A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants

Advanced age-related macular degeneration (AMD) is the leading cause of blindness in the elderly, with limited therapeutic options. Here we report on a study of >12 million variants, including 163,714 directly genotyped, mostly rare, protein-altering variants. Analyzing 16,144 patients and 17,832 controls, we identify 52 independently associated common and rare variants \((P < 5 \times 10^{-8})\) distributed across 34 loci. Although wet and dry AMD subtypes exhibit predominantly shared genetics, we identify the first genetic association signal specific to wet AMD, near \(MMP9\) (difference \(P\) value = \(4.1 \times 10^{-10}\)). Very rare coding variants (frequency \(<0.1\%\)) in \(CFH\), \(CFI\) and \(TIMP3\) suggest causal roles for these genes, as does a splice variant in \(SLC16A8\). Our results support the hypothesis that rare coding variants can pinpoint causal genes within known genetic loci and illustrate that applying the approach systematically to detect new loci requires extremely large sample sizes.

Advanced AMD is a neurodegenerative disease and the leading cause of vision loss among the elderly, affecting 5% of those \(>75\) years of age\(^{1,2}\). The disease is characterized by reduced retinal pigment epithelium (RPE) function and photoreceptor loss in the macula. Advanced AMD is classified as wet (choroidal neovascularization; when accompanied by angiogenesis) or dry (geographic atrophy; when angiogenesis is absent). These advanced stages of disease are typically preceded by clinically asymptomatic earlier stages\(^3\). Advanced AMD is estimated to affect 10 million patients worldwide, with the number of affected individuals reaching >150 million for earlier stages\(^5\). At present, understanding of disease biology and therapies remains limited\(^5\).

Identification of associated genetic variants can help uncover disease mechanisms and provide entry points into therapy. Analyses of common variation have identified numerous risk-associated loci for many complex diseases (GWAS Catalog; see URLs), including 21 loci for AMD\(^{6-12}\). However translation of these loci into biological insights remains a challenge, as the functional consequences of disease-associated common variants are typically subtle\(^{13}\) and hard to decipher.

With advances in sequencing technology, genetic analyses are gradually extending to rare variants, which often have more obvious functional consequences\(^{14,15}\) and can thus accelerate translation into biological understanding\(^{14,16}\). For example, identifying multiple disease-associated coding variants (particularly knockout alleles) in the same gene provides strong evidence that disrupting gene function leads to disease\(^{17}\). So far, studies that implicate specific rare variants in complex diseases either rely on special populations\(^{8,18,19}\), on targeted examinations of a few genes\(^{7,9-11,20,21}\) or on genome-wide assessments of relatively modest numbers of individuals\(^{22-25}\). In contrast, systematic analyses of common variation are now available in hundreds of thousands of phenotyped individuals\(^{26,27}\). Thus, there remains considerable uncertainty about the relative role of rare variants in complex disease and about the sample sizes and study designs that will enable systematic identification of association with these variants\(^{16}\).

Here we set out to systematically examine common and rare variation of AMD in the International AMD Genomics Consortium (IAMDGC). The preceding largest study of AMD examined \(\sim2.4\) million variants, including \(\sim18,000\) imputed or genotyped protein-altering variants, using meta-analysis\(^6\). Customizing a chip for \textit{de novo} centralized genotyping, we analyze >12 million variants, including 163,714 directly typed protein-altering variants, in 43,566 unrelated subjects of predominantly European ancestry. Our study constitutes a detailed simultaneous assessment of common and rare variation in a complex disease and a large sample, setting expectations for other well-powered studies.

RESULTS  
Study data and genomic heritability

We gathered advanced AMD cases with geographic atrophy and/or choroidal neovascularization, intermediate AMD cases and control subjects across 26 studies (Supplementary Table 1). Whereas recruitment and ascertainment strategies varied (Supplementary Table 2), DNA samples were collected and genotyped centrally. Making maximal use of genotyping technologies, we used a chip with (i) the usual genome-wide variant content, (ii) exome content comparable to an exome chip (adding protein-altering variants from across all exons), and a specific customization to add (iii) protein-altering variants detected by our previous sequencing of known AMD-associated loci (Online Methods) and (iv) previously observed and predicted variation in \(TIMP3\) and \(ARCA4\), two genes implicated in monogenic retinal dystrophies. After quality control, we retained 439,350 directly typed variants, including a grid of 264,655 primarily noncoding (93%) common...
variants (frequency among controls >1%) and 163,714 protein-altering variants (including 8,290 from known AMD-associated loci), which were mostly rare (88% with frequency among controls ≤1%). Imputation to the 1000 Genomes Project reference panel enabled examination of a total of 12,023,830 variants (Supplementary Table 3).

Our final data set included a total of 43,566 subjects, consisting of 16,144 patients with advanced AMD and 17,832 control subjects of European ancestry for our primary analysis, as well as 6,657 Europeans with intermediate disease and 2,933 subjects with non-European ancestry (Supplementary Fig. 1 and Supplementary Table 3).

Altogether, our genotyped markers accounted for 46.7% (ref. 28) of variability in advanced AMD risk in the European-ancestry subjects (95% confidence interval (CI) = 44.5–48.8%). With respect to AMD subtypes, heritability estimates for choroidal neovascularization (h² = 44.3%, CI = 42.2–46.5%) and geographic atrophy (h² = 52.3%, CI = 47.2–57.4%) were similar; bivariate analyses showed a genetic correlation of 0.85 (CI = 0.78–0.92) between these disease subtypes.

Thirty-four susceptibility loci for AMD

We first conducted a genome-wide single-variant analysis of the >12 million genotyped or imputed variants (applying genomic control λ = 1.13) comparing the 16,144 patients with advanced AMD and 17,832 controls of European ancestry (full results available online; see URLs). We obtained >7,000 variants with genome-wide significant association (P ≤ 5 × 10⁻⁸; Supplementary Fig. 2). Sequential forward selection (Supplementary Fig. 3) identified 52 independently associated variants at P ≤ 5 × 10⁻⁸ (Supplementary Table 4 and Supplementary Data Set 1). These variants are distributed across 34 ‘locus regions’ (Fig. 1a), each extending across the identified and correlated variants, including variants with r² ≥ 0.5 and ±500 kb with respect to the index SNP (Supplementary Table 5). The 34 loci include 16 loci that reached genome-wide significance for the first time (new loci; Table 1) and overlap genes with compelling biology, such as extracellular matrix genes (COL4A3, MMP19 and MMP9), an ABC transporter linked to high-density lipoprotein (HDL) cholesterol (ABCA1) and a key activator in immune function (PILRB). Also included are 18 of the 21 AMD susceptibility loci that reached genome-wide significance previously (known loci; Table 1); between-study heterogeneity was low, particularly for the new loci (Supplementary Tables 6 and 7, and Supplementary Note).

