BMC Genetics

BioMed Central

Research article

Ordered subset linkage analysis supports a susceptibility locus for age-related macular degeneration on chromosome 16p12

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Published: 06 July 2004

BMC Genetics 2004, **5**:18 doi:10.1186/1471-2156-5-18

Received: 21 January 2004
Accepted: 06 July 2004

This article is available from: http://www.biomedcentral.com/1471-2156/5/18

Abstract

Background: Age-related macular degeneration (AMD) is a complex disorder that is responsible for the majority of central vision loss in older adults living in developed countries. Phenotypic and genetic heterogeneity complicate the analysis of genome-wide scans for AMD susceptibility loci. The ordered subset analysis (OSA) method is an approach for reducing heterogeneity, increasing statistical power for detecting linkage, and helping to define the most informative data set for follow-up analysis. OSA assesses the linkage evidence in subsets of potentially more homogeneous families by rank-ordering family-specific lod scores with respect to trait-associated covariates or phenotypic features. Here, we present results of incorporating five continuous covariates into our genome-wide linkage analysis of 389 microsatellite markers in 62 multiplex families: Body mass index (BMI), systolic (SBP) and diastolic (DBP) blood pressure, intraocular pressure (IOP), and pack-years of cigarette smoking. Chromosome-wide significance of increases in nonparametric multipoint lod scores in covariate-defined subsets relative to the overall sample was assessed by permutation.

Results: Using a correction for testing multiple covariates, statistically significant lod score increases were observed for two chromosomal regions: 14q13 with a lod score of 3.2 in 28 families with average IOP $\leq$ 15.5 ($p = 0.002$), and 6q14 with a lod score of 1.6 in eight families with average BMI $\geq$ 30.1 ($p = 0.0004$). On chromosome 16p12, nominally significant lod score increases ($p \leq 0.05$), up to a lod score of 2.9 in 32 families, were observed with several covariate orderings. While less significant, this was the only region where linkage evidence was associated with multiple clinically meaningful covariates and the only nominally significant finding when analysis was restricted to advanced forms of AMD. Families with linkage to 16p12 had higher averages of SBP,

IOP and BMI and were primarily affected with neovascular AMD. For all three regions, linkage signals at or very near the peak marker have previously been reported.

Conclusion: Our results suggest that a susceptibility gene on chromosome 16p12 may predispose to AMD, particularly to the neovascular form, and that further research into the previously suggested association of neovascular AMD and systemic hypertension is warranted.

Background

Age-related macular degeneration (AMD) affects the central region of the retina (macula), which has the highest concentration of cone photoreceptors and is responsible for central visual acuity. In developed nations, it is the most common cause of irreversible blindness in older adults. Approximately 4% of individuals over 60 years of age and 10% of those over 75 years of age have advanced stages of the disorder, which include geographic atrophy (dry AMD) and neovascular (wet) AMD [1]. While both forms lead to loss of central vision, it is more common and occurs more rapidly with neovascular AMD. It is unknown whether the two clinical subtypes have a distinct etiology, but longitudinal studies have shown that the presence of large soft drusen, which are extensive extracellular protein/lipid deposits in the macula, increases the risk of progressing to either form of advanced AMD [2-4].

AMD has a complex etiology likely to result from the interplay of several risk factors, both genetic and environmental. A contribution of genetic susceptibility is supported by epidemiologic [5-7] and twin studies [8,9], as well as segregation analyses [10]. Candidate gene association studies have examined many genes responsible for retinal disorders with Mendelian inheritance, with generally negative results. One of the most intensely studied genes has been the ABCA4 gene on chromosome 1p, which causes juvenile-onset autosomal-recessive Stargardt disease. An initial study reporting an increased risk of AMD for carriers of two particular ABCA4 sequence variants [11] was followed by only one positive replication study [12] and multiple studies reporting an absence of this association [13-19]. At this point, the ABCA4 gene is not believed to be a major susceptibility gene for AMD, although it may account for a small proportion of the disease, possibly only the dry form. A candidate gene that has consistently been reported to be associated with AMD in multiple independent studies is the apolipoprotein E (APOE) gene on chromosome 19q. The APOE-4 allele, or a nearby allele in linkage disequilibrium with APOE-4, appears to be protective for AMD [20-28]. To date, only two studies failed to confirm this finding in a Caucasian [29] and a Chinese sample [30]. The APOE-2 allele may increase AMD risk in smokers [31].

Several research groups have collected multiplex families (2+ sampled family members with AMD) to perform a genome-wide screen for AMD susceptibility loci [32-37]. Despite variable phenotype definitions and different analysis approaches, these genome scans identified remarkably consistent regions of linkage on several chromosomes, including chromosome 1q25-31, 10q25-26, 12q21-23 and 16p11-12. None of the genes responsible for these linkage signals have thus far been identified. The hemicentin-1 (FIBL6) gene, located on 1q31, has been proposed as a rare cause of AMD [38], but awaits confirmation by other research groups.

