Patterns of gene expression and allele-specific expression vary among macular tissues and clinical stages of Age-related Macular Degeneration

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Abstract

Age-related macular degeneration (AMD) is a complex neurodegenerative disease and is the leading cause of blindness in the aging community. AMD progresses, has an early and intermediate stages (both dry and two late stages, neovascular and geographic atrophy (GA). It is the late stages that are responsible for the majority of visual impairment and blindness. Available medicines are directed against the less common wet form and do not cure or reverse vision loss. Therefore, it is imperative to identify preventive and therapeutic targets. As the mechanism for AMD is unclear, one, way is to interrogate well characterized disease affected tissue in the appropriate geographic region that is the macular neural retina and macular retina pigment epithelium (RPE)/choroid. We investigated differential gene expression (DEG) across the clinical stages of AMD in the macula of the primary affected tissue compared to well characterized normal using a standardized published protocol (Owen et al., 2019). Donor eyes (n=27) were Caucasian with an age range of 60-94 and 63% were male, and tissue from the macula RPE/choroid and macula neural retina were taken from the same eye. Donor eyes were recovered within 6 hours post mortem interval time and phenotyped by ophthalmic experts using multi modal imaging (fundus photos and SD-OCT) to ensure maximal preservation of RNA quality and accuracy of diagnosis. Utilizing DESeq2, followed PCA, Benjamini Hockberg Analysis to control for the FDR, Bonferonni for the paired comparisons: a total of 26,650 genes were expressed in the macula RPE/choroid and/or macula retina with 1,204 genes found to be statistically different between neovascular AMD and normal eyes, 40 genes found between intermediate AMD (AREDS3) and normal eyes, and 1,194 genes found between intermediate AMD and neovascular AMD. A comparison of the DEGs from intermediate AMD with normal eyes and neovascular AMD with normal eyes showed an overlap of six genes: *MTRNR2L1, CLEC2L, CCM2L, CYP4X1, GLDN,* and *SMAD7*. The top pathways of DEGs identified through Ingenuity Pathway Analysis (IPA) for intermediate AMD with normal eyes were the interferon signaling and Th1 and Th2 activation pathways, while for neovascular AMD with normal eyes it was the phototransduction and SNARE signaling pathways. Allele specific expression (ASE) revealed known coding regions in previously reported GWAS loci demonstrated that significant ASE for C3 rs2230199 and CFH rs1061170 occurred in the macula RPE/choroid for normal, and intermediate AMD while ASE for CFH rs1061147 occurred in occurred in the macula RPE/choroid for normal, and intermediate and neovascular AMD. An investigation of previously identified GWAS loci (Fritsche et al., 2016) revealed that 76% of the 34 loci previously identified were significantly differentially expressed between normal macular RPE/choroid and macular neural retina, with 77% of these identified loci expressed higher in the RPE. Pathway analysis was conducted on these DEGs to reveal the involvement of pathways previously implicated in AMD as well as being the first study to assess ASE across the clinical spectrum of AMD. By utilizing a systems biology approach that integrates clinical, experimental, genomic, and phenotypic data to aid in the understanding of the pathogenesis of AMD.

Introduction

Age-related macular degeneration (AMD), the leading cause of blindness in the aging population, is a complex neurodegenerative disease with both intermediate and late forms. The intermediate form is a clinical biomarker that can increase the risk of either of the two advanced forms; neovascular AMD (also referred to as macular neovascularization, MNV) and geographic atrophy (GA). In either form, this condition involves progressive degradation of the central retina – the macula which can lead to visual disability, where the individual is unable to read, recognize faces, and drive [1]. Thus, the primary geographical region and the tissues that are affected by AMD include the macular retina and macular RPE/choroid, however, the precise mechanisms contributing to disease pathogenesis and why the disease is generally localized to the macular region remain unclear. Though AMD is phenotypically heterogeneous, the Age-Related Eye Disease Study (AREDS) classifies AMD into four stages based on the number and size of drusen in addition to observable pathological abnormalities, such as the sharp loss of pigmentation characteristic of geographic atrophy. These stages are denoted AREDS 1 (no AMD/normal aging), AREDS 2 (early AMD), AREDS 3 (intermediate AMD), and AREDS 4 (end-stage/advanced AMD) [2]. Tools used to assist the ophthalmologist in the staging of AMD include fundus imaging and spectral domain optical coherence tomography (SD-OCT) to provide direct visualization and/or high-resolution, non-invasive cross-sectional, enface imaging of the retina/RPE/choroid. The wet form of AMD or macular neovascularization overall represents a small number of AMD cases and the development of anti-vascular endothelial growth factor (VEGF) treatment has helped to reduce the visual loss associated with the condition [3].

However, there is persistent progression of visual deterioration despite control of the neovascularization, despite the ability of the treatment to often reverse acute vision loss through VEGF inhibition [4]. Since dry AMD represents the majority of the AMD population there has been focus of the development of therapies for the late stage of dry. Currently the only approved treatment for intermediate AMD is supplementation of antioxidant AREDS2 formula, which has been demonstrated only to modestly reduce the rate of progression to advanced AMD [6]. As yet there are no FDA approved clinical trials for geographic atrophy, although an anti-C3 and ani-C3 agent slowed the growth of geographic atrophy, both increased incidence of neovascular AMD, it is unclear when and if these agonists will be FDA approved [7, 8]. Appropriate therapies to at least prevent, slow down and or stop disease progression are clearly needed. Although a study by the International Age-related Macular Degeneration Genomics Consortium (IAMDGC) identified genome-wide significant association at 34 loci [3]. Most of the significantly associated variants are within non-coding regions (such as introns, gene regulatory regions, or positioned distally from a functional gene) and have no known functional consequences. While this has directed us to potential pathways underlying disease mechanism(s) this has not moved us closer to druggable therapeutic targets for AMD. Therefore, as we and others have previously hypothesized an investigation of well characterized and geographically dissected tissues affected by the disease pathophysiology may be the appropriate first step in ascribing function to a gene in a complex disorder such as AMD [9, 10]. Though gene expression has been examined within tissues affected by AMD findings have not been consistent between studies [11-17]. Inconsistency between studies whether single cell, single nuclei and/or bulk

RNASeq, may include lack of separation of geographical regions (eg. macula retina and/or the macula RPE/choroid from the periphery), a lengthy death to preservation time in the processing of the tissue, and lack of pre and/or post mortem phenotyping for both eyes within the same donor [10,18, 20]. RNA-Seq is an agnostic and unbiased approach to examine gene expression [21-22]. Additionally, RNA-Seq can uncover differentially spliced genes and long intervening non-coding RNAs (lncRNAs). [23–27] .To date, studies that have employed RNA-Seq [28-30] have not evaluated gene expression in both macula neural retina and macula RPE/choroid within the same donor eye in intermediate and separately neovascular AMD. Since the precise mechanisms leading to AMD are unclear, we focused the present study on tissues specifically affected by AMD, the macula of the retina pigment epithelium/choroid (RPE)/choroid and the macula of the neural retina, in an effort to ascertain differential gene expression (DEG) between the clinical stages of AMD. To address the complexity of a multi-faceted disease, we utilized a systems biology approach that integrates basic experimental, genomic, phenotypic, and clinical data into a model for understanding AMD disease pathogenesis. [28] We employed a standardized phenotyping protocol of both donor eyes recovered within 6 hours post mortem [20], the maximum interval to achieve minimal RNA half-life variability [29] and enhanced RNA quality [34–36]. In addition, motivated by previous studies showing evidence of allele-specific expression (ASE) [37] in genes associated with risk of autism, stroke progression, Alzheimer disease and cancer [38-43], we interrogated the DNA of each donor at a genome wide level to determine whether an imbalance of expression between alleles may underlie phenotypic variation and hence the pathophysiology of AMD. To our knowledge, this is

the first study to assess ASE across the clinical spectrum of AMD at a genome-wide level.

Methods

Donor Eye Tissue Repository

Details of the donor eye repository ascertainment, collection and post-mortem phenotyping of both donor eyes have been previously published. [20] In the instance where the donor eyes are discordant for phenotype Briefly, a standardized protocol has been developed to collect, ascertain and phenotype the tissue that has been used in this study and the rest flash frozen for later use. [20]. For this study, the AMD phenotype employed the Age-Related Eye Disease Study severity grading scale, where AREDS category 0/1 was considered normal, AREDS category 3 intermediate AMD, and AREDS category 4b neovascular AMD [44]. The currently growing repository implements a standardized system for acquisition of eyes and peripheral blood (serum, plasma) $\leq$ 6 hours of death in addition to collection of retrospective epidemiological and clinical data from donors. features of AMD (drusen, MNV, fibrosis) as previously described. [20]. In the case of discordant phenotypes within the same donor, the more severe diseased eye was used for inclusion in the study. For example, if a patient had a diagnosis of AREDS 3 in one eye and AREDS 0/1 in the contralateral eye only the AREDS 3 eye was used in the study. Similarly, if a patient had a diagnosis of AREDS 3 in one eye and neovascular AMD in the contralateral eye only the neovascular eye was used in the study. Although AREDS category 2 early AMD, category 4a (geographic atrophy) and AREDS category 4c (both geographic atrophy and neovascular AMD)

were collected and ascertained as previously described they were not included in this study [9].

Eye Tissue Dissection

For this study, only the macular region of both the neural retina and separately the RPE/choroid was used for RNA-Seq Tissues are collected using an 8 mm punch centered over the fovea to collect the retina/RPE/choroid from the macular region (encompassing the fovea) and an 8mm as previously described by us and others [20,45,-46]. Retina is removed from each punch and placed in RNAlater (Ambion), an RNA stabilizing reagent. Additionally, a 6mm punch is used to cut a button of RPE/choroid from the 8 mm punch, which is immediately submersed into RNAlater at 4°C for 24 hours and then placed at -80° C for long term storage. In our experience, this smaller punch of 6 mm minimizes retina contamination to the RPE as previously described [20].