Most (45/52) associated variants are common, showing fully conditioned odds ratios (ORs; from one model including all 52 variants) from 1.1 to 2.9 (Fig. 1b and Supplementary Table 4) with two interacting variants (Supplementary Note). We also observed seven rare variants with frequencies between 0.01 and 1% and ORs between 1.5 and 47.6 (Fig. 1b and Supplementary Table 4). All of these variants were also rare in individuals of non-European ancestry (Supplementary Table 8; see extended association results for non-Europeans in Supplementary Data Set 2). All seven rare variants are located in or near complement genes; they comprise four previously described nonsynonymous variants (CFH, p.Arg1210Cys; CFI, p.Gly119Arg; C9, p.Pro1067Ser; C3, p.Lys155Gln)¹²–¹⁴ and three others (rs14855336, rs191281603 and rs35292876 in CFIH) described here for the first time, including two for which the rare allele decreases disease risk. To ensure the validity of our results, we verified associations of lead variants in sensitivity analyses that relied on alternate association tests, adjusted for age, sex or ten ancestry principal components, or that were restricted to population-based controls or controls ≥50 years of age (data not shown). Altogether, our genome-wide single-variant analysis nearly doubles the number of AMD-associated loci and variants.
Prioritizing variants within 52 association signals

It is often challenging to translate common variant association signals into a mechanistic understanding of biology; two key challenges are (i) variants with similar signals because of linkage disequilibrium (LD) and (ii) variants with subtle functional consequences. Without narrowing lists of candidate variants, follow-up functional experiments are complicated. To prioritize variants near index SNPs for follow-up, we computed each variant’s ability to explain the observed signal and derived, for each of the 52 signals, the smallest set of variants that included the causal variant with 95% probability. These 52 credible sets each included from one to >100 variants (total of 1,345 variants; Supplementary Data Set 3). Twenty-seven of the 52 sets were small, with ≤10 variants (19 sets had ≤5 variants; Supplementary Table 9); seven sets included only one variant. Among the 205 variants with >5% probability of being causal, we observe 11 protein-altering (all nonsynonymous) variants (versus two expected assuming that 1% of variants are protein altering; P value for enrichment = 8.7 × 10^{-8}; Supplementary Table 10). We recognize that the analysis has limitations (for example, when the signal is due to a combination of multiple variants, as in the counterexample in Supplementary Fig. 4).

Rare variant association signals

Analysis of rare variants that alter peptide sequences (nonsynonymous), truncate proteins (stop gain) or affect RNA splicing (splice site) can help to identify causal mechanisms—particularly when multiple associated variants reside in the same gene. We examined the cumulative effect of rare protein-altering variants in each ancestry group. Across the genome, no signal was detected with P ≤ 0.05/17,044 = 2.9 × 10^{-6} outside the 34 AMD-associated loci (Fig. 1c). Within the 34 loci, we found 14 genes with significant disease burden (P < 0.05/703 genes = 7.1 × 10^{-5}; Supplementary Table 11). To eliminate scenarios in which a finding of rare variant burden is an LD shadow of association for a nearby common variant, we reevaluated each signal of burden with conditioning on nearby single variants (from Supplementary Table 4). Four of the 14 genes...
retained burden at $P < 0.05/703 = 7.1 \times 10^{-5}$ in this analysis (CFH, CFI, TIMP3 and SLC16A8; conditioned $P = 1.2 \times 10^{-6}, 1.0 \times 10^{-8}, 9.0 \times 10^{-8}$ and $3.1 \times 10^{-6}$ respectively; Table 2). Sensitivity analyses provided similar (excluding previously sequenced subjects) and extended results (prioritizing variants with high predicted functionality; Supplementary Table 12 and Supplementary Note).

Several interesting patterns emerge, many of which we owe to our chip design. First, three of the four rare variant burden signals (CFH, CFI and TIMP3) are due to variants with frequency <0.1%, all genotyped (Supplementary Data Set 4). Many human genetic studies have used frequency thresholds of 1 to 5% as a working definition of ‘rare’, but our data suggest that trait-associated variants with clear function may often be much rarer—necessitating very large sample sizes for analysis. In two genes (CFH and CFI), the rare burden was detected because we enriched the array with variants from previous sequencing of AMD-associated loci (54 of 80 variants). The burden findings in CFH (new locus; Supplementary Note) and CFI, together with the CFH variant encoding p.Arg1210Cys and the CFI variant encoding p.Gly119Arg (refs. 7, 9), corroborate a causal role for these genes in AMD etiology.

![Figure 2](https://example.com/figure2.png)

**Table 2** Four genes within the 34 AMD loci with a significant rare variant burden independent of identified index variants

<table>
<thead>
<tr>
<th>Gene</th>
<th>Optimal RAC threshold</th>
<th>Number of variants below optimal RAC</th>
<th>Summed RAC (frequency as %)</th>
<th>Cases n = 16,144</th>
<th>Controls n = 17,832</th>
<th>$P_A$</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFH</td>
<td>10 (0.015)</td>
<td>37 (9 + 28)</td>
<td>88 (0.273)</td>
<td>38 (0.107)</td>
<td>1.2 $\times 10^{-6}$</td>
<td>2.94</td>
<td></td>
</tr>
<tr>
<td>CFI</td>
<td>46 (0.068)</td>
<td>43 (17 + 26)</td>
<td>213 (0.660)</td>
<td>82 (0.230)</td>
<td>1.0 $\times 10^{-8}$</td>
<td>2.95</td>
<td></td>
</tr>
<tr>
<td>TIMP3</td>
<td>14 (0.021)</td>
<td>9 (1 + 8)</td>
<td>29 (0.0898)</td>
<td>1 (0.00280)</td>
<td>9.0 $\times 10^{-8}$</td>
<td>31.21</td>
<td></td>
</tr>
<tr>
<td>SLC16A8</td>
<td>648 (0.954)</td>
<td>9 (7 + 2)</td>
<td>487 (1.51)</td>
<td>392 (1.10)</td>
<td>3.1 $\times 10^{-6}$</td>
<td>1.40</td>
<td></td>
</tr>
</tbody>
</table>

We computed a gene-based burden test of rare protein-altering variants comparing 16,144 advanced AMD cases and 17,832 controls. Shown are $P$ values from the variable-threshold test (from up to 100 million permutations) and odds ratios from the collapsed burden test, both adjusted for the other variants identified in each locus (locus-wide conditioning). Four genes (among the 703 genes in the 34 AMD locus regions) showed a significant ($P < 0.05/703 = 7.1 \times 10^{-5}$) burden. Details about the corresponding rare variants underlying the observed burden can be found in Supplementary Data Set 4. Results for the 14 genes that show significant burden within the 34 AMD loci without locus-wide conditioning are shown in Supplementary Table 11. Rare variants were defined here as variants with MAF in cases and controls <1% in each of the ancestry groups—European, Asian and African. RAC, rare allele count.

