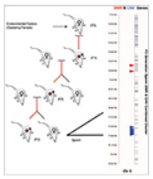
epigenetics



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RESEARCH PAPER

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Differential DNA methylation identified in the blood and retina of AMD patients

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Keywords: age-related macular degeneration, DNA methylation, genome-wide methylation, methyl-QTL, peripheral blood leukocytes, retina

Abbreviations: AMD, Age-related macular degeneration; AMD-MMAP, Michigan, Mayo; AREDS, and Pennsylvania; AREDS, Age-Related Eye Disease Study; DNAm, DNA methylation; GA, geographic atrophy; GWAS, genome-wide association study; KEC, Kellogg Eye Center; LCLs, lymphoblastoid cell lines; meQTL, methylation quantitative trait loci; NV, choroidal neovascularization; RPE, retinal pigment epithelium

Age-related macular degeneration (AMD) is a major cause of blindness in the western world. While genetic studies have linked both common and rare variants in genes involved in regulation of the complement system to increased risk of development of AMD, environmental factors, such as smoking and nutrition, can also significantly affect the risk of developing the disease and the rate of disease progression. Since epigenetics has been implicated in mediating, in part, the disease risk associated with some environmental factors, we investigated a possible epigenetic contribution to AMD. We performed genome-wide DNA methylation profiling of blood from AMD patients and controls. No differential methylation site reached genome-wide significance; however, when epigenetic changes in and around known GWAS-defined AMD risk loci were explored, we found small but significant DNA methylation differences in the blood of neovascular AMD patients near *age-related maculopathy susceptibility 2* (*ARMS2*), a top-ranked GWAS locus preferentially associated with neovascular AMD. The methylation level of one of the CpG sites significantly correlated with the genotype of the risk SNP rs10490924, suggesting a possible epigenetic mechanism of risk. Integrating genome-wide DNA methylation analysis of retina samples with and without AMD together with blood samples, we further identified a consistent, replicable change in DNA methylation in the promoter region of *protease serine 50* (*PRSS50*). These methylation changes may identify sites in novel genes that are susceptible to non-genetic factors known to contribute to AMD development and progression.

Introduction

Age-related macular degeneration (AMD), the leading cause of irreversible loss of central vision in developed countries, is a multifactorial disease that occurs in the elderly. AMD affects approximately 2 million Americans over the age of 50, and over 30–50 million people worldwide.[1] The AMD disease process can be divided into early/intermediate and late stages. Early/intermediate AMD [Age-Related Eye Disease Study (AREDS) grades 2 and 3] is the most common and least severe form, characterized by pigmentary abnormalities in the macula and the accumulation of yellowish deposits called drusen beneath the macula.[2] Late AMD (AREDS grades 4 and 5) is usually subdivided into geographic atrophy (GA) and choroidal neovascularization (NV).[2] In NV, new capillaries from the choriocapillaris, the vascular bed below the retinal pigment epithelium (RPE), invade the RPE and retina, and leakage and bleeding from these new vessels can cause loss of vision. NV can be partially treated with inhibitors of vascular endothelial growth factor, although this requires frequent intraocular injections.[3] In GA, retinal pigment epithelium and photoreceptor cells slowly degenerate and die, and no treatment option exists.[4]

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Genome wide association studies (GWAS) have successfully identified risk alleles that implicate the complement system as well as other immune responses in disease pathogenesis and severity of AMD;[5] however, only 40–60% of the disease is explained by known susceptibility loci.[6] The large proportion of disease that is currently not explained by genetics has led to an interest in non-genetic factors, such as epigenetic alterations, that contribute to common, late-onset disease.[7]

In addition to genetic risk factors, environmental factors contribute to the development of AMD. For example, strong associations have been established between AMD and environmental factors independent of genetic risk, such as cigarette smoking;[8] conversely, dietary antioxidants and fish consumption appear to have a protective effect.[9,10] Additionally, certain environmental exposures like smoking are also thought to modify the genetic risk of some SNP alleles more than others.[11-13] Such environmental factors are hypothesized to account, in part, for increasing changes in epigenetic marks over time.[14] These epigenetic changes can modulate the effect of genetic variation and may explain the late onset of common diseases like AMD. Recently, alterations in DNA methylation (DNAm), one type of epigenetic mark, have been demonstrated in the affected tissue in some common non-neoplastic human diseases, such as autism[15] and type 2 diabetes.[16]

One obvious barrier to accomplishing genome-wide methylation profiling for AMD is the difficulty of obtaining affected tissue from sufficient cases and controls for an adequately powered study. As a surrogate tissue, peripheral blood has been successfully used for some diseases;[17-19] for some systemic diseases, disease-associated differential methylation has been found in the blood of affected patients.[20,21] As building evidence suggests that AMD is an eye disease with a systemic contribution, we felt it was reasonable to test whether the blood of AMD patients might harbor epigenetic-related biomarkers of disease.[22]

To investigate the possibility that aberrant DNA methylation may contribute to the development or progression of AMD, we performed complementary genome-wide methylation analyses comparing AMD patients to carefully matched controls in both peripheral blood (N = 298) and retinal tissue (N = 19). The Michigan patients contributing peripheral blood were from the AMD-MMAP (Michigan, Mayo, AREDS, and Pennsylvania) GWAS.[23] Although we did not identify any genome-wide significant differentially methylated probes in the blood study, when we focused on epigenetic changes in and around genetic loci already reported to be associated with AMD, we identified subtle but locally significant DNAm differences in the blood of NV patients near *ARMS2*, a top-ranked GWAS risk locus,[5] which we then replicated in an independent blood cohort from Baltimore. In the independent blood cohort, we found that the methylation level of one of the differentially methylated probes in *ARMS2* correlated with the genotype of the *ARMS2* risk SNP rs10490924. We then performed genome-wide methylation analyses of retina samples with and without AMD, and, using an integrative analysis of the peripheral blood and retinal tissue datasets, we identified consistent change in DNAm at the promoter of *PRSS50*, which we again replicated in both sample types. These methylation changes may contribute to the underlying mechanisms by which the genetic risk factors contribute to the AMD disease process.

Results

Illumina 450K profiling of blood from individuals with and without AMD

We performed an epigenome-wide association study on peripheral whole blood from 100 AMD (GA and NV) case-control trios (a subset of the Michigan AMD-MMAP cohort; dbGaP phs000182) using the Illumina HumanMethylation450 (450K) platform. Each trio (1 individual with bilateral GA, 1 individual with bilateral NV, 1 control individual) was matched for sex and age (<1 year difference between the 3 individuals in each trio). We included a series of technical control samples (including technical, biological, and spike-in replicates) and performed several established quality control steps (removing 2 samples with low median intensities, across-sample normalization, dropping probes susceptible to genetic variation, and exploring effects of cellular composition)[24] to ensure high-quality genomic data (**Fig. S1**, see Methods). After removing samples with lower quality global DNAm data, the final sample group of 298 individuals (99 controls, 99 NV, and 100 GA subjects) comprised 63% females, with a mean ± SD age of 79.3 ± 5.6.

