Altered gene expression in dry age-related macular degeneration suggests early loss of choroidal endothelial cells

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Purpose: Age-related macular degeneration (AMD) is a major cause of blindness in developed countries. The molecular pathogenesis of early events in AMD is poorly understood. We investigated differential gene expression in samples of human retinal pigment epithelium (RPE) and choroid from early AMD and control maculas with exon-based arrays.

Methods: Gene expression levels in nine human donor eyes with early AMD and nine control human donor eyes were assessed using Affymetrix Human Exon ST 1.0 arrays. Two controls did not pass quality control and were removed. Differentially expressed genes were annotated using the Database for Annotation, Visualization and Integrated Discovery (DAVID), and gene set enrichment analysis (GSEA) was performed on RPE-specific and endothelium-associated gene sets. The complement factor H (CFH) genotype was also assessed, and differential expression was analyzed regarding high AMD risk (YH/HH) and low AMD risk (YY) genotypes.

Results: Seventy-five genes were identified as differentially expressed (raw p value < 0.01; ≥50% fold change, mean log expression level in AMD or control ≥ median of all average gene expression values); however, no genes were significant (adj. p value < 0.01) after correction for multiple hypothesis testing. Of 52 genes with decreased expression in AMD (fold change < 0.5; raw p value < 0.01), 18 genes were identified by DAVID analysis as associated with vision or neurologic processes. The GSEA of the RPE-associated and endothelium-associated genes revealed a significant decrease in genes typically expressed by endothelial cells in the early AMD group compared to controls, consistent with previous histologic and proteomic studies. Analysis of the CFH genotype indicated decreased expression of ADAMTS9 in eyes with high-risk genotypes (fold change = –2.61; raw p value = 0.0008).

Conclusions: GSEA results suggest that RPE transcripts are preserved or elevated in early AMD, concomitant with loss of endothelial cell marker expression. These results are consistent with the notion that choroidal endothelial cell dropout or dedifferentiation occurs early in the pathogenesis of AMD.

Age-related macular degeneration (AMD) is the leading cause of blindness among the elderly in developed countries [1]. AMD involves the progressive loss of photoreceptor cells from the macular region of the retina, resulting in impaired vision and, in advanced stages, blindness. At least three cell layers undergo changes in AMD, including the photoreceptor cells, retinal pigment epithelium (RPE), and choriocapillaris. The RPE regulates the activities of the photoreceptor cells and choriocapillaris. For example, RPE cells actively phagocytose photoreceptor outer segments, recycle vitamin A, shuttle debris from the photoreceptor cells to the bloodstream, and import glucose, oxygen, and other components to accommodate the high metabolic demands of the retina [2], in addition to providing trophic support to the choriocapillaris [3,4]. The choroid serves as a high-volume transportation courier, delivering nutrients to the RPE and accepting waste products for further processing elsewhere in the body. The preclinical and early stages of AMD are recognizable by increased formation of lipid-rich sub-RPE deposits termed drusen and altered RPE pigmentation [5,6].

The photoreceptor cells, RPE, and choriocapillaris endothelial cells form an interdependent complex. Injury or dysfunction in any of these layers leads to loss of the other two in several chorioretinal diseases. A more complete understanding of the early sequelae of events in AMD is necessary to guide new therapies. Numerous interdependent biologic processes have been implicated in the pathogenesis of AMD, including increased activity of the complement cascade, infiltration of cells mediating inflammatory responses, increased oxidative stress, and altered lipid metabolism [7,8]. Although RPE cells are typically viewed as the primary cells affected in AMD, changes in the microvasculature of the choroid (choriocapillaris) have also been reported in association with drusen, including dropout of vessels [9,10] and decreased blood flow [11]. In a subset of advanced AMD cases, choroidal neovascular membranes (CNVs) form as blood vessels from the choroid breach the RPE and proliferate either beneath the RPE or in the sub-retinal space. Expression

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### TABLE 1. DONOR INFORMATION.

| Sample | Batch | Gender | CFH genotype | Age | Cause of death                  | Age-related maculopathy                                                                 |
|--------|-------|--------|--------------|-----|---------------------------------|-----------------------------------------------------------------------------------------|
| ARM1   | A     | F      | HY           | 78  | Coronary artery disease         | RPE changes                                                                             |
| ARM2   | A     | F      | HY           | 80  | Intracerebral hemorrhage        | RPE changes                                                                             |
| ARM3   | A     | M      | HY           | 90  | Respiratory failure             | Macular drusen                                                                          |
| ARM4   | A     | F      | HY           | 91  | Pneumonia                       | Macular drusen                                                                          |
| ARM5   | A     | F      | YY           | 91  | Not available                   | RPE changes; neovascular membrane in contralateral eye                                   |
| ARM6   | A     | M      | HH           | 78  | Respiratory failure             | RPE changes                                                                             |
| ARM7   | B     | F      | YY           | 81  | Ischemic bowel                  | RPE changes                                                                             |
| ARM8   | B     | M      | HH           | 92  | Pneumonia                       | Numerous large drusen, no atrophy or exudate                                           |
| ARM9   | B     | M      | HH           | 77  | Not available                   | RPE changes                                                                             |
| CTRL1  | B     | M      | HY           | 93  | Cardiac arrest                  | Normal fundus exam <2 years                                                             |
| CTRL2  | A     | M      | YY           | 83  | Pneumonia                       | Normal fundus exam <2 years; large cup to disc ratio                                   |
| CTRL3* | B     | M      | HY           | 84  | Subdural hematoma               | Normal fundus exam >2 years                                                             |
| CTRL4  | B     | F      | HY           | 77  | Not available                   | Normal fundus exam but old records; normal gross appearance                             |
| CTRL5  | A     | M      | YY           | 81  | Respiratory failure             | Normal fundus exam <2 years                                                             |
| CTRL6  | A     | F      | HH           | 87  | Aortic stenosis                 | Normal fundus exam <2 years                                                             |
| CTRL7  | A     | M      | HY           | 77  | Renal failure                   | Normal fundus exam <2 years                                                             |
| CTRL8  | B     | F      | YY           | 83  | Not available                   | Normal fundus exam <2 years                                                             |
| CTRL9* | A     | M      | HY           | 77  | Brain tumor                     | Normal fundus exam <2 years                                                             |

Asterisks (*) denote samples that did not pass quality control metrics. Batch indicates the batch of array processing. RPE changes refer to regions where RPE is depigmented or hypopigmented [32].
of vascular endothelial growth factor (VEGF), a marker of hypoxia, has been implicated in the formation of CNVs [12]. In current medical practice, only after CNVs have appeared and photoreceptor cell death has occurred can therapeutic measures be taken to slow further vision loss [13].

Despite considerable progress in unraveling genetic risk factors for AMD, major challenges remain. The relationships between the biologic processes remain uncertain, and the initial molecular conditions driving development of AMD are poorly understood. Evaluating gene expression in early AMD, intermediate AMD, and advanced AMD is one approach to advancing exploration of these problems. The first large-scale study of gene expression in the AMD-affected retina and RPE and choroid tissue identified changes between various stages of AMD, including apoptotic and neovascular pathways in advanced AMD [14]. As part of a study examining the relationship between AMD and gene methylation, Hunter and colleagues examined gene expression in AMD and normal samples [15]. They found that expression of glutathione S-transferase isofrom mu1 (GSTM1) and mu5 (GSTM5), antioxidant isoenzymes, was reduced in the RPE and choroid of eyes affected by AMD compared to control eyes; this reduction was correlated with hypermethylation of the GSTM1 promoter [15]. However, only two of the samples were classified as early AMD, and analysis was performed without respect to AMD grade.