$^{a}$*P* values from the variable-threshold test with conditioning on other variants identified in the locus (locus-wide conditioned).
The third signal (TIMP3) was in a gene previously associated with Sorsby’s fundus dystrophy, a rare monogenic disease with early onset at <45 years of age but with clinical presentation strikingly similar to AMD. Because the majority of Sorsby’s-associated alleles disrupt cysteine-cysteine bonds in TIMP3, we arrayed all possible cysteine-disrupting sites together with other previously described Sorsby’s risk alleles. The nine rare TIMP3 variants were cumulatively associated with >30-fold increased risk of disease. TIMP3 resides in an established AMD-associated locus targeted in previous sequencing efforts that were too small to evaluate rare variation on this scale (one variant in 17,832 controls versus 29 variants in 16,144 cases).

Interestingly, although Sorsby’s-associated TIMP3 variants typically occur in exon 5, four of the unpaired cysteine residues we observed map to other exons—perhaps because unpaired cysteine residues in different locations impair protein folding in different ways. AMD cases with these rare TIMP3 risk alleles still exhibited higher counts of AMD risk alleles across the genome than controls, suggesting that TIMP3 is not a monogenic cause of AMD but contributes to disease together with alleles at other risk-associated loci. Our finding illustrates a locus where complex and monogenic disorders arise from variation in the same gene, similar to MC4R and POMC in obesity or UMOD in kidney function. In a similar approach, we analyzed 146 rare protein-altering variants in ABCA4, a gene underlying Stargardt disease, but found no association (P = 0.97).

The signal of rare variant burden in SLC16A8 was primarily driven by a putative splice-site variant (c.214+1G>C, rs77968014, minor allele frequency among controls, CAF = 0.81%, OR = 1.5, imputed with R² = 0.87; Supplementary Data Set 4). This is not a burden from multiple rare variants but from a single variant emerging as significant owing to the reduced multiple-testing burden from gene-wide testing (single-variant association P = 9.1 × 10⁻⁶, P value with conditioning on rs8135665 = 1.3 × 10⁻⁶). This variant is interesting because it is predicted to disrupt processing of the encoded transcript (as a variant at the +1 position; Human Splicing Finder 3.0). SLC16A8 encodes a cell membrane transporter involved in the transport of pyruvate, lactate and related compounds across cell membranes. This class of proteins mediates the acidity level in the outer retinal segments, and mice with knockout of Slc16a8 have changes in visual function and scotopic electroretinograms but not overt retinal pathology. Interestingly, progressive loss of SLC16A8 expression in eyes affected with geographic atrophy was reported with increasing severity of disease. In summary, our chip design and our large data set enabled us not only to detect interesting features of AMD genetics but also to provide guidance for future investigations on rare variants.

From disease-associated loci to biological insights

Many analyses can further narrow the list of candidate genes in our loci. We annotated the 368 genes closest to our 52 association signals (including the index variant and proxies with r² ≥ 0.5 and ±100 kb relative to index variants; Supplementary Data Set 5), noting among these genes those that contained associated credible set variants (Supplementary Data Set 3) or a rare variant burden (Table 2)—these genes represent the highest-priority candidates, consistent with previous analysis of putative cis-regulatory variants. We further checked whether genes were expressed in retina (82.6% of genes) or RPE/choroid (86.4%; Supplementary Data Set 6). We sought relevant eye phenotypes in genetically modified mice (observed for 32 of the 368 queried genes; Supplementary Data Set 7). We tagged genes in biological pathways enriched across loci, such as the alternative complement pathway, HDL transport, and extracellular matrix organization and assembly (Supplementary Table 13), highlighting genes that connect multiple pathways (COL4A3-COL4A4, ABCA1, MMP9 and VTN). We also highlighted genes that were approved or experimental drug targets (31 of the 368 queried; Supplementary Data Set 8). Finally, we prioritized genes where at least one of the credible set variants (Supplementary Data Set 3) was protein altering or located in a putative functional region (promoter, 3′ UTR or 5′ UTR).

All this information is summarized in the gene priority score (GPS) table (Supplementary Table 14, Supplementary Note and Supplementary Data Set 9), which uses a simple customizable scoring scheme to assign priority: the scheme, using equal weights for each column, assigns highest scores (Fig. 2a and Supplementary Table 15) to genes such as master regulators of immune function (PILRB), matrix metalloproteinase genes (MMP9 and MMP19), genes involved in lipid metabolism (ABCA1 and GPX4), an inhibitor of the complement cascade (VTN), another collagen gene (COLA43), a gene causing a developmental monogenic disorder (PPTN11) and a retinol dehydrogenase (RDHS). Six of these are current drug targets (ABCA1, MMP19, RDHS, PTPN11, VTN and GPX4). Among the known AMD-associated loci, the highest scores per locus were found in the ‘usual suspects’ (CFH, CFI, CFB, C3 and APOE) as well as TIMP3 and SLC16A8 (Fig. 2b). This summary of evidence is not amenable to formal statistical enrichment testing but may help prioritize genes for follow-up functional experiments.

Commonalities and differences of advanced AMD subtypes

Previously identified risk variants all contribute to the two advanced AMD subtypes—choroidal neovascularization and geographic atrophy. We compared association signals for the 10,749 cases with choroidal neovascularization and the 3,235 cases with geographic atrophy. Four of the 34 lead variants showed a significant difference (difference between subtypes, Pdifference < 0.05/34 = 0.00147) between disease subtypes (in the ARMS2-HTRA1, CETP, MMP9 and SYN3-TIMP3 loci; Fig. 3a and Supplementary Table 16). Variant rs42450006

Figure 3 Comparison of advanced AMD subtypes and intermediate versus advanced AMD. We compared associations of the 34 lead variants across different AMD phenotypes. Shown are effect sizes (log(OR)) per minor allele in controls, as well as 95% confidence intervals (widths and heights of diamonds). (a) Comparison of neovascular disease (10,749 cases versus 17,832 controls) and geographic atrophy (3,235 cases versus 17,832 controls) identifies four variants in the MMP9, ARMS2-HTRA1, CETP and SYN3-TIMP3 loci with significantly different association in the two subtypes (Pdifference < 0.05/34; red diamonds; Supplementary Table 16). (b) Comparison of intermediate AMD (6,657 cases versus 17,832 controls) with advanced AMD (16,144 cases versus 17,832 controls) identifies 24 variants with nominally significant (P < 0.05; red diamonds) association with intermediate AMD (Pnominal = 4.8 × 10⁻⁴), all of which have the same direction of effect and less extreme effect sizes in intermediate compared to advanced AMD (Supplementary Table 17).
upstream of MMP9 was the only one that was specific to one subtype, being exclusively associated with choroidal neovascularization (frequency in controls = 14.1%, $P = 8.4 \times 10^{-17}$; OR = 0.78) but not with geographic atrophy ($P = 0.39$, OR = 1.04; $P_{\text{difference}} = 4.1 \times 10^{-10}$; Supplementary Note). The MMP9 signal for neovascular disease fits well with previous evidence: upregulation of MMP9 appears to induce neovascularization and interacts with vascular endothelial growth factor (VEGF) signaling in the RPE. VEGF currently provides an effective therapy target for patients with choroidal neovascularization, but the struggle to keep vision continues. Beyond confirming shared genetic predisposition for the two subtypes, our data identify for the first time, to our knowledge, a variant that is specific to one subtype.