We recently genotyped 62 multiplex AMD families ascertained through Duke University Medical Center (DUMC) and Vanderbilt University Medical Center (VUMC) for 389 microsatellite markers distributed at 10 cM density across the human genome. These families were screened for the first time and were not included in previously published genome screens for AMD [32-37]. For all individuals enrolled in our study, an extensive array of clinical, anthropometric, demographic and environmental covariates were collected. The ordered subset analysis method [39] is one approach for incorporating such covariate information into nonparametric linkage analysis. The goal of the method is to test whether the evidence for linkage is significantly influenced by a trait-related covariate, which may define a genetically more homogeneous subset of families. In addition to family history and increasing age, there are several well-established risk factors for AMD. Smoking is considered a major modifiable risk factor and appears to increase the risk of both the atrophic and neovascular disease type [40]. Systemic hypertension is another risk factor, particularly for the neovascular form of AMD [41], and is potentially associated with increased intraocular pressure in some racial groups [42,43]. Increased systolic blood pressure (SBP) was shown to be a significant predictor of AMD incidence in two large prospective studies [44,45]. Other factors associated with an increased risk of hypertension can be considered indirect risk factors for AMD, but some of them have also been implicated as independent predictors of risk, such as obesity [46]. A recent study reported that overall obesity (measured as body mass index, BMI) and abdominal obesity (measured by waist-to-hip ratio and waist circumference) were the most significant variables associated with an increased risk of progressing from early to

Table 1: Clinical and demographic characteristics of study population. Data for 147 AMD patients (grade 3, 4 or 5) in 62 multiplex families included in genome screen are shown. 26 individuals with grade 1, 7 with grade 2 and 5 without available fundus photographs were also genotyped (n = 185 individuals total).

	AMD Grade			All
	3 (early AMD)	4 (geographic atrophy)	5 (neovascular AMD)	
N (%)	29 (19.7)	31 (21.1)	87 (59.2)	147 (100)
Age at exam: Mean (SD)	70.6 (9.2)	76.6 (8.1)	75.6 (7.7)	74.8 (8.3)
N (%) Female	22 (75.9)	19 (61.3)	54 (62.1)	95 (64.6)

advanced stages of AMD [2]. On the basis of these reported clinical associations, this study focused on incorporating the above covariates into a nonparametric linkage analysis of our multiplex families to reduce the phenotypic and genetic heterogeneity of AMD and potentially improve our ability to detect linkage.

Results

OSA analysis of multiplex families with early or advanced AMD

Descriptive characteristics of the study population are shown in Table 1. OSA-defined family subsets with lod score increases meeting uncorrected statistical significance ($p \leq 0.05$) are shown in Table 2. When a corrected significance level of 0.006 was used, only regions on chromosome 14q13 and 6q14 produced significant increases in lod scores. When ordering families by ascending average IOP, a maximum lod score of 3.2 was obtained between markers D14S608 and D14S599 (33 cM) in 28 families with average IOP ≤ 15.5. This was a significant increase from the baseline lod score for all families (0.31; p = 0.002). When ordering families by descending average BMI, a maximum lod score of 1.6 in eight families with average BMI ≥ 30.1 was obtained at marker D6S1031 (90 cM; p = 0.0004). While the third region of interest, chromosome 16p12, did not reach the corrected statistical significance level of 0.006, it met all of the other criteria for judging the clinical relevance of OSA results mentioned in the Methods section: Consistency across several clinically meaningful covariates, agreement with previously reported linkage signals, and continued significance when using a more stringent phenotype definition. A maximum lod score of 2.2 at marker D16S403 (44 cM) was obtained for a subset of six families with average SBP ≥ 153.5 (p = 0.04). Ordering families by descending average IOP produced a nominally significant lod score increase in a subset of 35 families with average IOP ≥ 15.4 very close to this map position (39 cM, p = 0.04). The peak marker (D16S403) is located only 7 cM distal to D16S769, which was reported as a significant marker (p = 0.009) in a genome-wide analysis of AMD as a quantitative trait in 102 nuclear families from Beaver Dam, Wisconsin [34], and was also significant (p = 0.005) in a second sample of 34 extended pedigrees ascertained from the same geographic region [37]. In both studies, the Wisconsin Age-Related Maculopathy Grading System, which is a 16-level severity scale for the extent of macular damage, was used as the trait in a Haseman-Elston regression applied to all genotyped sibling pairs [47].

OSA analysis of multiplex families with advanced AMD

To implement a more stringent binary phenotype definition for further evaluation of our OSA results, we excluded individuals with early AMD (grade 3) from the set of affected individuals, which reduced the number of families with at least one sampled affected relative pair to 45. When the OSA procedure was repeated on the chromosomes where lod score increases met nominal statistical significance in the initial analysis (Table 2), only the result for chromosome 16p12 maintained nominal significance. The maximum lod score for the IOP-defined subset of 32 families increased to 2.9 at 39 cM (p = 0.008). The maximum lod score for the SBP-defined subset was 2.3 near the same map position (44 cM, p = 0.05), and the number of families in this subset increased from 6 in the previous analysis to 25 (with average SBP ≥ 133). In addition, a nominally significant lod score increase at 44 cM was observed when families were rank-ordered by average BMI (9 families with average BMI ≥ 28.6 and lod score 2.0 at D16S403, p = 0.04). Eight of the nine families in the BMI subset and 20 of the 25 families in the SBP subset were part of the IOP subset. Multipoint lod score curves for the BMI, SBP and IOP subsets with significantly increased linkage evidence to chromosome 16p12, along with the baseline lod score for the data set, are displayed in Figure 1. Similar plots for chromosomes 14 and 6 are shown in Figures 2 and 3.

Clinical features of families identified by OSA

The clinical features of family subsets identified by OSA are shown in Table 3. The eight families in the BMI-defined subset linked to chromosome 6q14 were characterized by higher mean BMI (31.4) and pack-years (PKYRS) of cigarette smoking (24.5), compared to the

Table 2: OSA results with nominally significant lod score increases in covariate-based subgroup ($p \leq 0.05$). Significant results after correcting for testing multiple covariates on the same chromosome are shown in bold ($p \leq 0.05/8 = 0.006$). "Max LOD" denotes maximum lod score in covariate-based subgroup of families identified by OSA. Baseline lod score in entire data set is difference between "Max LOD" and "Change from Baseline" columns. See text for covariate abbreviations.

