

Common variant near the endothelin receptor type A (*EDNRA*) gene is associated with intracranial aneurysm risk

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The pathogenesis of intracranial aneurysm (IA) formation and rupture is complex, with significant contribution from genetic factors. We previously reported genome-wide association studies based on European discovery and Japanese replication cohorts of 5,891 cases and 14,181 controls that identified five disease-related loci. These studies were based on testing replication of genomic regions that contained SNPs with posterior probability of association (PPA) greater than 0.5 in the discovery cohort. To identify additional IA risk loci, we pursued 14 loci with PPAs in the discovery cohort between 0.1 and 0.5. Twenty-five SNPs from these loci were genotyped using two independent Japanese cohorts, and the results from discovery and replication cohorts were combined by meta-analysis. The results demonstrated significant association of IA with rs6841581 on chromosome 4q31.23, immediately 5' of the *endothelin receptor type A* with $P = 2.2 \times 10^{-8}$ [odds ratio (OR) = 1.22, PPA = 0.986]. We also observed substantially increased evidence of association for two other regions on chromosomes 12q22 (OR = 1.16, $P = 1.1 \times 10^{-7}$, PPA = 0.934) and 20p12.1 (OR = 1.20, $P = 6.9 \times 10^{-7}$, PPA = 0.728). Although endothelin signaling has been hypothesized to play a role in various cardiovascular disorders for over two decades, our results are unique in providing genetic evidence for a significant association with IA and suggest that manipulation of the endothelin pathway may have important implications for the prevention and treatment of IA.

subarachnoid hemorrhage | stroke | genetic risk loci

Intracranial aneurysms (IAs) are balloon-like dilations of cerebral arteries and affect 2–5% of the population (1). Although most of these lesions are clinically silent, their rupture and consequent subarachnoid hemorrhage usually occurs between ages 40 and 60 without prior warning, resulting in substantial morbidity and mortality (2, 3).

Aside from the well-established risk factors, such as hypertension, smoking, female sex (4), and high shear stress imposed on the cerebrovasculature (5), there is evidence for significant genetic contribution to IA pathogenesis (6). As is the case for other multifactorial diseases, both common and rare variants are thought to contribute to IA. In an attempt to identify common variants that confer risk of IA, we previously completed two genome-wide association studies of IA (6, 7). The larger, second

genome-wide association study (7) implemented a discovery and a replication phase using samples of European and Japanese descent, respectively. Using a discovery cohort of 2,780 cases and 12,515 controls, we analyzed 831,532 genotyped and imputed autosomal SNPs for association with IA. We used a Bayesian measure of the strength of association—the posterior probability of association (PPA)—to prioritize SNPs by calculating to what extent the data supports association with IA (7). This analysis revealed five loci with PPA > 0.5. Following replication genotyping using two independent Japanese cohorts and combining the discovery and replication cohort results, all five loci surpassed the genome-wide significance level of 5×10^{-8} (observed P values $\leq 2.5 \times 10^{-9}$) with PPA ≥ 0.998 , suggesting that each locus contains a variant that confers risk of developing IA. The five loci were on chromosomes 8q12.1 (*SOX17*), 9p21.3 (*CDKN2A/CDKN2B*), 10q24.32 (*CNNM2*), 13q13.1 (*KL/STARD13*), and 18q11.2 (*RBBP8*) (7).

Because these five loci explained only ~5% of the IA genetic risk and the number of SNPs showing P values < 0.001 was greater than that expected by chance alone (7), we hypothesized the presence of additional true IA risk loci among a range of SNPs showing weaker evidence of association in the discovery cohort. We tested this hypothesis using the two Japanese replication cohorts.

Results

Analysis of Previously Uninvestigated Intervals. The statistical analysis of the discovery cohort was described in detail previously (7). Following strict sample and SNP quality control (QC) measures, we matched cases and controls of the same sex based

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on inferred genetic ancestry to eliminate potential confounding because of population stratification and sex. We then tested for association of 831,529 QC-passed SNPs with IA and evaluated the strength of association using PPA (7). In addition to the five previously investigated loci with PPA > 0.5, there were 15 additional loci with PPA > 0.1 (observed values between 0.1 and 0.31) (Fig. 1 and Table S1). One of these intervals, on chromosome 7 (PPA = 0.31), was detected only by imputed SNPs without support from nearby genotyped SNPs in linkage disequilibrium (LD), suggesting an imputation error (8). Therefore, we pursued the two-stage follow-up genotyping for the remaining 14 loci (Table 1).