Differential methylation of CpG sites in the promoter of *ARMS2* in blood

We first analyzed the differential methylation data by comparing patients with AMD to controls (N = 456,655 CpGs/probes). None of the probes showed differential methylation when corrected for multiple testing (at FDR < 10%). However, using a more liberal cutoff of $P < 10^{-6}$, 9 probes (8 genes) in GA patients and 15 probes (15 genes) in NV patients were differentially methylated (**Table 1**). Two of the 9 probes in the GA list were in the same gene, *ectonucleoside triphosphate diphosphohydrolase 1* (*ENTPD1*), in an intron near an alternative transcription start site. These probes were 1.2% and 1.3% less methylated in GA patients. *ENTPD1*, also known as *CD39*, is a ubiquitously expressed integral immune and vascular ectonucleotidase, and the 2 differentially methylated probes are less than 500 bp upstream of the alternative transcription start site of the smaller of 2 isoforms. The tenth most genome-wide significant probe, comparing NV patients to controls, was in the promoter of *ARMS2*, in a region that is strongly associated with the risk of developing AMD. The gene for *ARMS2*, a protein with a yet unknown function, has no homology to other non-primate mammalian genomic sequences. The same differentially methylated probe associated with *lectin, mannose-binding 2* (*LMAN2)* was differentially methylated in both NV patients compared to controls, as well as GA patients compared to controls, and had decreased methylation (1.5% less methylation) in both GA and NV individuals.

Twenty-eight genes map closest to the 19 AMD index SNPs reaching genome-wide significance ($P < 5 \times 10^{-8}$) for clinical

Table 1. Differentially methylated probes and corresponding CpG sites in individuals

Probe	Mean Meth Δ	*P*-value	q-value	Mean % Meth Control	Chr	Position	Gene Symbol
GA							
cg10381771	−0.013	3.5E-07	0.16	0.244	chr10	97515398	ENTPD1
cg09076123	−0.010	2.2E-06	0.30	0.136	chr1	183559527	NCF2
cg13471990	−0.012	2.4E-06	0.30	0.189	chr10	97515222	ENTPD1
cg13446753	−0.016	2.7E-06	0.30	0.852	chr19	2199988	DOT1L
cg09848114	−0.016	4.9E-06	0.35	0.778	chr6	30130698	TRIM15
cg04771938	−0.015	5.2E-06	0.35	0.120	chr2	97405809	LMAN2L
cg07388903	−0.010	5.3E-06	0.35	0.084	chr17	38574180	TOP2A
cg16786178	−0.009	7.7E-06	0.41	0.247	chr14	75764329	LOC731223
cg16739686	−0.025	8.1E-06	0.41	0.553	chr11	2721336	KCNQ1
NV							
cg09163117	−0.010	2.1E-07	0.10	0.091	chr2	239148697	HES6
cg04771938	−0.015	4.2E-07	0.10	0.119	chr2	97405809	LMAN2L
cg06889484	−0.007	1.9E-06	0.24	0.067	chr19	18304021	MPV17L2
cg03442545	−0.011	2.2E-06	0.24	0.100	chr11	68228164	PPP6R3
cg05927274	0.014	3.2E-06	0.24	0.624	chr1	153457181	S100A7
cg20596433	0.013	3.5E-06	0.24	0.789	chr13	112237366	TEX29
cg18386131	−0.006	3.7E-06	0.24	0.069	chr11	67797936	NDUFS8
cg12273231	−0.008	5.2E-06	0.26	0.075	chr2	128784973	SAP130
cg00955451	0.017	5.2E-06	0.26	0.649	chr15	29213640	APBA2
cg24296920	−0.020	6.0E-06	0.27	0.680	chr10	124214120	ARMS2
cg25801292	0.010	7.2E-06	0.29	0.140	chr19	10614272	KEAP1
cg10556106	−0.008	7.9E-06	0.29	0.865	chr22	25344750	TMEM211
cg25877086	−0.010	8.3E-06	0.29	0.095	chr17	45726922	KPNB1
cg20481789	−0.006	9.1E-06	0.29	0.067	chr19	45458522	CLPTM1
cg09598596	0.011	9.4E-06	0.29	0.756	chr7	157463734	PTPRN2
cg03623097	−0.042	1.3E-05	0.29	0.439	chr10	124213466	ARMS2

GA – geographic atrophy; Mean Meth Δ – mean difference in methylation (disease - control); Mean % Meth Control – mean methylation level in control samples; Chr – chromosome; NV – neovascular; light gray shading – genes with more than one differentially methylated probe; dark gray shading – gene that was present in both the GA and NV lists

risk of AMD (see **Table S5** of Fritsche et al., 2013[5]). If the differential methylation cutoff was relaxed to $P < 0.01$, when all AMD samples were analyzed together, 12 of those 28 genes had at least one associated differentially methylated probe ($P = 0.007$, 2-tail). When the GA and NV AMD samples were analyzed separately, 13 of the 28 genes were represented on at least one of the differentially methylated gene lists (see **Table S1**).

Next, we further examined the areas in and around (within 10 kb) the 19 genetic loci that showed genome-wide significance.[5] There were 1,408 probes on the Illumina 450K microarray within 10 kb of the genes associated with these 28 genomic loci. Among these loci, 2 probes (cg03623097 and cg24296920), both in the *ARMS2* locus—the top GWAS signal—demonstrated multiple testing-corrected significance ($P < 0.05/1408$, corresponding to Bonferroni significance within this set of probes) for decreased methylation in patients with NV compared to controls (**Fig. 1**, 4.2% less methylation, $P = 1.26 \times 10^{-5}$, and 2.0% less, $P = 5.97 \times 10^{-6}$, respectively). There were also significant differences combining both NV and GA patients together compared to controls (3.5% less methylation, $P = 2.05 \times 10^{-5}$, and 1.6% less, $P = 4.14 \times 10^{-5}$, respectively). Considering only patients with GA, there was directionally consistent lower methylation compared to controls at these 2 probes, but these differences were non-significant (2.5% less methylation, $P = 0.008$ and 1.1% less, $P = 0.013$). We then used bisulfite pyrosequencing on a cohort of Baltimore AMD patients and controls to validate the differential methylation in *ARMS2*. The hypomethylation of the 2 *ARMS2* CpGs was validated in the blood of this second cohort of GA and NV patients and controls (chr10:124213466, corresponding to cg03623097: $P = 0.010$ and chr10:124214120, corresponding to cg24296920: $P = 4.1 \times 10^{-3}$, **Figure S2**, probe design described in the Methods section). This finding may be specific to blood, as pyrosequencing of peripheral retina from 6 control eyes and 10 eyes with AMD failed to identify a significant difference in DNAm levels at these loci (**Fig. S2**).

QTL analysis of DNA methylation level and ARMS2 risk genotype

We next compared the methylation level of the 2 significantly hypomethylated *ARMS2* probes to genotypes of individuals at risk SNP rs10490924 *(ARMS2/HTRA1)*. The risk allele was strongly associated with AMD status [odds ratio (OR) = 3.47 per copy, $P = 1.73 \times 10^{-7}$], particularly in NV patients compared to controls (OR = 4.57 per copy, $P = 6.46 \times 10^{-8}$) in our samples. We identified significant association between the risk allele (T) at rs10490924 and the DNAm levels at both cg03623097 (6.2% less methylation per risk allele copy, $P = 1.31 \times 10^{-23}$, **Fig. 2A**) and cg24296920 (0.9% less methylation per risk allele copy, $P = 5.6 \times 10^{-3}$, **Fig. 2B**) in AMD patients