To further investigate the molecular events initiating AMD, we evaluated gene expression in early AMD and normal RPE and choroid tissues using exon-based microarrays (Affymetrix). Our analysis identified a statistically significant decrease in expression of endothelial genes in early AMD with preservation of RPE-specific transcripts, suggesting that vessel loss or dedifferentiation may precede advanced damage in AMD.

**METHODS**

**Tissue acquisition:** Donor eyes were obtained through the Iowa Lions Eye Bank (Iowa City, IA) after receiving informed consent in accordance with the tenets of the Declaration of Helsinki. Donor age, sex, cause of death, and diagnostic status are listed in Table 1. Eyes were dissected, and 6 mm punches
were taken from the macula, centered on the fovea centralis. Macular RPE and choroid were separated from the neural retina, flash frozen in liquid nitrogen, and stored at −80 °C. The neural retina was used for separate experiments. In this

Figure 2. Quality control plots generated by arrayQualityMetrics before and after removal of CTRL3 and CTRL9. A and B are a false color heatmaps indicating the distances between arrays, computed as the absolute mean distance between the data. C and D indicate the sum of distances computed for A and B. For C the outlier threshold was 7.14 (vertical bar), and for D the outlier threshold was 5.79 (vertical bar). When analyzing all 18 samples, both CTRL3 and CTRL9 were flagged as exceeding the outlier threshold (A and C). When CTRL3 and CTRL9 were omitted, only ARM7 exceeded the threshold (D). E and F are boxplots of the gene-level signal intensity distribution across the arrays. For E and F, the Kolmogorov-Smirnov statistic $K_a$ was calculated based on the distances between each individual array and the pooled distribution of all arrays. G and H show $K_a$ for each array with outlier thresholds (vertical bars) of 0.0509 and 0.049, respectively. Only CTRL3 was flagged by this metric (G).
manner, RNA was stabilized within 4 h of death. RNA was extracted from the frozen RPE and choroids using a commercially available kit (RNeasy Mini Kit; Qiagen, Valencia, CA).

**CFH risk-allele genotyping:** To characterize how the risk allele in complement factor H (CFH; single nucleotide polymorphism [SNP] rs1061170) affects gene expression in the RPE and choroid, genotyping was performed on DNA from either whole blood (collected in EDTA-coated tubes) or extraocular muscle. DNA was isolated using established methods with the Gentra system (Qiagen, Valencia, CA) or the Qiagen DNA Blood & Tissue Kit, for blood and muscle, respectively. Genotyping of this polymorphism was performed using TaqMan predesigned SNP genotyping assays (Applied Biosystems) in a high-throughput system (Fluidigm, San Francisco, CA).

**Microarray processing:** Exon expression levels of the RPE and choroid RNA were determined using Affymetrix GeneChip Human Exon 1.0 ST arrays processed in two batches at the University of Iowa DNA Facility. Samples were used essentially as previously described [16]. Briefly, single primer isothermal amplification was used to convert 25 ng total RNA to cDNA with the WT-Ovation Pico RNA Amplification System (NuGEN Technologies, San Carlos, CA). The resulting cDNA was purified using a Qiagen QIAquick PCR Purification column, converted to sense target (ST)-cDNA using the WT-Ovation Exon Module v1 (NuGEN Technologies), and purified once more. ST-cDNA was then fragmented (85 nt mean length), and the NuGEN FL-Ovation cDNA Biotin Module, v2 (NuGEN Technologies) was used to biotin-label the fragments according to the manufacturer’s guidelines. This product was combined with Affymetrix eukaryotic hybridization buffer (Affymetrix, Santa Clara, CA) and hybridized to Affymetrix Human Exon 1.0 ST arrays (Affymetrix). An Affymetrix Model 3000 scanner with 7G upgrade was used to scan the arrays. Data were collected with GeneChip operating software (ver. 1.4; Affymetrix) and saved as CEL files.

**Microarray analysis:**

**Preprocessing, expression analysis, and quality control**—To assess differential expression and alternative splicing, CEL files were normalized by robust multiarray average (RMA) [17] to facilitate comparisons across arrays using AltAnalyze (ver. 2.0.7 beta) with gene and transcript data from the Ensembl 65 database and human genome build Hg19 [18]. RMA, a common algorithm for processing microarray data, corrects for background fluorescence, normalizes data for comparison between arrays, and produces log₂-transformed estimates of probeset expression. Technical variation among microarrays is well known and often arises...
Table 2. Differentially expressed genes in early AMD versus control samples.