For the first stage, we genotyped 25 SNPs (SI Methods) located within these 14 intervals using the larger of the two Japanese cohorts (JP2), comprising 2,282 cases and 905 controls (Table S2). All of these SNPs passed QC filters. Association tests revealed that three of these loci, on chromosomes 4q31.23, 12q22, and 20p12.1, supported association with IA [i.e., Bayes Factor (BF) > 1] (Table 1). Although the data also supported association with IA at SNPs on chromosomes 1p36.31 and 2q33.1, the risk alleles were different from those found in the discovery cohort (Table 1). Further study of these latter loci will be needed to determine whether this might be because of allelic heterogeneity between European and East Asian populations.

In the second stage, using the JP1 cohort, we genotyped 13 SNPs in a total of nine loci that showed BF > 0.5 in the JP2 cohort with the same risk allele as the discovery cohort (Table 1 and Table S2). Two of the genotyped SNPs (rs2282652 and rs1132274) failed to pass the QC filters and were excluded from further analysis, leading to coverage of eight of the nine loci. JP1 data supported association with IA at SNPs located in two intervals, 4q31.23 and 12q22 (Table 1 and Table S2).

Meta-analysis of JP1 and JP2 cohorts revealed that the combined replication data strengthened the association with IA at SNPs located within two of the eight loci on chromosomes 4q31.23 and 12q22 (rs6841581 and rs6538595, respectively) by increasing the odds of association 85.1- and 24.0-fold, respectively ($P = 0.00042$ and 0.0017) (Table 2 and Table S2). One more SNP, rs1132274, for which the JP1 cohort data were not available, showed $P = 0.021$ and BF = 4.9 in the JP2 cohort.

Combined Results. We performed meta-analysis to combine the results from the discovery and replication cohorts (Fig. 2 and Table S2). The new genotyping results substantially increased the strength of the evidence of association for three loci on chromosomes 4q31.23, 12q22, and 20p12.1 compared with the discovery data (Figs. 2 and 3, Table 2, and Table S2).

The strongest association was detected at rs6841581, located at 148,620,640 base pairs on chromosome 4q31.23, with $P = 2.2 \times 10^{-8}$ [odds ratio (OR) = 1.22, PPA = 0.986] (Figs. 2 and 3). Only a single gene, *Endothelin Receptor Type A (EDNRA)*, is

located within the LD interval containing this SNP (Fig. 2). SNP rs6841581 lies only 1,129 bases from the 5' end of the *EDNRA* transcript (NM_001957.3), located within an interval predicted to have regulatory functions (University of California at Santa Cruz genome browser, <http://www.genome.ucsc.edu>). The encoded protein, EDNRA, plays an important role in the cerebrovascular physiology (see below). An examination of the publicly available eQTL databases (<http://eqtl.uchicago.edu>) did not reveal any SNPs that significantly alter *EDNRA* expression.

The second strongest association was at rs6538595 on chromosome 12q22 (OR = 1.16, $P = 1.1 \times 10^{-7}$, PPA = 0.934) (Figs. 2 and 3). A cluster of SNPs strongly correlated with rs6538595 is associated with IA and is mapped within the introns of the *FYVE*, *RhoGEF*, and *PH domain-containing 6 (FGD6)* gene (Fig. 2). Three other genes, *NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 12 (NDUFA12)*, *nuclear receptor subfamily 2, group C, member 1 (NR2C1)*, and *Vezatin, Adherens Junctions Transmembrane (VEZT)*, are located within the same LD interval as rs6538595.

Finally, although the JP1 cohort data were not available, a missense variant, rs1132274, within the *Ribosome Binding Protein 1 (RRBP1)* gene located on chromosome 20p12.1 showed moderate evidence of association with IA (OR = 1.20, $P = 6.9 \times 10^{-7}$, PPA = 0.728) (Figs. 2 and 3). Another gene, *Destrin (DSTN)*, is also contained in the same LD interval.

There was no evidence for a two-locus interaction that was consistent across all cohorts between various SNPs that were found to be associated with IA (Table S3).

