Identification of Urinary Biomarkers for Age-Related Macular Degeneration

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PURPOSE. Age-related macular degeneration (AMD) can be considered as a chronic low-grade systemic inflammatory disease. This study was undertaken to test the associations of AMD with the urinary proinflammatory cytokines transforming growth factor (TGF)-β1, macrophage chemoattractant protein (MCP)-1 and C3a-desArg, as potential noninvasive biomarkers for monitoring AMD.

METHODS. A cross-sectional study of 103 AMD cases, comprising early AMD ($n = 51$), geographic atrophy (GA; $n = 19$), or choroidal neovascularization (CNV; 33), and 54 unrelated controls, aged 73 ± 9 years, who attended the Royal Victorian Eye and Ear Hospital and private practice in Victoria, Australia. AMD status was determined from the bilateral retinal digital photographs and through angiography and optical coherence tomography images when confirmation of CNV was needed. Serum and urine cytokine levels were measured by immunoassay and the rs1061170 (Y402H) single-nucleotide polymorphism of the complement factor H (*CFH*) gene was determined.

RESULTS. Multivariate logistic regression analyses demonstrated significant associations of urinary TGF-β1 levels (odds ratio [95% confidence interval]: OR = 1.24 [1.02–1.50]; $P < 0.031$) and MCP-1 levels (OR = 1.07 [1.02–1.12]; $P < 0.008$), in early AMD, and also MCP-1 levels with GA (OR = 1.10 [1.03–1.17]; $P < 0.003$). There was no correlation between urinary and serum cytokine levels. Individuals with one or more copies of the *C* allele (Y402H) were 2.5 times more likely to have urinary MCP-1 above median levels ($P < 0.040$).

CONCLUSIONS. This study demonstrates a novel finding of an association between elevated urinary cytokines TGF-β1 and MCP-1 and AMD. Further development of a urinary biomarker profile could provide a practical tool for detection of early AMD, progression monitoring, and assessment of treatment efficacy. (*Invest Ophthalmol Vis Sci.* 2011;52:4639–4644) DOI:10.1167/iovs.10-7120

Recent advances in the treatment of choroidal neovascularization (CNV) in age-related macular degeneration (AMD) have dramatically improved the outcome in this late-stage complication. However, there is still no effective treatment for geographic atrophy (GA), and apart from general lifestyle advice, there are no specific interventions to slow or reverse progression from early AMD to late stages of the disease. Advances in these areas are severely hampered by a lack of our ability to monitor progression of early disease, to predict risk of progression to late vision-threatening stages, or to assess the efficacy of treatment in this common, devastating disease.

Over the past decade, an accumulation of evidence has implicated the complement cascade and immune mechanisms in AMD and has led to a general consensus that inflammation is a key driver to the development of AMD.[1–5] As in several other chronic diseases of aging such as Alzheimer's disease, it is postulated that AMD is the result of an ongoing low-grade chronic inflammatory process that results in tissue damage at a local level, which in the retina can result in the breakdown of the blood–retina barrier and the release of circulating inflammatory mediators.[3,5] This finding has led to several studies that investigated serum levels of systemic markers of inflammation in AMD and their use as possible indicators of the risk of developing AMD. These markers have included C-reactive protein (CRP), interleukin (IL)-6, interleukin (IL)-2, tumor necrosis factor (TNF)-α, soluble intercellular adhesion molecule (sICAM)-1 and C3a-desArg, where higher levels were found by some to be associated with AMD in comparison to controls.[6–11] It has also been suggested that those with more advanced AMD, have higher systemic levels of inflammatory markers, compared with those who do not; however, others have failed to show such an incremental association.[6–8]

Major disadvantages of using serum as a source of potential biomarkers relate to its wide range in protein abundance levels and the difficulty in recognizing small relative changes in levels. A novel approach taken in other nonrenal diseases has been to include the analysis of urine because of its relative abundance, ease of collection, relative stability, and fewer proteins compared with serum.[12–15]