Commonalities and differences of advanced and early AMD

We evaluated our association signals in 6,657 individuals with intermediate AMD, defined as having more than five macular drusen with a diameter of greater than 63 μm and/or pigmentary changes in the RPE. Examining all genotyped variants, we found a correlation of 0.78 (95% CI = 0.69–0.87) between intermediate and advanced AMD, indicating substantial overlap between genetic determinants of advanced and intermediate AMD. Of our 34 index variants, 24 showed nominally significant association ($P_{\text{intermediate}} \leq 0.05$) with intermediate AMD (two expected, $P_{\text{nominal}} = 4.8 \times 10^{-24}$; all had odds ratios in the same direction but smaller in magnitude (Fig. 3b and Supplementary Table 17). The other ten variants showed no association with intermediate AMD ($P_{\text{intermediate}} > 0.05$), despite sufficient power to detect association (Supplementary Table 18). Interestingly, these ten variants point to seven extracellular matrix genes (COL1A1, COL3A1, MMP9, POCOLCE, MMP19, CTRB1-CTRB2 and ITGAV; Supplementary Table 19), on the basis of which one may hypothesize that extracellular matrix variation represents a disease subtype without early-stage manifestation or with extremely rapid progression. If this hypothesis is confirmed, a group of rapidly progressing patients or patients without early symptoms might eventually derive maximum benefit from genetic diagnosis and future preventive therapies.

An accounting of AMD genetics

To account for progress made here in understanding AMD genetics, we estimated the proportion of disease risk explained by our 52 independent variants and compared it to our initial estimates of heritability obtained by examining all genotyped variants. We computed a weighted risk score of the 52 variants and modeled a population risk score distribution (Online Methods). Individuals in the top decile of genetic risk have 44-fold increased risk of developing advanced AMD in comparison to those in the lowest decile; of those in the highest decile, 22.7% are predicted to have AMD in an elderly general population above 75 years of age with ~5% disease prevalence (Fig. 4a, Supplementary Table 20). Altogether, the 52 variants explain 27.2% of disease variability (Fig. 4b, also showing results based on other prevalence assumptions), including a contribution of 1.4% from rare variants. The 52 identified variants thus explain more than half of the genomic heritability; the balance might be attributed to additional variation not studied here or to genetic interaction with environmental factors such as smoking, diet or sunlight exposure.

DISCUSSION

We set out to improve understanding of rare and common genetic variation for macular degeneration biology, to thereby guide the development of therapeutic interventions and facilitate early diagnosis, monitoring and prevention of disease. We systematically examine rare variation (through direct genotyping) and common variation (through genotyping and imputation) for AMD in a study designed to discover >80% of associated protein-altering variants with an allele frequency of >0.1% and >3-fold increased disease risk (or a frequency >0.5% and >1.8-fold increased disease risk). Our study provides a simultaneous assessment of common and rare variation, enabling us to understand the relative roles of rare and common variants and the scientific insights to be gained from rare variation.

Rare protein-altering variants are an attractive target for genetic studies because most of these variants are expected to damage gene function. Furthermore, observing that many rare variants in a gene are, together, associated with a change in disease risk strongly suggests that the gene is causally implicated in disease biology and, further, suggests the consequences of mimicking or blocking gene action using a drug. Our study demonstrates that, when rare variants are systematically assessed across the genome, significant signals can be assigned to single rare variants as well as to individual genes with rare variant disease burden.

Our study also demonstrates the challenges of these analyses. For three of the genes for which we identified rare variant burden, the accumulated evidence was spread across very rare variants with frequencies <0.1% in controls. Most of these variants were derived from sequencing patients with AMD. This emphasizes the value of a hybrid approach with direct targeted sequencing of patient samples for variant discovery followed by genotyping in larger samples for association analysis. Another conclusion is related to required sample sizes: although rare variants such as these are expected to exist in nearly all genes, no rare variant burden was observed in most of the 34 loci we studied. For these loci, identifying causal mechanisms through the study of rare protein-altering variants will require a combination of more sequencing and even larger sample sizes. Although our findings of rare variant burden are predominantly from targeted enrichment, knowledge of the effect sizes and frequencies of contribut ing variants illustrates that applying the approach across the genome to detect new loci requires extremely large sample sizes. In our view, a recent estimate that sequencing of 25,000 patients will be needed to identify genes where rare variants have a substantial impact on disease risk is likely to be optimistic, particularly given the fact that effect sizes for AMD risk alleles appear to be larger than for many other complex traits.
In addition to corroborating previous reports of rare variants that disrupt genes in the complement pathway and lead to large increases in disease risk, our study also includes two unexpected rare variant findings. First, we show that a putative splicing variant in SLC16A8 can greatly increase the risk of AMD, providing strong evidence that this gene is directly involved in disease biology. SLC16A8 encodes a lactate transporter expressed specifically by the RPE; a deficit in lactate transport results in acidification of the retina and photoreceptor dysfunction in Slc16a8-knockout mice. Second, we show a >30-fold excess of rare TIMP3 mutations among putative cases of macular degeneration. TIMP3 is an especially attractive candidate that has been the subject of previous underpowered genetic association studies.

Although it has been hypothesized that studies of rare and low-frequency genetic variants will greatly increase the proportion of genetic risk that can be explained, our results do not support this notion. Our study and others successfully identify many low-frequency disease risk alleles, and these provide clues about disease biology; however, our results also show that common variants make a much larger contribution to disease risk. Common variants also suggest interesting leads and pathways for future analysis (Fig. 2a and Supplementary Table 15), including attractive candidates such as immune regulators (PILRB), genes implicated in mouse ocular phenotypes (MMP9, MMP19, COLA33, PTEN11, GPX4 and RH5) and proven drug targets (ABCA1, MMP19, RDH5, PTN11, VTN and GPX4). In a literature search, we identified no previous candidate gene association studies targeting our newly associated loci, although several model organism, cellular and functional studies evaluated potential links between genes in these loci and AMD (highlights from several model organism, cellular and functional studies evaluated and proven drug targets (phenotypes as immune regulators.