| Ensembl ID       | Symbol   | Mean AMD | Mean Control | Fold change | Raw p value | FDR adj. P value | Name                                                      |
|------------------|----------|----------|--------------|-------------|-------------|-----------------|----------------------------------------------------------|
| ENSG00000138207  | RBP4     | 9.01     | 7.92         | 2.12        | 0.0089      | 0.4149          | retinol binding protein 4, plasma                         |
| ENSG00000198759  | EGFL6    | 7.78     | 6.77         | 2.01        | 0.0089      | 0.4149          | EGF-like-domain, multiple 6                              |
| ENSG00000158201  | ABHD3    | 8.01     | 7.13         | 1.84        | 0.0044      | 0.3607          | abhydrolase domain containing 3                           |
| ENSG00000186480  | INSIG1   | 9.25     | 8.44         | 1.76        | 0.0024      | 0.3582          | insulin induced gene 1                                   |
| ENSG00000112072  | HMGCS1   | 8.39     | 7.58         | 1.76        | 0.0095      | 0.4149          | 3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)       |
| ENSG00000116791  | CRYZ     | 9.20     | 8.39         | 1.75        | 0.0002      | 0.2057          | crystallin, zeta (quinone reductase)                     |
| ENSG00000135324  | MRP2     | 7.41     | 6.61         | 1.75        | 0.0006      | 0.2515          | melanocortin 2 receptor accessory protein 2               |
| ENSG00000165124  | SVEP1    | 8.16     | 7.37         | 1.74        | 0.0052      | 0.3792          | sushi, von Willebrand factor type A, EGF and pentraxin    |
|                  |          |          |              |             |             |                 | domain containing 1                                        |
| ENSG00000149485  | FADS1    | 9.22     | 8.43         | 1.73        | 0.0035      | 0.3582          | fatty acid desaturase 1                                   |
| ENSG00000112394  | SLC16A10 | 7.43     | 6.67         | 1.70        | 0.0001      | 0.1976          | solute carrier family 16, member 10 (aromatic amino acid  |
|                  |          |          |              |             |             |                 | transporter)                                              |
| ENSG00000109452  | INPP4B   | 7.41     | 7.04         | 1.69        | 0.0031      | 0.3582          | inositol polyphosphate-4-phosphatase, type II, 105 kDa   |
| ENSG00000091137  | SLC26A4  | 7.80     | 8.04         | 1.69        | 0.0030      | 0.3582          | solute carrier family 26, member 4                        |
| ENSG00000134824  | FADS2    | 9.08     | 9.34         | 1.67        | 0.0089      | 0.4149          | fatty acid desaturase 2                                   |
| ENSG00000153790  | C7orf31  | 8.06     | 7.32         | 1.67        | 0.0089      | 0.4149          | chromosome 7 open reading frame 31                       |
| ENSG00000251606  | CTD-2215E18.1 | 8.28 | 7.55        | 1.66        | 0.0006      | 0.2515          | Uncharacterized protein                                  |
| ENSG00000178896  | EXOSC4   | 9.03     | 9.32         | 1.63        | 0.0039      | 0.3582          | exosome component 4                                       |
| ENSG00000125931  | CITED1   | 8.14     | 7.47         | 1.60        | 0.0013      | 0.3582          | Cbp/p300-interacting transactivator, with Glu/Asp-rich    |
|                  |          |          |              |             |             |                 | C-terminal domain, 1                                      |
| ENSG00000184270  | HIST2H2AB| 8.04     | 7.41         | 1.55        | 0.0034      | 0.3582          | histone cluster 2, H2ab                                    |
| ENSG00000121316  | PLBD1    | 7.40     | 6.77         | 1.54        | 0.0001      | 0.1976          | phospholipase B domain containing 1                      |
| ENSG00000134202  | GSTM3    | 8.04     | 7.42         | 1.53        | 0.0095      | 0.4149          | glutathione S-transferase mu 3 (brain)                    |
| ENSG00000165996  | PTPLA    | 7.81     | 7.21         | 1.51        | 0.0000      | 0.1157          | protein tyrosine phosphatase-like (proline instead of     |
|                  |          |          |              |             |             |                 | catalytic arginine), member A                             |
| ENSG00000147459  | DOCK5    | 7.25     | 6.65         | 1.51        | 0.0062      | 0.4088          | dedicator of cytokinesis 5                                |
| ENSG00000169084  | DHR5X    | 7.68     | 7.09         | 1.51        | 0.0035      | 0.3582          | dehydrogenase/reductase (SDR family) X-linked             |
| ENSG00000070961  | ATP2B1   | 7.75     | 8.37         | −1.53       | 0.0041      | 0.3582          | ATPase, Ca\(^{2+}\) transporting, plasma membrane 1      |
| ENSG00000186260  | MKL2     | 8.50     | 9.12         | −1.54       | 0.0038      | 0.3582          | MKL/myocardin-like 2                                       |
| ENSG00000125834  | STK35    | 7.66     | 8.29         | −1.54       | 0.0057      | 0.4062          | serine/threonine kinase 35                               |
| ENSG00000155749  | ALS2CR12 | 6.77     | 7.39         | −1.54       | 0.0004      | 0.2281          | amyotrophic lateral sclerosis 2 (juvenile) chromosome      |
|                  |          |          |              |             |             |                 | region, candidate 12                                       |
| ENSG00000115350  | POLE4    | 8.41     | 9.04         | −1.55       | 0.0028      | 0.3582          | polymerase (DNA-directed), epsilon 4 (p12 subunit)        |
| Ensembl ID     | Symbol     | Mean AMD | Mean Control | Fold change | Raw p value | FDR adj. P value | Name                                                                                      |
|---------------|------------|----------|--------------|-------------|-------------|------------------|-----------------------------------------------------------------------------------------|
| ENSG00000130559 | CAMSAP1    | 7.34     | 7.98         | −1.56       | 0.0028      | 0.3582           | calmodulin regulated spectrin-associated protein 1                                      |
| ENSG00000132932 | ATP8A2     | 6.46     | 7.13         | −1.59       | 0.0030      | 0.3582           | ATPase, aminophospholipid transporter, class I, type 8A, member 2                      |
| ENSG00000181004 | BBS12      | 7.07     | 7.75         | −1.60       | 0.0098      | 0.4191           | Bardet-Biedl syndrome 12                                                               |
| ENSG00000149294 | NCAM1      | 7.06     | 7.74         | −1.60       | 0.0068      | 0.4149           | neural cell adhesion molecule 1                                                      |
| ENSG0000254528  | RP11–728F11.4 | 7.98   | 8.67         | −1.62       | 0.0018      | 0.3582           |                                                                                          |
| ENSG00000156194 | PPEF2      | 7.19     | 7.89         | −1.63       | 0.0024      | 0.3582           | protein phosphatase, EF-hand calcium binding domain 2                                  |
| ENSG00000132026 | RTBDN      | 6.52     | 7.25         | −1.66       | 0.0032      | 0.3582           | retbindin                                                                                 |
| ENSG0000080511  | RDH8       | 6.82     | 7.56         | −1.66       | 0.0046      | 0.3582           | retinol dehydrogenase 8 (all-trans)                                                   |
| ENSG00000139220 | PPFIA2     | 6.46     | 7.21         | −1.68       | 0.0043      | 0.3582           | protein tyrosine phosphatase, receptor type, F polypeptide (PTPRF), interacting protein (liprin), alpha 2 |
| ENSG00000082126 | MPP4       | 7.27     | 8.02         | −1.68       | 0.0088      | 0.4149           | membrane protein, palmitoylated 4 (MAGUK p55 subfamily member 4)                    |
| ENSG00000153944 | MSI2       | 6.61     | 7.36         | −1.68       | 0.0064      | 0.4141           | musashi homolog 2 (Drosophila)                                                        |
| ENSG00000239474 | KBTBD10    | 7.54     | 8.30         | −1.69       | 0.0025      | 0.3582           | kelch repeat and BTB (POZ) domain containing 10                                      |
| ENSG00000131711 | MAP1B      | 8.24     | 9.04         | −1.74       | 0.0002      | 0.2057           | microtubule-associated protein 1B                                                    |
| ENSG00000102755 | FLT1       | 9.15     | 9.97         | −1.76       | 0.0059      | 0.4088           | fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) |
| ENSG00000172519 | OR10H5     | 6.51     | 7.33         | −1.77       | 0.0011      | 0.3582           | olfactory receptor, family 10, subfamily H, member 5                                  |
| ENSG00000059804 | SLC2A3     | 7.88     | 8.77         | −1.86       | 0.0003      | 0.2094           | solute carrier family 2 (facilitated glucose transporter), member 3                   |
| ENSG00000128052 | KDR        | 8.08     | 8.99         | −1.89       | 0.0015      | 0.3582           | kinase insert domain receptor (a type III receptor tyrosine kinase)                  |
| ENSG00000198515 | CNGA1      | 8.87     | 9.90         | −2.05       | 0.0005      | 0.2515           | cyclic nucleotide gated channel alpha 1                                              |
| ENSG00000108370 | RGS9       | 6.12     | 7.19         | −2.10       | 0.0059      | 0.4088           | regulator of G-protein signaling 9                                                   |
| ENSG00000074621 | SLC24A1    | 6.62     | 7.71         | −2.13       | 0.0062      | 0.4088           | solute carrier family 24 (sodium/potassium/calcium exchanger), member 1               |
| ENSG00000143995 | MEIS1      | 7.49     | 8.59         | −2.14       | 0.0000      | 0.1172           | Meis homeobox 1                                                                     |
| ENSG00000149489 | ROM1       | 7.80     | 8.94         | −2.20       | 0.0036      | 0.3582           | retinal outer segment membrane protein 1                                             |
| ENSG00000146350 | C6orf170   | 6.09     | 7.26         | −2.25       | 0.0001      | 0.2057           | chromosome 6 open reading frame 170                                                  |
| ENSG00000114279 | FGF12      | 6.20     | 7.38         | −2.27       | 0.0004      | 0.2281           | fibroblast growth factor 12                                                          |
| ENSG00000134183 | GNAT2      | 6.85     | 8.08         | −2.35       | 0.0036      | 0.3582           | guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2 |
| ENSG00000158445 | KCNB1      | 6.13     | 7.37         | −2.37       | 0.0047      | 0.3608           | potassium voltage-gated channel, Shab-related subfamily, member 1                    |
| Ensembl ID          | Symbol    | Mean AMD | Mean Control | Fold change | Raw p value | FDR adj. P value | Name                                                                 |
|---------------------|-----------|----------|--------------|-------------|-------------|-----------------|----------------------------------------------------------------------|
| ENSG00000118473     | SGIP1     | 7.24     | 8.50         | −2.38       | 0.0004      | 0.2281          | SH3-domain GRB2-like (endophilin) interacting protein 1               |
| ENSG00000182985     | CADM1     | 7.50     | 8.86         | −2.58       | 0.0047      | 0.3608          | cell adhesion molecule 1                                              |
| ENSG00000078018     | MAP2      | 7.25     | 8.63         | −2.60       | 0.0016      | 0.3582          | microtubule-associated protein 2                                      |
| ENSG00000205683     | DPF3      | 5.87     | 7.26         | −2.62       | 0.0012      | 0.3582          | D4, zinc and double PHD fingers, family 3                             |
| ENSG00000047639     | ANO2      | 6.73     | 8.16         | −2.70       | 0.0043      | 0.3582          | anoctamin 2                                                          |
| ENSG00000158234     | FAIM      | 6.41     | 7.96         | −2.92       | 0.0020      | 0.3582          | Fas apoptotic inhibitory molecule                                    |
| ENSG00000118402     | ELOVL4    | 6.78     | 8.35         | −2.97       | 0.0039      | 0.3582          | ELOVL fatty acid elongase 4                                           |
| ENSG00000111837     | MAK       | 6.18     | 7.76         | −3.00       | 0.0020      | 0.3582          | male germ cell-associated kinase                                     |
| ENSG00000203756     | C6orf191  | 5.94     | 7.56         | −3.07       | 0.0092      | 0.4149          | chromosome 6 open reading frame 191                                  |
| ENSG00000114349     | GNAT1     | 5.42     | 7.12         | −3.24       | 0.0034      | 0.3582          | guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1 |
| ENSG00000100362     | PVALB     | 6.18     | 7.88         | −3.26       | 0.0084      | 0.4149          | parvalbumin                                                          |
| ENSG00000152578     | GRIA4     | 5.84     | 7.55         | −3.28       | 0.0031      | 0.3582          | glutamate receptor, ionotrophic, AMPA 4                               |
| ENSG00000132639     | SNAP25    | 6.20     | 7.96         | −3.38       | 0.0087      | 0.4149          | synaptosomal-associated protein, 25 kDa                               |
| ENSG00000148798     | INA       | 5.36     | 7.17         | −3.49       | 0.0074      | 0.4149          | internexin neuronal intermediate filament protein, alpha              |
| ENSG00000130561     | SAG       | 6.03     | 7.84         | −3.51       | 0.0049      | 0.3688          | S-antigen; retina and pineal gland (arrestin)                         |
| ENSG00000112619     | PRPH2     | 6.61     | 8.50         | −3.73       | 0.014       | 0.3582          | peripherin 2 (retinal degeneration, slow)                             |
| ENSG00000116703     | PDC       | 5.93     | 7.86         | −3.83       | 0.0096      | 0.4149          | phosducin                                                             |
| ENSG00000163914     | RHO       | 7.26     | 9.23         | −3.93       | 0.0051      | 0.3754          | rhodopsin                                                            |
| ENSG00000132915     | PDE6A     | 5.87     | 7.88         | −4.02       | 0.0045      | 0.3607          | phosphodiesterase 6A, cGMP-specific, rod, alpha                      |
| ENSG00000185518     | SV2B      | 5.43     | 7.60         | −4.50       | 0.0045      | 0.3607          | synaptic vesicle glycoprotein 2B                                     |
| ENSG00000112706     | IMPG1     | 5.29     | 7.50         | −4.62       | 0.0060      | 0.4088          | interphotoreceptor matrix proteoglycan 1                             |