				62 families: 2+ sampled relatives with early or late AMD				45 families: 2+ sampled relatives with late AMD			
Chrom.	Kosambi cM	Nearest marker(s)	Variable and rank order	Max LOD	Change from baseline	P-value	No. of families in subset	Max LOD	Change from baseline	P-value	No. of families in subset
2	87	D2S441	BMI Ascending	2.2	2.1	0.03	20	-	-	>0.05	-
6	**90**	**D6S1031**	**BMI Descending**	**1.6**	**1.6**	**0.0004**	**8**	-	-	>0.05	-
9	129	D9S934	IOP Descending	1.7	1.2	0.05	42	-	-	>0.05	-
12	50	D12S1042	PKYRS Descending	1.5	1.5	0.04	12	-	-	>0.05	-
14	**33**	**D14S608, D14S599**	**IOP Ascending**	**3.2**	**2.9**	**0.002**	**28**	-	-	>0.05	-
15	106	D15S657	IOP Ascending	1.6	1.6	0.05	7	-	-	>0.05	-
16	39	D16S403	IOP Descending	2.3	1.7	0.04	35	2.9	2.1	0.008	32
	44	D16S403	SBP Descending	2.2	2.0	0.04	6	2.3	1.7	0.05	25
	44	D16S403	BMI Descending	-	-	>0.05	-	2.0	1.4	0.04	9
17	11	D17S1298	PKYRS Descending	1.5	1.3	0.03	14	-	-	>0.05	-
20	67	D20S481	PKYRS Ascending	1.9	1.5	0.03	26	-	-	>0.05	-
21	40	D21S2055	DBP Descending	1.6	1.6	0.04	11	-	-	>0.05	-

entire data set of 62 multiplex families (averages of 26.2 and 21.1, respectively). They also included a higher proportion of early AMD (33.3%, versus 19.7% in the entire data set). The nine families in the BMI-defined subset linked to 16p12 were also characterized by higher mean BMI (29.3) and PKYRS (25.5), compared with the entire data set of 45 multiplex families (averages of 25.7 and 22.5, respectively). Only three families were in the BMI-defined subset contributing to both linkage signals on 6q14 and 16p12. Due to the opposite rank ordering of the IOP covariate in OSA (lowest to highest for 14q13 and highest to lowest for 16p12), the 28 families with linkage to 14q13 and the 32 families with linkage to 16p12 had IOP averages below (13.9) and above (17.3) the population average of about 15.0, as expected. Seven families with IOP averages between 15 and 15.5 contributed to both linkage signals. Finally, the 25 families in the SBP-defined subset with linkage to 16p12 had higher mean SBP (145.0) than the entire data set of 45 multiplex families (137.8). As mentioned above, most of these families (20/25) were also part of the IOP-defined subset. In all three covariate-based subsets with linkage to 16p12, as well as the baseline data set of 45 multiplex families with advanced AMD, the proportion of neovascular AMD was much higher than the proportion of geographic atrophy (71–83%). While there is some overlap in families included in the various covariate-based subsets, the five variables used for OSA showed limited correlation in the AMD patient data set included in this analysis. Only the Pearson correlations of IOP and BMI ($r = 0.20$, $p = 0.03$), and, as expected, SBP and DBP ($r = 0.51$, $p < 0.0001$) were statistically significant. BMI was inversely correlated with age at exam ($r = -0.26$, $p = 0.004$), while IOP ($r = -0.12$, $p = 0.18$), SBP ($r = 0.19$, $p = 0.06$), DBP ($r = -0.03$, $p = 0.77$) and PKYRS ($r = -0.11$, $p = 0.25$) were not significantly correlated with age.

Discussion

Our analysis supports the possibility of distinct AMD susceptibility loci on three chromosomal regions: 6q14, with a lod score of 1.6 in a subset of eight overweight families; 14q13, with a lod score of 3.2 in 28 families with lower-than-average IOP values; and 16p12, with a lod score of 2.9 in 32 families with higher-than-average IOP values, most of which also had above-average values of SBP and BMI. Of these three regions, we believe that the 16p12 linkage is the most interesting finding since there is consistency across several clinically meaningful covariates, agreement with prior studies, and increased statistical significance with a more stringent phenotype definition. Based on our results, a gene on 16p12 may be associated with an increased risk of primarily neovascular AMD. The peak marker, D16S403, is located very close to marker D16S769, which was implicated in two prior genome screens of the Beaver Dam Study based on different phenotype definitions and statistical analysis methods ($p = 0.009$ and $p = 0.005$, respectively). However, the families included in these prior screens were not described with respect to the vascular risk factors we have evaluated here. It is possible that the significant result for the 16p12