Although very large samples will be needed, our results also show that the effort to extend genetic studies to rare variants is worthwhile, as these variants can pinpoint causal genes and advance understanding of disease biology.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Data permitted for sharing by respective institutional review boards and summary statistics reported in the paper have been deposited in the database of Genotypes and Phenotypes (dbGaP) under accession phs001039.v1.p1. Full GWAS summary statistics are available at http://amdgeneics.org/.

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AUTHOR CONTRIBUTIONS

Deep resequencing of GWAS loci identifies independent rare penetrant mutation in obesity biology.

Whole-exome sequencing identifies rare and low-frequency variants burden analysis.

New genetic loci link adipose and insulin biology to body fat age-related macular degeneration.


1Center for Statistical Genetics, Department of Biostatistics, University of Michigan, Ann Arbor, Michigan, USA. 2Department of Genetic Epidemiology, University of Regensburg, Regensburg, Germany. 3Department of Epidemiology and Biostatistics, Case Western Reserve University School of Medicine, Cleveland, Ohio, USA. 4Institute of Human Genetics, University of Regensburg, Regensburg, Germany. 5Kidney Epidemiology and Cost Center, Department of Internal Medicine-Nephrology, University of Michigan, Ann Arbor, Michigan, USA. 6School of Medicine, Menzies Research Institute Tasmania, University of Tasmania, Hobart, Tasmania, Australia. 7Center for Human Genetics, Marshfield Clinic Research Foundation, Marshfield, Wisconsin, USA. 8Department of Ophthalmology, University of California, San Diego and Veterans Affairs San Diego Health System, La Jolla, California, USA. 9Retina Service, Massachusetts Eye and Ear, Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, USA. 10Department of Ophthalmology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA. 11Department of Ophthalmology, Wilmot Eye Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. 12Department of Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. 13Department of Neurosciences, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. 14Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. 15Institut de la Vision, Université Pierre et Marie Curie, Paris, France. 16Hôpital Intercommunal de Créteil, Hôpital Henri Mondor, Université Paris Est Créteil, Créteil, France. 17Department of Ophthalmology, University of Bonn, Bonn, Germany. 18Louis Stokes Cleveland Veterans Affairs Medical Center, Cleveland, Ohio, USA. 19Department of Ophthalmology and Visual Sciences, University of Wisconsin, Madison, Wisconsin, USA. 20Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts, USA. 21Department of Nutrition, Harvard School of Public Health, Boston, Massachusetts, USA. 22John P. Hussman Institute for Human Genomics, Miller School of Medicine, University of Miami, Miami, Florida, USA. 23Centre for Vision Research, Department of Ophthalmology and Westmead Millennium Institute for Medical Research, University of Sydney, Sydney, New South Wales, Australia. 24Department of Ophthalmology, Columbia University, New York, New York, USA. 25University College London Institute of Ophthalmology, University College London, London, UK. 26Moorfields Eye Hospital, London, UK. 27Centre for Human Genetics Research, Vanderbilt University Medical Center, Nashville, Tennessee, USA. 28Department of Ophthalmology, University Hospital of Cologne, Cologne, Germany. 29Department of Ophthalmology, Radboud University Medical Centre, Nijmegen, the Netherlands. 30Centre for Eye Research Australia, University of Melbourne, Royal Victorian Eye and Ear Hospital, East Melbourne, Victoria, Australia. 31South Texas Diabetes and Obesity Institute, School of Medicine, University of Texas, San Antonio, Texas, USA.
University of Texas Rio Grande Valley, Brownsville, Texas, USA. 32Department of Biostatistics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania, USA. 33Medical Research Council (MRC) Human Genetics Unit, Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, UK. 34Department of Ophthalmology, Erasmus Medical Center, Rotterdam, the Netherlands. 35Department of Epidemiology, Erasmus Medical Center, Rotterdam, the Netherlands. 36Quantitative Biomedical Research Center, Department of Clinical Science, University of Texas Southwestern Medical Center, Dallas, Texas, USA. 37Center for the Genetics of Host Defense, University of Texas Southwestern Medical Center, Dallas, Texas, USA. 38Neurobiology, Neurodegeneration and Repair Laboratory (N-NRL), National Eye Institute, US National Institutes of Health, Bethesda, Maryland, USA. 39Department of Ophthalmology and Visual Sciences, University of Michigan, Kellogg Eye Center, Ann Arbor, Michigan, USA. 40Department of Ophthalmology, Flinders Medical Centre, Flinders University, Adelaide, South Australia, Australia. 41Centre for Ophthalmology and Visual Science, Lions Eye Institute, University of Western Australia, Perth, Western Australia, Australia. 42Sichuan Provincial Key Laboratory for Human Disease Gene Study, Hospital of the University of Electronic Science and Technology of China and Sichuan Provincial People’s Hospital, Chengdu, China. 43Sichuan Translational Medicine Hospital, Chinese Academy of Sciences, Chengdu, China. 44Molecular Medicine Research Center, State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, China. 45Eye Centre South West, Stuttgart, Germany. 46University Eye Clinic, Ludwig Maximilians University, Munich, Germany. 47Department of Ophthalmology, University Hospital Regensburg, Regensburg, Germany. 48Department of Ophthalmology and Visual Sciences, University of Utah, Salt Lake City, Utah, USA. 49Department of Medicine (Biomedical Genetics), Boston University Schools of Medicine and Public Health, Boston, Massachusetts, USA. 50Department of Ophthalmology, Boston University Schools of Medicine and Public Health, Boston, Massachusetts, USA. 51Department of Neurology, Boston University Schools of Medicine and Public Health, Boston, Massachusetts, USA. 52Department of Epidemiology, Boston University Schools of Medicine and Public Health, Boston, Massachusetts, USA. 53Department of Biostatistics, Boston University Schools of Medicine and Public Health, Boston, Massachusetts, USA. 54Department of Ophthalmology, Seoul Metropolitan Government Seoul National University Boramae Medical Center, Seoul, Republic of Korea. 55Centre for Experimental Medicine, Queen’s University, Belfast, UK. 56Department of Ophthalmology, University of Thessaly, School of Medicine, Larissa, Greece. 57Department of Ophthalmology, Seoul National University Bundang Hospital, Seongnam, Republic of Korea. 58Department of Ophthalmology, Weill Cornell Medical College, New York, New York, USA. 59Schiee Eye Institute, Department of Ophthalmology, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA. 60Department of Biostatistics and Epidemiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania, USA. 61Department of Ophthalmology, University of Alabama at Birmingham, Birmingham, Alabama, USA. 62INSERM, Paris, France. 63Institut de la Vision, Department of Genetics, Paris, France. 64Centre National de la Recherche Scientifique (CNRS), Paris, France. 65Centre Hospitalier National d’Ophthalmologie des Quinze-Vingts, Paris, France. 66Fondation Ophtalmologique Adolphe de Rothschild, Paris, France. 67Académie des Sciences–Institut de France, Paris, France. 68Department of Molecular Genetics, Institute of Ophthalmology, London, UK. 69Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, UK. 70University Hospital Southampton, Southampton, UK. 71Department of Ophthalmology, Hadassah Hebrew University Medical Center, Jerusalem, Israel. 72Center for Human Disease Modeling, Duke University, Durham, North Carolina, USA. 73Department of Cell Biology, Duke University, Durham, North Carolina, USA. 74Department of Pediatrics, Duke University, Durham, North Carolina, USA. 75Centre d’Etude du Polymorphisme Humain (CEPH) Fondation Jean Dausset, Paris, France. 76Commissariat à l’Energie Atomique et aux Energies Alternatives (CEA), Institut de Génomique, Centre National de Génotypage, Evry, France. 77Cole Eye Institute, Cleveland Clinic, Cleveland, Ohio, USA. 78Department of Ophthalmology, Duke University Medical Center, Durham, North Carolina, USA. 79Department of Medicine, Duke University Medical Center, Durham, North Carolina, USA. 80Bascom Palmer Eye Institute, University of Miami Miller School of Medicine, Naples, Florida, USA. 81Department of Ophthalmology, New York University School of Medicine, New York, New York, USA. 82Department of Ophthalmology, Royal Perth Hospital, Perth, Western Australia, Australia. 83Department of Medical Genetics, Cambridge Institute for Medical Research, University of Cambridge, Cambridge, UK. 84Department of Ophthalmology, Cambridge University Hospitals National Health Service (NHS) Foundation Trust, Cambridge, UK. 85Department of Ophthalmology, University of California San Francisco Medical School, San Francisco, California, USA. 86Department of Ophthalmology and Visual Sciences, Vanderbilt University, Nashville, Tennessee, USA. 87Casey Eye Institute, Oregon Health and Science University, Portland, Oregon, USA. 88Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania, USA. 89School of Clinical Sciences, University of Edinburgh, Edinburgh, UK. 90Center for Inherited Disease Research (CIDR) Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA. 91Department of Ophthalmology, David Geffen School of Medicine, Stein Eye Institute, University of California, Los Angeles, Los Angeles, California, USA. 92Department of Human Genetics, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, California, USA. 93Department of Human Genetics, Radboud University Medical Centre, Nijmegen, the Netherlands. 94Department of Pathology and Cell Biology, Columbia University, New York, New York, USA. 95Center for Translational Medicine, Moran Eye Center, University of Utah School of Medicine, Salt Lake City, Utah, USA. 96Division of Preventive Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts, USA. 97Division of Epidemiology and Clinical Applications, Clinical Trials Branch, National Eye Institute, US National Institutes of Health, Bethesda, Maryland, USA. 98Institute for Computational Biology, Case Western Reserve University School of Medicine, Cleveland, Ohio, USA. 100These authors contributed equally to this work. 101These authors jointly supervised this work. Correspondence should be addressed to I.M.H. (iris.heid@klinik.uni-regensburg.de), G.R.A. (goncalo@umich.edu) or S.K.I. (skil@case.edu).
**ONLINE METHODS**