Furthermore, there are other findings that suggest that the kidney itself may be affected in AMD and as such could reveal a distinct pattern of proteins that would not necessarily be reflected systemically in the serum. The renal glomeruli share structural similarity to the choroidal vasculature complex, and there are several examples of kidney disease that affect the retina.[16,17] The rare kidney disease membranoproliferative glo-

merulonephritis type II (MPGN Type II) is associated with variants in the complement factor H (*CFH*) gene, the gene implicated in AMD, and along with dense deposition of substance within the glomerular capillary walls, there are also drusenoid deposits in the macula.[2,17,18] Evidence of kidney involvement in AMD has also come from the Blue Mountains Eye Study (BMES), which reported reduced renal function in AMD cases, with a decreased estimated glomerular filtration rate (eGFR) and creatinine clearance when compared with age-matched controls.[19] This suggests the possibility that AMD indeed is manifestation of a systemic disease that includes mild abnormalities in renal function. The possibility of renal involvement in AMD added weight to the potential for urine to be a source of unique profiles of proinflammatory cytokines.

In this study, we investigated the level of urinary proinflammatory cytokines in subjects with AMD and control participants. Specifically, both transforming growth factor (TGF)-β1 and monocyte chemoattractant protein (MCP)-1 are proinflammatory cytokines, which are upregulated in response to tissue injury, have been implicated in AMD, and are involved in the inflammatory responses in the kidney.[20,21] These, along with C3a-desArg, a breakdown product of the complement cascade that reflects the degree of inflammation and is known to be involved in AMD,[22] were measured.

METHODS

Participants in this cross-sectional study, were recruited from July 2007 to November 2009 from the Royal Victorian Eye and Ear Hospital (RVEEH) Medical Retinal Outpatient Clinic (Melbourne, Australia) and from private practice and were assessed for their AMD status and urinary cytokine levels.

Ethics approval was obtained from the RVEEH Human Research and Ethics Committee (95/283H/05). The project adhered to the tenets of the Declaration of Helsinki, and all subjects and controls had to read a plain language statement and provide written informed consent.

After giving informed consent, the patients underwent a standard health interview, with particular attention to any history of renal or systemic inflammatory disease, as well as treated hypertension. The inclusion criteria were the diagnosis of early or late AMD for cases and the absence of AMD for controls (<10 hard drusen seen on the retinal image). Patients with any co-existing retinal disease, other ocular diseases, known renal disease, uncontrolled hypertension, any systemic inflammatory disease, poor command of English, and low cognitive function were excluded from the study.

Body measurements (blood pressure [BP], height, weight, waist and hip circumferences) were taken, and body mass index was calculated. Three consecutive BP measurements were taken, and the lowest was recorded.

An ocular examination included dilated slit lamp examination and digital fundus photography (C6-45NM; Canon, Tokyo, Japan). Fundus fluorescein angiography (FFA) and optical coherence tomography (OCT) were used to confirm cases of CNV. Each image was graded based on the international classification and grading system for AMD.[23] The following categories of AMD severity were used to classify AMD cases in the present study: early AMD, drusen >63 μm in diameter; GA, late AMD, with a sharply delineated area of hypopigmentation >175 μm in diameter, in which choroidal vessels can be visualized; and CNV, late AMD, with choroidal neovascularization secondary to AMD, based on a medical retinal consultant's interpretation of the FFA and OCT. Once graded, patients were categorized into one of the four categories, control, early AMD, GA, or CNV. If both GA and CNV were present, they were categorized in the CNV group. Unrelated volunteers were used as control subjects if their fundus photographs were graded with <10 hard drusen.

Venous blood samples were collected, for DNA extraction, standard hematology and biochemistry analysis, including serum creatinine for subsequent eGFR determination, with the remainder centrifuged

within half an hour of collection, at 2000g at 4°C for 10 minutes and then stored in a −80°C freezer. Midstream urine samples of 50 mL each were also collected and centrifuged within 1 hour after collection at 1200g at 8°C for 10 minutes and stored in a −80°C freezer. The frozen urine was thawed as required in a −20°C freezer overnight and then in a 4°C refrigerator. Urine samples were concentrated using centrifugal 10-kDa filter devices (Ultra-4; Amicon; Millipore Australia Pty Ltd., Kilsyth, VIC, Australia) at 4000g at 25°C for 10 minutes. Urinary protein levels of TGF-β1, MCP-1, and C3a-desArge were strictly measured, in duplicate by ELISA assays (Quantikine Human TGF-β1 ELISA kits; R&D Systems, Inc. Minneapolis, MN; Human MCP-1 ELISA Development Kit; PromoCell, Heidelberg, Germany; BD OptEIA Human C3a ELISA Kit; BD Biosciences, San Diego, CA). The urinary levels of each cytokine were standardized to the urinary creatinine levels measured using Jaffe's method.