*Genes were filtered based on at least 50% fold change between AMD and control and raw p value <0.01.*
when arrays are processed in separate batches (i.e., prepared by different people, on different days, etc.) [19]. To address this issue in our data set, initial probeset-level expression estimates were corrected for batch effects while preserving AMD status, CFH genotype, and sex covariates using the ComBat algorithm [20]. ComBat removes batch effects more effectively than several other procedures [21]. The batch-corrected data were reimported into AltAnalyze and processed as follows: masking of cross-hybridizing probesets; filtering probesets expressed below non-log level (i.e., expression intensity before log_2 transformation) of 70; gene-level differential expression using a moderated t test; and computation of false discovery rate (FDR) adjusted p values to account for multiple hypothesis testing correction [22]. Heatmap generation and hierarchical clustering were performed using the R statistical software (ver. 3.0.0) [23]. To cluster genes based on the similarity of the expression pattern across samples, we used a Pearson-based distance metric (1 minus Pearson’s correlation coefficient) [24]. To assess alternative splicing, the splicing index and MIDAS [25] procedures in AltAnalyze were used. Putatively alternatively spliced transcripts were visualized using DomainGraph (ver. 3.0) [18], a plugin for Cytoscape (ver. 2.8.1) [26]. Quality control metrics, including distance between arrays and comparison of array intensity distributions, were calculated using the arrayQualityMetrics

Figure 4. Heatmap of differentially expressed genes shown in Table 2. Dark shading indicates low expression; light shading indicates high expression.
| Ensembl ID                  | Symbol | Mean AMD | Mean Control | Fold change | Raw p value | FDR adj. P value | Name                                                                 |
|----------------------------|--------|----------|--------------|-------------|-------------|-----------------|-----------------------------------------------------------------------|
| ENSG00000100156            | SLC16A8| 8.26     | 7.88         | 1.31        | 0.0129      | 0.4358          | solute carrier family 16, member 8 (monocarboxylic acid transporter 3) [43] |
| ENSG00000166278            | C2     | 7.49     | 7.19         | 1.24        | 0.1434      | 0.6643          | complement component 2 [43,45,46]                                    |
| ENSG00000125730            | C3     | 8.80     | 8.55         | 1.19        | 0.1226      | 0.6470          | complement component 3 [40,47-49]                                     |
| ENSG00000000971            | CFH    | 9.64     | 9.43         | 1.16        | 0.2662      | 0.7440          | complement factor H [41,50-52]                                        |
| ENSG00000100234            | TIMP3  | 11.37    | 11.22        | 1.11        | 0.3122      | 0.7823          | TIMP metallopeptidase inhibitor 3 [40,49,53,54]                       |
| ENSG00000243649            | CFB    | 7.93     | 7.81         | 1.09        | 0.4199      | 0.8396          | complement factor B [43,45,46]                                        |
| ENSG00000205403            | CFI    | 8.93     | 8.84         | 1.07        | 0.6688      | 0.9296          | complement factor I [43,55]                                           |
| ENSG00000225830            | ERCC6  | 7.34     | 7.29         | 1.03        | 0.7487      | 0.9492          | excision repair cross-complementing rodent repair deficiency, complementation group 6 [56] |
| ENSG00000137331            | IER3   | 7.72     | 7.67         | 1.03        | 0.8162      | 0.9636          | immediate early response 3 [43]                                       |
| ENSG00000168386            | FILIPIL| 8.14     | 8.17         | -1.02       | 0.8496      | 0.9718          | filamin A interacting protein 1-like [43]                              |
| ENSG00000144810            | COLS8A1| 9.15     | 9.20         | -1.04       | 0.7052      | 0.9370          | collagen, type VIII, alpha 1 [43,57]                                  |
| ENSG00000166033            | HTRA1  | 8.36     | 8.50         | -1.10       | 0.4118      | 0.8344          | HtrA serine peptidase 1 [58-60]                                       |
| ENSG00000104689            | TNFRSF10A| 6.88   | 7.12         | -1.18       | 0.0136      | 0.4398          | tumor necrosis factor receptor superfamily, member 10a [43,61]        |
| ENSG00000112715            | VEGFA  | 7.11     | 7.36         | -1.19       | 0.0735      | 0.5904          | vascular endothelial growth factor A [12,62]                           |
package (ver. 3.16.0) [27] for R. After outlying arrays were removed, the final AltAnalyze results were recomputed.