Nucleic Acid Extraction

Macular retina and RPE/choroid samples from 27 unrelated eye tissue donors were selected for transcriptome analysis via RNA-sequencing. Only one eye per donor was used, which collectively included 10 eyes with intermediate AMD, 5 eyes with neovascular AMD, and 12 normal eyes. DNA and RNA were extracted from 8 mm punches of macular retina and 6mm of RPE/choroid tissue from each donor using the Qiagen All-prep DNA/RNA mini kit (cat #80204) according to manufacturer’s protocol (a

total of 54 samples). Quality of RNA samples was assessed with an Agilent Bioanalyzer.

RNA-Sequencing

Total RNA samples were poly-A selected and cDNA libraries were constructed using the Illumina TruSeq Stranded mRNA Sample Preparation Kit (cat# RS-122-2101, RS-122-2102) according to the manufacturer's protocol. Sequencing libraries (18 pM) were chemically denatured and applied to an Illumina TruSeq v3 single read flow cell using an Illumina cBot. Hybridized molecules were clonally amplified and annealed to sequencing primers with reagents from an Illumina pTruSeq SR Cluster Kit v3-cBot-HS (GD-401-3001). Following transfer of the flowcell to an Illumina HiSeq instrument (HCS v2.0.12 and RTA v1.17.21.3), a multiplexed, 50 cycle single read sequence run was performed using TruSeq SBS v3 sequencing reagents (FC-401-3002).

Primary Analysis of RNA Sequencing Data

Each of the 54 sample 50bp, poly-A selected, non-stranded, Illumina HiSeq fastq datasets were processed as follows: Reads were aligned using NovoCraft's novoalign 2.08.03 software (http://www.novocraft.com/) with default settings plus the -o SAM -r All 50 options to output multiple repeat matches. The genome index used contained human hg19 chromosomes, phiX (an internal control), and all known and theoretical splice junctions based on Ensembl transcript annotations. Additional details for this aspect of the protocol are described elsewhere (http://useq.sourceforge.net/usageRNA-Seq.html). Next, raw novoalignments were processed using the open source USeq

SamTranscriptiomeParser (http://useq.sourceforge.net) to remove alignments with an alignment score greater than 90 (~ 3 mismatches), convert splice junction coordinates to genomic, and randomly select one alignment to represent reads that map equally well to multiple locations. Relative read coverage tracks were generated using the USeq Sam2USeq utility (http://useq.sourceforge.net/cmdLnMenus.html#Sam2USeq) for each sample and sample type (Normal Retina, Intermediate AMD Retina, Neovascular AMD Retina, Normal RPE/choroid, Intermediate AMD RPE, and Neovascular AMD RPE/choroid). These data tracks are directly comparable in genome browsers and good tools to visualize differential expression and splicing. Estimates of sample quality were determined by running the Picard CollectRNA-SeqMetrics application (http://broadinstitute.github.io/picard/) on each sample. These QC metrics were then merged into one spreadsheet to identify potential outliers. Agilent Bioanalyzer RNA integrity number (RIN) and library input concentration columns were similarly added for QC purposes (http://www.genomics.agilent.com). Out of the 54 samples, 48 samples passed QC analysis.

Differential Gene Expression and Splicing Analysis

Sample sets were analyzed using the DefinedRegionDifferentialSeq (DRDS) utility of USeq to detect differentially expressed and differentially spliced genes. This application accepts as input a conditions directory containing folders with biological replicas from each sample type (Normal Retina, Intermediate AMD Retina, Neovascular AMD Retina, Normal RPE, Intermediate AMD RPE, and Neovascular AMD RPE) and an Ensembl gene table in UCSC refFlat format. Gene models were created by merging gene

transcripts into a single composite “gene” with the USeq MergeUCSCGeneTable utility. A table containing alignment counts from each sample for each gene was created with DRDS. Data in this table provided the basis for estimating count-based differential abundance using the DESeq2 Bioconductor package (http://www.bioconductor.org/packages/release/bioc/ html/DESeq2.html) [47]. This program estimates the over-dispersion in the count data and calculates p-values using a negative binomial test. The Benjamini-Hochberg p-value correction was applied to control for multiple testing. DESeq2 also generates a log2 ratio estimate of difference in gene abundance using variance corrected counts as well as rLog values for clustering and principal component analysis (PCA). Library size and within replica variance were estimated for each sample. Pairwise comparisons were made between the normal and disease subgroups. Differences in splicing were assessed for merged replica counts for each exon with $\geq$10 counts in each gene in each subgroup by a chi-square test. A Bonferroni multiple testing correction was applied and the exon with the biggest absolute log2 normalized gene count ratio was noted. A per base normalized gene count read coverage log2 ratio graph was created enabling visualization of the relative exon coverage difference for each pairwise comparison. To identify potential outlier samples, unsupervised hierarchical clustering (HC) and PCA were performed with the aid of the Partek Genomic Suite (http://www.partek.com/pgs) using the default settings. DESeq2 rLog values from genes with $\geq$20 counts were included in this procedure. For HC visualization, row values were mean centered at zero and scaled to a standard deviation of one.

SNP Genotyping

Genome-wide SNP genotyping was performed on DNA obtained from peripheral blood from each donor eye subject using Illumina's HumanOmni2.5-8 BeadChip Kit according to the manufacturer's protocol **(**http://www.illumina.com/products/humanomni25-8_beadchip_kits.ilmn**).** AMD associated SNPs that were not present on the HumanOmni2.5-8 BeadChip were genotyped using a combination of pre-designed and Custom Taqman SNP Genotyping Assays (Applied Biosystems). Each assay was run in a 15 µl reaction containing 2x Taqman GTXpress master mix, 40x probe, and 10 ng of DNA. Thermal cycling was performed according to the manufacturer's protocol. The ABI 7500 Real-Time PCR System, with the accompanying software, was used to analyze the genotypes.

Differential Expression Validation by Real-Time PCR

RNA was reverse transcribed using oligo-dT primers (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. The cDNA was used as a template for real-time PCR reactions run in triplicate using pre-designed Taqman Gene Expression Assays (Life Technologies) for *UCHL1, PFKP, LPCAT1, PDPN, GAS1, CST3* and for *UBC* as an endogenous control. Assays were run on the Taqman 7500 Real Time PCR system (Life technologies). Mean Ct values were normalized to *UBC* and analyzed using REST 2009 Software (http://www.gene-quantification.de/rest.html).

Allele-Specific Expression (ASE)

SNPs previously identified by GWAS as being associated with AMD (determined using the GWAS Catalog https://www.ebi.ac.uk/gwas/) were investigated for allele-specific expression (ASE) in our dataset. Specifically, we genotyped the exonic AMD SNPs using either the genotypes from the HumanOmni2.5-8 BeadChip Kit or TaqMan assays. Bam files of individuals showing a heterozygous genotype were examined to determine the number of reads for each of the two alleles. Genotypes of heterozygotes determined from the SNP Chip showing monoallelic expression were confirmed using proxies ($r^2 \geq 0.8$) as determined by 1000GENOMES:phase 3:CEU. Only individuals with ≥ 10 reads were used. The binomial test, corrected using Benjamini-Hochberg, was used to determine statistically significant allelic imbalance within each individual.[48]

Bioinformatic Analysis

QIAGEN Ingenuity Pathway Analysis (IPA) (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA) was employed to identify pathways our DEGs function in between AMD normal, Intermediate AMD, and Neovascular AMD [49].

Results

Numbers and characteristics of subjects available for analysis of each tissue type after QC are shown in **Table 1**. For clustering analysis, using both hierarchal clustering (HCA) and principal component analysis (PCA) based on the samples' whole transcriptome expression, samples split into two primary groups comprised of Retina and RPE/choroid samples. Clear separation of RPE/choroid and retina tissue types was observed. Within each tissue type, there was not clear separation between the disease

subtypes. Greater variability was observed among the RPE/choroid samples than among the retina samples (**Fig 1**). None of the samples differed substantially to warrant flagging as an outlier.

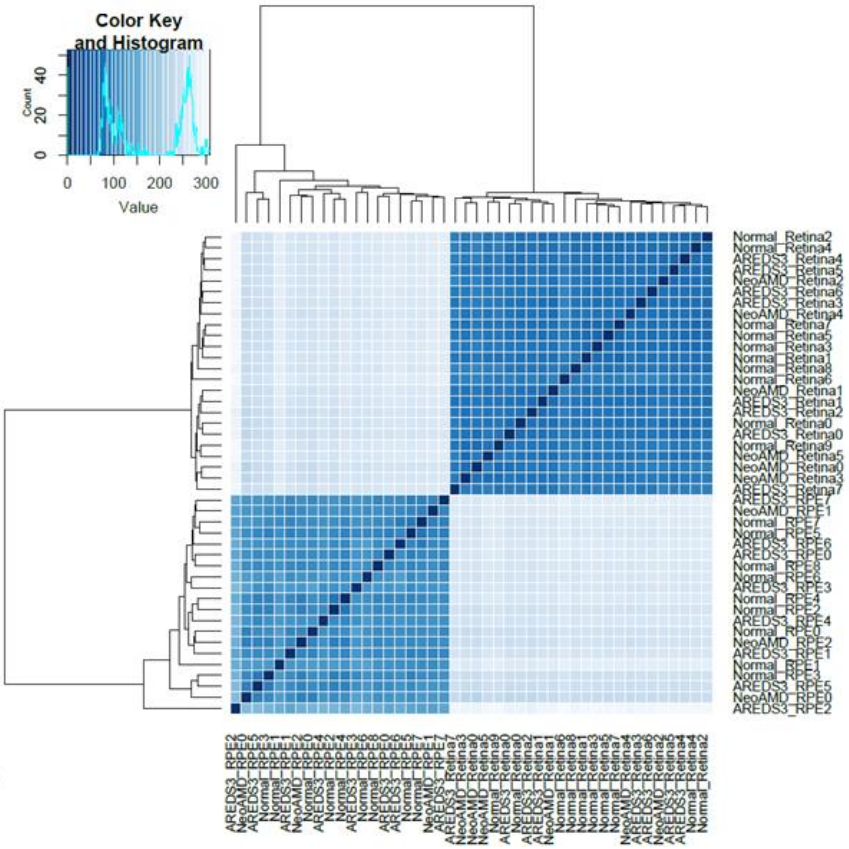

Fig 1. Hierarchal clustering analysis (HCA) of 47 donor eye samples from the RPE/choroid and retina. Rows and columns represent each individual sample against the rest of the samples.