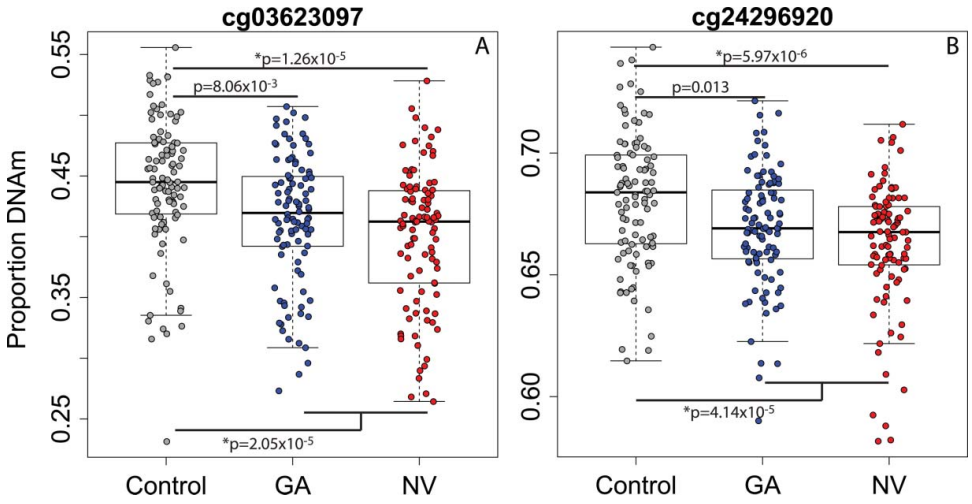

Figure 1. Differential methylation of CpG sites in the promoter of *ARMS2* in blood of AMD patients. Significant differential hypomethylation of 450K probes in NV AMD patients compared to controls. (**A**) cg03623097 (4.2% less methylation, $P = 1.26 \times 10^{-5}$) and (**B**) cg24296920 (2.0%, less methylation, $P = 5.97 \times 10^{-6}$). GA, geographic atrophy; NV, neovascular; *P, Bonferroni significant *P*-values.

combined across NV and GA. In subgroup analysis, the rs10490924:cg03623097 methylation quantitative trait loci (meQTL) was significant in separate analyses within both NV (5.8% less methylation per risk allele, $P = 1.06 \times 10^{-5}$) and GA (6.8% less methylation, $P = 6.11 \times 10^{-10}$) but rs10490924: cg24296920 only showed significance in patients with GA (1.6% less methylation per allele, $P = 7.00 \times 10^{-3}$). Neither meQTL was significant in only the controls ($P = 0.086$ and $P = 0.72$, respectively) but only a few control samples had 2 copies of the risk allele. We identified 3 additional meQTLs (at $P < 1 \times 10^{-4}$) that were not significantly differentially methylated in AMD patients: rs10490924: cg00676728 (1.5% less methylated per allele, $P = 3.74 \times 10^{-13}$, 688 bp upstream, **Fig. S3A**), rs10490924:cg25446361 (1.67% more methylated per allele, $P = 5.2 \times 10^{-11}$, 6056 bp downstream, **Fig. S3B**) and rs10490924: cg24433738 (0.8% more methylated per allele, $P = 7.81 \times 10^{-5}$, 6186 bp downstream, **Fig. S3C**). The index SNP rs10490924 is in strong disequilibrium ($r^2 = 0.90$) with a downstream SNP, rs11200638, that regulates *HtrA serine peptidase 1* (*HTRA1*)[25], so it has not been clear whether *HTRA1* or *ARMS2* is the causal gene in this locus. Interestingly, the 2 downstream meQTLs are in the promoter of *HTRA1*. Within only controls, these 3 meQTLs were directionally consistent but only the first was significant ($P = 2.2 \times 10^{-4}$).

We further noted that the cg03623097 *ARMS2* probe has a SNP (rs72631113) in the 50-bp probe sequence (but not at the target CpG or single base extension site). SNPs within probes have been shown to influence DNAm levels,[40] and genotype at this SNP strongly correlated with those at the risk SNP rs10490924 in our data ($r^2 = 0.678$, D'=1). Joint statistical analyses initially suggested that the probe SNP was likely driving the association (rs10490924: $P = 0.25$, rs72631113: $P = 3.4 \times 10^{-28}$) but fully untangling these associations *in silico* was difficult due to the potential measurement error induced by having a SNP in the probe sequence. We therefore performed bisulfite pyrosequencing and sequencing in 48 individuals (24 control samples and 24 NV samples) to determine CpG methylation levels at cg03623097 and cg24296920 as well as the genotypes at rs10490924 and rs72631113.

Using the PCR-based genotypes and the pyrosequencing-based DNAm levels in these independent samples, there was again a significant association between DNAm levels of chr10:124214120 (corresponding to 450K probe cg24296920) and rs10490924 (2.02% less methylation per risk allele, $P = 0.013$) and a marginally significant association with rs72631113 (1.85% less methylation per risk-associated allele, $P = 0.051$) – these 2 SNPs were also in high linkage disequilibrium in this

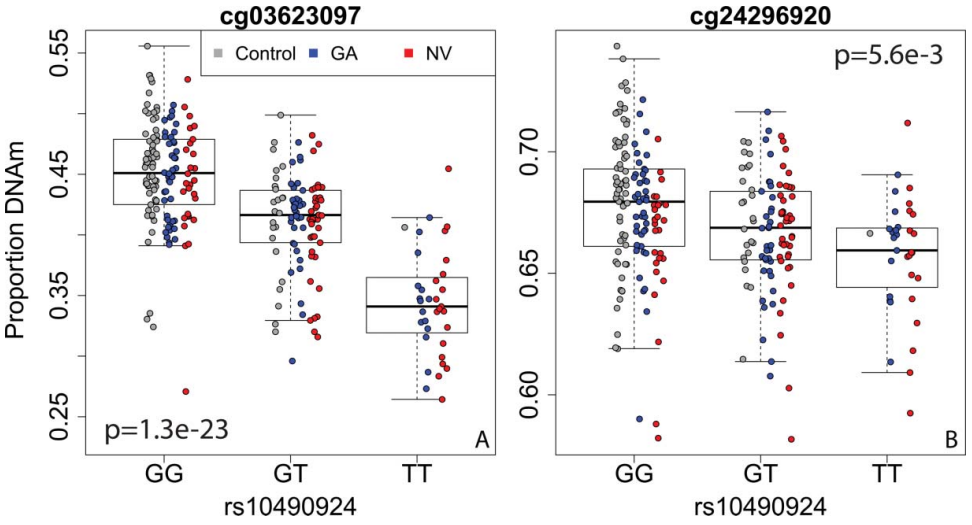

Figure 2. QTL analysis of DNA methylation level and *ARMS2* risk genotype shows a relationship between genotype and differential methylation. Hypomethylation of 450K probes (**A**) cg03623097 and (**B**) cg24296920 in GA and NV AMD patients and controls is associated with the T risk allele at rs10490924. GA, geographic atrophy: NV, neovascular.

replication sample ($r^2 = 0.629$, D'=1). We found directionally consistent, but statistically negative, association between DNAm levels of chr10:124213466 (corresponds to the less differentially methylated cg03623097) and these SNPs ($P = 0.18$ and $P = 0.20$, respectively), suggesting that the original associations in the Illumina 450K data may have been driven by technical variability from the probe SNP or that larger sample sizes may be required to fully replicate the findings. In contrast, in the peripheral retina from 6 control eyes and 10 eyes with AMD, albeit in a much smaller sample, we found that the risk allele significantly associated with DNAm at chr10:124213466 (1.6% less methylation in the T-carriers, $P = 0.049$) but not chr10:124214120 (1.25% more methylation in the T-carriers, $P = 0.24$) (**Fig. S4**). These results suggest that the genetic risk for AMD at rs10490924 associates with nearby DNAm levels at some CpG sites, perhaps mediating risk of illness through epigenetic mechanisms.

