Methods for Testing Sera for Presence of Autoantibody Recognizing Human Macular Tissue Antigens in the Health ABC ARMA Study

Western Blotting:

Sera were assessed for reactivity against normal human tissue homogenates comprising neuroretina (nRet), retinal pigment epithelium (RPE), Bruch's membrane (BM) and choroid (Ch), harvested from the macular region of human donor eyes provided to us by the Mid-South Eye Bank. In brief, after resecting and removing the anterior segment of the donor eyes, the vitreous gel was gently removed, the remaining posterior pole were further dissected at the 3, 6, 9 and 12 o'clock meridians and 10 mm full-thickness punches were obtained from the macula of fresh donor eyes with a dermal biopsy punch (Miltex, York, PA). The isolated tissues from the macula areas were ground and sonicated. Due to the high content of collagen fibers in the BM/Ch layers, the BM/Ch/RPE macular punch was also subjected to bead beating before being pooled with the nRet macular punch. The homogenates from various donor eyes were pooled, and used as source of antigens for Western blots (WB).

Macular lysates (15 ug) were loaded into 12 % SDS-PAGE gel and resolved using a Mini-PROTEAN Tetra Cell apparatus (Bio-Rad Laboratories, Hercules, CA) run at 150 V for approximately 1.5 hrs. The proteins were then transferred to nitrocellulose membranes. Transfer was confirmed by visualization with Ponceau-S staining. The nitrocellulose membranes were cut into strips, blocked with 5% milk powder in 2% mixture of Tris-Buffered Saline and Tween 20 TBS-T for 1 hour, and incubated with human sera, diluted 1:150 in 2.5% milk in 2% TBS-T, overnight at 4ºC. Membranes were washed and incubated with a 1:30,000 dilution of goat anti-human IgG conjugated to horseradish peroxidase (Southern Biotech, Birmingham, AL), in 2.5% milk in 2% TBS-T, for 1 hr at room temperature. Membranes were washed after incubations with 2% TBS-T and 1x TBS for 10 minutes. Strips were then incubated for 2 min in the dark with approximately 400µl of Plus-ECL Enhanced Chemiluminescence Substrate (Pierce), transferred to an autoradiography cassette, and exposed to film in a dark room. Three films were generated for each blot with exposure times of 5, 15, or 30 sec. Films were then evaluated to determine the presence of bound Ab in a semi-quantitative manner and graded.

Immuno-reactivity Analysis:

WBs were graded by trained lab staff in a masked fashion for presence and intensity of reactive bands. The kilo-Dalton (kD) value for each band of reactivity was determined using measurements from the standard SDS-PAGE ladder (BioRad) run on the same gel. In each region of the ladder, 1 kD was calculated to correspond to a given distance in millimeters, thus the distance of each band from the nearest marker could be used to obtain an approximate molecular weight value. The presence or absence of reactivity, and its relative level of intensity were recorded in 2 kilo-Dalton intervals from 1 to 199 for each patient and control. These values were recorded in a database and statistical analysis was performed as described below. Once the molecular weights were determined, the relative intensity of reactivity was recorded using a 6-step (grades 0-5) classification. The method was developed and tested for repeatability in the PI's lab and based on a similar scale previously developed to assess other images (**Table 1**). This intensity scale for was constructed based on visual inspection across the three exposure levels.

Data analysis

We accounted for multiple comparisons using the Benjamini-Hochberg (B-H) multiple test correction and $p<0.05$. In brief, the B-H correction limits the type-I error, or false discovery rate (FDR). Starting from an initial $\alpha=0.05$ cut-off, the B-H method allows for application of sequentially more stringent α values for each of the comparisons made until the observed p-value exceeds the critical B-H value (which, for our dataset was 0.0065). This method, customarily utilized in contrasting large arrays of data, avoids the overcorrection (and, therefore, the risk of incurring a Type-II error) caused

when classical methods such as the Bonferroni correction as applied to exploratory datasets that require by design multiple comparisons. Two levels of preplanned analysis were performed by comparing the AMD and control dataset via unpaired t-test for samples with unequal variances and by constructing 2x2 tables and computing odds ratios (ORs), 95% confidence intervals (CI), χ^2 statistic, and p-values, for each significant IR band.

Table 1. Immunoreactivity classification criteria of western blotting bands exposed to chemi-luminescent substrate and imaged at 5, 15, 30 seconds (sec). Scores (0-5) represent IR intensity.

SCORE (0-5)	**IMMUNOREACTIVITY (IR)**	**EXPOSURE TIME**		
		5 sec	**15 sec**	**30 sec**
0	No reactivity	No bands	No bands	No bands
1	Trace positive	No bands	No bands	Faint band intensity
2	Weakly positive	No bands	Faint band intensity	Moderate band intensity
3	Moderately positive	Faint band intensity	Moderate band intensity	Strong band intensity [a]
4	Strongly positive	Moderate band intensity	Strong band intensity [a]	Greater intensity than at 15 sec [b]
5	Maximal reactivity	Strong band intensity [a]	Greater intensity than at 5 sec [b]	Greater intensity than at 15 sec [b]

[a] Approximately identical to strong heavy Ig chain reactivity seen around 50-kDa
[b] Typically saturating intensity seen at this exposure