To evaluate the quality of our tissue dissection we calculated the number of reads mapped to genes known to be expressed exclusively in the neural retina and RPE/choroid, respectively, using an approach as previously described for the retina [61]. Retina genes involved in phototransduction (*GNGT1, GUCA1A, PDE6A, GNB1, CNGB1, GNAT1, CNGA1, PDE6B, PDE6G, PRPH2, RHO, ROM1, SAG*, and *SLC24A1*) accounted for an average of 2.3% of reads in the total normal retina library

and accounted for 0.06% of our normal RPE/choroid tissue reads, proportions which are similar to those reported in a previous study.[50] In our study RPE/choroid genes (*BEST1, RDH5*, and *RPE6*,) accounted for an average of 0.65% of reads in the total RPE/choroid library and only 0.02% of total reads in the retina library. These findings demonstrate that neither the retina or the RPE/choroid is likely contaminated (e.g., if there was contamination of retina genes in the RPE/choroid library, reads would be greater than 1% compared to the observed proportion of 0.06%).

Table 1. Subject Characteristics

	Intermediate AMD				Neovascular AMD				Normal			
Group	**n**	**Ave. RIN**	**Age (range)**	**Males (%)**	**n**	**Ave. RIN**	**Age (range)**	**Males (%)**	**n**	**Ave. RIN**	**Age (range)**	**Males (%)**
All Samples (Retina)	10	6.89	76.0 (60-87)	6 (60.0%)	5	6.7	83.4 (74-94)	2 (40.0%)	12	6.65	74.0 (60-94)	9 (75.0%)
All Samples (RPE/Choroid)	10	6.7	76.0 (60-87)	6 (60.0%)	5	7.06	83.4 (74-94)	2 (40.0%)	12	6.66	74.0 (60-94)	9 (75.0%)
Clean Samples (Retina)	9	6.91	75.0 (60-87)	6 (66.7%)	5	6.7	83.4 (74-94)	2 (40.0%)	10	6.76	74.4 (60-94)	8 (80.0%)
Clean Samples (RPE/Choroid	9	6.76	75.0 (60-87)	7 (66.7%)	5	7.06	83.4 (74-94)	2 (40.0%)	9	6.93	74.2 (60-94)	7 (77.8%)

Abbreviations: n, number; Ave., average; RIN, RNA Integrity Number; RPE, Retinal Pigment Epithelium.

Gene Expression Differences

A total of 26,650 genes were expressed in RPE/choroid and/or retina. Within phenotypically documented normal eyes, 16,638 genes showed significant (FDR <= 0.05) differential expression between RPE/choroid and retina tissues with a minimum fold change >= |1.5|. Within macular RPE/choroid tissues, significant differential expression was observed for 1,204 genes between neovascular AMD and normal eyes, 40 genes between intermediate AMD (AREDS 3) and normal eyes, and 1,194 genes between Intermediate AMD and neovascular AMD eyes (**Fig 2a; S1 Table**). Within macular neural retina tissues, 41 genes were differentially expressed between

neovascular AMD and normal eyes, 30 genes between Intermediate AMD and normal eyes, and 50 genes between Intermediate AMD and neovascular AMD eyes (**Fig 2b**). Of these differentially expressed genes, 29 were unique to Intermediate AMD vs. normal RPE/choroid, 285 were unique to neovascular AMD vs. normal, and 276 were unique to intermediate AMD vs. neovascular AMD. Of the 40 significant differentially expressed genes in the normal versus Intermediate AMD RPE/choroid and the 1204 significant differentially expressed genes in the normal versus neovascular AMD RPE/choroid, six genes found in common (**Fig 3**). These six common genes were *MTRN2L1, CLEC2L, CCM2L, CYP4X1, GLDN,* and *SMAD7* (**Fig 3**). The top 10 differentially expressed genes between disease states within the RPE/choroid and Retina are listed in **Table 2a-f**. Of note, the only gene, that overlapped between any RPE/choroid and retina disease comparisons, was mitochondrial-derived peptide humanin (*MTRNR2L1*). *MTRNR2L1* was found to be differentially expressed between the intermediate AMD versus normal state. Of interest in the context of AMD, *MTRNR2L1* has been previously reported to protect human RPE cells against oxidative stress and restore mitochondrial function *in vitro* [51]. *CHD7* which was found to be downregulated in those with intermediate AMD compared to normal RPE/choroid has been previously associated with AMD in non-smokers by GWAS [52]. *TIMD4*, upregulated in those with intermediate AMD compared to normal RPE is associated with regulation of blood level lipids (LDL, cholesterol and triglycerides) at the GWAS level [53-55]. Also noteworthy, a unique lncRNA (AC000124.1) was down regulated in intermediate AMD compared to neovascular RPE/choroid, while lincRNA (RP11-

240M16.1) and PIWL1 were both upregulated in neovascular AMD compared to normal RPE/choroid (Tables 2 and 2b, respectively).

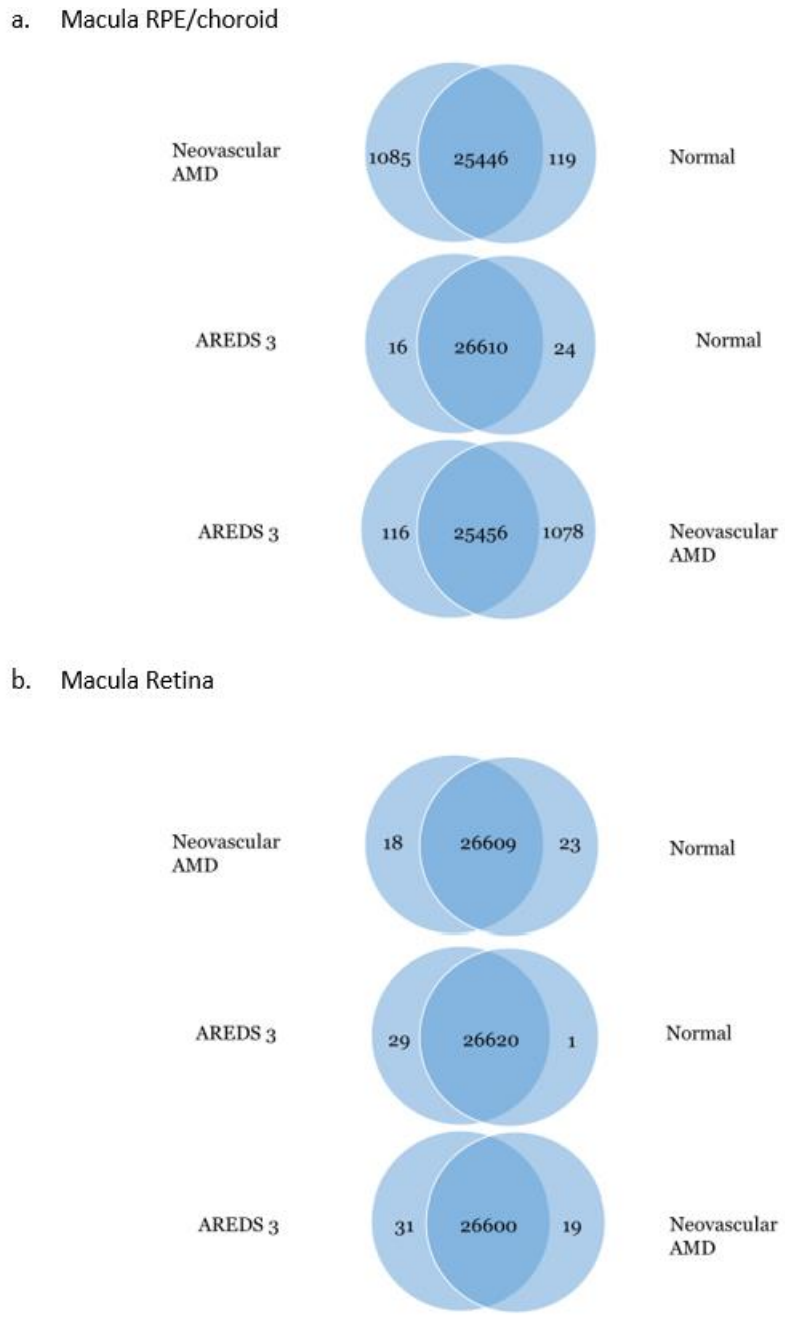

Fig 2. Differentially expressed genes. Each circle shows the number of genes with increased expression in that disease state, i.e., 1085 genes were upregulated in the macula RPE/choroid of neovascular eyes when compared to normal eyes and 119 genes were upregulated in the macula RPE/choroid of normal eyes compared to neovascular eyes.

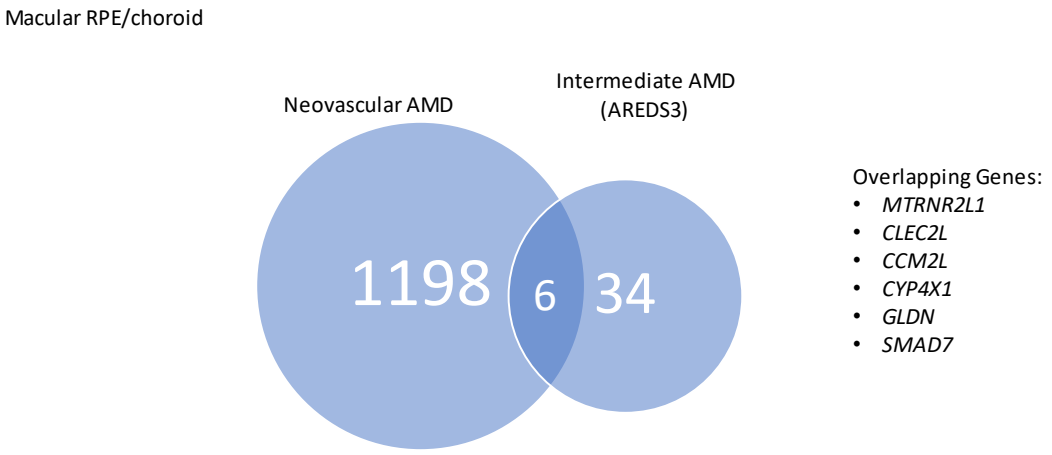

Fig 3. Overlap of differentially expressed genes. Each circle represents the number of significant differentially expressed genes in RPE/choroid when comparing normal eyes with both neovascular AMD and AREDS3. The overlap between these two circles shows the number of common genes between these two conditions.