Illumina 450K profiling of retinal tissue

The Illumina 450K platform was used to analyze retina from 9 eyes with gross and histological evidence of AMD (average age 88.3 years) and retina from 9 control eyes (average age 83.2 years) that did not come from individuals in the discovery blood cohort. We note high genome-wide concordance between mean methylation levels across the blood and eye DNAm data sets – 59.5% and 73.3% of probes had mean proportion DNAm methylation levels (i.e., Illumina "Beta" scale) within 0.05 and 0.1 comparing blood to eye, respectively, suggesting that many probes overall had similar absolute levels of proportion methylation across these 2 tissue types. Similarly, 76.4% of probes were in the same general methylation level bin, where the bins were "unmethylated," containing CpGs with methylation proportions between 0–0.2, "partially methylated" with mean proportion methylation levels in the 0.2–0.8 range, and "methylated" which were proportions in the range of 0.8–1.0. This global similarity is likely due to the CpG island-focused design of the microarray where ~63% of probes are in or near CpG islands (within 5 kb, e.g., in shores or shelves).[26]

Not surprisingly given the number of samples, none of the array CpGs showed genome-wide significant differential methylation in the analysis of the retina samples or when restricting to only probes near the GWAS-positive genes. We then performed meta-analysis combining the differential methylation analysis using eye tissue alone and the surrogate variable analysis (SVA) for all 3 blood models (All, GA, NV) with the differential methylation analysis of the blood alone using SVA. The corresponding t-statistics across blood and eye were combined using the Stouffer's method for each of the 3 models. The meta-analysis-based t-statistics were adjusted for the false discovery rate; 3 probes, cg02834909 (chr3:46759438), cg19668234 (chr3: 46759449) and cg01788113 (chr3: 46759472), in a 34 bp region of the *PRSS50* promoter were significantly hypermethylated in NV blood samples at an FDR < 10% in this meta-analysis (**Fig. 3**).

A 41 bp region encompassing the 3 hypermethylated CpGs and 4 additional CpGs in the promoter of *PRSS50* were interrogated by bisulfite pyrosequencing in the blood of the Baltimore AMD cohort. The methylation level of all 7 sites was higher in all AMD samples compared to controls, and 3 and 2 of the CpGs were statistically significant in GA and NV, respectively (**Fig. 5S**). The same region was interrogated in retina of eyes with AMD and control eyes (**Fig. 5S**). Similarly, in AMD eyes, all 7 sites were hypermethylated compared to controls, 2 of which were statistically significant ($P < 0.05$). These results suggest that differential methylation in blood at certain individual loci may serve as surrogates of DNAm in retina tissue, which can be more fully characterized in larger retina tissue samples.

Discussion

Much progress has been made identifying risk alleles and biological pathways that appear to be involved in the development of AMD,[5] yet a large proportion of disease is not explained by these genetic risk factors. As many of the environmental factors likely to contribute to AMD are also known to contribute to epigenetic modifications, and the underlying mechanism by which the genetic risk factors contribute to the disease process remains largely elusive, we sought to investigate possible epigenetic differences present in individuals with AMD that might modulate the disease process. Using the Illumina 450K platform and peripheral blood of AMD patients and controls, we identified 2 differentially methylated CpGs in the *ARMS2* gene promoter. The methylation level of at least one of these CpGs correlated with the risk polymorphism associated with *ARMS2/HTRA1*. We also identified a gene never before associated with AMD, *PRSS50*, which is differentially methylated in both blood and retina from 2 populations of AMD patients.

Demonstrating functionality of the differentially methylated regions will be important in future studies. Two differentially methylated probes in the *ARMS2* promoter were less methylated in the NV blood samples than controls. Interestingly, these 2 probes are located very close to the *ARMS2/HTRA1* risk SNP (G/T) rs10490924 located in the first exon of *ARMS2*. The differentially methylated probe cg23296920, which is located in the promoter of *ARMS2* (52 bp upstream from TSS) is 328 bp upstream from rs10490924, and the other differentially methylated *ARMS2* probe cg03623097 (713 bp upstream from TSS) is 982 bp upstream from rs10490924. In other studies, a direct relationship between the genetic sequence and DNAm at specific sites has been found. meQTLs, and mediators such as DNA binding factors or secondary chromatin structures, are thought to be the functional link between the genetic sequence and the differential methylation.[27,28] In the case of *ARMS2*, the lower methylation level of chr10:124214120 in the blood correlated with the risk allele, T, at rs10490924, whereas in the retina of AMD patients, the risk allele significantly associated with the methylation level of chr10:124213466, possibly reflecting either true tissue-specific differences or the result of a much smaller retinal sample size.

The association between chromosome 10q26, which surrounds *ARMS2* and *HTRA1*, has been demonstrated in many studies since it was first identified; however, because of linkage disequilibrium in the region, the debate is ongoing as to the causal allele and underlying pathogenic mechanism. Among the