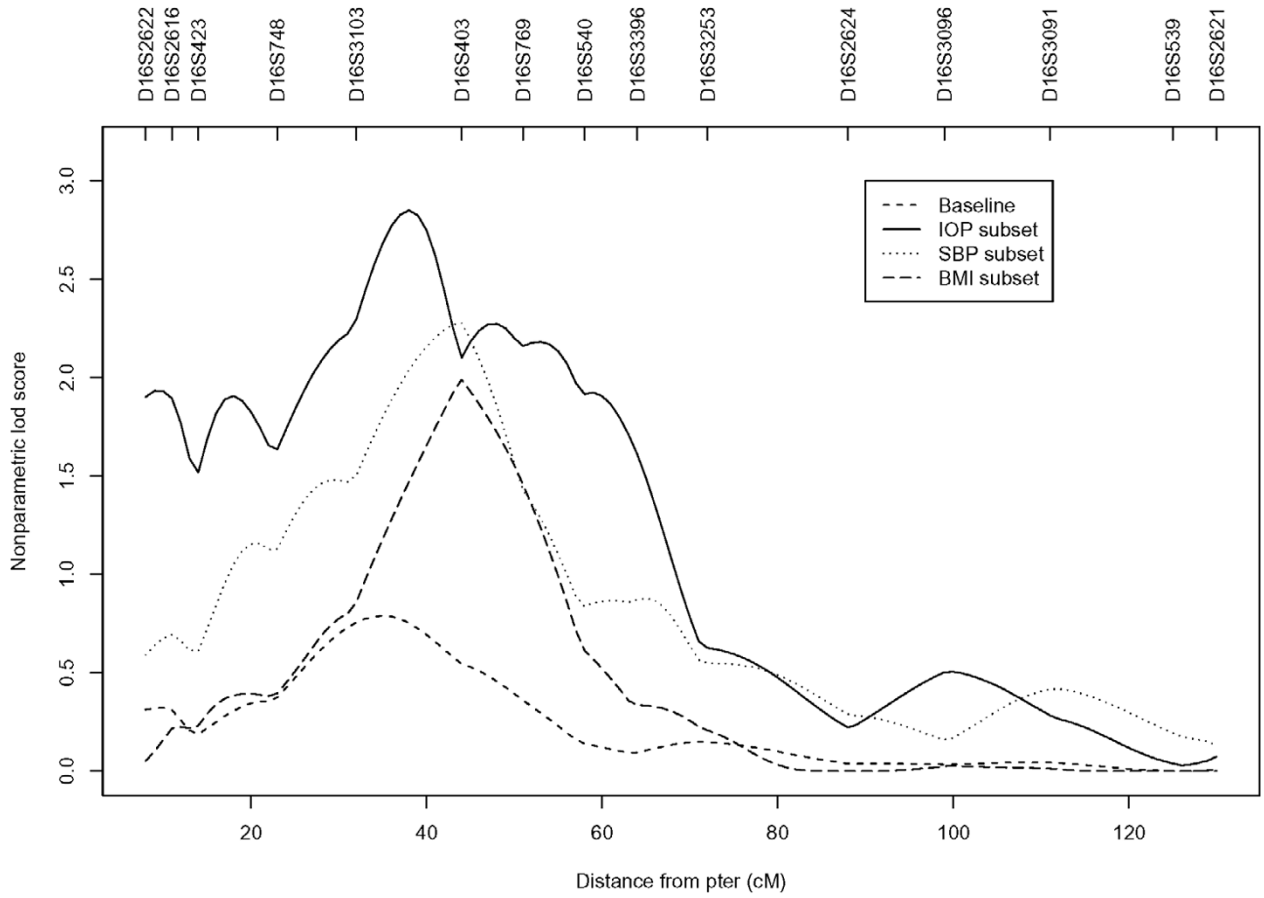

Figure 1
Multipoint lod scores for chromosome 16. The baseline score for 45 multiplex families (2+ sampled relatives affected with late AMD, grade 4 and 5) and scores for OSA subsets defined by IOP, SBP and BMI (ordered from highest to lowest family average, see text and Table 2) are shown.

marker in the Beaver Dam Study is primarily due to a contrast in identity-by-descent sharing of individuals with neovascular versus other forms of AMD, although a more detailed analysis would be necessary to confirm this hypothesis.

The BMI-defined subgroup with linkage to 16p12 fits the definition of being overweight. Increased BMI has previously been associated with a greater risk of AMD [48,49]. It was proposed that overweight and obese individuals may have lower macular pigment optical density (MPOD), which is a measure of retinal levels of the carotenoids lutein and zeaxanthin [50]. Carotenoids may be protective for AMD, implying that lower MPOD may confer a greater risk of AMD [51]. Our results suggest that individuals in the BMI-defined subset with linkage to 16p12 were also heavier smokers, as indicated by a greater average of pack-years of smoking. Epidemiologic studies have consistently reported an increased risk of both dry and wet AMD due to smoking [40], but little is known about the underlying mechanism. A recent study reported that nicotine increased the size and severity of experimental choroidal neovascularization in a mouse model of wet AMD and suggested that nicotinic receptor activation may mediate this harmful effect [52].

The SBP-defined subset of families meets the definition of systemic hypertension (SBP ≥ 140 mmHG or DBP ≥ 90 mmHG according to the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure, [53]). Elevated SBP is a more important predictor of cardiovascular risk than elevated DBP in per-