Genomic DNA was isolated from venous blood leukocytes using a standard phenol/chloroform extraction procedure. Genotyping was performed after DNA amplification (MassARRAY platform; Sequenom, San Diego, CA) through the Australian Genome Research Facility (Brisbane, QLD, Australia), as previously described.[24] Results were validated by performing unidirectional di-deoxy sequencing on a representative set of clinical samples.[24]

Covariates assessed for inclusion in a multivariate model of the analysis included: age in years, renal function measured by eGFR as a binary variable with the cutoff at 60 mL/min/1.73 m^2 (this level has been used in clinical practice for the diagnosis of chronic kidney disease), sex, treated hypertension, smoking, family history of AMD, and use of angiotensin converting enzyme inhibitors (ACEis) or angiotensin receptor blockers (ARBs), which are known to have the potential to affect renal function. Age as a continuous variable, treated hypertension, eGFR below cutoff point, and the use of ACEi or ARB, as binary variables remained associated with outcome in multivariate multinomial logistic regression analysis (SPSS; IBM, New York, NY). The group of control participants was used as the reference. In addition, logistic regression analysis, adjusted for age, was used to test the associations between the presence of the AMD risk-conferring *C* allele (Y402H) in the *CFH* gene and elevated above median urinary protein levels.

RESULTS

A total of 157 subjects were recruited into the study, comprising 103 AMD cases and 54 controls. The subjects were aged between 42 and 89 years, with a mean age of 73 years (SD 9.34). The cases consisted of 51 early AMD, 19 GA, and 33 CNV patients. Characteristics of the study groups are summarized in Table 1.

In univariate logistic regression analysis, age was significantly associated with late AMD ($P < 0.001$ for GA; $P < 0.001$ for CNV), and treated hypertension was significantly associated with CNV ($P < 0.049$). No other covariates, including measured blood pressure, were associated with any of the AMD subtypes. Although in the multivariate analyses of associations between urinary cytokine levels and risk of AMD, age remained the only covariate associated with outcome, we included treated hypertension, eGFR level as a measure of renal function and the use of ACEi or ARB in the final regression model, as they could potentially affect the levels of predictors and their influence on outcomes.

The distributions of urinary TGF-β1, MCP-1, and C3a-desArg levels had a positive skew and contained 0 values (Figs. 1, 2, 3); therefore, logistic regression analysis was applied to assess the strength of the associations of each urinary cytokine level with AMD status. The urinary TGF-β1 and MCP-1 levels were presented as continuous variables, in 10-ng/mmol units. The urinary C3a-desArg levels were also presented as a continuous variable, in micrograms per millimole.

TABLE 1. Characteristics of the Study Participants

Characteristics	Control	Early AMD	GA	CNV
Subjecs, *n*	54	51	19	33
Age, y	69 (53, 89)	74 (42, 83)	82 (68, 88)	78 (57, 87)
Female	30/54 (56)	36/51 (71)	13/19 (68)	21/33 (64)
eGFR, <60 mL/min/1.73 m^2	8/54 (15)	8/51 (16)	6/19 (32)	9/33 (27)
Treated hypertension	21/54 (39)	22/50 (44)	11/19 (58)	19/31 (61)
Smoking history	17/48 (35)	21/44 (48)	7/17 (41)	13/30 (43)
Family history of AMD	12/44 (27)	16/44 (36)	6/19 (32)	6/27 (22)
Use of ACEi/ARB	16/54 (30)	38/51 (75)	14/19 (74)	24/33 (73)

Data are the number (%) of subjects or median (min, max).

We found a significant association between "any AMD," which included early AMD, GA, and CNV, and elevated urinary MCP-1 levels ($P < 0.02$), but not between any AMD and the levels of urinary TGF-β1 ($P < 0.062$) or C3a-desArg ($P < 0.99$; Table 2).

