloid-β in the brain. However, whether a similar effect occurs for ASC is yet to be tested.

Taken together, here we extend this knowledge to AMD. We suggest that ASC and IL-18 can be used as part of a platform of biomarkers that in conjunction with other proteins can be used to better diagnose and prognose AMD since here we show that ASC and IL-18 are associated with the diagnosis of AMD. Linear regression analysis between ASC and the pro-inflammatory cytokine IL-18 shows that 19% of IL-18 present in the serum of AMD patients is due to levels of ASC. This suggests that nearly a quarter of IL-18 can be accounted for as a result of ASC-dependent inflammasome activation, with other signaling pathways not included in this study being responsible for the remainder of IL-18 present in blood. The production of IL-18 is not completely dependent on inflammasome activation, because IL-18 has been shown to be expressed constitutively at basal levels.[43] In addition, the suppression of VEGF has been demonstrated to show a distinct increase in IL-18 levels.[21] Moreover, logistic regression analysis suggests that ASC is significantly associated with the pathology of AMD.

AMD is responsible for 8.7% of legal blindness around the world.[44,45] The early stages of AMD are devoid of symptoms, making the necessary early diagnosis challenging.[23] Studies indicate the necessity of starting treatments such as anti-VEGF as soon as possible after the detection of signs and visual symptoms in patients with wet AMD.[46] However, early detection of the predevelopment of CNV and AMD may be difficult and atypical outside of controlled research settings.[47] Thus the development of more effective screening technologies to diagnose AMD before symptoms appear or in earlier stages of the disease would greatly improve outcomes for sufferers of the disease.

The following study is limited in that patients have not been stratified with regard to how severe the macular degeneration is. Therefore it is important to understand how inflammasome protein expression is altered in response to anti-VEGF therapies and to understand how the presence of drusen in different dry AMD severities contributes to inflammasome expression changes, and how macular edema correlates with inflammasome protein expression. Such findings offer the promise of potential monitoring biomarkers that can be used to evaluate response to treatment in AMD patients. Thus future studies should aim at identifying differences in inflammasome signaling proteins between patients with wet AMD and those with the dry form of the disease. Importantly, more studies are needed to understand the relationship between AMD and systemic inflammation, for this study and previous work highlights that AMD is not just a disease limited to eye.[13]

Conclusions

Here we show that ASC and IL-18 are contributors to the pathology of AMD and that these two proteins are good candidates as inflammatory biomarkers of the disease. Future studies will look at inflammasome signaling proteins at different stages of the disease for the wet and dry forms of AMD.

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