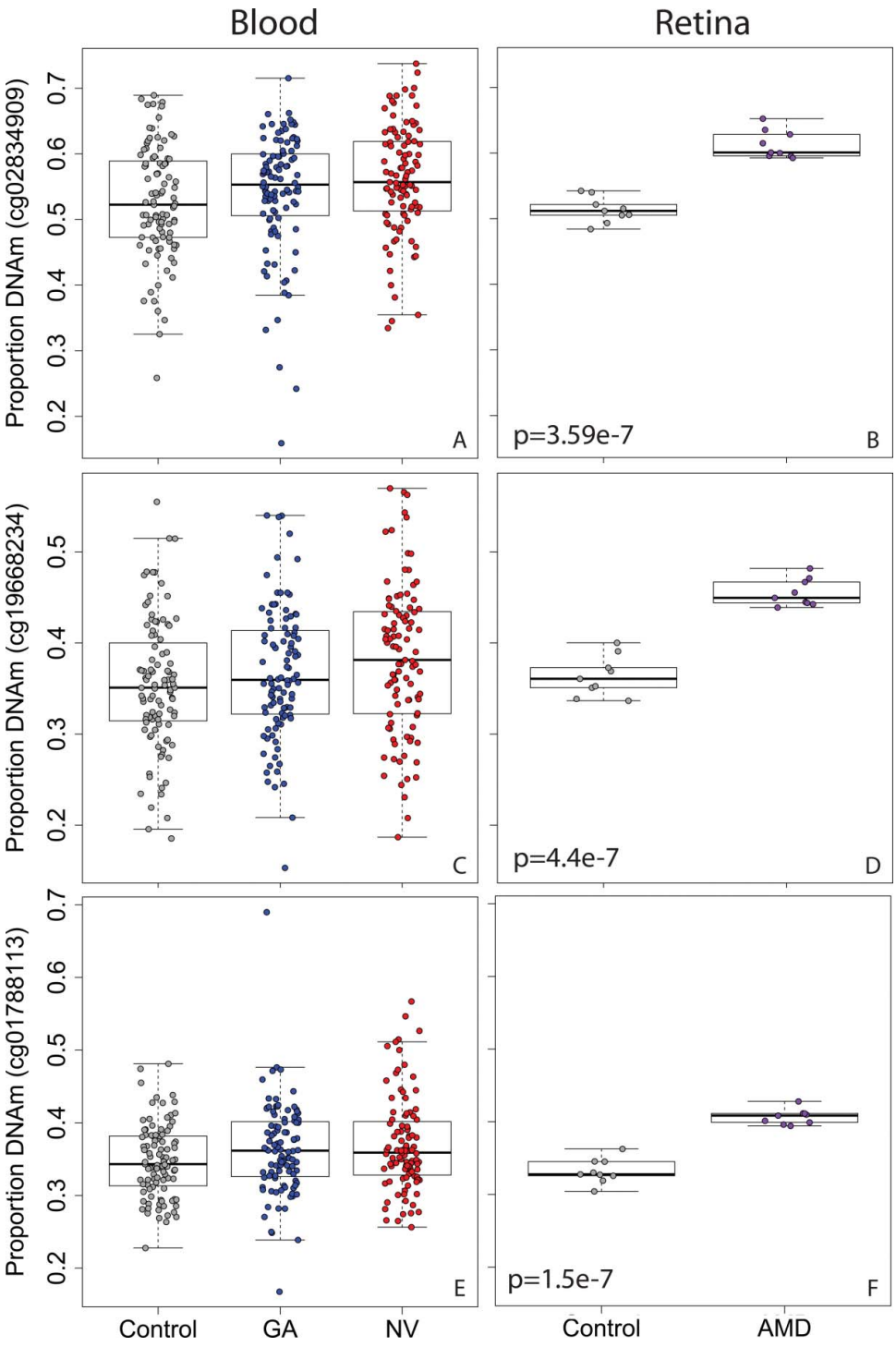

Figure 3. Differential methylation of CpG sites within the promoter of *PRSS50*. Hypermethylation of 450K probes cg02834909, cg19668234, and cg01788113 in the promoter of *PRSS50* in (**A**, **C**, **E**) blood of GA and NV patients with AMD compared to controls, and in (**B**, **D**, **F**) peripheral retina from eyes with AMD. GA, geographic atrophy; NV, neovascular,

genetic loci that showed genome-wide significance for clinical risk of AMD, only the 2 probes in the *ARMS2* locus, cg03623097 and cg24296920, demonstrated multiple testing-corrected significance ($P < 0.05/1408$). The methylation level of the 2 probes was correlated with the rs10490924 SNP status, arguing for a pathogenic role for *ARMS2*. However, 2 additional meQTLs were found to be associated with rs10490924, both of which are in the *HTRA1* promoter, and although neither was significantly differentially methylated, cg05723130 was more methylated in blood from AMD patients ($P = 0.002$) suggesting a potentially smaller contribution of *HTRA1* differential methylation to AMD.

In many large epidemiological studies, whole blood is the only biological material that has been archived. The extent to which the DNA methylation patterns of easily accessible tissues like whole blood represent the epigenetic phenotype in inaccessible tissues is unclear, although recent studies are showing a concordance. Epigenetic variation arising before disease could be inherited and thus be present in all adult tissues, or it could arise stochastically during one's lifetime and be limited to one or a few tissues. Epigenetic variation can also be environmentally induced by life-style related factors, such as diet or smoking.[29] Some of the DNA methylation changes that occur during aging and as a result of environmental exposures like smoking may be reflected in multiple tissues, e.g., blood and retina which are both exposed to oxidative stress from smoking, while others may be tissue specific. Studies are ongoing to investigate the true concordance between blood methylation levels and those of the RPE and retina. In this study, 3 CpGs in a 34 bp region of the *PRSS50* promoter (chr3:46759438–46759472) were hypermethylated in AMD blood and retina samples compared to controls (7–13% more methylated). Hypermethylation of selective CpGs within the probe sequences was replicated in a second cohort of blood and in retina of AMD patients. *PRSS50* is a serine protease whose mRNA is highly expressed in testes and at a lower level in retina.[30] The differentially methylated region has strong CTCF occupancy, which has previously been linked to DNA methylation.[31] Additionally, it is also a DNAseI hypersensitive region, suggesting a regulatory role. Previous studies have

demonstrated that DNA methylation might regulate *PRSS50* gene expression in different tissues and spermatic cells.[32] More interestingly, the gene was abnormally reactivated in many breast cancer biopsies,[33] and knockdown of *PRSS50* induced cell apoptosis.[34] Understanding how increased promoter methylation and presumably decreased retinal expression of *PRSS50* play a role in AMD pathogenesis will require further study.

The difference in methylation levels observed in the blood of AMD patients was less than 10%, and the methylation differences in the *PRSS50* promoter were comparable in both the blood and retina. The biological implications of such small alterations in DNA methylation in terms of gene expression are unknown, although the significant overlap with genes already associated with AMD and the observed meQTLs suggest that the methylation differences may be functional. These methylation differences are on the order of changes observed in other recent studies. For example, significant methylation differences of ~5% were seen in the brains of multiple sclerosis patients,[35] and the blood of patients with schizophrenia[36] and asthma,[37] suggesting that very small differences could have important functional implications in disease pathology. Likewise, relatively small methylation differences have be linked to changes in gene expression, which hint at potential functional effects of our observed methylation differences.[16]

This is one of the first studies to show a relationship between DNAm and AMD,[38-40] but many questions remain. Did the disease-associated differential methylation observed in the blood and retina arise prior to the onset of AMD and contribute to the disease phenotype or was it a secondary effect of the disease process or treatment? Determining whether the variation was present before obvious signs of AMD would help to answer this question. What is the functional significance of the methylation changes in the *ARMS2* promoter and are similar methylation changes present in the macular RPE of AMD patients? Additional studies are needed to answer these questions. Ultimately, a better characterization of epigenetic-disease mechanisms associated with the development of AMD will be important for a better understanding of the pathogenic process and the rational design of future epigenetic-based therapeutic strategies.

Materials and Methods

Ethical approval

All aspects of this project were conducted in accordance with the principles of the Declaration of Helsinki, with informed consent being obtained from all participants. This project was approved by the Johns Hopkins University School of Medicine Institutional Review Board and the University of Michigan Institutional Review Board.

Sample collection

DNA samples from peripheral blood of 298 age- and sex-matched samples [comprising 100 bilateral geographic atrophy (GA), 99 bilateral neovascularization (NV) and 99 controls] were obtained from a subset of the Michigan patients from the AMD-MMAP study cohort (dbGaP: phs000182). DNA was extracted from whole blood using the Puregene Blood Core Kit C (Qiagen). The sample group comprised 63% females, with a mean ± SD age of 79.3 ± 5.6. All samples were derived from the Kellogg Eye Center (KEC) at the University of Michigan.[23] From the original KEC cohort, patients with bilateral GA or NV were selected. Control patients were examined and accepted if they had no retinal changes or small drusen and/or pigment changes in one eye only with family history of one or no family members affected with AMD. All cases and controls were ≥60 years of age. Donor eyes were obtained from National Disease Research Interchange (Philadelphia, PA) with limited clinical history. Macular photos were taken prior to the isolation of macular calottes from each eye. Macular tissue was passed through a sucrose gradient and frozen. Sections were stained with hematoxylin and eosin and were reviewed for histologic evidence of AMD by an ocular pathologist. Only those eyes with a clinical history of AMD, gross evidence of late AMD in the macula (comparable to the degree of disease found in the patients from whom the blood samples were obtained) and histologic evidence of AMD were used as AMD samples. Peripheral retina was isolated from AMD and control eyes and was snap frozen. DNA was isolated from the tissue using AllPrep DNA/RNA Mini Kit (Qiagen, Germantown, Maryland). Ten control eyes (average age 83.6) and 9 AMD eyes (average age 88.3) were used for the 450K analysis. Six control eyes (average age 86.3) and 13 AMD eyes (average age 86.4) were used for pyrosequencing.

Illumina infinium human methylation450 (450K) bead array

DNA samples (1.3 μg) were processed for the Illumina Infinium HumanMethylation450 Bead Array platform[24] (Illumina 450K) at the Center for Inherited Disease Research (CIDR; Johns Hopkins University) as per the manufacturers' protocols. All matched samples (GA-NV-control) were run within a single BeadChip array. A series of technical controls were incorporated in the experimental design: 1) lymphoblastoid cell lines (LCLs); inputs that were 2) 0%; 3) 50%; and 4) 100% methylated (included in each processing plate); and 5) 8 of the blood samples, which were run in duplicate. Data was deposited to dbGaP, http://www.ncbi.nlm.nih.gov/gap, MMAP-Methylation in AMD (phs000457.v1.p1).