**Gene set analysis:** The Ensembl BioMart tool was used to map identifiers from previous gene expression studies to Ensembl IDs [28]. Gene set enrichment analysis (GSEA; ver. 14) [29] implemented with GenePattern (ver. 3.6.0) [30] was used to evaluate the overrepresentation of custom gene sets with AMD or control samples. For GSEA, phenotype permutation was performed using 1,000 permutations and two gene sets, an RPE-specific set and an endothelium-associated set, compiled based on literature search. Annotation of differentially expressed genes was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Functional Annotation Tools with default parameters [31].

**RESULTS**

**Quality control:** We defined early AMD as RPE changes (depigmentation or hypopigmentation) and/or macular drusen without geographic atrophy (GA) or CNV [32] as determined by chart review. Eyes with CNV or geographic atrophy were excluded. Since early AMD was classified in the donors by ophthalmoscopy, completely ruling out a rare event such as occult CNV is impossible in the early AMD samples. With the exception of one donor who later did not pass quality control, all unaffected control donors were required to have had a normal fundus exam within two years of death. Based on these criteria, nine samples were classified as early AMD (designated ARM1-9) and nine samples were classified as controls (designated CTRL1-9; Table 1). To assess the statistical equivalence of donor ages in each group, we performed Welch’s two-sample t test, which gave a p value of 0.54. The mean RNA integrity numbers for samples processed in batch A was 6.76 (standard deviation [SD] 0.34) and for batch B was 6.22 (SD 0.59; Table 1). Following normalization in AltAnalyze (Figure 1), samples CTRL3 and CTRL9 were flagged as potential outliers by arrayQualityMetrics analysis [27] based on their sum of distances to other arrays (Figure 2A,C). Additionally, the gene-level signal intensity distribution of CTRL3 was lower than that of other arrays and was flagged by arrayQualityMetrics’s Kolmogorov–Smirnov-based outlier detection module (Figure 2E,G). Given the magnitude of the difference between these two arrays and the other arrays, we removed these two arrays from the data set and reprocessed the remaining 16 samples (Figure 2B,D,F,H). With this reduced data set, ARM7 was flagged as an outlier...
### Table 4. Genes highly expressed in the RPE.

| Ensembl ID       | Symbol     | Mean AMD     | Mean Control | Fold change | Raw p value | FDR adj. P value | Name                                                                 |
|------------------|------------|--------------|--------------|-------------|-------------|------------------|----------------------------------------------------------------------|
| ENSG00000147003  | TMEM27     | 9.25         | 8.47         | 1.72        | 0.0130      | 0.4358           | transmembrane protein 27 [33]                                        |
| ENSG00000084453  | SLCO1A2    | 7.82         | 7.06         | 1.69        | 0.0232      | 0.4779           | solute carrier organic anion transporter family, member 1A2 [33]     |
| ENSG00000150656  | CNDP1      | 8.76         | 8.04         | 1.64        | 0.0813      | 0.5984           | carnosine dipeptidase 1 (metallopeptidase M20 family) [33]            |
| ENSG00000010144  | BMP7       | 7.29         | 6.64         | 1.57        | 0.0361      | 0.5074           | bone morphogenetic protein 7 [33]                                   |
| ENSG000000105855 | ITGB8      | 9.46         | 8.86         | 1.52        | 0.0627      | 0.5757           | integrin, beta 8 [33]                                                |
| ENSG000000157193 | LRP8       | 9.09         | 8.52         | 1.48        | 0.0333      | 0.4942           | low density lipoprotein receptor-related protein 8, apolipoprotein e receptor [33] |
| ENSG00000139155  | SLCO1C1    | 10.15        | 9.68         | 1.39        | 0.0425      | 0.5262           | solute carrier organic anion transporter family, member 1C1 [33]     |
| ENSG00000122481  | RWDD3      | 8.31         | 7.84         | 1.38        | 0.0942      | 0.6178           | RWD domain containing 3 [33]                                         |
| ENSG00000136541  | ERMN       | 9.28         | 8.83         | 1.36        | 0.1648      | 0.6759           | ermin, ERM-like protein [33]                                        |
| ENSG00000167995  | BEST1      | 9.29         | 8.84         | 1.36        | 0.0621      | 0.5736           | bestrophin 1 [33]                                                   |
| ENSG00000180287  | PLD5       | 10.21        | 9.77         | 1.35        | 0.0695      | 0.5816           | phospholipase D family, member 5 [33]                               |
| ENSG00000170011  | MYRIP      | 8.46         | 8.03         | 1.35        | 0.1063      | 0.6341           | myosin VIIA and Rab interacting protein [33]                         |
| ENSG00000187889  | Clorf168   | 7.15         | 6.75         | 1.33        | 0.2653      | 0.7427           | chromosome 1 open reading frame 168 [33]                            |
| ENSG00000148482  | SLC39A12   | 8.60         | 8.20         | 1.32        | 0.1679      | 0.6783           | solute carrier family 39 (zinc transporter), member 12 [33]          |
| ENSG0000016745   | RPE65      | 11.29        | 10.90        | 1.32        | 0.0376      | 0.5105           | retinal pigment epithelium-specific protein 65 kDa [33]              |
| ENSG00000183715  | OPCML      | 7.74         | 7.04         | 1.28        | 0.2173      | 0.7119           | opioid binding protein/cell adhesion molecule-like [33]              |
| ENSG00000114115  | RBPI       | 9.29         | 8.93         | 1.28        | 0.1930      | 0.6977           | retinol binding protein 1, cellular [33]                             |
| ENSG00000108231  | LGI1       | 8.58         | 8.23         | 1.27        | 0.2161      | 0.7105           | leucine-rich, glioma inactivated 1 [33]                              |
| ENSG00000128578  | FAM40B     | 9.07         | 8.73         | 1.26        | 0.3842      | 0.8192           | family with sequence similarity 40, member B [33]                   |
| ENSG00000159212  | CLIC6      | 10.51        | 10.19        | 1.25        | 0.2284      | 0.7221           | chloride intracellular channel 6 [33]                                |
| ENSG00000141526  | SLC16A3    | 7.97         | 7.67         | 1.23        | 0.0985      | 0.6231           | solute carrier family 16, member 3 (monocarboxylic acid transporter 4) [33] |
| ENSG00000121207  | LRAT       | 10.84        | 10.57        | 1.21        | 0.2578      | 0.7374           | lecithin retinol acyltransferase (phosphatidylecholine-retinol O-acyltransferase) [33] |
| ENSG00000140522  | RLBP1      | 9.18         | 8.94         | 1.18        | 0.2469      | 0.7340           | retinaldehyde binding protein 1 [33]                                |
| ENSG00000132386  | SERPINF1   | 10.54        | 10.31        | 1.17        | 0.2352      | 0.7252           | serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1 [34] |
| ENSG00000010379  | SLC6A13    | 7.31         | 7.11         | 1.14        | 0.3584      | 0.8067           | solute carrier family 6 (neurotransmitter transporter, GABA), member 13 [33] |
| Ensembl ID        | Symbol  | Mean AMD | Mean Control | Fold change | Raw p value | FDR adj. P value | Name                                                                 |
|-------------------|---------|----------|--------------|-------------|-------------|------------------|----------------------------------------------------------------------|
| ENSG00000083067   | TRPM3   | 11.43    | 11.25        | 1.13        | 0.3586      | 0.8067           | transient receptor potential cation channel, subfamily M, member 3 [33] |
| ENSG00000142611   | PRDM16  | 7.28     | 7.12         | 1.12        | 0.0982      | 0.6231           | PR domain containing 16 [33]                                          |
| ENSG00000139318   | DUSP6   | 9.18     | 9.10         | 1.06        | 0.6565      | 0.9264           | dual specificity phosphatase 6 [33]                                   |

Genes in this list were previously reported as highly expressed in RPE compared to retina and choroid [33] or known to be RPE-specific (i.e., SERPINF1) [34].
based on its sum of distances to other arrays (Figure 2B,D). However, as the magnitude of this difference was marginal, we retained this sample in the data set. After CTRL3 and CTRL9 were removed, the p value for age between the two groups was 0.70.