Table 2. Top ten significant differentially expressed genes in each disease comparison

2a- Intermediate AMD vs Normal RPE/choroid

Gene Name	Fold Change	p-value	Function
MTRNR2L1	59.045	1.75462E-05	Negative regulaiton of apoptosis
PON1	4.606	0.0003	Calcium ion binding and phospholipid binding
IFI6	3.613	0.0033	Reguation of apoptosis
TIMD4	23.277	0.0033	enable phosphatidylserine binding activity
GPR123	-3.811	0.0040	G-protein couples receptor activity and transmembrane signaling receptor activity
RN7SKP186	2.105	0.0041	Pseudogene
CDH7	-3.263	0.0128	Calcium ion binding
TCN2	3.753	0.0144	Cobalamin binding
CLEC2L	-4.781	0.0230	Carbohydrate binding
DPP6	-2.197	0.0230	Serine-type peptidase activity

2b- Neovascular AMD vs Normal RPE/choroid

Gene Name	Fold Change	p-value	Function
RP11-58B17.2	8.857	1.01735E-14	Unknown
RP11-240M16.1	31.631	3.15544E-14	Unknown
SLC1A2	12.297	3.15544E-14	Glutamate transmembrane transporter and symporter activity
SLC24A1	12.288	3.15544E-14	Calcium and postssium/sodium symporter
SCAMP5	7.473	5.13906E-13	Calcium-dependent exocytosis
PTPRN	11.977	7.34743E-13	Phosphatase activity and spectrin binding
RHO	47.223	7.34743E-13	Photoreceptor and G-protein coupled receptor activity
RP11-215G15.5	8.640	1.27666E-12	Unknown
SEMA7A	8.749	2.14117E-12	Integrin binding and signaling receptor activity
CPE	6.373	2.27007E-12	Cell adhesion molecule binding and carboxypeptidase activity

2c- Intermediate AMD vs Neovascular AMD RPE/choroid

Gene Name	Fold Change	p-value	Function
CALB1	-13.385	1.17108E-10	Calcium and vitamin D binding
RP11-58B17.2	-5.688	1.17108E-10	Unknown
AC000124.1	-16.266	8.11468E-10	Unknown
AC114803.3	-9.014	8.11468E-10	Unknown
CADM2	-7.240	8.11468E-10	Synaptic cell adhesion
CHRNB2	-4.518	8.11468E-10	Protein heterodimerization and extracellular ligand-gated ion channel activity
PTPRN	-9.354	8.11468E-10	Phosphatase activity and spectrin binding
SEZ6L2	-4.310	8.11468E-10	Endoplasmic reticulum functions in neurons
SLC24A1	-8.444	8.11468E-10	Calcium and postssium/sodium symporter
TM4SF2	-3.642	8.11468E-10	Cellular proliferation and motility

2d- Intermediate AMD vs Normal Retina

Gene Name	Fold Change	p-value	Function
MTRNR2L1	68.021	0.0000	Negative regulaiton of apoptosis
ERN1	2.121	0.0038	Degrading misfolded proteins
ANO3	3.706	0.0053	Calcium-dependent phospholipid scramblase activity
CASP7	2.223	0.0053	Execution phase of cellular apoptosis
CXCL10	25.072	0.0053	Signaling receptor binding and chemokine activity
HELZ2	2.847	0.0053	RNA binding and nuclear receptor activity
RNU4-1	2.566	0.0053	RNA coding associated with the snRNA class
SNORA12	2.126	0.0053	Small non-coding RNA involved in RNA processing
SNORD89	2.620	0.0053	RNA coding associated with the snoRNA class
SNORA21	2.573	0.0084	RNA coding associated with the snoRNA class

2e- Neovascular AMD vs Normal Retina

Gene Name	Fold Change	p-value	Function
NME3	-1.839	3.41E-05	Nucleoside diphosphate kinase activity
CTD-2571L23.6	-3.642	9.41E-05	RNA gene affiliated with the lncRNA class
MTRNR2L1	62.680	1.00E-04	Negative regulaiton of apoptosis
RNU11	3.658	0.0004	RNA gene assocaited with the snRNA class
RPS25	1.537	0.0005	RNA binding
RN7SKP185	-5.475	0.0008	Small nuclear pseudogene
RPL14P1	1.732	0.0028	Pseudogene
GRIN3B	-2.177	0.0044	Calcium channel activity and ionotropic glutamate receptor activity
AC083873.4	2.623	0.0103	Unknown
RP11-56B16.5	-2.297	0.0103	Unknown

2f- Intermediate AMD vs Neovascular AMD Retina

Gene Name	Fold Change	p-value	Function
AC004943.1	9.961	0.0003	Unknown
NME3	1.779	0.0003	Nucleoside diphosphate kinase activity
CTD-2571L23.6	3.390	0.0005	RNA gene affiliated with the lncRNA class
RP11-90H3.1	-2.179	0.0005	Unknown
RN7SL263P	8.049	0.0006	Pseudogene
AC013470.6	-3.211	0.0015	Unknown
NSA2	-1.662	0.0015	RNA binding
AC010761.8	1.617	0.0020	Unknown
AL353629.1	-2.141	0.0034	Unknown
RPL14P1	-1.725	0.0034	Pseudogene

Analysis of Genes previously associated with AMD

Among genes demonstrated to be associated with AMD risk in previous studies [10,56-57] and the GWAS conducted by the International AMD Genetics Consortium

(IAMDGC) [1], expression was statistically significantly *higher* for *ABCA4* (3.1 fold increase, p-value = 0.0001), *ABCA7* (3.4 fold increase, p-value = 0.0018), and *RORA* (1.9 fold increase, p-value = 0.004) in neovascular RPE/choroid tissue compared with normal RPE/choroid tissue. (fold change > 2, p-value ≤ 0.01; **Table 3**). *VTN* expression (fold change = 3.3, p-value = 0.02) was also found to be significantly *higher* in neovascular RPE/choroid tissue compared with normal RPE/choroid tissue. Consistent with these results, we found that expression of *ABCA4* (fold change = -3.1, p-value = 0.0002) and *ABCA7* (fold change = -3.2, p-value = 0.0018) was significantly *lower* in intermediate AMD RPE/choroid tissue compared with neovascular RPE/choroid tissue. (fold change > 2, p-value ≤ 0.01). Expression was also found to be significantly *higher* for *TNFRSF10B* (fold change = 1.7, p-value = 0.02) and *TRPM1* (fold change = 1.7, p-value = 0.04) in intermediate AMD RPE/choroid tissue compared with neovascular RPE/choroid tissue. Additionally, *SPEF2* expression (fold change = -1.5, p-value = 0.0165) was significantly *lower* in intermediate AMD RPE/choroid tissues compared to neovascular RPE/choroid tissue. None of the associated AMD risk susceptibility genes were significantly differentially expressed between intermediate AMD and normal RPE/choroid tissues.

Table 3. Comparisons of Expression levels in RPE of previously established AMD risk loci among AMD stages*

AMD GWAS Loci	Normal RPE vs. Normal Retina		Neo vs. Normal Retina		AREDS3 vs. Normal Retina		AREDS3 vs. Neo Retina		Neo vs. Normal RPE		AREDS3 vs. Normal RPE		AREDS3 vs. Neo RPE	
Threshold Values	*Fold Change*	*p-value*	*Fold Change*	*p-value*	*Fold Change*	*p-value*	*Fold Change*	*p-value*	*Fold Change*	*p-value*	*Fold Change*	*p-value*	*Fold Change*	*p-value*
ABCA1	13.865	1.39E-83	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ABCA4	-2.284	9.52E-05	N/A	N/A	N/A	N/A	N/A	N/A	3.142	0.0001	N/A	N/A	-3.076	0.0002
ABCA7	-6.374	6.44E-13	N/A	N/A	N/A	N/A	N/A	N/A	3.375	0.0018	N/A	N/A	-3.231	0.0032
ABHD2	4.298	3.86E-21	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ACAD10	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ADAMTS9-AS1	11.727	2.85E-24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ADAMTS9-AS2	16.646	1.16E-46	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
APOE	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ARHGAP21	-1.53	8.32E-07	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ARMS2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
B3GALTL	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
C10orf88	-2.16	6.79E-35	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
C2	17.818	1.71E-21	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
C3	16.823	3.73E-24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
C4A	19.678	2.28E-19	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
C9	14.424	6.47E-31	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CCT3	-1.523	6.66E-06	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CD63	4.9501	3.07E-65	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CETP	146.22	1.97E-35	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CFB	24.826	6.07E-24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CFH	62.528	4.79E-179	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CFHR3-CFHR1	44.624	1.56E-16	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CFI	3.5126	1.44E-12	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CNN2	8.0194	6.84E-60	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
COL4A3	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
COL5A1	7.6473	7.78E-32	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
COL8A1	90.435	3.09E-106	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Corf85	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CTRB1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CTRB2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CXCL13	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CYP24A1	-5.642	0.000112	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
HTRA1	-1.523	0.011715	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ITGA7	3.6065	1.66E-32	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
KM2E	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
LIPC	13.302	2.41E-15	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
MIR6130	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
MMP19	5.3998	1.86E-31	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
MMP7	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
MMP9	5.7203	4.07E-05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
NLRP2	-4.161	0.001664	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
NPLOC4	1.7751	3.47E-16	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
PCOLCE	24.56	4.88E-85	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
PELI3	-2.506	8.96E-22	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
PILRA	3.6966	1.01E-06	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
PILRB	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
PKP2	6.1432	1.04E-62	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
PLA2G4A	6.8626	6.81E-33	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
PRLR	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
RAD51B	1.6343	7.50E-05	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
RDH5	22.822	4.47E-34	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
RGS13	9.175	1.40E-07	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
RLBP1	4.797	1.63E-09	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
ROBO1	1.7187	2.80E-07	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
RORA	-4.953	1.19E-30	N/A	N/A	N/A	N/A	N/A	N/A	1.867	0.0043	N/A	N/A	N/A	N/A
RORB	-3.035	3.68E-23	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
RPS6KA2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
SKIV2L	1.6319	1.72E-14	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
SLC16A8	54.981	6.76E-54	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
SPEF2	-2.981	3.60E-25	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	-1.536	0.0165
SRPK2	-1.767	2.29E-39	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
STON1-GTF2A1L	1.5378	0.045064	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
SYN3	-17.08	1.16E-74	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
TGFBR1	3.7317	9.59E-24	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
TIMP3	54.192	3.04E-129	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
TMEM97	-3.67	3.66E-12	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
TNFRSF10A	17.434	3.57E-58	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
TNFRSF10B	8.2059	8.67E-50	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.717	0.0183
TRPM1	2.8839	1.20E-12	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	1.702	0.041
TRPM3	7.2432	3.97E-40	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
TSPAN10	67.019	2.60E-80	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
UNC93B1	25.512	1.38E-47	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
VDR	4.976	1.29E-07	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
VEGFA	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
VTN	-4.777	8.35E-07	N/A	N/A	N/A	N/A	N/A	N/A	3.334	0.0206	N/A	N/A	N/A	N/A