Cumulative Effect. Analysis of the cumulative effect of the seven IA risk loci including the two SNPs (rs6841581 and rs6538595) replicated here, as well as the previously identified five SNPs (see Methods), explains 6.1%, 4.4%, and 4.1% of the familial risk in the Finnish, European, and Japanese cohorts, respectively (Table S4). The ORs of developing IA between the top and bottom 1% risk groups representing the tails of the distribution of genetic profiles ranged between 5.74 and 8.39 for the Japanese, European, and Finnish cohorts analyzed (Table S4).

Discussion

In this study, we demonstrated the association of SNPs located within three intervals on chromosomes 4q31.23, 12q22, and 20p12.1 with IA. Among these, rs6841581, located on chromosome 4q31.23 near the *EDNRA* gene, showed the most significant association, with a P value of 2.2×10^{-8} (PPA = 0.986). The data for another SNP, rs6538595, on chromosome 12q22 also supported association with IA; replication data increased the probability of association from 0.114 to 0.934 ($P = 1.1 \times 10^{-7}$). The evidence of association for the third SNP, rs1132274, on chromosome 20p12.1 was less. Although the replication data from the JP2 cohort increased the support for association with

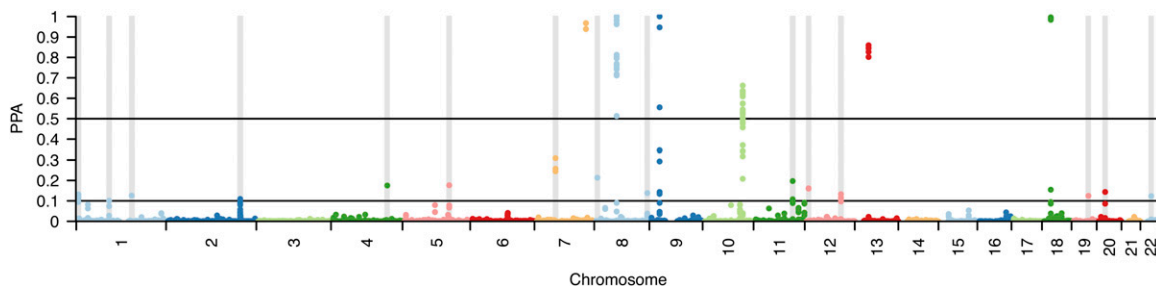


Fig. 1. Distribution of the PPA along the genome. The PPAs for SNPs analyzed in ref. 7 are plotted along the genomic coordinates [National Center for Biotechnology Information (NCBI) build 36]. We omitted 767,877 SNPs with PPA < 0.0001 from a total of 831,532 SNPs. Fifteen regions analyzed in this article (i.e., those regions containing a SNP with $0.1 < PPA < 0.5$ but no SNPs with PPA > 0.5) are shaded in gray.

Table 1. Cohort-wise association test results for 14 representative SNPs

Chromosome	SNP	Position	Discovery			JP2 (Replication 1)			JP1 (Replication 2)		
			RA	P	PPA	RA	P	log ₁₀ (BF)	RA	P	log ₁₀ (BF)
1p36.31	rs1876848	6,876,262	G	2.0E-05	0.1128	A	0.016	0.61	NA		
1p22.2	rs1725390	91,031,160	A	2.0E-05	0.1011	A	0.14	-0.11	A	0.59	-0.40
1q21.3	rs905938	153,258,013	T	1.7E-05	0.1252	T	0.62	-0.18	T	0.82	-0.15
2q33.1*	rs787994	197,931,366	T	2.1E-05	0.0988	C	8.6E-05	2.51	T	0.070	0.19
4q31.23	rs6841581	148,620,640	G	1.1E-05	0.1750	G	0.0066	0.93	G	0.023	0.53
5q23.2	rs2287696	122,488,231	A	1.1E-05	0.1760	A	0.27	-0.32	NA		
8p23.2	rs2045637	2,963,188	A	8.6E-06	0.2139	G	0.36	-0.38	NA		
8q24.23*	rs1554349	139,604,536	A	7.9E-05	0.0349	A	0.15	-0.12	G	0.33	-0.28
11q22.2	rs2124216	101,644,113	A	9.1E-06	0.1963	A	0.20	-0.24	G	0.65	-0.43
12p13.31	rs728342	5,577,633	G	1.2E-05	0.1601	G	0.33	-0.30	G	0.56	-0.33
12q22	rs6538595	94,030,754	A	1.8E-05	0.1136	A	0.0051	1.01	A	0.13	-0.012
19q13.12	rs1688005	40,340,205	G	1.6E-05	0.1244	T	0.16	-0.069	NA		
20p12.1	rs1132274	17,544,155	A	1.5E-05	0.1435	A	0.012	0.69	NA [†]		
22q12.1	rs133885	24,489,289	G	1.6E-05	0.1230	G	0.67	-0.42	NA		