**Differentially expressed genes:** AltAnalyze mapped the exon array probesets to 42,187 unique Ensembl identifiers. To reduce false positive observations, we filtered this set by selecting only protein coding genes with mean log2 expression greater than or equal to the median expression of all protein-coding genes (7.11; Figure 3), which resulted in 10,286 genes. We found 75 genes differentially expressed in AMD compared to controls (at least 50% difference in expression; moderated t test raw p value <0.01; Table 2; Figure 4). None of the genes were significantly differentially expressed after FDR correction (adj. p value <0.1). We submitted the 52 downregulated genes and the 23 upregulated genes to DAVID for annotation. Of genes upregulated in early AMD, three genes (*FADS1, FADS2, PTPLA*) were identified as implicated in biosynthesis of unsaturated fatty acids (fold enrichment = 86.68; Benjamini corrected p value = 0.0052). Of genes downregulated in early AMD, 28 terms were significantly enriched (fold enrichment from 2 to 128; Benjamini-corrected p value <0.01). These terms were associated with vision, sensory perception, and the plasma membrane. No transcripts flagged by AltAnalyze as putative splicing events appeared to be alternatively spliced upon close visual inspection of the probeset data in DomainGraph.
| Ensembl ID     | Symbol | Mean AMD | Mean Control | Fold change | Raw p value | FDR adj. P value | Name                                                                 |
|---------------|--------|----------|--------------|-------------|-------------|-----------------|----------------------------------------------------------------------|
| ENSG00000115380 | EFEMP1 | 10.25    | 10.05        | 1.15        | 0.3126      | 0.7823          | EGF containing fibulin-like extracellular matrix protein 1 [35]       |
| ENSG00000152818 | UTRN   | 9.66     | 9.50         | 1.12        | 0.2251      | 0.7199          | utrophin [35]                                                        |
| ENSG00000118523 | CTGF   | 8.17     | 8.01         | 1.11        | 0.3471      | 0.8008          | connective tissue growth factor [35]                                 |
| ENSG00000078401 | EDN1   | 7.28     | 7.18         | 1.07        | 0.5917      | 0.9029          | endothelin 1 [35]                                                   |
| ENSG00000164035 | EMCN   | 9.22     | 9.26         | −1.02       | 0.9159      | 0.9858          | endomucin [35]                                                      |
| ENSG00000101000 | PROCR  | 8.44     | 8.50         | −1.04       | 0.6952      | 0.9347          | protein C receptor, endothelial [35]                                |
| ENSG00000111341 | MGP    | 10.96    | 11.05        | −1.06       | 0.3872      | 0.8200          | matrix Gla protein [35]                                             |
| ENSG00000205542 | TMSB4X | 10.57    | 10.67        | −1.07       | 0.5172      | 0.8821          | thymosin beta 4, X-linked [35]                                      |
| ENSG00000174175 | SELP   | 7.27     | 7.41         | −1.10       | 0.6747      | 0.9312          | selectin P (granule membrane protein 140 kDa, antigen CD62) [63,64] |
| ENSG00000108622 | ICAM2  | 7.53     | 7.68         | −1.11       | 0.1335      | 0.6557          | intercellular adhesion molecule 2 [35]                              |
| ENSG00000131471 | AOC3   | 7.21     | 7.38         | −1.12       | 0.2033      | 0.7008          | amine oxidase, copper containing 3 (vascular adhesion protein 1) [65]|
| ENSG00000174751 | ECSCR  | 7.77     | 7.94         | −1.12       | 0.1685      | 0.6783          | endothelial cell-specific chemotaxis regulator [35]                   |
| ENSG00000167434 | CA4    | 7.52     | 7.72         | −1.15       | 0.3435      | 0.7992          | carbonic anhydrase 1V [66]                                          |
| ENSG00000179776 | CDH5   | 7.55     | 7.81         | −1.20       | 0.0621      | 0.5736          | cadherin 5, type 2 (vascular endothelium) [35]                        |
| ENSG00000168497 | SDPR   | 6.92     | 7.25         | −1.26       | 0.0529      | 0.5567          | serum deprivation response [35]                                     |
| ENSG00000003436 | TFPI   | 7.65     | 8.01         | −1.28       | 0.1613      | 0.6758          | tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor) [35] |
| ENSG00000178726 | THBD   | 7.41     | 7.82         | −1.34       | 0.1061      | 0.6340          | thrombomodulin [35]                                                 |
| ENSG00000120156 | TEK    | 7.40     | 7.83         | −1.35       | 0.1085      | 0.6363          | TEK tyrosine kinase, endothelial [67]                                |
| ENSG00000106991 | ENG    | 8.57     | 9.01         | −1.35       | 0.1025      | 0.6275          | endoglin [35]                                                       |
| ENSG00000110799 | VWF    | 9.34     | 9.87         | −1.45       | 0.0680      | 0.5797          | von Willebrand factor [35]                                           |
| ENSG00000138722 | MMRN1  | 7.25     | 7.96         | −1.64       | 0.0551      | 0.5579          | multimerin 1 [35]                                                   |
| ENSG00000091879 | ANGPT2 | 6.94     | 7.72         | −1.72       | 0.0420      | 0.5244          | angiopoietin 2 [67]                                                  |
| ENSG00000102755 | FLT1   | 9.15     | 9.97         | −1.76       | 0.0059      | 0.4088          | fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor) [35] |
| ENSG00000128052 | KDR    | 8.08     | 8.99         | −1.89       | 0.0015      | 0.3582          | kinase insert domain receptor (a type III receptor tyrosine kinase) [35] |

Genes in this list were previously reported as associated with endothelial cells.
Comparison with previous studies: Genome-wide association studies have identified several genes harboring polymorphisms associated with AMD. We compiled a list of these genes and examined their expression levels in our data set (Table 3; Figure 5). Some genes (e.g., ADAMTS9, APOE, ARMS2, B3GALT1, CCR3, CETP, CFHR2, CFHR3, COL10A1, DDR1, LIPC, RAD51B, TGFBR1, TLR3) did not meet our filtering criteria (i.e., mean expression in AMD or control groups > median expression of all protein coding genes). Of the genes previously associated with AMD, none were differentially expressed (raw p value <0.01).

Analysis of genes associated with either RPE or vascular endothelium: We next evaluated genes with cell-type-specific expression in either the RPE or endothelium. For RPE genes, we took the top 35 genes identified as highly expressed in the RPE versus the retina and choroid in the recent manuscript by Booij and colleagues [33]. We added SERPINF1 (PEDF), another marker of RPE, to this set [34]. Of these, 28 mapped to Ensembl IDs in our filtered set and were included for analysis. Thirteen RPE-expressed genes showed between 12% and 72% increased expression (raw p value <0.1; Table 4; Figure 6).