Table 3. A Fold change larger than two with a p-value ≤ 0.01 is indicated with yellow, and values significant using a lower threshold (p-value ≤ 0.05) are shown in light blue.

Abbreviations: Int, intermediate; RPE, RPE/choroid.

*No significant differences were seen between any disease comparisons within the retina tissues

Within normal macular tissues, *ABCA4, ABCA7, C10orf88, CYP24A1, NLRP2, PELI3, RORA, RORB, SPEF2, SYN3, TMEM97,* and *VTN* were significantly up-regulated in the neural retina compared to the RPE/choroid, whereas *ABCA1, ABHD2, ADAMTS9-AS1, ADAMTS9-AS2, C2, C3, C4A, C9*, *CD63, CETP, CFB, CFH, CFHR3-CFHR1, CFI, CNN2, COL5A1, COL8A1, ITGA7, LIPC, MMP19, MMP9, PCOLCE, PILRA, PKP2, PLA2G4A, RDH5, RGS13, RLBP1, SLC16A8, TGFBR1, TIMP3, TNFRSF10A, TNFRSF10B, TRPM1, TRPM3, TSPAN10, UNC93B1,* and *VDR* were significantly up-regulated in the RPE/choroid compared to the retina (threshold: fold change > 2, p-value ≤ 0.01; **Table 3**). *ARHGAP21, CCT3, HTRA1,* and *SRPK2* were also found to be up-regulated in the neural retina compared to the RPE/choroid, whereas *NPLOC4, RAD51B, ROBO1, SKIV2L,* and *STON1-GTF2A1L* were found to be marginally up-regulated in the RPE/choroid compared to the neural retina (**Table 3**). Overall, of the 34 GWAS loci (5), 75% were found to be statistically differentially expressed between the macular Retina and the macular RPE/choroid, of these genes 75% were significantly upregulated in the RPE/choroid.

Pathway Analysis for Differentially Expressed Genes

IPA pathway analysis was conducted on significant differentially expressed genes found in the RPE/choroid across all disease states. For each comparison, the ten most significant pathways for each condition was identified (**Table 4**). When comparing intermediate AMD and normal, 21 significant pathways for significant differentially

expressed genes were revealed (**Table 4**). A total of 63 significant pathways were identified when looking into significant differentially expressed genes in neovascular AMD with normal, and 52 significant pathways when comparing neovascular AMD with intermediate AMD (**Table 4**). Interestingly, the calcium signaling pathway was significant across all comparisons, while 47 pathways were in common between the neovascular AMD and normal with the neovascular and intermediate AMD (**Table 4**). A pathway analysis of the six overlapping genes between intermediate AMD and normal with neovascular AMD and normal revealed 14 significant pathways, with BMP signaling and TGF-β signaling being of particular interest.

Table 4. IPA pathway analysis of significant differentially expressed genes.

Neovascular AMD vs Intermediate AMD		Intermediate AMD vs Normal		Neovascular AMD vs Normal		Comparison of Neovascular AMD vs Normal with Neovascular AMD vs Intermediate AMD
Pathway Name	**P-Value**	**Pathway Name**	**P-Value**	**Pathway Name**	**P-Value**	**Pathway Name**
Phototransduction Pathway	8.41E-30	Interferon Signaling	2.68E-05	Phototransduction Pathway	1.08E-29	Phototransduction Pathway
SNARE Signaling Pathway	6.06E-08	Th1 and Th2 Activation Pathway	1.68E-04	SNARE Signaling Pathway	3.37E-07	SNARE Signaling Pathway
Cardiac β-adrenergic Signaling	1.5E-06	Th1 Pathway	1.01E-03	Cardiac β-adrenergic Signaling	1.72E-06	Cardiac β-adrenergic Signaling
Synaptogenesis Signaling Pathway	2.45E-06	nNOS Signaling in Neurons	2.62E-03	G-Protein Coupled Receptor Signaling	5.51E-06	G-Protein Coupled Receptor Signaling
Protein Kinase A Signaling	5.95E-05	Role of JAK2 in Hormone-like Cytokine Signaling	4.52E-03	Endocannabinoid Neuronal Synapse Pathway	5.69E-06	Endocannabinoid Neuronal Synapse Pathway
GABA Receptor Signaling	6.26E-05	Activation of IRF by Cytosolic Pattern Recognition Receptors	4.96E-03	Role of NFAT in Cardiac Hypertrophy	5.04E-05	Role of NFAT in Cardiac Hypertrophy
Endocannabinoid Neuronal Synapse Pathway	7.18E-05	Multiple Sclerosis Signaling Pathway	4.58E-03	Synaptogenesis Signaling Pathway	6.1E-05	Synaptogenesis Signaling Pathway
G-Protein Coupled Receptor Signaling	3.81E-04	JAK/STAT Signaling	7.78E-03	GABA Receptor Signaling	6.88E-05	GABA Receptor Signaling
Role of NFAT in Cardiac Hypertrophy	3.69E-04	Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza	8.53E-03	Protein Kinase A Signaling	6.95E-05	Protein Kinase A Signaling
Relaxin Signaling	3.76E-04	IL-15 Production	1.69E-02	Relaxin Signaling	1.27E-04	Relaxin Signaling

Table 4. Shows the top ten most significant pathways of the significant differentially expressed genes across all disease conditions. A comparison of the significant pathways in two comparisons, Neovascular AMD vs Normal and Neovascular AMD vs Intermediate AMD with the top ten shown.

Gene splicing differences among AMD stages and tissue types

Differential splicing was observed for 1,154 genes within the retina and for 629 genes within the RPE/choroid in a comparison of neovascular AMD and normal tissues (**Fig 4**). Fewer differentially spliced genes were observed for comparisons of intermediate AMD vs. neovascular AMD (810 in retina, 608 in RPE/choroid) and intermediate AMD vs. normal (210 in retina, 177 in RPE/choroid). Only a small proportion of the 629 differentially spliced genes in the RPE choroid were also differentially expressed in group comparisons: 113 neovascular AMD vs. normal (18.0%), 98 intermediate AMD vs. neovascular AMD (16.1%), and 6 intermediate AMD vs. normal (3.4%). There were no overlapping differentially expressed and spliced genes within the retina. A much larger number of genes in the retina were differentially spliced than in the RPE for all group comparisons.

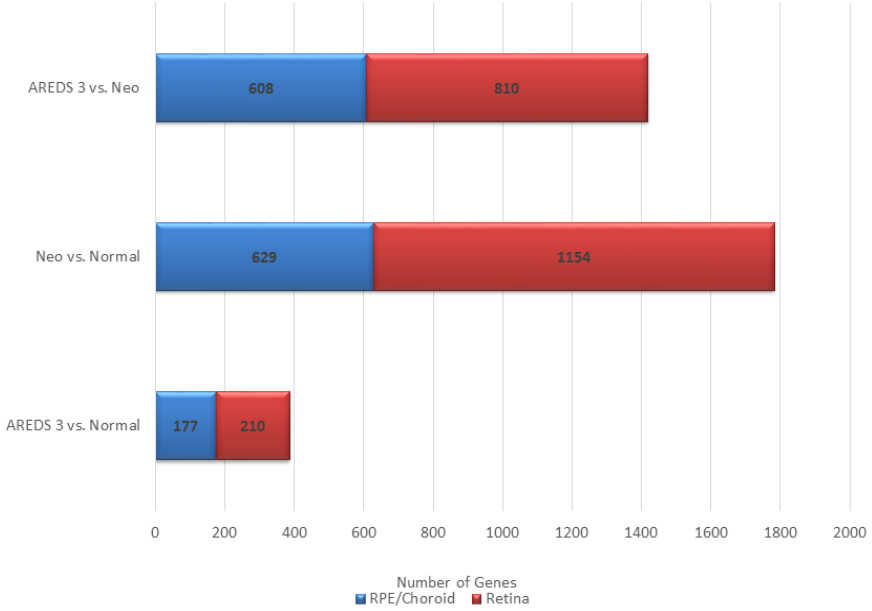

Fig 4. Differentially spliced genes. The number of genes showing differential splice sites among each of the comparisons examined.