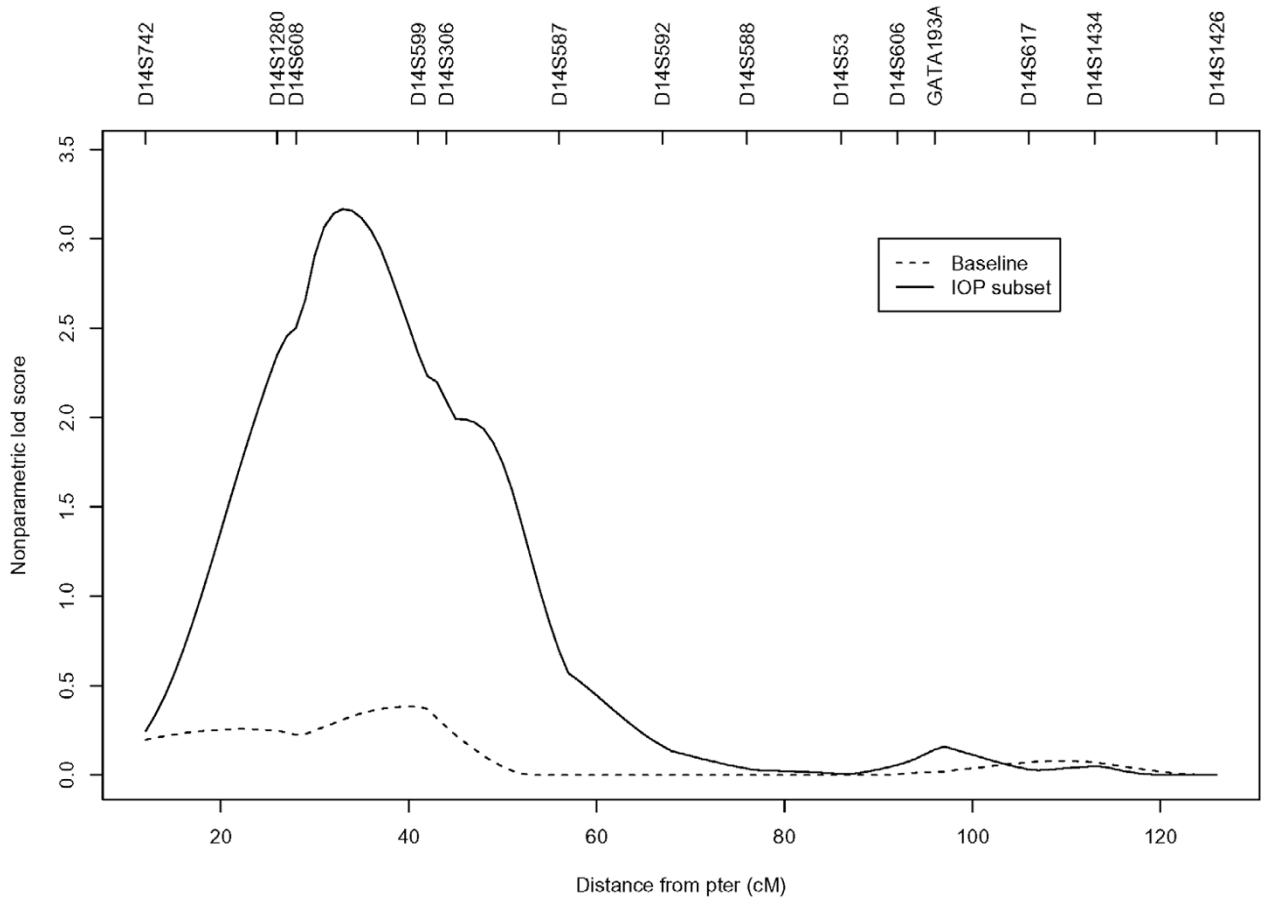

Figure 2
Multipoint lod scores for chromosome 14. The baseline score for 62 multiplex families (2+ sampled relatives affected with early or late AMD, grade 3, 4 and 5) and the score for the OSA subset defined by IOP (ordered from lowest to highest family average, see text) are shown.

sons older than 50 years. Consistent with our results, the Beaver Dam Study reported that higher SBP at baseline and longitudinal increase of SBP were significantly associated with the 10-year incidence of neovascular, but not atrophic AMD [45]. The Rotterdam Study reported an increased incidence of early AMD with higher baseline SBP [44]. The shorter follow-up time precluded the assessment of whether SBP specifically influenced the progression of early to neovascular, rather than atrophic, AMD. A large case-control study also reported a positive association of hypertension and neovascular, but not atrophic AMD [41]. The 16p12 region harbors genes for monogenic forms of hypertension (SCNN1B [MIM 600760], SCNN1G [MIM 600761]). However, until the region responsible for the linkage signal in our data set can be substantially narrowed down by association mapping, we believe it would be premature to consider these particular genes as promising locational candidate genes for neovascular AMD.

The IOP-defined subgroup with linkage to 16p12 is more difficult to interpret, since there is little evidence to suggest that elevated IOP is a risk factor for AMD. In African-American samples, cross-sectional associations between SBP and IOP as well as positive associations of elevated SBP and DBP at baseline with longitudinal IOP increases have been reported [42,43]. However, the general relationship of systemic hypertension and IOP is not well documented, and in the data set used here, IOP was only significantly correlated with BMI. It is unknown whether the mechanisms that contribute to hypertension or increased IOP have anything in common with the ang-