Illumina 450K microarray data preprocessing

We processed the Illumina 450K data using the minfi Bioconductor package.[25] We first used the raw methylated (M) and unmethylated (U) channel intensities to perform quality control checks that also used the technical control samples (LCLs and duplicates) described above. We then performed a modified version of quantile normalization (*preprocessQuantile* in the minfi package) to normalize across samples, which, briefly, forces the distribution of type I and type II probes on the Illumina 450K microarray to be the same across samples. This normalization is performed separately on the M and U channels, and the resulting normalized DNA methylation proportions were derived based on the logit (base 2) transform: $y = \log_2(M/U)$, which was

converted to the proportion methylation ("β" scale referred to by Illumina) by: $2^y/(1+2^y)$. We estimated cell composition in the peripheral blood data set using the *estimateCellComposition* function in the *minfi* package.[26,27] Probes annotated to the sex chromosomes (chrX = 11,232 and chrY = 416) and probes containing an annotated SNP (via dbSNP v137) at the target CpG site (N = 16,756) and at the single base extension site (N = 7,880) were removed prior to statistical analyses. The data from retinal tissue and blood were preprocessed separately, given the large imbalance of sample sizes between the 2 datasets.

Statistical analysis

We performed differential methylation analysis using 3 different subsets of the blood data: 1) either GA or NV – "all" model; 2) just NV – "NV" model; and 3) just GA – "GA" model, compared to the shared control population. Within the eye data set, we fit a single model comparing patients with AMD to controls. For each of these AMD outcomes in blood and within eye, we fit the following statistical model:

$$Y_{ij} = \alpha_i + \beta_i AMD_j + \zeta_i SV_j + \varepsilon_{i,j}$$

where Y_{ij} is the proportion DNA methylation levels at probe i and subject j, α_i is the mean methylation level for the controls, AMD_J is the AMD status for subject j, and β_i is the effect of each AMD outcome on DNAm levels. We further adjusted for estimated surrogate variables estimated from each model (blood: 47 for all, 32 for NV, and 35 for GA; eye: 5) to control for potential unmeasured technical and/or biological confounding,[28,29] including cellular composition of blood.[26,30] See Jaffe et al., 2012 for additional details on this statistical framework.[31] We observed no difference in cell composition between NV patients and controls, but slight increases of CD4+ ($P = 0.034$) and CD8+ ($P = 0.001$) cell counts between GA patients; however, the first 2 surrogate variables were associated with estimated cell composition.

We first conducted an epigenome-wide search to identify potentially novel loci associated with AMD, and controlled for multiple testing via the false discovery rate (FDR).[32] Next, we limited the analysis to probes mapping near genes (using RefSeq coordinates for the genes and a 10 kb window for overlapping Illumina 450K probes) within regions previously associated with AMD in GWAS (1,408 probes within the 28 GWAS-positive regions), and utilized a Bonferroni-based correction to control for multiple testing ($P < 0.05/1408$).

We then performed an integrative meta-analysis across DNAm data from blood and eye tissue using Stouffer's method [33] to combine resulting moderated t-statistics at each CpG. We generated meta-analysis test statistics by combining SV-corrected t-statistics from the 1) "all," 2) "NV," and 3) "GA" models separately with the T-statistics from the eye data. To obtain measures of statistical uncertainty for these meta-analysis statistics, we resampled the T-statistics from the eye data 500 times (without replacement, across the probes, which removes any correlation between eye and blood statistics) and calculated null Stouffer meta-analysis Z-scores from the observed blood T-statistics with each of the 500 sets of the resampled eye T-statistics. Empirical *P*-values were calculated comparing the observed combined Z-scores to the pooled distribution of null Z-scores across all 500 permutations, and these *P*-values were controlled for multiple testing using the FDR approach described above.

GWAS enrichment analysis

We tested for statistical enrichment between the genes mapping to the marginally significant differentially methylated probes (DMPs) and genes associated with clinical risk for AMD based on GWAS results from Fritsche et al.[5] The observed number of overlapping genes was contrasted to the number of genes covered by the Illumina 450K design. Genes were mapped to probes using a distance cutoff of 10 kb. This test forms a 2 × 2 table for each marginally significant DMP list (for all, NV, and GA models), where the cells were: gene both contains a DMP and GWAS-positive, gene only GWAS-positive, gene only contains a DMP, and the gene overlaps neither. We calculated corresponding odds ratio of enrichment, and *P*-value based on a chi-squared distribution with 1 degree of freedom.

Sample selection from the Baltimore cohort

DNA samples derived from peripheral whole blood were obtained from a cohort collected at Johns Hopkins University School of Medicine in Baltimore as previously described.[34,35] In brief, diagnosis of advanced AMD was based on the presence of GA or NV (equivalent to AREDS category 4 or 5). Controls were identified as >60 y of age, having fewer than 5 small drusen (<63 μm) and no RPE abnormalities. See **Table S2** for demographics of the validation cohorts. Genomic DNA was isolated from peripheral whole blood using Puregene Blood Kit chemistry on an Autopure LS automated DNA purification instrument (Qiagen, Valencia, CA).

Bisulfite pyrosequencing of *ARMS2* and *PRSS50*

Pyrosequencing assays were designed using algorithms built into the PyroMark Assay Design Software (Version 2.0.1, Qiagen, Germantown, Maryland). Briefly, primers designed to target specific CpG sites within the regions of interest containing the differentially methylated probes were chosen from a list generated by the software on the basis of the algorithms' predicted assay quality. Genomic DNA (500 ng) was bisulfite converted using the EZ DNA Methylation Gold kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. Following bisulfite treatment all previously unmethylated cytosine residues are converted to uracil, whereas methylated cytosine residues remain unconverted. The regions of interests were amplified from the bisulfite-treated DNA using PCR primer sets #1 (*ARMS2*) or #2 (*PRSS50*) as shown in **Table S3**.

PCR products were bound to Streptavidin Sepharose High Performance beads (GE Healthcare Life Sciences, Rydalmere, Australia) and a single-strand template was isolated using the Pyrosequencing Vacuum Prep Tool (Qiagen, Maryland USA). The beads were transferred to an optically clear, 24 well sequencing plate in 0.3 μM of the sequencing primer (**Table S3**). Serial pyrosequencing was performed where multiple sequencing

primers were contained within a single PCR amplicon.[36] Pyrosequencing was performed on a PyroMark 24 Pyrosequencing System (Qiagen, Germantown, Maryland) as per the manufacturer's instructions. Data were analyzed on the PyroMark Q24 software (Qiagen, Germantown, Maryland). This software calculates the C peak as a percentage of the T plus C peak at each CpG site taking into account sequence length and signal strength. The methylation level for each sample was calculated for 6 CpG sites in *ARMS2* and 7 CpG sites in *PRSS50*. An unpaired Student's t-test was used to compare the methylation difference between the disease and control groups.

meQTL analysis in Illumina 450K samples

Observed high-quality genotypes on 290 subjects with Illumina 450k data (out of 298, 97.3%) were obtained from dbGaP (accession: phs000182.v2.p1). SNPs on chromosome 10 were phased using ShapeIT,[37] and the 6 megabase area flanking the AMD risk SNP (chr10:121214448–127214448) was imputed up to the latest 1000 Genomes Phase 3 reference panel using Impute2[38] to obtain genotype calls for rs10490924 and rs72631113. Using the previously calculated surrogate variables (SVs, see above), we performed meQTL analyses separately within AMD patients (NV and GA together) and controls, at both SNPs, using the linear model of the form: $Meth_j = \alpha + \beta SNP_j + \zeta SV_j + \varepsilon_j$ for person j. We considered all Illumina 450k probes within 20kb of each SNP for these analyses using the MatrixEQTL package.[41]