Allele Specific Expression (ASE)

According to annotation information for published AMD genome-wide association studies included in the NHGRI-EBI Catalog (https://www.ebi.ac.uk/gwas/home; accessed 3/28/17), 12 AMD-associated SNPs are located in coding regions of APOE (1), ARMS2 (1), C2 (1), C3 (2), CFB (1), CFH (4), CFI (1), and PLA2G12A (1), and therefore were investigated for allele specific expression (**Table 5**). No heterozygotes were found in our sample for CFH rs121913059, CFI rs141853578, C3 rs147859257, or APOE rs429358. We found no expression of ARMS2 in either the RPE/choroid or neural retina and therefore we could not investigate the coding SNP rs10490924. For those heterozygotes showing mono-allelic expression (n=6), CFH rs10754199 was used to confirm heterozygotes for CFH coding SNPs rs10661170 and rs1061147 (r2 = 1 for rs10754199 and both coding SNPs), CFB rs2242572 was used to confirm heterozygous genotypes for CFB rs641153 (r2 = 1), and C3 rs1047286 was used to confirm the heterozygous genotype of C3 rs2230199 (r2 = 0.843). Significant ASE was detected within individuals at 4 SNPs: CFH rs1061170 (Y402H), CFH rs1061147, CFB rs641153, and C3 rs2230199. Specifically, for CFH rs1061170 we found significant ASE within 2/6 intermediate AMD RPE samples, and 1/7 normal RPE samples. None of the four neovascular AMD RPE heterozygotes showed ASE, noting that there were 10 or fewer reads for these samples in the retina data. For rs1061147, significant ASE was observed within 5/6 intermediate AMD RPE samples, 3/4 neovascular RPE samples, and 7/7 normal RPE samples. These same heterozygotes had 10 or fewer reads among the retina data. The single heterozygote for CFB rs641153 (a normal sample) showed

significant ASE within the RPE tissue. There were 10 or fewer reads for this SNP in macula retina. There was significant ASE for C3 rs2230199 within 2/3 intermediate AMD RPE samples, 0/1 neovascular AMD RPE samples, and 2/2 normal RPE samples. These same heterozygotes had 10 or fewer reads in retina tissue.

Table 5. Allele Specific Expression (ASE) of known AMD associated SNPs

			Individuals with Significant ASE (p<.05)*					
SNP	**Location**	**#Hets**	**Intermediate AMD Retina**	**Neovascular Retina**	**Normal Retina**	**Intermediate AMD RPE**	**Neovascular RPE**	**Normal RPE**
CFH rs1061147	chr1:196654324	18	0/0	0/0	0/0	5/6	3/4	7/7
CFH rs1061170	chr1:196659237	18	0/0	0/0	0/0	2/6	0/4	1/7
CFH rs35292876	chr1:196706642	1	0/0	0/0	0/0	0/1	0/0	0/0
CFH rs121913059	chr1:196716375	0	0/0	0/0	0/0	0/0	0/0	0/0
PLA2G4A rs2285714	chr4:110638810	15	0/1	0/1	0/3	0/3	0/0	0/1
CFI rs141853578	chr4:110685820	0	0/0	0/0	0/0	0/0	0/0	0/0
C2 rs9332739	chr6:31903804	4	0/0	0/0	0/0	0/1	0/0	0/2
CFB rs641153	chr6:31914180	6	0/0	0/0	0/0	0/0	0/0	1/1
ARMS2 rs10490924	chr10:124214448	7	0/0	0/0	0/0	0/0	0/0	0/0
APOE rs429358	chr19:45411941	0	0/0	0/0	0/0	0/0	0/0	0/0
C3 rs147859257	chr19:6718146	0	0/0	0/0	0/0	0/0	0/0	0/0
C3 rs2230199	chr19:6718387	6	0/0	0/0	0/0	2/3	0/1	2/2

Abbreviations: *, Only individuals with greater than 10 reads are counted; SNP, Single Nucleotide Polymorphism; Hets, heterozygotes; RPE, Retinal Pigment Epithelium.

Validation and replication of RNA-Seq findings

We validated our RNA-Seq methodology by choosing genes that varied in fold expression from a range of +20 to -20 (FDR of $p < 0.05$) between the normal RPE/choroid and retina -- *UCHL1, PFKP*, and *LPCAT1* (down-regulated in RPE/choroid vs. retina) and *PDPN, GAS1*, and *CST3* (up-regulated in RPE/choroid vs. retina) -- by real-time qPCR reactions run in triplicate on a subset of samples that were used for the RNA-Seq experiments. We confirmed the direction of effect for five of the six genes examined (data not shown). We were unable to detect *PFKP* expression in the

RPE/choroid tissue and therefore this gene could not be validated. Additionally, we were able to replicate all of our top 20 genes from the normal RPE/choroid vs. normal retina by the Human Eye Integration data (https://eyeintegration.nei.nih.gov/). This database is a collection of healthy human RNA-Seq datasets generated from various studies of human eye tissue. To the best of our knowledge, no public database is yet available that contains gene expression data for the macular RPE/choroid tissues for different AMD clinical stages compared with normal macular RPE/choroid.

Discussion

This is the first study using RNA-Seq to demonstrate genes, including a few of those previously associated with AMD risk, and lncRNAs are expressed differently in the macular retina compared to the macular RPE/choroid layers of eyes obtained within six hours of death from persons with intermediate or macular neovascularization or age-matched controls using a standardized protocol. Evaluation of known coding regions in previously reported GWAS loci demonstrated that significant ASE for C3 rs2230199 and CFH rs1061170 occurred in the macula RPE/choroid for normal, and intermediate AMD while ASE for CFH rs1061147 occurred in occurred in the macula RPE/choroid for normal, and intermediate and neovascular AMD. The protective variant for CFB, rs641153 only demonstrated ASE in the normal macular RPE/choroid. This is also underscored that these genes which function in the complement pathway were shown to be expressed statistically significantly higher in the normal macula RPE choroid compared to the normal macula retina. Our comprehensive analysis demonstrated differential patterns of gene expression and splicing between diseased and non-

diseased macula retina and RPE/choroid. It is clear that both the retina and RPE/choroid are important to AMD pathophysiology, however the relationship between the tissue types at given stages of disease is not entirely clear [58-59]. It has been hypothesized that RPE cell function is more significantly related to AMD pathophysiology than retinal cell function [58, 60-62]. It is interesting to note that while we did not find the majority of the IAMDGC GWAS loci [5] differentially expressed between intermediate AMD and/or neovascular AMD, we did find that 75% of the genes were statistically significantly higher in the macular RPE/choroid compared to the macular retina thus underscoring the importance of tissue and geographic location in elucidating AMD etiology [62].

Of the known previously associated AMD loci [1, 10, 57, 63], none of them showed differential expression between disease states within the macular neural retina tissues. Of the 34 GWAS loci [5], expression differences between disease states were observed for SPEF*2* (between AREDS3 vs. Neo RPE) and *VTN* (between Neo vs. normal RPE/choroid). This suggests that variants in the gene regions showing heritable differences in AMD risk may influence the expression of tissue. A recent proteomic study on serums from patients with and without all types of late AMD (eg both neovascular and GA), found that ZBPB*, KREMEN2,* and *LINGO1*A were upregulated in diseased patients [64]. We found the expression of these genes to be significantly upregulated when comparing neovascular AMD or intermediate AMD with the normal macula RPE/choroid.

Absence of differential expression of the two genes previously reported to have the largest effect on AMD risk, *CFH* and *ARMS2/HTRA1*, is in agreement with studies

demonstrating that *HTRA1* may influence AMD risk at the tRNA level.[65-66]. Moreover, while *CFH* did not demonstrate differential gene expression between disease states in our study, CFH did demonstrate allele specific expression with both intermediate AMD and neovascular AMD depending on the SNP being interrogated. It may be this unequal expression of alleles at a given variant within the CFH gene to the disease pathophysiology of AMD. The mechanisms that underlie ASE are under active investigation and include epigenetics [67] In general, the differentially expressed genes reported here belong to pathways that have been shown to have important roles in AMD pathogenesis, including cholesterol and lipid metabolism (*DPP6, PON1, TIMD4*), mitochondrial regulation (*MTRNR2L1, TIMD4, IFI27, DISC1FP1*), and pro-inflammatory pro-macrophagic signaling in intermediate AMD.[68-71]. Thus, our results also substantiate the possible contribution of macrophages in the late neovascular stage of the disease, as well as the contribution of mitochondrial dysfunction in the onset and progression of neurodegenerative diseases [68, 72]. Furthermore, *TCN2* regulates Vitamin B12 levels by encoding a protein that is part of the Vitamin B pathway, and was found in this study to be upregulated in those with intermediate AMD compared to normal macular RPE/choroid. Circulating low vitamin B12, high total homocysteine, and low folic acid levels are associated with AMD risk, suggesting that a defect in *TCN2* may reduce its ability to bind its partner proteins correctly, thus resulting in overexpression in the RPE/choroid and potentially a role in the subsequent development of large pathogenic drusen [73-75].

A trend was observed when comparing the top ten differentially expressed genes between each RPE comparison. In both the ‘intermediate AMD versus normal

RPE/choroid' and 'neovascular AMD versus normal RPE/choroid' comparisons, upregulation of *MTRNR2L1* was observed. Between both the 'neovascular AMD versus normal RPE/choroid' and 'intermediate versus neovascular AMD RPE/choroid' comparisons, three differentially expressed genes were shared, all expressed in opposite magnitudes (ie. upregulated in neovascular AMD and intermediate AMD, respectively; downregulated in normal and neovascular AMD, respectively): *RHO, RBP3,* and *RP51092A11.2.* Further investigation of these loci could prove useful for identifying novel biomarkers to differentiate between, as well as to track disease progression from, the intermediate form of AMD to the neovascular form.