Genomic positions were based on the human genome build 36. NA, the SNP was not genotyped. RA, risk allele aligned to the forward strand of the reference genome.

*For these loci, the lead SNPs were not genotyped (see *Methods*).

[†]Genotyping of rs1132274 in the JP1 cohort failed.

IA, we could not reliably genotype the JP1 cohort using the available platforms, thereby limiting the evidence for replication at this locus to a single Japanese cohort. Thus, we considered only rs6841581 and rs6538595 on chromosomes 4q31.23 and 12q22, respectively, as previously undetected risk loci for IA. Although the addition of these two loci remarkably increased the difference in the odds of disease between the highest and lowest risk groups to 5.7- and 8.4-fold in Japanese and Finnish cohorts, respectively, this only slightly improved the predictability of the disease risk (Table S4).

The most significant locus, 4q31.23, contains a single gene, *EDNRA*, which has been of great interest in various cardiovascular pathologies. Indeed, the endothelin system has been implicated in the pathogenesis of cardiovascular disorders, including pulmonary and primary hypertension (9). *EDNRA*, along with *EDNRB*, are G protein-coupled receptors for endothelins, with the 21-aa endothelin-1 (EDN1) being the predominant isoform (10, 11). EDN1 is produced primarily by the vascular endothelium and smooth-muscle cells and is involved in maintaining vasomotor control and vascular homeostasis (10). *EDNRA* is found on vascular smooth-muscle cells, including the cerebrovasculature, along with the heart, kidney, and neuronal cells (12), and mediates the vasoconstriction and mitogenic effects of EDN1 (13, 14). On the other hand, *EDNRB* reside on both smooth-muscle and endothelial cells, with downstream signaling through nitric oxide (15). Thus, EDN1 and its receptors, *EDNRA* and *EDNRB*, play

key roles in the maintenance of the vasculature by controlling the balance between vasoconstriction and vasodilation in response to hemodynamic stress.

EDNRA may play a role in IA pathogenesis in two distinct ways, depending on whether the *EDNRA*-mediated signaling is up- or down-regulated by the causal variant captured by the established SNP rs6841581. Increased *EDNRA*-mediated signaling might predispose to progression and rupture of IA through a mechanism analogous to the one that has been implicated for the development of atherosclerosis (16). Endothelin signaling has been shown to be activated at the site of vascular injury (17). Immediately after the injury, this might be beneficial with recruitment of endothelial cells to repair the damage (18). Following this initial response, however, prolonged, excessive endothelin signaling might be harmful by leading to atherosclerosis. *EDNRA* mediates this vascular mitogenic effect of EDN1 by promoting cell cycle progression and proliferation, both of which might play a role in IA progression and rupture (13, 14, 19). Consistent with this role of endothelins, increased EDN1 and *EDNRA* levels have been reported following rupture of IAs (20–22). In addition, hypertension and smoking, both well-established risk factors of IA pathogenesis, have been shown to alter the expression of endothelins (23).

Alternatively, if *EDNRA*-mediated signaling were attenuated, the risk allele might predispose to the formation of IA because of the failure of the repair mechanism mentioned above (17, 18).

Table 2. Summary of results for SNPs located in three unique genomic intervals on chromosomes 4q31.23, 12q22, and 20p12.1