**Study data and phenotype.** In IAMDGC, we gathered 26 studies with each including (i) advanced AMD cases with geographic atrophy and/or choroidal neovascularization in at least one eye and age at first diagnosis ≥50 years, (ii) intermediate AMD cases with pigmented changes in the RPE or more than five macular drusen greater than 63 µm in diameter and age at first diagnosis ≥50 years, or (iii) controls without known advanced or intermediate AMD. Recruitment and ascertainment strategies varied by study (Supplementary Tables 1 and 2, and Supplementary Note). All groups collected data according to Declaration of Helsinki principles. Study participants provided informed consent, and protocols were reviewed and approved by local ethics committees.

**DNA and chip design.** We gathered DNA samples from more than 50,000 individuals. Groups with very limited amounts of available DNA contributed aliquots after whole-genome amplification (8% of subjects).

We used a custom-modified HumanCoreExome array by Illumina, which includes (i) tagging variants across the genome (genome chip content) and (ii) a catalog of protein-altering variants (exome chip content). Our customization of the array included three additional tiers to enrich for variants from 22 AMD loci implicated by our previous genome-wide association analysis, including 19 index variants with genome-wide significance and three variants with consistent direction of effect in the replication stage and 4 × 10^(-7) ≤ P ≤ 2 × 10^(-9), selecting (iii) tagging variants (pairwise tagging r^2 > 0.8) from Phase 1 10,000 Genomes Project or HapMap2.3 common variants (MAF ≥ 1% in European or East Asian individuals) using Tagger implemented in Haploviz54 within 100 kb of the 22 index variants expanded to cover all correlated variants (r^2 (EUR) > 0.5) and the complete gene (transcript ± 1 kb), (iv) protein-altering variants within 500 kb of the 22 index variants identified from public general population databases (dbSNP55, the NHLBI Exome Sequencing Project56 and Phase I of the 100 Genomes Project; see URLs), and (v) protein-altering variants within 500 kb of the 22 index variants identified by resequencing AMD case-control study data (targeted resequencing of 2,335 AMD cases and 789 controls56,57 and whole-genome sequencing of 60 AMD cases and 60 controls; A.M.K., X.Z., L.G.F., G.R.A. and A.S. et al., unpublished data). The customization further included (vi) the 1,000 top independent (>2 Mb distant) variants from the previous analysis and 100 additional top variants each from the previous choroidal neovascularization only and geographic atrophy only analyses, (vii) and 375 variants in ABCA4, including known variants causing Stargardt disease58, benign variants and those with unknown consequences, as well as ten known and 43 predicted cysteine-altering mutations in TIMP3, whose selection was motivated by the known variants causing Sorsby’s fundus dystrophy59-61.

**Annotation.** Variant identifiers were based on NCBI dbSNP v137. Chromosomal position and functional annotation of the variants was based on the NCBI RefSeq hg19 reference genome59 (downloaded December 2012) and SeattleSeq Annotation 138 (ref. 60; see URLs). We particularly focus on protein-altering variants, including nonsynonymous coding variants (missense, stop loss, in-frame insertion or deletion, frameshift and stop gain) and splice-site variants. We converted the description of splice-site variants to HGVS nomenclature using Mutalyzer version 2.0.beta-33 (ref. 61; see URLs).