As a proxy for choroidal endothelial cells, we compiled a list of 24 genes with known expression in the endothelium and compared the fold change between the AMD and control samples. Endothelium-associated genes were based on gene
| Ensembl ID     | Symbol | Mean AMD | Mean Control | Fold change | Raw p value | FDR adj. P value | Name                                                   |
|----------------|--------|----------|--------------|-------------|-------------|-----------------|--------------------------------------------------------|
| ENSG00000127083| OMD    | 7.19     | 5.97         | 2.33        | 0.0047      | 0.8828          | osteomodulin                                           |
| ENSG00000146374| RSPO3  | 8.03     | 6.88         | 2.21        | 0.0058      | 0.8828          | R-spondin 3                                            |
| ENSG00000011465| DCN    | 8.86     | 7.72         | 2.19        | 0.0008      | 0.6616          | decorin                                                 |
| ENSG00000090104| RGS1   | 8.10     | 7.10         | 2.00        | 0.0076      | 0.8828          | regulator of G-protein signaling 1                     |
| ENSG00000186439| TRDN   | 8.04     | 7.08         | 1.95        | 0.0068      | 0.8828          | triadin                                                 |
| ENSG00000176971| FIBIN  | 8.87     | 7.98         | 1.86        | 0.0004      | 0.6215          | fin bud initiation factor homolog (zebrafish)          |
| ENSG00000146233| CYP39A1| 7.31     | 6.46         | 1.80        | 0.0065      | 0.8828          | cytochrome P450, family 39, subfamily A, polypeptide 1 |
| ENSG00000196344| ADH7   | 7.66     | 6.94         | 1.65        | 0.017       | 0.7811          | alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide |
| ENSG00000182230| FAM153B| 7.23     | 6.54         | 1.61        | 0.0056      | 0.8828          | family with sequence similarity 153, member B          |
| ENSG00000116667| C1orf21| 10.29    | 9.65         | 1.56        | 0.0000      | 0.1533          | chromosome 1 open reading frame 21                     |
| ENSG00000121769| FABP3  | 7.48     | 6.85         | 1.55        | 0.0088      | 0.8828          | fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor) |
| ENSG00000071189| SNX13  | 8.46     | 7.82         | 1.55        | 0.0020      | 0.7811          | sorting nexin 13                                       |
| ENSG00000115607| IL18RAP| 7.46     | 8.05         | −1.50       | 0.0083      | 0.8828          | interleukin 18 receptor accessory protein              |
| ENSG00000089157| RPLP0  | 7.00     | 7.59         | −1.51       | 0.0029      | 0.7833          | ribosomal protein, large, P0                           |
| ENSG00000177076| ACER2  | 6.82     | 7.41         | −1.51       | 0.0027      | 0.7833          | alkaline ceramidase 2                                  |
| ENSG00000146592| CREB5  | 6.66     | 7.26         | −1.51       | 0.0070      | 0.8828          | cAMP responsive element binding protein 5              |
| ENSG00000182853| VMO1   | 6.58     | 7.18         | −1.52       | 0.0051      | 0.8828          | vitelline membrane outer layer 1 homolog (chicken)     |
| ENSG00000141753| IGFBP3 | 9.07     | 9.71         | −1.56       | 0.0082      | 0.8828          | insulin-like growth factor binding protein 4            |
| ENSG00000167772| ANGPTL4| 7.66     | 8.32         | −1.58       | 0.0003      | 0.6215          | angiopoietin-like 4                                    |
| ENSG00000198959| TGM2   | 8.87     | 9.61         | −1.68       | 0.0006      | 0.6616          | transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase) |
| ENSG00000104332| SFRP1  | 6.96     | 7.77         | −1.76       | 0.0067      | 0.8828          | secreted frizzled-related protein 1                    |
| ENSG00000113083| LOX    | 6.29     | 7.14         | −1.80       | 0.0055      | 0.8828          | lysyl oxidase                                          |
| ENSG00000167236| CCL23  | 7.10     | 8.00         | −1.86       | 0.0066      | 0.8828          | chemokine (C-C motif) ligand 23                        |
| ENSG00000068366| ACSL4  | 7.33     | 8.33         | −2.00       | 0.0028      | 0.7833          | acyl-CoA synthetase long-chain family member 4         |
| ENSG00000138135| CH25H  | 6.20     | 7.21         | −2.01       | 0.0057      | 0.8828          | cholesterol 25-hydroxylase                             |
| ENSG00000102265| TIMP1  | 10.09    | 11.11        | −2.03       | 0.0035      | 0.8828          | TIMP metallopeptidase inhibitor 1                      |
| ENSG00000156804| FBXO32 | 6.83     | 7.96         | −2.19       | 0.0000      | 0.1533          | F-box protein 32                                        |
| ENSG00000179431| FJX1   | 8.10     | 9.28         | −2.27       | 0.0008      | 0.6616          | four jointed box 1 (Drosophila)                        |
| Ensembl ID    | Symbol    | Mean AMD | Mean Control | Fold change | Raw p value | FDR adj. P value | Name                                                                 |
|--------------|-----------|----------|--------------|-------------|-------------|-----------------|----------------------------------------------------------------------|
| ENSG00000163638 | ADAMTS9  | 5.74     | 7.12         | −2.61       | 0.0008      | 0.6616          | ADAM metallopeptidase with thrombospondin type 1 motif, 9             |
| ENSG00000124102 | PI3       | 6.40     | 7.79         | −2.63       | 0.0006      | 0.6616          | peptidase inhibitor 3, skin-derived                                  |
| ENSG00000205362 | MT1A      | 8.34     | 9.75         | −2.66       | 0.0082      | 0.8828          | metallothionein 1A                                                   |
| ENSG00000159167 | STC1      | 9.09     | 10.62        | −2.90       | 0.0027      | 0.7833          | stanniocalcin 1                                                      |
| ENSG00000064886 | CHI3L2    | 6.20     | 7.83         | −3.10       | 0.0070      | 0.8828          | chitinase 3-like 2                                                   |
| ENSG00000162992 | NEUROD1   | 5.41     | 7.30         | −3.69       | 0.0092      | 0.8828          | neurogenic differentiation 1                                         |
| ENSG00000115602 | IL1RL1    | 5.35     | 7.33         | −3.93       | 0.0001      | 0.3640          | interleukin 1 receptor-like 1                                       |
lists identified as specific to the endothelium [35] or from a literature search. Seven of these genes showed decreased expression in early AMD between 20% and 189% (raw p value <0.1; Table 5; Figure 7), which suggested decreased numbers of vascular endothelial cells in the choroid and/or dedifferentiation of extant endothelial cells. Thus, there appeared to be a trend toward increased expression of RPE-expressed genes and decreased expression of endothelium-expressed genes in eyes with early AMD.

To calculate the enrichment of the RPE and endothelium gene sets, we performed GSEA [29]. For each gene set, GSEA calculates (a) an enrichment score (ES) based on the rank distribution of individual genes within the set from among all unique genes symbols in our filtered set (n=10,286), (b) a normalized enrichment score (NES) that accounts for the size of the set, (c) a nominal p value based on phenotype permutation, e.g., AMD or control status of samples, and (d) an FDR q value to control for the gene set size and multiple hypothesis testing. GSEA suggests a greater trend for the overall enrichment of the endothelium-associated gene set in AMD samples (ES = –0.78; NES = –1.39; nominal p value=0.133; FDR q value = 0.08) than the enrichment of RPE-specific genes in the control samples (ES = 0.81; NES = 1.3; nom. P value = 0.167; FDR q value = 0.191). Since an FDR q value of less than 0.25 is considered significant for GSEA [29], these data suggest a significant decrease in endothelial cell transcripts.