We also observed differential expression of two distinct lncRNAs (*lncRNA AC00124.1* and *lincRNA RP11-240M16.1*) in the RPE/choroid of both intermediate and late stage AMD patients in comparison to neovascular and normal eyes respectively, although in opposite directions (downregulated and upregulated, respectively). Since one lncRNA (*lncRNA AC000124.1*) was downregulated in intermediate AMD compared to neovascular AMD patients, it may be used as a biomarker for further validating disease progression from intermediate AMD to neovascular AMD. The lncRNA (*lincRNA RP11-240M16.*1) that we observed to be overexpressed in neovascular RPE/choroid compared to normal RPE/choroid may be a biomarker for late stage disease. Further investigations could include comparing *lncRNA RP11-240M16.1* expression in neovascular versus GA AMD samples. If found to be differentially expressed between neovascular and GA forms of AMD, it be used as a biomarker to further distinguish between the two late stage forms and could potentially contribute to pathogenesis.

Many of our findings substantiate current ideas regarding AMD pathophysiology. Specifically, significant differential expression between disease states was observed for genes involved in lipid metabolism (*PON1*)[76] and inflammation (*TIMD4*, *PON1, DPP6*, *GPR123*)[55,77-78]. In addition, several of these genes have been associated with disease states hypothesized to co-occur more with AMD such as Alzheimer disease (AD) (*PON, SLC1A2, CPE, NME3*)[78-84] and epilepsy (*CHRNB2, SEZ6L2*) [84-86]. This suggests such diseases may have co-occurring pathophysiology as well as occurrence with AMD.[86-88] Our data also suggest a possible overlap in pathophysiology within the vitamin B12 synthesis pathway[89-90] and amyotrophic lateral sclerosis (ALS).[90-91] Finally, we describe differential expression between intermediate and normal donor eyes of *CDH7,* a gene that was previously found to interact with smoking and be associated with AMD risk.[52] One gene, *MT2L1* (humanin), overlapped between RPE/choroid and retina, and was overexpressed in the intermediate stage of AMD compared to normal. Further studies on *MT2L1* have found it to serve a protective role in AD [92-94]. A similar circumstance has been noted for the inverse pattern of association of the APOE alleles: the ε4 allele increases risk of AD and the ε2 allele is protective, whereas the effects of these alleles on AMD risk are opposite [5,95-98].

Several genes and pathways were differentially expressed in comparisons of neovascular AMD with both intermediate AMD and normal macula, suggesting that clinical AMD phenotypes may represent a spectrum of molecular pathophysiology with a similar “core” of dysfunction with superimposed changes that dictate end-stage pathology. The majority of genes that were significantly differentially expressed in

comparisons of normal macular tissue and intermediate AMD with end-stage neovascular disease participate in injury response (*SLC1A2, SCAMP5, RHO, PIWIL1, TM4SF2, SLC24A1, PTPRN, CPE, CHRNB2, CALB1*, and *CADM2*) and apoptosis (*CPE* and *PTPRN*) [98–104]. Targeting molecules involved in photoreceptor apoptosis, has been suggested as an avenue for potential therapies to improve vision in neovascular AMD [105]. Pathway analysis of the overlapping DEGs from intermediate AMD vs normal AMD in the RPE/choroid and neovascular AMD vs normal AMD in the RPE/choroid showed the involvement of previously implicated GWAS hits TGF β signaling as well as BMP4 [106]. Thus, our findings may provide insight into the pathways modifying disease severity and or progression to neovascular end-stage disease.

Notch Signaling, WNT Beta Catenin Signaling, and TGF β signaling genes, as well as genes down-regulated in response to UV light, were down-regulated in the intermediate clinical stage macula compared to normal RPE/choroid macula. Of note, these pathways were not identified in the pathway analysis of differentially expressed genes in the retina comparing eyes from normal donors and persons with intermediate AMD (**Table 4**), and have all been implicated in AMD [57, 66 ,107-120]. IL2/STAT5 signaling, IL6/JAK/STAT3 signaling, and the inflammatory response and epithelial mesenchymal transition were uniquely differentially expressed and upregulated in macular intermediate AMD compared to normal macular retina. Stat3 activation has previously been implicated in AMD pathophysiology, specifically in the neovascular subtype [121-126]. Taken together, these findings suggest that particular genes that function in the pathways of the RPE/choroid are protective in the development of the

advanced forms of AMD while expression of STAT3 and/or other genes in inflammatory pathways in the retina may precede the development of late stage AMD.

Within the RPE/choroid tissues, we identified several genes that were differentially expressed between normal donor eyes and both Intermediate AMD and neovascular AMD, but also, more importantly, gene expression profiles and pathways that were unique to these AMD stages. These observations are consistent with the idea that mechanisms governing the development of intermediate AMD are different from those influencing progression to neovascular AMD, [57, 127-133] and thus unique therapeutic interventions may be necessary to treat effectively these different forms of AMD. Our findings also underscore the importance of studying both macular tissue types to gain a full understanding of mechanisms leading to AMD. Future studies should focus on single cell and/or single nuclei from well characterized fresh tissue particularly.

In summary, this RNA-Seq experiment identified novel and established factors contributing to development of intermediate and neovascular AMD. It also expands upon previous RNA studies that demonstrated gene expression differences in affected tissues Our results may provide insight into why some but not all individuals with intermediate AMD progress to the severe forms. If our results are confirmed in larger independent samples, differential gene expression could be implemented as an adjunct to a prognostic scheme (eg., blood). In order to make this a clinically useful tool, future studies will need to demonstrate equivalency in the same donors of pertinent gene expression patterns in eye tissue and other tissue that is easily accessible in living persons (e.g., blood).

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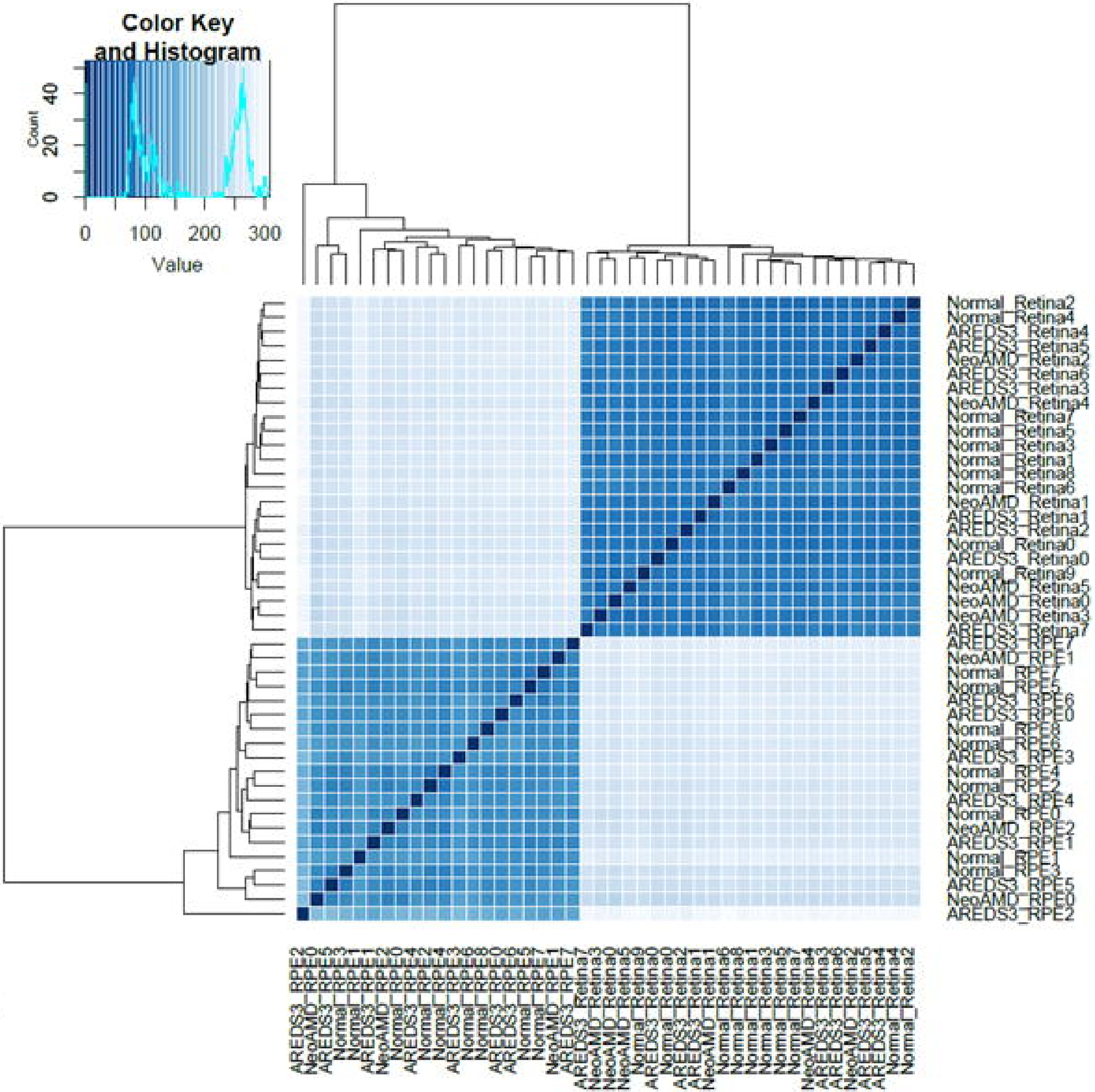
Supporting Information

S1 Table. Significant differential gene expression. Tables indicating all significant differential gene expression using a medium significance threshold (fold change > |1.5| and $p < .05$). There are separate tabs for each tissue and disease comparison.