Chromosome	SNP	Position	Gene	RA	Cohort	P value	log ₁₀ (BF)	PPA	OR (95% CI)
4q31.23	rs6841581	148,620,640	<i>EDNRA</i>	G	Discovery	1.1E-05	3.33	0.1750	1.25 (1.13–1.39)
					Replication	0.00042	1.93		1.20 (1.08–1.32)
					Combined	2.2E-08	5.84	0.9857	1.22 (1.14–1.31)
12q22	rs6538595	94,030,754	<i>NDUFA12/INR2C1/FGD6/VEZT</i>	A	Discovery	1.8E-05	3.11	0.1136	1.16 (1.08–1.24)
					Replication	0.0017	1.38		1.16 (1.06–1.28)
					Combined	1.1E-07	5.15	0.9343	1.16 (1.10–1.23)
20p12.1	rs1132274	17,544,155	<i>RRBP1</i>	A	Discovery	1.5E-05	3.22	0.1435	1.22 (1.11–1.33)
					Replication (JP2)	0.012	0.69		1.16 (1.03–1.30)
					Combined	6.9E-07	4.43	0.7279	1.20 (1.11–1.28)

Genomic positions were based on the human genome build 36. OR, the per-allele odds ratio of the risk allele; RA, risk allele aligned to the forward strand of the reference genome. Replication and combined results are based on the fixed-effects model.

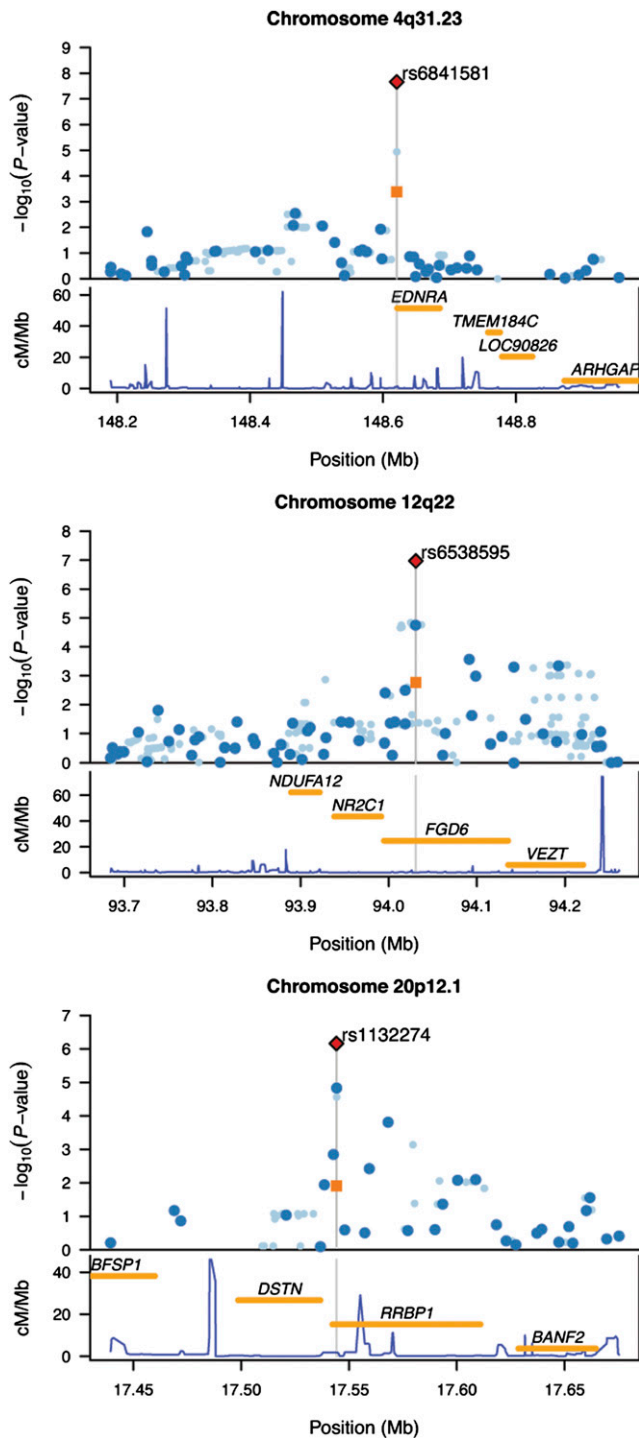


Fig. 2. Regional plots for associated regions. For each chromosomal interval, $-\log_{10} P$ values for association test are plotted against the genomic coordinates (NCBI build 36) (Upper); the recombination rates obtained from the HapMap database and the RefSeq genes (hg18) within the regions (Lower). The rs identifier of the SNP listed in Table 2 is shown for each chromosomal interval, and its position is indicated by the gray vertical line (Upper). Dark and light blue dots represent results of the genotyped and imputed SNPs for the discovery cohort, respectively. Orange squares represent the association result from the replication cohort using the JP1 plus JP2 (rs6541581 and rs6538595) or JP2-only (rs1132274); combined P values of the discovery and replication cohorts based on the fixed-effects model are shown by red diamonds.