In the analysis of associations of these biomarkers with different stages of AMD, we found a statistically significant 24% increased odds for early AMD per 10 ng/mmol increase in urinary TGF-β1 levels ($P < 0.031$), adjusted for age, eGFR levels, and the use of ACEis or ARBs (Table 2). The increases in the odds ratios (ORs) for either GA or CNV did not reach significance.

Furthermore, results revealed significant associations between elevated urinary MCP-1 levels and both early AMD ($P < 0.008$) and GA ($P < 0.003$) after adjustment for age, renal eGFR levels, and the use of ACEis or ARBs.

Thus, there was a 7% increase in the odds for early AMD and 10% increase in the odds for GA per 10-ng/mmol increase in the urinary level of MCP-1 (Table 2).

No association between urinary C3a-desArg levels and any of the three AMD subtypes was detected ($P < 0.983$ for early AMD, $P < 0.162$ for GA, and $P < 0.105$ for CNV). There was no correlation between any of the three urinary protein levels and their corresponding serum levels ($P < 0.057$, $P < 0.589$, and $P < 0.953$ for TGF-β1, MCP-1, and C3a-desArg levels, respectively).

Urinary cytokine excretion results were further analyzed according to the allelic status of the *Y402H* variant of the *CFH* gene, available from 126 (82%) participants. The presence of at least one risk-conferring allele (C) of this SNP was significantly associated with elevated urinary cytokine levels of MCP-1, conferring twice the odds of having higher than median levels of urinary MCP-1 measurement than subjects who did not have any risk allele (OR = 2.47; $P < 0.040$; Table 3). In addition, patients who were heterozygous or homozygous for the risk allele of this SNP had a sixfold increase in the odds of having AMD compared with those who did not have the risk-conferring allele (OR = 6.05 [2.42–15.14]; $P < 0.001$) which is consistent with the literature.

DISCUSSION

This study represents a novel approach in the search for new biomarkers in AMD. It is the first to explore urinary cytokine excretion, with analysis of the data demonstrating a statistically
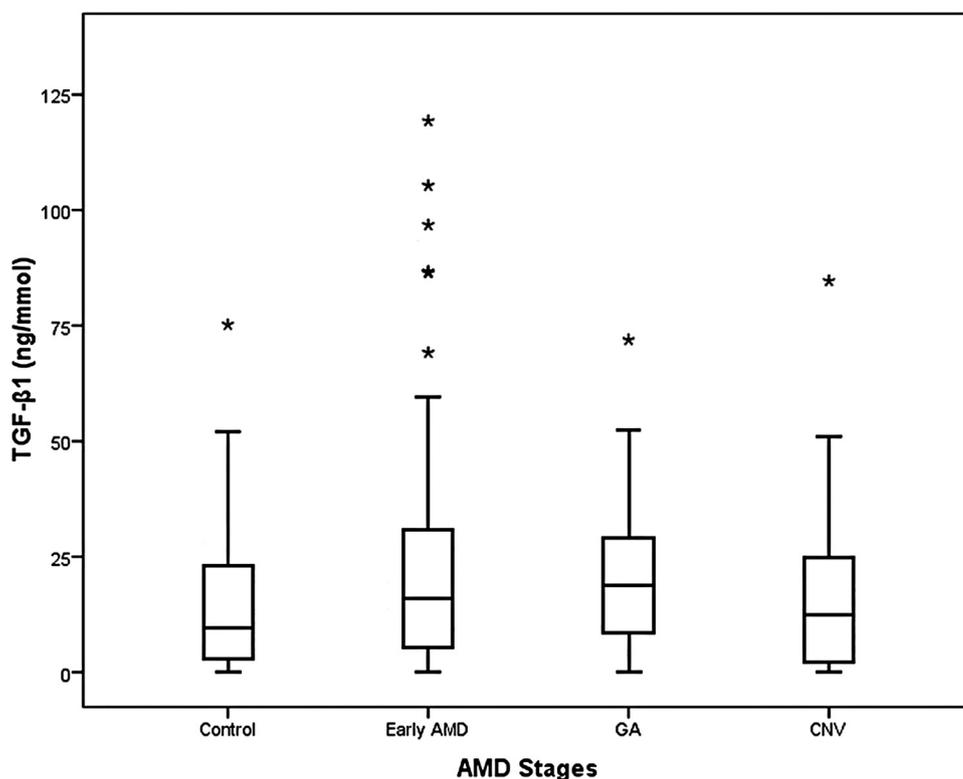