Color Key
and Histogram
Count
0
20
40
0
100
200
300
Value
Normal_Retina2
Normal_Retina4
AREDS3_Retina4
AREDS3_Retina5
NeoAMD_Retina2
AREDS3_Retina6
AREDS3_Retina3
NeoAMD_Retina4
Normal_Retina7
Normal_Retina5
Normal_Retina3
Normal_Retina1
Normal_Retina8
Normal_Retina6
NeoAMD_Retina1
AREDS3_Retina1
AREDS3_Retina2
Normal_Retina0
AREDS3_Retina0
Normal_Retina9
NeoAMD_Retina5
NeoAMD_Retina0
NeoAMD_Retina3
AREDS3_Retina7
AREDS3_RPE7
NeoAMD_RPE1
Normal_RPE7
Normal_RPE5
AREDS3_RPE6
AREDS3_RPE0
Normal_RPE8
Normal_RPE6
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Normal_RPE2
AREDS3_RPE4
Normal_RPE0
NeoAMD_RPE2
AREDS3_RPE1
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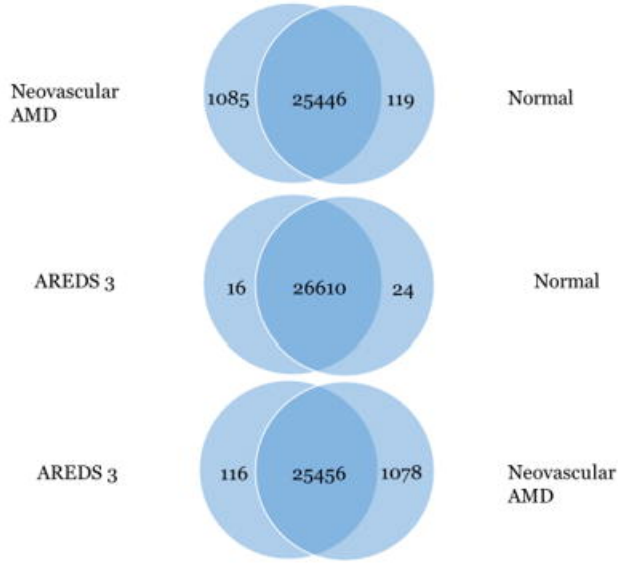
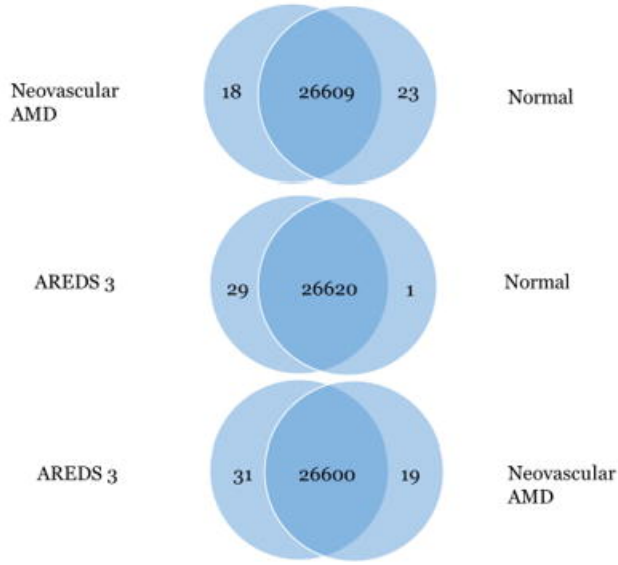
Normal_RPE3
AREDS3_RPE5
NeoAMD_RPE0
AREDS3_RPE2

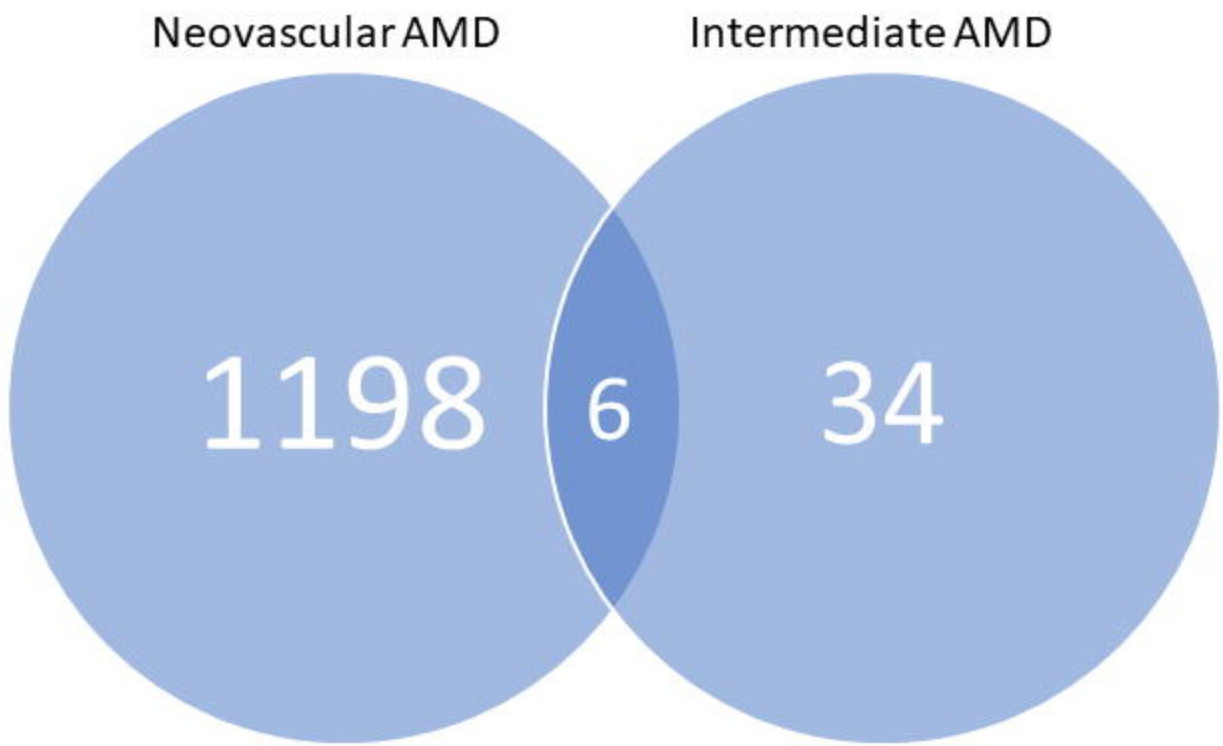
a. Macula RPE/choroid



b. Macula Retina

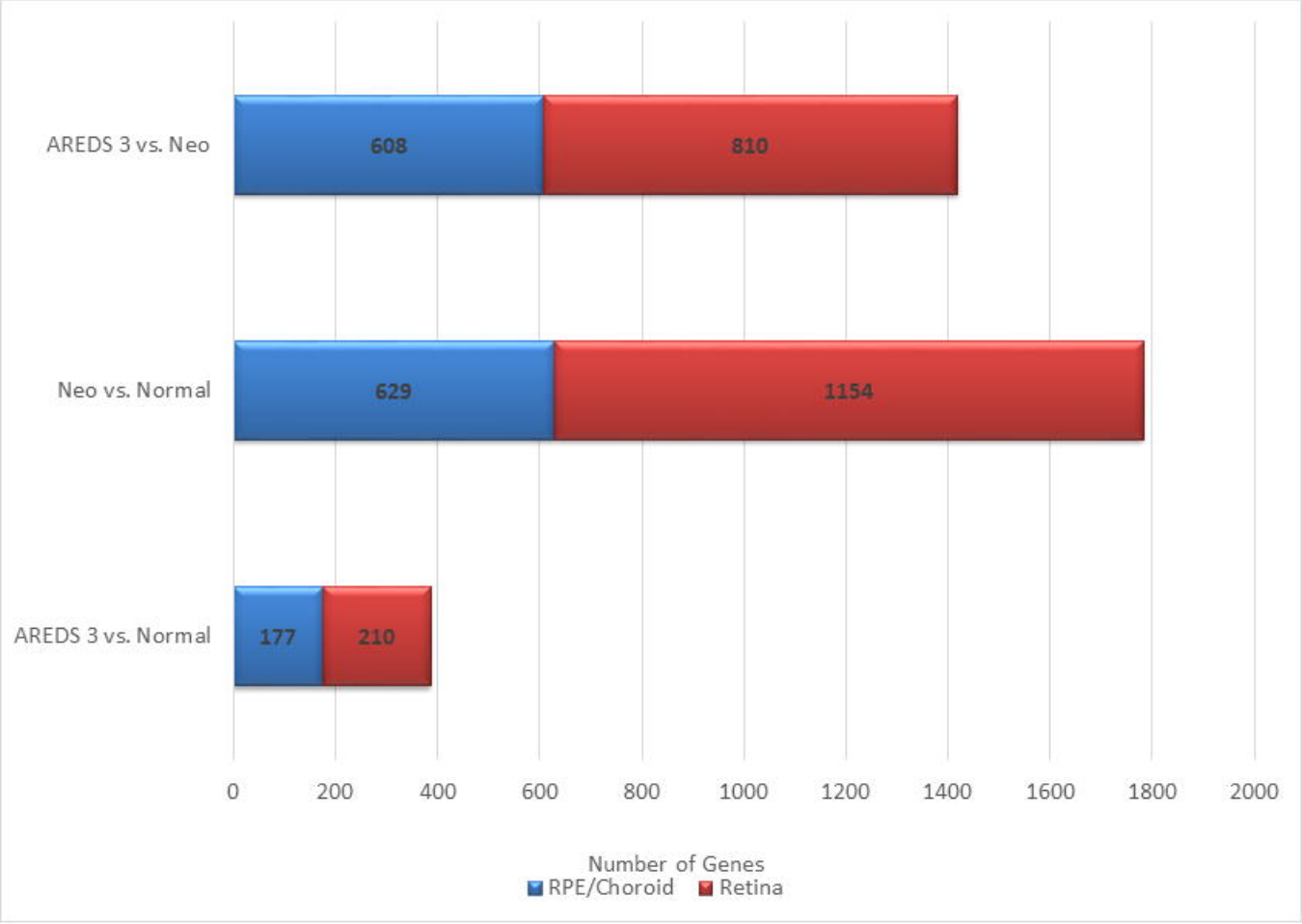


Macular RPE/choroid



Overlapping Genes:

- *MTRNR2L1*
- *CLEC2L*
- *CCM2L*
- *CYP4X1*
- *GLDN*
- *SMAD7*


AREDS 3 vs. Neo
608
810
Neo vs. Normal
629
1154
AREDS 3 vs. Normal
177
210
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Number of Genes
RPE/Choroid
Retina