Analysis of CFH risk genotypes: Last, we reanalyzed the data set by stratifying based on genotypes at the rs1061170 SNP in CFH (11 high-risk, YH/HH samples; five low-risk, YY samples), independent of AMD affection status. The same filtering criteria were applied as in the previous analysis, retaining 10,288 protein-coding genes. Thirty-five genes were identified as differentially expressed (at least 50% difference in expression; moderated t test raw p value <0.01; Table 6). None of the genes were significantly differentially expressed after FDR correction (p value <0.1).

DAVID analysis of the 12 genes with increased expression in the high-risk genotype revealed no significantly enriched terms (with Benjamini corrected p value <0.01). In the DAVID analysis of the 23 genes with lower expression in the high-risk genotype, DAVID identified eight terms with significant enrichment (fold enrichment from 3 to 8; Benjamini corrected p value <0.01). These terms were associated with extracellular secretion, signal peptides, and disulfide bonds.

**DISCUSSION**

AMD is a complex disease that shows altered function and viability of photoreceptor cells, RPE, and choriocapillaris endothelial cells. Although our understanding of the genetics of AMD has progressed in the last decade, many basic questions about the pathogenesis of this disease remain. In the current study, we evaluated gene expression at the exon level in AMD and control eyes and found a loss of transcripts expressed by choroidal endothelial cells.

Limitations of the current study include small sample size (due to stringent inclusion criteria) and the lack of strong signatures of differential expression between the case and control samples, obviating validation of single genes. Increased sample size may reveal some genes with a small difference between the cases and controls, which remain significant after multiple hypothesis testing. However, this is unlikely. Previous analysis of AMD-affected tissues with microarray technologies required the use of non-standard methods to identify altered gene expression (expression correlation between two data sets with p<0.1 and 25% fold change) [15] or the use of sensitive clustering algorithms to identify patterns across many AMD grades simultaneously [14]. At the tissue level in our data set, the apparent trends among the RPE and endothelial genes (overall direction of fold change and p<0.1) are supported by the GSEA results. These gene set-level changes are small in our samples.

**Cross-contamination by photoreceptor-specific transcripts:** We observed an elevation of select photoreceptor-specific genes (i.e., RHO, PDC) across all control samples compared to the AMD-affected samples, particularly noticeable in CTRL4 and CTRL5. High expression of retinal transcripts is commonly reported in gene expression studies of the RPE and choroid [14,36,37], and various strategies have been taken to deal with cross-contamination (e.g., laser capture microdissection [33], flagging genes with high expression in one tissue that appear in another tissue [14]). Cross-contamination is not surprising, as photoreceptor cell outer segments are partially interdigitated and ensheathed by the apical microvilli of the RPE and supported by the interphotoreceptor matrix [38]. Although most photoreceptor cell transcripts are expected to be within the outer nuclear layer and inner segments, Van Soest and colleagues suggest that these transcripts may be present at the interface between the photoreceptor outer segments and apical RPE [37]. The photoreceptor cell-specific gene expression we observed could be an artifact of mechanical separation or a stochastic individual variation in adhesion, and this random event might have occurred to a higher degree in the control eyes. However, we hypothesize that the pattern of elevated retinal contamination in
the controls, which was absent from AMD cases, may be due to decreased adhesion of the retina to the RPE in the AMD samples compared to the controls. Although adhesion between the neural retina and the RPE has been examined in primates [39], to our knowledge this has not been investigated in the context of early AMD. Alternatively, if neural retina components are uniformly present in all samples, then the decreased expression of retina-specific genes in the AMD samples could indicate that expression within photoreceptors is decreased even at the earliest stages of AMD.

Intriguingly, genes with lower expression in AMD than in controls included KDR and F11T1, known VEGF receptors. Hierarchical clustering reveals that these genes do not segregate with the neural retina-specific genes and that expression of this sub-cluster of genes is consistent across controls and is not affected by CTRL4 and CTRL5 (Figure 1).

Loss of endothelial-specific gene expression: Our group has previously shown that the density of microvasculature decreases as the volume of sub-RPE drusen increases [9]. Similarly, studies of blood flow in eyes with drusen show susceptibility of the choroidal vasculature to degenerative changes in AMD [11]. In elegant whole mount studies of eyes with advanced AMD, McLeod and colleagues found a linear relationship between the RPE and choriocapillaris, with the loss of either layer affecting the integrity of the other [10]. From these studies, with a sample size similar to that in the current report, the authors concluded that RPE loss precedes endothelium loss in GA, whereas endothelium loss precedes RPE loss in CNV [10]. Our data indicate that gene expression of endothelium-related genes decreases in early AMD before any atrophy or neovascular change develops.

Our results are also consistent with state-of-the-art proteomics studies of Bruch’s membrane–choroid preparations, in which the authors found the endothelial cell proteins von Willebrand factor (VWF) and carbonic anhydrase 4 (CA4) are reduced in early and mid-stage AMD and advanced dry AMD, respectively [40]. The same authors showed no loss of RPE proteins RPE65 and CRALBP (RLBP1). In fact, the levels of these proteins were elevated in Bruch’s membrane, consistent with our findings at the mRNA level. Taken together, these studies suggest loss or dedifferentiation of choroidal endothelial cells before loss of the RPE in eyes with AMD. In addition, expression of the choroidal endothelial genes ICAM1, SELE, and PLVAP was decreased in some AMD classes in the report by Newman et al. (supplemental data) [14].

Decreased expression of ADAMTS9 in samples with high AMD risk CFH genotype: Eyes with high-risk CFH genotypes were also assessed for gene expression changes. We previously found increased membrane attack complex formation in eyes homozygous for the high-risk allele [41], and complement activation may affect gene expression in nearby cells. Although not significant after multiple hypothesis testing correction, expression of ADAMTS9 transcripts was lower in samples with high-risk CFH genotypes than in samples with the low-risk genotype. This gene is a member of the ADAMTS (a disintegrin-like and metalloprotease domain with thrombospondin type I motifs) protein family with the ability to cleave aggrecan and versican [42]. Recently a SNP (rs6795735) located 32.5 kb upstream of the ADAMTS9 transcription start site was associated with increased risk of AMD in a meta-analysis of genome-wide association (GWA) data [43]. ADAMTS9 is expressed in ARPE-19 cells [44] and several types of microvascular endothelial cells [42], although to our knowledge localization of ADAMTS9 has not been examined in human RPE and choroid samples. In microvascular endothelial cells, ADAMTS9 suppresses angiogenesis, albeit not via sequestration of VEGF165 [42]. Further experimentation is necessary to elucidate the relationship between CFH activity and ADAMTS9 function in early AMD.

In conclusion, we performed microarray analysis of human donor maculas and found early loss of choriocapillaris endothelial cell markers in early AMD, with preservation of RPE cell transcripts. These results have potential importance for therapy. Recently, the replacement of RPE cells in AMD has been contemplated as a treatment for AMD. These studies, as well as proteomic [40], anatomic [9,10], and clinical [11] studies, strongly suggest caution in this type of approach, since transplanting healthy RPE into a macula with a degenerated choriocapillaris may be fruitless. In addition to replacing photoreceptor cells and RPE, strategies for replacing lost choriocapillaris are necessary to fully restore function in AMD.

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