FIGURE 1. Urinary levels of TGF-β1. Box plots of data for urinary protein levels in the study groups with different stages of AMD. The length of the box is correspondent to the interquartile range (IQR), the difference between the 75th and 25th percentiles. The ends of the whiskers represent the lowest datum still within a 1.5 IQR of the lower quartile and the highest datum still within a 1.5 IQR of the upper quartile; *stars* beyond the whisker represent the outliers.
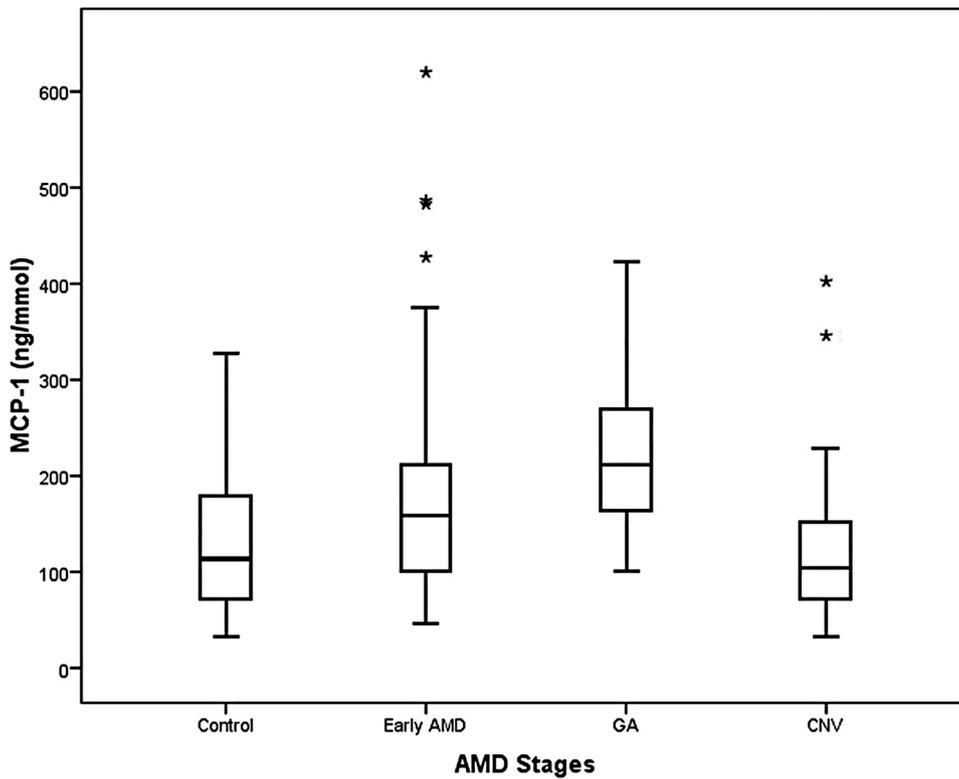
FIGURE 2. Urinary levels of MCP-1. The description of the plots is as in Figure 1.

significant association between levels of specific proinflammatory urinary cytokines and different stages of AMD, with urinary TGF-β1 levels being significantly elevated in early AMD and urinary MCP-1 levels being elevated in early AMD and GA.