Decreased signaling might interfere with the repair process in response to vascular injury, limiting the recruitment of vascular progenitor cells to the site of the damage with resultant defective repair, which in turn might result in the formation of arterial aneurysms. In support of this hypothesis, the use of at least one EDNRA antagonist in primates has been shown to be associated with the formation of preaneurysmal coronary artery lesions, characterized by fragmentation of the internal elastic lamina and loss of the medial smooth muscle (24).

Finally, endothelins, specifically EDNRA-mediated signaling, have been implicated in the pathogenesis of cerebral vasospasm, the pathologic vasoconstriction of the cerebral blood vessels that can often result in delayed ischemic neurologic deficits following IA rupture (22, 25). Several animal studies suggested a beneficial effect of EDNRA inhibition in treating vasospasm, leading to human clinical trials (26). The use of clazosentan, a selective EDNRA antagonist with a predilection for the central nervous system, has demonstrated reduction in angiographically demonstrated vasospasm, even though there was no improvement in clinical outcome (27, 28). Given the potentially biphasic role of endothelin signaling in IA pathogenesis, therapeutic strategies involving EDNRA have to be considered cautiously. As mentioned above, the use of at least one EDNRA antagonist in primates has been shown to be associated with the formation of preaneurysmal coronary artery lesions (24).

The discovery of a significant association of IA with a risk allele at immediate proximity to *EDNRA* within a predicted regulatory region is unique in providing genetic evidence linking endothelins to IA pathogenesis. Further studies will be needed to understand the effects of this newly discovered risk variant on EDNRA-mediated signaling, and how these effects ultimately predispose to IA. Once accomplished, this understanding can lead to pharmacological interventions that may have a potential therapeutic value in the treatment of aneurysms before rupture.

Methods

Subjects. Consent was obtained from all study participants. The study cohorts were described in detail elsewhere (7). Briefly, these cohorts included a genetically and sex-matched Finnish (FI) cohort of 808 cases and 4,393 controls, and a combined European (CE) cohort of 1,972 cases and 8,122 controls. The latter cohort consisted of three subcohorts based on the centers that ascertained the case samples: the Netherlands (NL), Germany (DE), and a pan-European (AN: @neurIST) cohort. The replication cohorts included two independent Japanese case-control samples (JP1 and JP2). JP1 consisted of 829 cases and 761 controls; JP2 consisted of 2,282 cases and 905 controls.

Replication Strategy. We used a two-stage design to follow-up 14 candidate intervals. In the first stage, we analyzed all of these loci using the JP2 cohort. For the second stage, we chose SNPs that showed $BF > 0.5$ in the JP2 cohort with the same risk allele as the discovery cohort, and analyzed them using the JP1 cohort.

Genotyping and QC. For SNPs reported in Table 1 and Table S2, we performed genotyping of the JP1 cohort using either the MassARRAY (Sequenom) assay or the Taqman (Applied Biosystems) platform. JP2 cases were genotyped using the multiplex PCR-based Invader assay (Third Wave Technologies); JP2 controls were genotyped using the Illumina platform (29). We excluded SNPs if any of the following three conditions were met in either cases or controls: fraction of missing genotypes > 0.1 , P value of the exact test of Hardy-Weinberg equilibrium < 0.001 , or minor allele frequency < 0.01 .

Statistical Analysis. We tested for association between each SNP and IA by fitting a logistic regression model with an additive effect of allele dosage and a sex covariate. For multilocus analysis, we combined genotypes from JP1 and JP2 and adjusted for the cohort label and sex, and analyzed the discovery cohort using the conditional logistic regression (7). We performed meta-analysis to combine the cohort-wise results (SI Methods). We report the results based on fixed-effects model in Fig. 2 and Table 2.