Genotyping of *ARMS2* in validation samples

SNP rs72631113 and rs10490924 located in the promoter and first exon, respectively of the *ARMS2* gene were bisulfite pyrosequenced and genotyped. Briefly, blood DNA samples were collected from 48 unrelated individuals from the Baltimore cohort (24 controls, 24 NV) as described above. Blood and eye genomic DNA samples were pyrosequenced to determine the methylation level of the 6 *ARMS2* sites described above. For genotyping, PCR fragments were amplified in 50 μL volumes with 30 ng genomic DNA using PyroMark PCR kit (Qiagen, Germantown, Maryland), and 12.5 pmol each of primer (**Table S4**). PCR conditions were as follows: 95°C for 3 min followed by 45 cycles of 94°C, 30 sec; 58°C, 30 sec; 72°C 30 sec, ending with 72°C for 2 min. PCR products were recovered using 1% agarose gel (Sigma Aldrich, USA) and Qiaquick Gel Extractionkit (Qiagen, Germantown, Maryland). A total of 180 ng recovered PCR product (12 μl volume) and a final concentration of 1 μM sequencing primer were used for Sanger sequencing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

References

1. Coleman HR, Chan CC, Ferris FL, 3rd, Chew EY. Age-related macular degeneration. Lancet 2008; 372:1835–45; PMID:19027484; http://dx.doi.org/10.1016/S0140-6736(08)61759-6
2. Ferris FL, Davis MD, Clemons TE, Lee LY, Chew EY, Lindblad AS, Milton RC, Bressler SB, Klein R. A simplified severity scale for age-related macular degeneration: AREDS Report No. Eighteen. Arch Ophthalmol 2005; 123:1570–4; PMID:16286620; http://dx.doi.org/10.1001/archopht.123.11.1570
3. Martin DF, Maguire MG, Ying GS, Grunwald JE, Fine SL, Jaffe GJ. Ranibizumab and bevacizumab for neovascular age-related macular degeneration. N Engl J Med 2011; 364:1897–908; PMID:21526923; http://dx.doi.org/10.1056/NEJMicm1005605
4. Meleth AD, Wong WT, Chew EY. Treatment for atrophic macular degeneration. Curr Opin Ophthalmol 2011; 22:190–3; PMID:21427574; http://dx.doi.org/10.1097/ICU.0b013e32834594b0
5. Fritsche LG, Chen W, Schu M, Yaspan BL, Yu Y, Thorleifsson G, Zack DJ, Arakawa S, Cipriani V, Ripke S, et al. Seven new loci associated with age-related macular degeneration. Nat Genet 2013; 45:433–9; PMID:23455636; http://dx.doi.org/10.1038/ng.2578
6. Fritsche LG, Fariss RN, Stambolian D, Abecasis GR, Curcio CA, Swaroop A. Age-related macular degeneration: genetics and biology coming together. Annu Rev Genomics Hum Genet 2014; 15:151–71; PMID:24773320; http://dx.doi.org/10.1146/annurev-genom-090413-025610
7. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ, McCarthy MI, Ramos EM, Cardon LR, Chakravarti A, et al. Finding the missing heritability of complex diseases. Nature 2009; 461:747–53; PMID:19812666; http://dx.doi.org/10.1038/nature08494
8. Delcourt C, Diaz JL, Ponton-Sanchez A, Papoz L. Smoking and age-related macular degeneration. The POLA Study. Pathologies Oculaires Liees a l'Age. Arch Ophthalmol 1998; 116:1031–5; PMID:9715683; http://dx.doi.org/10.1001/archopht.116.8.1031
9. Tan JS, Wang JJ, Flood V, Mitchell P. Dietary fatty acids and the 10-year incidence of age-related macular degeneration: the Blue Mountains Eye Study. Arch Ophthalmol 2009; 127:656–65; PMID:19433717; http://dx.doi.org/10.1001/archophthalmol.2009.76
10. van Leeuwen R, Boekhoorn S, Vingerling JR, Witteman JC, Klaver CC, Hofman A, de Jong PT. Dietary intake of antioxidants and risk of age-related macular degeneration. Jama 2005; 294:3101–7; PMID:16380590; http://dx.doi.org/10.1001/jama.294.24.3101
11. Naj AC, Scott WK, Courtenay MD, Cade WH, Schwartz SG, Kovach JL, Agarwal A, Wang G, Haines JL, Pericak-Vance MA. Genetic factors in nonsmokers with age-related macular degeneration revealed through genome-wide gene-environment interaction analysis. Ann Hum Genet 2013; 77:215–31; PMID:23577725; http://dx.doi.org/10.1111/ahg.12011
12. Schmidt S, Haines JL, Postel EA, Agarwal A, Kwan SY, Gilbert JR, Pericak-Vance MA, Scott WK. Joint effects of smoking history and APOE genotypes in age-related macular degeneration. Mol Vis 2005; 11:941–9; PMID:16288198
13. Schmidt S, Hauser MA, Scott WK, Postel EA, Agarwal A, Gallins P, Wong F, Chen YS, Spencer K, Schnetz-Boutaud N, et al. Cigarette smoking strongly modifies the association of LOC387715 and age-related macular degeneration. Am J Hum Genet 2006; 78:852–64; PMID:16642439; http://dx.doi.org/10.1086/503822
14. Feinberg AP. Phenotypic plasticity and the epigenetics of human disease. Nature 2007; 447:433–40; PMID:17522677; http://dx.doi.org/10.1038/nature05919
15. Grafodatskaya D, Chung B, Szatmari P, Weksberg R. Autism spectrum disorders and epigenetics. J Am Acad Child Adolesc Psychiatry 2010; 49:794–809; PMID:20643313; http://dx.doi.org/10.1016/j.jaac.2010.05.005
16. Dayeh T, Volkov P, Salo S, Hall E, Nilsson E, Olsson AH, Kirkpatrick CL, Wollheim CB, Eliasson L, Ronn T, et al. Genome-wide DNA methylation analysis of human pancreatic islets from type 2 diabetic and non-diabetic donors identifies candidate genes that influence insulin secretion. PLoS Genet 2014; 10:e1004160;