A limitation to our study was the small sample in the GA group ($n = 19$). Having so few subjects may have restricted power in the analyses for TGF-β1 and C3a-desArg, both of which appeared to be positively correlated with disease, but not statistically significantly. The finding for MCP-1 was statistically significant, indicating that there was sufficient power for this analysis. Another limitation of our study was that the control group was younger than the GA and CV groups. (The
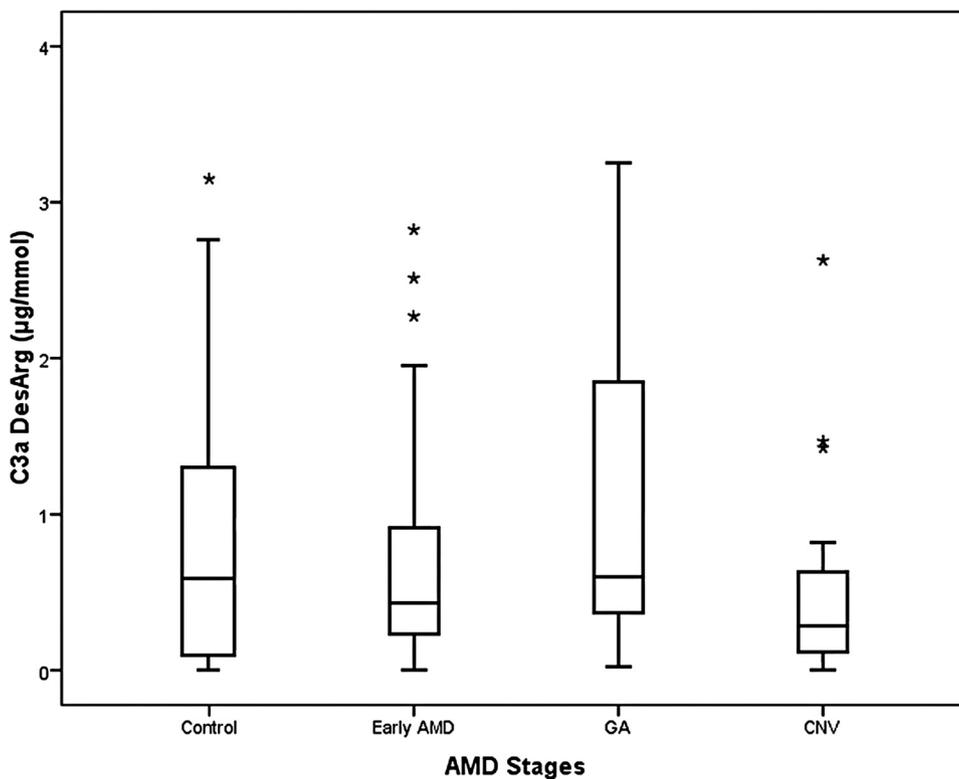

FIGURE 3. Urinary levels of C3a-desArg. The description of the plots is as in Figure 1.

TABLE 2. OR for the Association between AMD and the Increases in Urinary Cytokine Levels

Characteristics	AMD Subtypes			
	Early AMD OR (95% CI)	GA OR (95% CI)	CNV OR (95% CI)	Any AMD OR (95% CI)
TGF-β1 (10 ng/mmol)	**1.24 (1.02–1.50)**	1.16 (0.87–1.55)	1.04 (0.81–1.35)	1.19 (0.99–1.44)
MCP-1 (10 ng/mmol)	**1.07 (1.02–1.12)**	**1.10 (1.03–1.17)**	0.98 (0.92–1.05)	**1.05 (1.01–1.10)**
C3a-desArg (μg/mmol)	1.01 (0.62–1.62)	1.53 (0.85–2.75)	0.50 (0.22–1.16)	1.00 (0.66–1.51)

Data are adjusted for age, treated hypertension, renal function, and use of ACEi or ARB medications. Bold indicates statistically significant results.

age difference between the control and early AMD group was not statistically significant.) However, potential confounding by age was accounted for by inclusion of age in all multivariate analyses.

Participants with one or more copies of the AMD risk-conferring allele of the Y402H SNP of the *CFH* gene are considered to be predisposed to having a chronic low-grade inflammatory reaction, It was in these people that we found an association with higher urinary levels of TGF-β1 and MCP-1, as might be expected in those with an underlying background of inflammation.

TGF-β1 was found to be significantly elevated in the urine in early, but not late disease. It is known to play a pivotal role in the synthesis and deposition of extracellular matrix after renal tissue injury. Its elevation may reflect kidney changes early in the systemic inflammatory disease process. Similarly, MCP-1 was found to be significantly elevated in early AMD, but also in the late GA stage. Association between MCP-1 and AMD has been reported in a mouse model of AMD where the MCP-1 gene is knocked out alone or in association with its cognate CCR-2 receptor.[25] MCP-1 has also been reported to be upregulated in the retina of aged mice and in particular have increased expression in the retinal pigment epithelium.[5] Recently MCP-1 has also been reported to be elevated in the aqueous of patients with CNV secondary to AMD.[26] Urinary MCP-1 levels have also been used as potential biomarkers in late stage diabetic nephropathy[27] and ANCA-associated vasculitis.[28] We hypothesize that the increase in urinary MCP-1 level reflects local renal changes occurring in AMD, perhaps as a result of local inflammation. Findings of significant associations between elevated urinary TGF- β1 and MCP-1 levels and early AMD make these markers potentially very useful as biomarkers in monitoring early disease progression, and potential improvements with intervention. A marker of late disease would be of little benefit.