In addition to calculating the test P values, we also quantitatively measured the strength of the association using the BF and PPA, where the latter provides a probabilistic measure of the strength of the evidence (30). We regarded the association between an SNP and IA as replicated if $BF > 10$ in

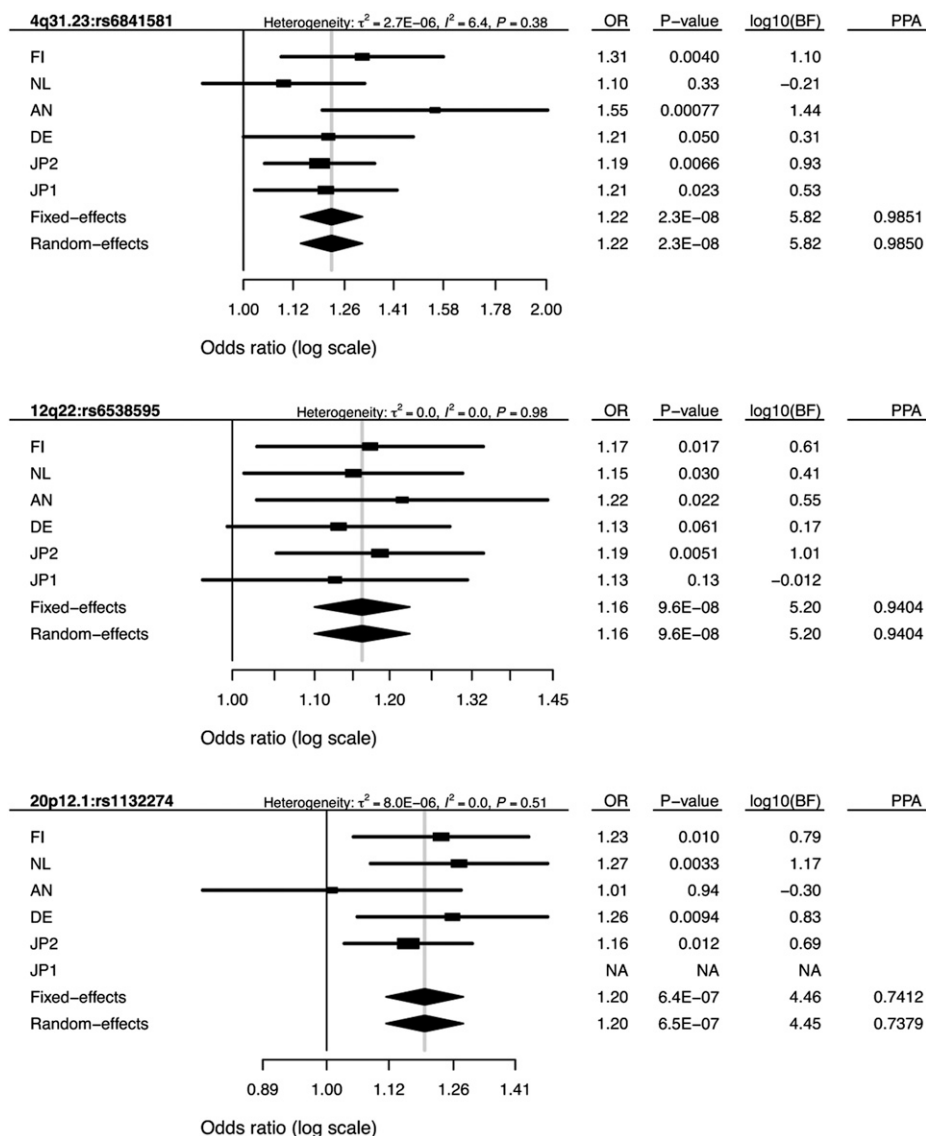


Fig. 3. Consistency of association across cohorts. Forest plots are shown for meta-analysis of SNPs listed in Table 2. Squares and horizontal segments represent estimated per-allele ORs and 95% confidence intervals (CIs) for individual cohorts. Diamonds represent the summary OR estimates and 95% CIs for the meta-analyses of six cohorts (fixed- and random-effects models). A $\log_{10}(\text{BF}) > 0$ supports association with IA, and a $\log_{10}(\text{BF}) < 0$ supports no association with IA.

the combined cohort of JP1 and JP2 (i.e., 10-fold increase in the odds of association after observing replication data) (7).

We tested for deviation from a linear model in which two SNPs combine to increase the log-odds of disease in an additive fashion. We also evaluated potential clinical implications of the genetic profiles of the IA risk loci following the approach described by Clayton (31). See *SI Methods* for a detailed description of the statistical analysis.

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