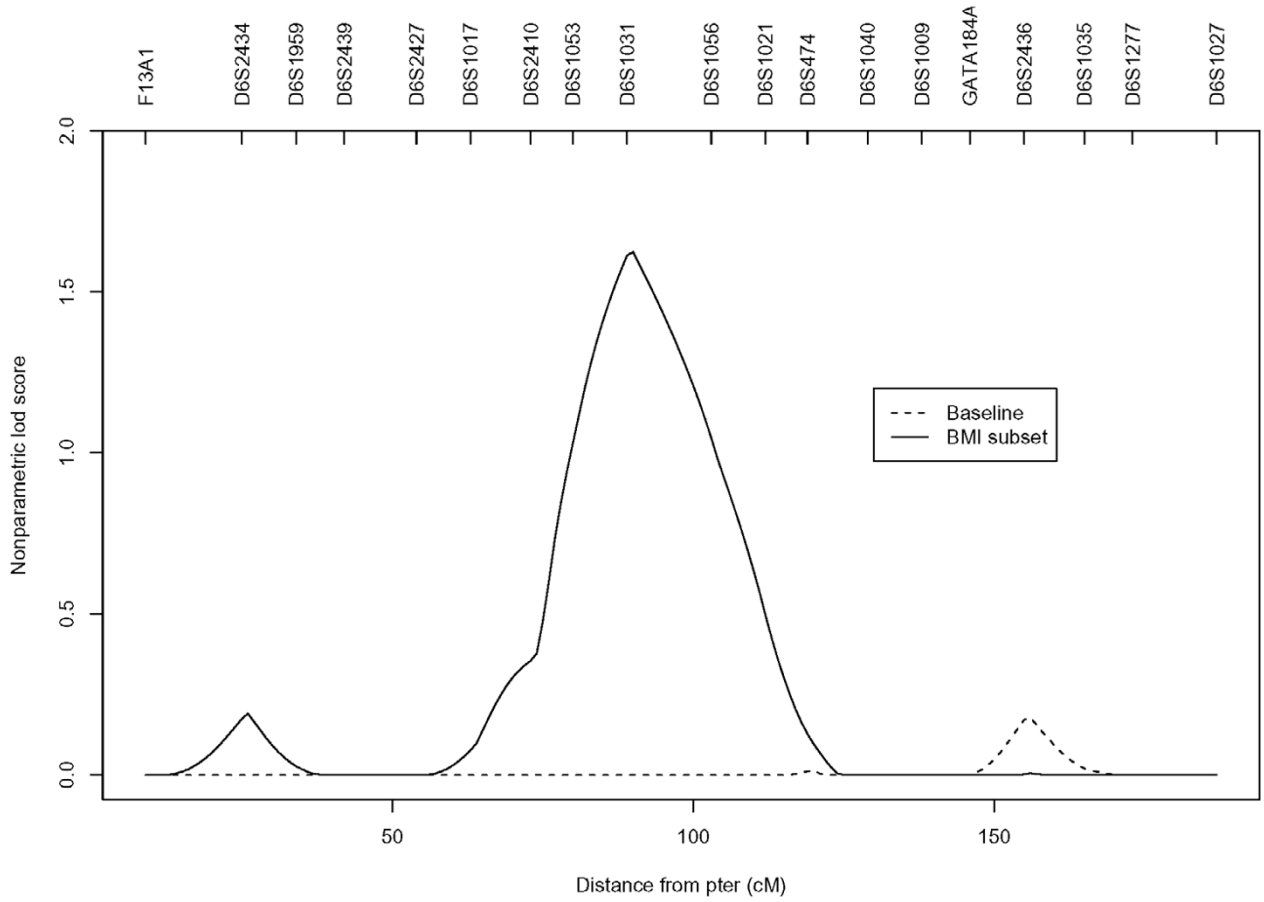

Figure 3
Multipoint lod scores for chromosome 6. The baseline score for 62 multiplex families (2+ sampled relatives affected with early or late AMD, grade 3, 4 and 5) and the score for the OSA subset defined by BMI (ordered from highest to lowest family average, see text) are shown.

iogenic processes observed in neovascular AMD. It is conceivable that both IOP and SBP values in the subgroup of families linked to 16p12 may be correlated with unmeasured markers of retinal vascular or choroidal blood flow characteristics. Correlations between elevated systemic blood pressure and retinal microvascular changes have been reported in multiple large-scale studies [54-56]. Retinal arteriolar narrowing in particular has been associated with increased risk of coronary heart disease [57] and with AMD progression in the Blue Mountains Eye Study [58]. Due to the limited size of our study, further interpretation of the SBP- and IOP-related results from this analysis awaits replication in a larger study population.

A linkage signal on 14q13 near the peak markers in our data set (D14S608, D14S599) was previously reported by one other study with a two-point parametric lod score of 1.5 [35]. While we observed the highest subset-based lod score in our data set (3.2) in this region, it is not clear whether the particular subset of 28 families with lower-than-average IOP values is defined by a clinically meaningful covariate. As mentioned above, little is known about the relationship of IOP and AMD risk. A statistically significant heritability of IOP was recently reported by the Beaver Dam Study [59], which gives some support to the use of IOP as a family covariate. In general, IOP is known to be influenced by many factors, including age, sex, refractive error, serum cholesterol, SBP, DBP, and BMI [60], although in our data set, IOP was only significantly correlated with BMI. The IOP-defined subset with linkage to 14q13 may represent individuals with a distinct profile of AMD risk factors that were not captured by the covari-

Table 3: Clinical features of family subsets identified by OSA. For chromosome 16p12, OSA-defined subsets were obtained from an analysis of 45 multiplex families (at least 2 sampled relatives with late AMD, grade 3 not considered affected). For the other two regions, OSA-defined subsets were obtained from an analysis of all 62 multiplex families (at least 2 sampled relatives with early or late AMD).

Region	OSA covariate	No. fams in subset	Avg. no. affecteds/ family	No. affected indiv.	Grade 3	Grade 4	Grade 5	Average covariate value in affected individuals					
								Age	BMI	SBP	DBP	IOP	PKYRS
6q14	BMI	8	2.3	18	6 (33.3)	1 (5.6)	11 (61.1)	70.3	31.4	133.9	74.5	16.8	24.5
14q13	IOP	28	2.3	65	17 (26.2)	12 (18.5)	36 (55.4)	75.8	25.9	135.2	75.1	13.9	24.9
16p12	SBP	25	2.4	61	-	18 (29.5)	43 (70.5)	75.0	25.6	145.0	78.2	16.2	21.3
	IOP	32	2.5	79	-	19 (24.1)	60 (75.6)	75.7	26.2	139.6	77.7	17.3	19.6
	BMI	9	2.7	24	-	4 (16.7)	20 (83.3)	74.5	29.3	137.2	74.4	17.2	25.5
					62 families: 2+ sampled relatives with early or late AMD								
			2.4	147	29 (19.7)	31 (21.1)	87 (59.2)	74.8	26.2	136.8	76.6	16.0	21.1
					45 families: 2+ sampled relatives with late AMD								
			2.4	106	-	27 (25.5)	79 (74.5)	76.3	25.7	137.8	76.4	16.1	22.5

ates considered here, but could be correlated with lower IOP. Alternatively, this result may be due to type I error, despite the correction for multiple testing we applied.