**Genotypes.** We genotyped all subjects centrally at the Center for Inherited Diseases Research (CIDR) at Johns Hopkins University School of Medicine. From the 569,645 genotyped variants, our quality control excluded variants with poor genotyping as evidenced by genotype call rates <98.5% (5.8%), deviation from Hardy-Weinberg equilibrium with P = 1 × 10^(-6) (0.34%), mapping to multiple genome locations (0.25%) or failure of other criteria, resulting in 521,950 (91.6%) variants passing all quality criteria. After excluding monomorphic variants (15.8%), we retained 264,655 common variants distributed across autosomes, sex chromosomes and mitochondrial DNA, as well as 163,714 directly genotyped protein-altering variants, including 8,290 from previously implicated AMD loci (Supplementary Table 3a).

We phased the autosomal and X-chromosomal genotype data using SHAPEIT (200 states, 2.5-Mb windows)62 and then imputed genotypes on the basis of the 1000 Genomes Project63 reference panel (1000 Genomes Project Phase I, version 3, SHAPEIT2 Reference) using Minimac64 (reference-based 2.5-Mb chunks, 500-kb buffer regions). We then merged study variants that were excluded during imputation (not found in the reference panel) back into the final data set. We excluded common variants (CAF ≥1%) with poor imputation quality, R^2 <0.3, and adopted a more stringent exclusion criterion for rare variants (CAF <1%), R^2 <0.8, for the initial identification of lead variants. This filtering yielded a total of 12,023,830 genotyped (439,350) or imputed (11,584,480) quality-controlled variants (Supplementary Table 3a).

**Analyzed subjects.** Using the genomic information for subject-level quality control, we excluded duplicated and related individuals (kinship coefficient \( \phi \geq 0.0884 \), indicating third-degree relatives or closer)62, subjects with discrepancies between reported sex and sex chromosomal information or with atypical sex chromosome configurations62, and subjects with genotyping call rates <89.5%; we derived ancestry from the first two principal components using autosomal genotyped variants together with genotype information for the samples from the Human Genome Diversity Project (HGDP).65 Our final data set contained 43,566 successfully genotyped unrelated subjects, including 16,144 advanced AMD cases and 17,832 controls of European ancestry, 6,657 intermediate AMD cases of European ancestry, and 2,933 subjects (advanced AMD or controls) of Asian or African ancestry (Supplementary Table 3b).

**Genomic heritability and genomic correlation.** Combined contribution of genotyped variants to disease was evaluated using a variance component–based heritability analysis66. This analysis used genotypes to build a similarity matrix, summarizing the overall genetic kinship for each pair of individuals, and then examined the correspondence between genetic and phenotypic similarity. We estimated the variance explained by all genotyped autosomal variants using restricted maximum likelihood (REML) analysis implemented in GCTA28 (see URLs). We jointly estimated the contributions of rare (CAF <1%) and common (CAF ≥1%) genotyped variants by first separately calculating their genetic relationship matrices (GRMs) before adding both to the model. Obtained estimates of variance explained were transformed from the observed scale to the liability scale, assuming various levels of disease prevalence68.

We estimated the genomic correlation between disease subphenotypes using bivariate REML analyses implemented in GCTA and only included common (CAF ≥1%) genotyped variants29. We compared 10,749 cases with choroidal neovascularization to 3,325 cases with geographic atrophy (excluding the 2,070 cases with mixed choroidal neovascularization and geographic atrophy), and we compared 6,657 intermediate AMD cases with 16,144 advanced AMD cases. For both analyses, we used the control subjects as a reference and avoided shared controls between traits by randomly splitting the 17,832 unrelated European control individuals into two subsamples of 8,916 individuals each.

**Genome-wide single-variant association analysis.** Single-variant association tests analyzing the 16,144 advanced AMD cases and 17,832 controls of European ancestry were based on the Firth bias-corrected likelihood-ratio test69, which is recommended for genetic association studies that include rare variants70, as implemented in EPACTS (see URLs). Analyses were adjusted for two principal components and source of DNA (whole-blood or whole genome–amplified DNA). Allele dosages were used for the imputed data. Sensitivity analyses were conducted to evaluate the influence of alternative association tests and alternative covariate adjustment including with age or sex or with up to ten principal components instead of two, as well as the influence of restricting to population-based controls or to controls aged 50 years or older. Genomic control correction was used to account for potential population stratification using all genotyped variants with minor allele count ≥20 outside of 20 previously described AMD loci6,69. As usual for GWAS, we considered \( P \leq 5 \times 10^{-8} \) to indicate genome-wide significance.

To identify independently associated variants, we adopted a sequential forward selection approach. We first computed single-variant association for each of the >12 million variants. Then, for each significantly associated locus, we selected the variant with the smallest P value and the 10-Mb region centered on this variant, repeating the process until no genome-wide significant variant \( (P \leq 5 \times 10^{-8}) \) was left, thereby identifying a number of 10-Mb regions.
Within each of these large regions, we reanalyzed each variant, conditioning on the top variant, and repeated this process by adding the previously identified genome-wide significant variant(s) within the respective 10-Mb region. This yielded one or more independently associated genome-wide significant variant(s) per 10-Mb region.

A locus region was defined by a genome-wide significant variant and the region spanned by the correlated variants ($r^2 \geq 0.5$) adding a further 500 kb to both sides; overlapping locus regions were merged into one locus, and some loci therefore contained more than one index variant (details in Supplementary Fig. 3).

To derive independent effect sizes (log-transformed odds ratios) for all identified variants, we computed a fully conditioned logistic regression model including all identified variants.

Bayesian approach to prioritize variants. To summarize the statistical evidence of association strength, for each variant, we computed the Bayes factor, which is a measure of the strength of the association that is comparable between variants irrespective of variant frequency or study sample size. The Bayes factor provides the probability of the genotype configuration at a variant (in cases and controls) under the alternative hypothesis (association) divided by the probability of the genotype configuration under the null hypothesis (no association). It is computed using the association results for each variant\(^\text{57}\). The posterior probability of each variant is then computed as the Bayes factor for the variant relative to the sum of the Bayes factors for all variants across one locus region and can be thought of as the relative strength of evidence in favor of each SNP studied in the respective region. This approach assumes that there is one causal variant per region and that the causal variant is in the analyzed data set.

Expanding to loci with multiple association signals and thus a single alleged causal variant per signal, we used the association results for each SNP obtained by conditioning on the other independent variants at that locus to compute the Bayes factor.

For each signal, we derived the 95% credible set of variants, constituting the minimal set of variants for which the sum of the posterior probabilities exceeds 95%. This approach was recommended for fine mapping of association signals and for prioritizing variants\(^\text{40}\). Assuming that there is only one causal variant for each association signal and that the causal variant is contained among the analyzed variants, such a credible set of variants contains the causal variant with 95% probability.

We annotated the functionality of the variants in each of the 95% credible sets.