PMID:24603685; http://dx.doi.org/10.1371/journal.pgen.1004160
17. Gavin DP, Sharma RP. Histone modifications, DNA methylation, and schizophrenia. Neurosci Biobehav Rev 2010; 34:882–8; PMID:19879893; http://dx.doi.org/10.1016/j.neubiorev.2009.10.010
18. Gervin K, Vigeland MD, Mattingsdal M, Hammero M, Nygard H, Olsen AO, Brandt I, Harris JR, Undlien DE, Lyle R. DNA methylation and gene expression changes in monozygotic twins discordant for psoriasis: identification of epigenetically dysregulated genes. PLoS Genet 2012; 8:e1002454; PMID:22291603; http://dx.doi.org/10.1371/journal.pgen.1002454
19. Nimmo ER, Prendergast JG, Aldhous MC, Kennedy NA, Henderson P, Drummond HE, Ramsahoye BH, Wilson DC, Semple CA, Satsangi J. Genome-wide methylation profiling in Crohn's disease identifies altered epigenetic regulation of key host defense mechanisms including the Th17 pathway. Inflamm Bowel Dis 2012; 18:889–99; PMID:22021194; http://dx.doi.org/10.1002/ibd.21912
20. Liu Y, Aryee MJ, Padyukov L, Fallin MD, Hesselberg E, Runarsson A, Reinius L, Acevedo N, Taub M, Ronninger M, et al. Epigenome-wide association data implicate DNA methylation as an intermediary of genetic risk in rheumatoid arthritis. Nat Biotechnol 2013; 31:142–7; PMID:23334450; http://dx.doi.org/10.1038/nbt.2487
21. Wang X, Zhu H, Snieder H, Su S, Munn D, Harshfield G, Maria BL, Dong Y, Treiber F, Gutin B, et al. Obesity related methylation changes in DNA of peripheral blood leukocytes. BMC Med 2010; 8:87; PMID:21176133; http://dx.doi.org/10.1186/1741-7015-8-87
22. Scholl HP, Charbel Issa P, Walier M, Janzer S, Pollok-Kopp B, Borncke F, Fritsche LG, Chong NV, Fimmers R, Wienker T, et al. Systemic complement activation in age-related macular degeneration. PLoS ONE 2008; 3:e2593; PMID:18596911; http://dx.doi.org/10.1371/journal.pone.0002593
23. Chen W, Stambolian D, Edwards AO, Branham KE, Othman M, Jakobsdottir J, Tosakulwong N, Pericak-Vance MA, Campochiaro PA, Klein ML, et al. Genetic variants near TIMP3 and high-density lipoprotein-associated loci influence susceptibility to age-related macular degeneration. Proc Natl Acad Sci U S A 2010; 107:7401–6; PMID:20385819; http://dx.doi.org/10.1073/pnas.0912702107
24. Michels KB, Binder AM, Dedeurwaerder S, Epstein CB, Greally JM, Gut I, Houseman EA, Izzi B, Kelsey KT, Meissner A, et al. Recommendations for the design and analysis of epigenome-wide association studies. Nat Methods 2013; 10:949–55; PMID:24076989; http://dx.doi.org/10.1038/nmeth.2632
25. Dewan A, Liu M, Hartman S, Zhang SS, Liu DT, Zhao C, Tam PO, Chan WM, Lam DS, Snyder M, et al. HTRA1 promoter polymorphism in wet age-related macular degeneration. Science 2006; 314:989–92; PMID:17053108; http://dx.doi.org/10.1126/science.1133807
26. Sandoval J, Heyn H, Moran S, Serra-Musach J, Pujana MA, Bibikova M, Esteller M. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics 2011; 6:692–702; PMID:21593595; http://dx.doi.org/10.4161/epi.6.6.16196
27. Bell JT, Pai AA, Pickrell JK, Gaffney DJ, Pique-Regi R, Degner JF, Gilad Y, Pritchard JK. DNA methylation patterns associate with genetic and gene expression variation in HapMap cell lines. Genome Biol 2011; 12:R10; PMID:21251332; http://dx.doi.org/10.1186/gb-2011-12-1-r10
28. Drong AW, Nicholson G, Hedman AK, Meduri E, Grundberg E, Small KS, Shin SY, Bell JT, Karpe F, Soranzo N, et al. The presence of methylation quantitative trait loci indicates a direct genetic influence on the level of DNA methylation in adipose tissue. PLoS ONE 2013; 8:e55923; PMID:23431366; http://dx.doi.org/10.1371/journal.pone.0055923
29. Christensen BC, Houseman EA, Marsit CJ, Zheng S, Wrensch MR, Wiemels JL, Nelson HH, Karagas MR, Padbury JF, Bueno R, et al. Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CpG island context. PLoS Genet 2009; 5:e1000602; PMID:19680444; http://dx.doi.org/10.1371/journal.pgen.1000602
30. Liu X, Yu X, Zack DJ, Zhu H, Qian J. TiGER: a database for tissue-specific gene expression and regulation. BMC Bioinformatics 2008; 9:271; PMID:18541026; http://dx.doi.org/10.1186/1471-2105-9-271
31. Wang H, Maurano MT, Qu H, Varley KE, Gertz J, Pauli F, Lee K, Canfield T, Weaver M, Sandstrom R, et al. Widespread plasticity in CTCF occupancy linked to DNA methylation. Genome Res 2012; 22:1680–8; PMID:22955980; http://dx.doi.org/10.1101/gr.136101.111
32. Huang Y, Wang Y, Wang M, Sun B, Li Y, Bao Y, Tian K, Xu H. Differential methylation of TSP50 and mTSP50 genes in different types of human tissues and mouse spermatic cells. Biochem Biophys Res Commun 2008; 374:658–61; PMID:18662669; http://dx.doi.org/10.1016/j.bbrc.2008.07.087
33. Shan J, Yuan L, Xiao Q, Chiorazzi N, Budman D, Teichberg S, Xu HP. TSP50, a possible protease in human testes, is activated in breast cancer epithelial cells. Cancer Res 2002; 62:290–4; PMID:11782390
34. Zhou L, Bao YL, Zhang Y, Wu Y, Yu CL, Huang YX, Sun Y, Zheng LH, Li YX. Knockdown of TSP50 inhibits cell proliferation and induces apoptosis in P19 cells. IUBMB Life 2010; 62:825–32; PMID:21086474; http://dx.doi.org/10.1002/iub.390
35. Huynh JL, Garg P, Thin TH, Yoo S, Dutta R, Trapp BD, Haroutunian V, Zhu J, Donovan MJ, Sharp AJ, et al. Epigenome-wide differences in pathology-free regions of multiple sclerosis-affected brains. Nature Neurosci 2014; 17:121–30; PMID:24270187; http://dx.doi.org/10.1038/nn.3588
36. Dempster EL, Pidsley R, Schalkwyk LC, Owens S, Georgiades A, Kane F, Kalidindi S, Picchioni M, Kravariti E, Toulopoulou T, et al. Disease-associated epigenetic changes in monozygotic twins discordant for schizophrenia and bipolar disorder. Hum Mol Genet 2011; 20:4786–96; PMID:21908516; http://dx.doi.org/10.1093/hmg/ddr416
37. Acevedo N, Reinius LE, Greco D, Gref A, Orsmark-Pietras C, Persson H, Pershagen G, Hedlin G, Melen E, Scheynius A, et al. Risk of childhood asthma is associated with CpG-site polymorphisms, regional DNA methylation and mRNA levels at the GSDMB/ORMDL3 locus. Hum Mol Genet 2015; 24:875–90; PMID:25256354; http://dx.doi.org/10.1093/hmg/ddu479
38. Hunter A, Spechler PA, Cwanger A, Song Y, Zhang Z, Ying GS, Hunter AK, Dezoeten E, Dunaief JL. DNA methylation is associated with altered gene expression in AMD. Invest Ophthalmol Vis Sci 2012; 53:2089–105; PMID:22410570; http://dx.doi.org/10.1167/iovs.11-8449
39. Oliver VF, Franchina M, Jaffe AE, Branham KE, Othman M, Heckenlively JR, Swaroop A, Campochiaro B, Vote BJ, Craig JE, et al. Hypomethylation of the IL17RC Promoter in Peripheral Blood Leukocytes Is Not A Hallmark of Age-Related Macular Degeneration. Cell Rep 2013; 5:1527–35; PMID:24373284; http://dx.doi.org/10.1016/j.celrep.2013.11.042
40. Wei L, Liu B, Tuo J, Shen D, Chen P, Li Z, Liu X, Ni J, Dagur P, Sen HN, et al. Hypomethylation of the IL17RC promoter associates with age-related macular degeneration. Cell Rep 2012; 2:1151–8; PMID:23177625; http://dx.doi.org/10.1016/j.celrep.2012.10.013
41. Shabalin AA. Matrix eQTL: ultra fast eQTL analysis via large matrix operations. Bioinformatics 2012; 28:1353–8; PMID:22492648; http://dx.doi.org/10.1093/bioinformatics/bts163