A linkage signal on 6q14 for the same peak marker as in our data set (D6S1031) was reported by two prior genome screen studies, with a p-value of 0.04 in Haseman-Elston regression [34] and an NPL_{pairs} p-value of 0.00001 [35]. However, since the number of families contributing to the linkage evidence in our data set is very small, it is difficult to speculate about the plausibility of our finding. The observation of a higher proportion of early AMD in the BMI-defined subset linked to 6q14, relative to the overall data set, is consistent with previous reports of an increased risk of early, but not late, AMD for both underweight and overweight individuals [61] and of a specific association between RPE abnormalities and BMI [62]. Alternatively, the lod score in this region may reflect identity-by-descent sharing of a gene that predisposes to obesity, rather than to AMD itself. One of the most consistently reported regions linked to BMI as a quantitative trait is chromosome 6q23-25 [63], however, this region is located at least 50 cM away from our region. It has been suggested that the incorporation of continuous covariates into binary trait linkage analysis may identify linkage signals that are distinct from those detected by analyzing such covariates as quantitative traits [64,65], but more methodological research is needed to further explore this question.

Conclusions

Our results, particularly those for chromosome 16p12, illustrate the utility of OSA for incorporating continuous covariates into linkage analysis of complex traits. The method is a conceptually and computationally simple approach to evaluating linkage evidence in subsets of families that are more homogeneous with respect to clinical features and/or non-genetic risk factors for the disease under study [64,66].

Phenotypically more similar families may be genetically more homogeneous as well, in which case OSA can greatly improve the power of linkage analysis. In contrast to the admixture test for parametric lod score analysis implemented in the program HOMOG [67], which simply allows for a proportion of families to be unlinked to a particular region under study, OSA may provide insight into the reasons for the underlying heterogeneity. It can have better power than the admixture test to detect linkage in subsets of families when the overall genetic effect is low or when the families are small, while the admixture test tends to be more powerful when the families are larger and provide more variability in family-specific lod scores [39]. It may also provide better localization of the putative susceptibility gene. The reduction of genetic heterogeneity is especially important in the analysis of complex disorders, for which AMD is a prime example. Limitations of OSA include the inability to incorporate more than one covariate at a time. The power of the method largely

depends on the degree of correlation between the evidence for linkage and the levels of the OSA covariate, i.e., on the extent to which phenotypic heterogeneity between families, as measured by averaged covariate values of affected family members, reflects underlying genetic heterogeneity [39].

In summary, our data support the presence of an AMD susceptibility locus on chromosome 16p12 that may predispose primarily to neovascular AMD. While it would be premature to speculate about biological relationships between SBP, IOP, and genetic predisposition in AMD etiology, our findings suggest that further research into the previously suggested association of neovascular AMD and systemic hypertension is warranted. The question of whether wet and dry AMD may have a different pathogenesis has long been debated. Distinct risk factor associations for the two disease forms were recently reported by the Beaver Dam Study [45]. At the genetic level, our data provide some support of a distinct etiology. Larger family data sets with sufficiently large proportions of both dry and wet AMD, particularly those for which baseline linkage results already exist, should be characterized with respect to the clinical variables considered here to further investigate the possibly distinct genetic basis of the two advanced stages of AMD.

Methods

Family ascertainment

Multiplex AMD families were ascertained at DUMC and VUMC via a proband with early or advanced AMD, as previously described [26]. All individuals included in this analysis were white, and their demographic and clinical characteristics are shown in Table 1. The assignment of AMD affection status was based on the clinical evaluation of stereoscopic color fundus photographs of the macula (EAP, AA, MADLP), according to a system described previously [5,68]. This system is a slight modification of the Age-Related Eye Disease Study (AREDS) grading system [69], using example slides from the Wisconsin Grading System [70] and the International Classification System [71] as guides. Briefly, individuals were assigned an AMD grade ranging from 1 through 5 based on the macular characteristics found within a 3000 μm-radius centered on the fovea. Eyes with extensive (≥ 15) small drusen (< 63 μm), non-extensive intermediate (≥ 63 μm) drusen or pigment abnormalities were assigned grade 2; eyes with extensive intermediate or any large (≥ 125 μm) drusen, with or without drusenoid (non-fluid) RPE detachments, were assigned grade 3; eyes with geographic atrophy were assigned grade 4, and eyes with serous or hemorrhagic RPE detachments, or choroidal neovascular membrane, were assigned grade 5. Eyes without any drusen and pigment abnormalities, or only small non-extensive drusen, were assigned grade 1.

For the purposes of performing a genome-wide screen for AMD susceptibility loci, multiplex families were defined as those with at least two sampled first- or second-degree relatives with grade 3 (early AMD/ARM), grade 4 (atrophic AMD), or grade 5 (neovascular AMD) in at least one eye. The resulting data set included 62 families, 147 affected individuals, 38 additional family members, and a total of 119 affected sibling pairs. Sixteen families had three or more affected siblings. Only three families had affected relative (avuncular) pairs other than sibling pairs. The number of affected siblings ranged from 2 to 5, with an average of 2.4.