Gene-based burden analysis. Single-variant analyses have limited power to detect rare variants with association. Gene-based burden tests evaluating accumulated association from multiple rare variants per gene have been shown to complement such analyses and improve power to detect a burden of disease. We computed the burden of disease using the variable-threshold test\(^\text{51}\) as implemented in EPACTS. These analyses assume that all variants in a gene either increase or decrease disease risk. When variants with opposite directions of effect reside in the same gene, power will be reduced. An analysis with SKAT and SKAT-O, which both allow for variants with opposite directions of effect to reside in the same gene, did not identify additional signals (data not shown).

We focused this analysis on protein-altering variants, as we assumed that other variants (not protein altering) would far outnumber these predicted deleterious variants and would thus dilute disease burden due to the deleterious variants. Assuming negative selection against such deleterious variants that causes their frequency to be low across ancestry groups, we restricted our rare variant definition to variants with MAF <1% (cases and controls combined) in each of our ancestry groups (African, Asian and European). We used the genotypes of these rare protein-altering variants, if genotyped directly, or rounded imputed allele dosages to the next best genotype, if imputed; imputed variants were restricted to those with the highest imputation quality ($R^2 \geq 0.8$) (ref. 51). If rare variants appear on a haplotype associated with disease through a common variant allele already identified for AMD, the rare variant burden would depict a mere shadow of the signal for the already-identified variant.

Therefore, we repeated the variable-threshold test with conditioning on the variant(s) identified in the respective locus by single-variant analysis (locus-wide conditioning) to unravel a gene-based burden of rare variants independent of risk variants identified in single-variants tests.

First, we searched for rare variant disease burden across the genome, applying a genome-wide Bonferroni-corrected significance threshold of 0.05/17,044 = 2.9 × 10\(^{-8}\) (17,044 genes across the genome with at least one variant included in the analysis, that is, with ≥1 rare protein-altering variant). In a second view on this, we focused on our 34 identified AMD loci and here applied a significance threshold based on the 703 genes overlapping the locus regions ($P < 0.05/703 = 7.1 \times 10^{-5}$). Odds ratio estimates of the burden were derived by logistic regression using the Wald test on the collapsed burden. As there was an overlap of the sequenced subjects with the subjects for whom chip data were used, we conducted a sensitivity analysis for the burden test excluding overlapping subjects (Supplementary Note).

Follow-up queries for genes overlapping the association signals. To derive information for all genes overlapping our 52 identified association signals (spread across the 34 AMD loci), we built a gene list containing all genes that overlapped a more narrow definition of locus regions. We used a particularly comprehensive definition of the locus region during the signal identification step (index variants and proxies, $r^2 \geq 0.5, \pm 500$ kb), to avoid far-reaching LD that may generate shadow signals (particularly in light of strong associations in the CFH, C3, C2-CFI and ARMS2-HTRA1 loci) and to optimally differentiate independent signals within a locus. We have also used this broad locus region definition for the rare variant burden test, again to fully correct for independent signals in the respective wider locus regions and to be conservative in multiple-testing corrections for the AMD locus-wide burden test search. However, this broad definition is less adequate when prioritizing genes around the identified signals under the assumption that most protein-altering or regulatory variants exert their effects in cis\(^\text{42}\). We thus focused the gene list for further queries to a more narrow locus region definition (index variants and proxies, $r^2 \geq 0.5, \pm 100$ kb) and yield 368 overlapping RefSeq genes (Supplementary Data Set 5).

Gene expression. For the 368 genes in our gene list, we sought to determine gene expression in relevant tissues—retina, RPE and choroid—in two independent data sets (see details in the Supplementary Note). A consensus rating of gene expression observed in the two laboratories performing the analysis was derived as follows. Expression of a gene in one set of tissues (retina or RPE/choroid) was inferred if both laboratories detected expression in the respective set of tissues; if at least one of the laboratories did not observe expression, the gene was considered to be not expressed. Gene expression of all other genes (with one laboratory observing expression and the other with missing data or where both laboratories had missing data) was regarded as missing.

Mouse model phenotypes. For the 368 genes in our gene list, we queried the Mouse Genome Informatics (MGI)\(^\text{73}\) and International Mouse Phenotyping Consortium (IPMC)\(^\text{74}\) databases (see URLs) and manually curated results by information from published literature. We determined whether a gene exhibited a relevant eye phenotype (retina, RPE or choroid phenotype) in established genetic mouse models (knockout, knock-in or transgenic mice).

Enrichment for molecular pathways. For the 368 overlapping genes, we performed functional enrichment analysis using INRICH\(^\text{75}\) with default settings unless stated otherwise (Supplementary Note). The target intervals for this analysis were the narrow AMD locus regions (index variants and proxies, $r^2 \geq 0.5$ and ±100 kb; Supplementary Table 8). As there is no consensus approach to pathway analysis, we queried multiple databases: (i) the Kyoto Encyclopedia of Genes and Genomes (KEGG)\(^\text{59}\); (ii) Reactome\(^\text{77}\) and (iii) the Gene Ontology (GO) Consortium\(^\text{78}\) (see URLs). For example, whereas KEGG is a manually curated database on metabolic pathways, the GO database also includes automatic annotations and a more comprehensive set of cellular processes and molecular functions.

Drug pathways and targets. To derive information on whether the product of a gene among the 368 genes in our gene list was a direct drug target, we
searched the DrugBank database (version 4.1), which contains 4,207 drug targets (genes) and 7,740 drugs[9] (see URLs).

Explained variability in disease liability. On the basis of the 52 identified AMD variants, we estimated the variance in disease liability explained by these variants (see URLs)[9] using the log-transformed odds ratio estimates from the model including all 52 identified variants (fully conditioned) to derive independent effect sizes. We compared this proportion explained by the 52 variants with the earlier derived genomic heritability based on all genotyped variants.

Genetic risk score and relative and absolute genetic risk of AMD. For each individual, we computed a GRS as the effect size–weighted sum of the AMD risk-increasing alleles for all 52 independent variants divided by the sum of all effect sizes. To derive a realistic GRS distribution, we modeled a general population on the basis of our case-control data, with this model requiring an assumption of the prevalence of advanced AMD (Supplementary Note). For this modeled general population, we derived the GRS distribution and its deciles. For weighting, the log-transformed odds ratio for each of the 52 variants was derived from the fully adjusted model (including all 52 variants) to assure independence of the effect sizes.

We derived relative risk estimates (as odds ratios) for each GRS decile, with the first decile as a reference. This relative risk estimate is independent of disease prevalence, except that the decile used to form the genetic risk groups was based on the GRS distribution as expected in a general population (which requires a prevalence assumption). We also computed absolute risk estimates for each GRS decile as the proportion of advanced AMD cases, applying weights and prevalence assumptions as described above.