TABLE 3. OR for Subjects with Elevated Median Urinary Cytokine Levels According to *CFH* (Y402H) Gene Polymorphism

Urinary Cytokine/*CFH* Allelic Status	Elevated above Median Urinary Cytokine Levels	OR (95% CI)
TGF-β1		
TT	11/30 (37)	Reference
CT or CC	53/96 (55)	2.219 (0.915–4.954)
MCP-1		
TT	10/30 (33)	Reference
CT or CC	43/96 (45)	**2.465 (1.044–5.820)**
C3a desArg		
TT	15/30 (50)	Reference
CT or CC	47/96 (49)	1.043 (0.459–2.367)

$n = 126$. Data are number of subject/total group (% of total group) and are adjusted for age. Bold represents significant results.

Clearly the role of underlying renal pathology leading to excess proteinuria or impaired renal function is a major concern in any study aiming to determine the presence of urinary biomarkers in nonrenal disease. In this study, all samples measured had normal levels of urinary albuminuria. In addition, although we had sought to exclude major underlying renal disease, a number of the subjects tested had reduced renal function (eGFR < 60 mL/min), not an unexpected finding, given the age of our cohort and the prevalence of reduced GFR in the community as a whole. Despite the number of subjects with reduced GFR, the level of renal function bore no relation to the measured urinary cytokine, further adding to the possible utility of these cytokines as potential biomarkers for AMD. The same was true of the presence of treated hypertension, blood pressure measured at the time of urine collection, and drug therapy with ACEis or ARBs.

Of interest is the lack of correlation between the serum and urinary levels of any of the three cytokines measured. Further understanding of the mechanisms behind this disparity between urinary excretion and circulating levels in the serum may help in our understanding of the processes that occur in AMD. The complex nature of protein behavior may prevent accurate measurement of free or bound peptides in the serum. Higher urine levels could be a result of renal secretion, as part of a response to maintain homeostasis preventing elevated serum levels. It is possible that the kidney is also a site for local production of the cytokines, in keeping with the notion that AMD is a systemic disease, with renal response to systemic inflammation or local complement deposition, thus reflected in elevated levels of inflammatory factors in the urine but not in the serum. Whatever the underlying reason for the difference, it appears that urine may prove more useful in the search for biomarkers of AMD than serum. Detected differences in urinary biomarker levels between early disease and controls allows for the possibility of using them as potential biomarkers of disease and its progression or regression with interventions.

With the high prevalence and devastating consequences of AMD in our aging communities, we urgently need more easily available and effective tools to determine who is at risk of the disease and to monitor the progression from the common early stage to the more severe vision-threatening late stages and also to judge treatment efficacy when new strategies are implemented. Even more enticing is the potential to have a marker that could pick up disease before it becomes clinically apparent in those with a family history or carrying risk-associated genotypes. Identification of increased levels of proinflammatory cytokines in genetically predisposed individuals offers a promising prospect of targeted intervention well in advance of clinical disease manifestation, which may offer the best chance of preventing vision loss from AMD.

As with the development of any biomarker, the importance of robust longitudinal studies cannot be understated. This is of importance, not only to determine the utility of the biomarker in prediction of progression, but also to better understand the

pathogenesis of the disease. Furthermore, the cytokines targeted in this study were selected by referring to existing knowledge in the area. Given this initial demonstration of the potential utility of urinary peptide analysis as a marker of disease, broader proteomic analysis of urine of people with AMD may give rise to recognition of additional markers of disease and progression. The identification of a panel of urinary biomarkers would allow even greater utility in monitoring progression of diseases, predicting vision-threatening complications, and also measuring response to new treatments.

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