Laboratory and statistical analysis

A total of 389 microsatellite markers spaced at an average 10 cM density across the human genome were genotyped on 185 individuals by the Center for Inherited Disease Research (CIDR). Prior to removal of genotypes that were inconsistent with Mendelian inheritance, pedigree relationships were verified with the programs RELPAIR [72] and PREST [73], both of which use multipoint identity-by-descent sharing estimates to infer the most likely relationship between pairs of individuals in the data set. No misspecified relationships were detected. For analysis, inconsistent genotypes detected by the program PEDCHECK [74] were removed. Inter-marker distances and marker order were obtained from the genetic linkage maps developed by the Marshfield Medical Research Foundation [75].

Motivated by successful applications of the ordered subset analysis (OSA) method in studies of other complex disorders [64,66], the primary goal of the analyses presented here was to incorporate AMD-associated clinical covariates into the nonparametric linkage analysis of the genome screen data. Given the extensive phenotypic heterogeneity of AMD, this may help identify more homogeneous subsets of families for follow-up analysis and has the potential to replicate previously published linkage signals that could be obscured in an analysis of the entire data set. To this end, the OSA method [39] was applied as follows: First, families were rank-ordered by the average covariate value of affected family members. Family-specific multipoint lod scores, which can in principle be parametric or nonparametric, were added one at a time in the covariate-based rank order, at each position on the chromosome map. Since the vast majority (59 of 62, 95%) of our multiplex families were nuclear families with two or more affected siblings, but no other affected relative pairs, we used the nonparametric MLS method for affected sibling pair data [76] to compute family-specific lod scores. This method has been implemented in the program SIBLINK [77]. Families with n affected siblings were weighted by a factor of $n-1$, and an additive model was assumed. For each ordered subset of families, the maximum lod score

anywhere on the chromosome was determined, the next family was added, and the procedure was repeated until all families had been analyzed in this way. The maximum subset-based lod score for each covariate ordering, along with the map position at which it occurred, was obtained. To evaluate whether the covariate-based subset of families provided significantly increased evidence of linkage, the observed maximum OSA lod score was compared to an empirical distribution of lod scores. This distribution was generated by randomly permuting the order in which families with available covariate information were added and computing the maximum lod score for each permutation as described above. The empirical p-value thus computed indicates how likely it is to obtain a subset-based lod score of the same or greater size than the observed OSA maximum lod score. It corresponds to a test of the null hypothesis of no increase in linkage evidence by rank-ordering families with respect to their covariate values. For the results presented here, we used a minimum of 10,000 permutations to compute empirical p-values.

We applied the OSA procedure with the following covariates: Body mass index (BMI), defined as self-reported weight (in kilograms) divided by squared height (in meters); systolic (SBP) and diastolic (DBP) blood pressure, defined as the average of two sequential measurements taken with the Hawksley random zero sphygmomanometer at the time of the clinical exam; intraocular pressure (IOP), measured by Goldmann applanation tonometry; and pack-years of self-reported cigarette smoking (PKYRS). To compute this combined measure of duration and dosage of cigarette smoking prior to study enrollment, we asked study participants (i) whether they had ever smoked cigarettes at least once per week, (ii) at which age they started and, if applicable, stopped smoking, and (iii) how many cigarettes, on average, they smoked per day. From this information, pack-years of cigarettes were computed as the product of smoking duration (in years), relative to a reference age 10 years prior to study enrollment, and dosage (number of cigarettes per day divided by 20). For never-smokers, zero pack-years were used. For BMI, IOP and PKYRS, we applied OSA with two independent ranking orders, lowest to highest and highest to lowest. For the two blood pressure variables, it was not clear how to best correct for the potential use of anti-hypertensive medication. We felt that only the presence of higher blood pressure at the time of the clinical exam indicated possible hypertension, while lower pressures may be observed for true normotensives as well as hypertensives on blood pressure-lowering medication. Therefore, we only applied the highest to lowest covariate ordering for SBP and DBP in OSA. Thus, eight maximum nonparametric lod scores, corresponding to two covariates with one ranking order and three covariates with two ranking orders, were obtained for each of the 22 autosomes, and a corrected chromosome-wide significance level of 0.006 (= 0.05/8) was used. Results with p-values ≤ 0.0003 (= 0.006/22) may be considered as having genome-wide significance. In addition to correcting for multiple testing, we applied the following considerations to help identify the most promising results of our OSA analysis: (i) Consistency of results across multiple clinically plausible covariates; (ii) agreement with prior published genome screens of AMD; (iii) increase or persistence in statistical significance when using a more stringent phenotype definition. Clinical features for family subsets with a statistically significant increase in nonparametric lod score were analyzed with the Statistical Analysis System (SAS Institute, Cary, NC, version 8).

Authors' contributions

SS participated in the design of the study, performed all statistical analyses and drafted the manuscript. WKS designed the study questionnaire used to collect environmental risk factor information (such as smoking history) and assisted in the interpretation of results. EAP, AA and MADLP were responsible for the clinical evaluation of all study participants. ERH developed the OSA method and assisted in the interpretation of results. JRG participated in the design of the study. DEW and MBG coordinated the genomic screening of study samples performed at CIDR. JLH and MAP-V conceived of the study and oversaw its overall design and coordination. All authors read and approved the final manuscript.

Acknowledgements

We thank the patients with AMD and their family members for participating in this research, and the personnel at the Duke Center for Human Genetics for assistance in patient and family ascertainment, data management, and preparation of this manuscript. Grant support from the National Eye Institute (EY12118, to MAP-V; EY09859, to MBG), the National Institute on Aging (AG11268, to Harvey Cohen, MD), The Eye and Ear Foundation, Pittsburgh, PA (to MBG), and Research to Prevent Blindness (to MBG) is gratefully acknowledged. The OSA software is available at http://wwwchg.duhs.duke.edu/software/osa.html.

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