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No Genetic Overlap Between Circulating Iron Levels and Alzheimer's Disease

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Abstract. Iron deposition in the brain is a prominent feature of Alzheimer's disease (AD). Recently, peripheral iron measures have also been shown to be associated with AD status. However, it is not known whether these associations are causal: do elevated or depleted iron levels throughout life have an effect on AD risk? We evaluate the effects of peripheral iron on AD risk using a genetic profile score approach by testing whether variants affecting iron, transferrin, or ferritin levels selected from GWAS meta-analysis of approximately 24,000 individuals are also associated with AD risk in an independent case-control

[1]Data used in the preparation of this article were obtained from the Genetic and Environmental Risk for Alzheimer's disease (GERAD1) Consortium. As such, the investigators within the GERAD1 consortia contributed to the design and implementation of GERAD1 and/or provided data but did not participate in analysis or writing of this report (except those who are named authors). Membership of the GERAD1 Consortium is provided in the Acknowledgments section.

[2]Data used in preparing this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (http://adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators may be found at: http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf

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cohort (n∼10,000). Conversely, we test whether AD risk variants from a GWAS meta-analysis of approximately 54,000 account for any variance in iron measures (n∼9,000). We do not identify a genetic relationship, suggesting that peripheral iron is not causal in the initiation of AD pathology.

Keywords: Alzheimer's disease, apolipoproteins E, dementia, ferritin, genetic profile scores, genome-wide association study, iron, population genetics, transferrin

INTRODUCTION

Iron is the most abundant metal in the brain, where it is vital for neurotransmitter synthesis, myelination of neurons, and energy generation by mitochondria [1]. However excess iron contributes to the generation of reactive oxygen species, and consequent tissue damage [2]. Dysfunctional brain iron homeostasis is believed to play an important role in Alzheimer's disease (AD) [3]. Iron accumulation is seen in the AD postmortem brain [4] and iron content correlates with disease duration and Mini-Mental State Examination (MMSE) score [5, 6]. Individuals with mild cognitive impairment (MCI) with high risk of AD, showed higher cortical iron *in vivo* using MRI (measured using quantitative susceptibility mapping techniques), which spatially co-localized with Aβ plaques and correlated with higher plaque load [7]. In addition, transferrin (an iron transport protein) and ferritin (an intracellular iron storage protein) are both elevated in AD brain tissue in neurodegenerative regions [8]. Ferritin levels in cerebrospinal fluid (CSF) negatively correlated with cognitive performance and predicted conversion from MCI to AD [9]. Ferritin levels were also associated with CSF apolipoprotein E levels and were elevated by the AD risk allele, APOE ε4, suggesting that ferritin may reflect the mechanism by which APOE ε4 is a risk factor for AD.

Iron trafficking across the blood-brain barrier is tightly regulated and early studies suggested that the brain is protected from systemic fluctuations in iron, with a lack of correlation between liver and brain iron concentrations postmortem [10, 11]. Animal studies went on to challenge this view, showing that excess dietary iron increased brain iron levels in specific brain regions [12]. Quantitative MRI studies measuring the proton transverse relaxation rate (R_2) now allow iron concentrations to be assessed in the brain *in vivo*. One such study in cognitively normal elderly men found that iron levels in basal ganglia structures were correlated with serum iron measures [13]. In an investigation in the large Australian Imaging Biomarker and Lifestyle (AIBL) cohort of healthy controls, MCI and AD patients had disturbed brain iron metabolism reflected in the periphery by a decrease in plasma iron and hemoglobin [14], which was due to a deficiency of iron-loading onto transferrin [15]. Several mechanisms have been suggested to cause dysregulation of iron transport across the blood-brain barrier in AD including the involvement of amyloid-β protein precursor fragments and chronic inflammation [11]. A deficit in brain iron trafficking, which is essential for heme formation, neurotransmitter synthesis, and myelination of axons, could contribute to the pathophysiology of AD. But results are inconsistent, with two meta-analyses having differing conclusions on whether differences in circulating iron levels can be detected between AD cases and controls, and reporting heterogeneity between studies [16, 17].

It is clear that iron dysregulation has a role in AD, and that to a limited extent plasma iron might reflect changes in brain iron levels, but there has been little investigation of whether peripheral iron levels over the long-term affect risk of AD. Apart from the lack of suitable and adequately powered prospective studies, a limitation of observational studies is the inability to distinguish between causal associations and those due to confounding and reverse causation. A systematic review found that, in a limited number of trials, testing whether depletion or supplementation of iron changed a person's risk of AD provided no conclusive evidence, and that additional studies are necessary [18].

Drug development and randomized clinical trials are expensive and take many years to reach fruition, especially for a slowly progressive disease where treatment needs to start early in the disease course. An alternative approach, which overcomes the problem of reverse causation, is Mendelian Randomization (MR). Here the genetic variants affecting the putative causal variable are used as instrumental variables to test for an effect on disease risk. A demonstration that genetic polymorphisms known to modify the phenotype level also modify disease risk provides indirect evidence of a causal association between phenotype and disease. MR analysis has the following

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assumptions: firstly, the genetic variant used is only associated with the risk factor of interest; secondly, it is independent of all confounding variables; and, finally, there is no causal pathway leading from the genetic variant to the disease except through the risk factor of interest. For highly polygenic traits, a large number of genetic polymorphisms can be combined to explain a larger proportion of the variance of the trait. The large numbers of variants included means that some are likely to violate the assumptions for a MR analysis. But a lack of association between appropriate SNPs and the outcome, given a dataset large enough to give reasonable power suggests that there is no causal relationship. A shared genetic basis indicates either, pleiotropy where a variant affects multiple traits independently, or a causal relationship between the two correlated traits; with the requirement that any potential confounders must be taken into account. If a shared genetic basis is found, then a quantitative MR approach would then be required to compare direct and mediated paths between variants affecting the postulated causal variables and the outcome. This method has been widely used, both confirming and refuting suggested causal relationships based on epidemiological findings [19]. For example, this approach has had significant success in clarifying relationships between lipid levels and ischemic heart disease [20]. In addition, a recent study compared 42 traits or diseases with available large genome-wide association studies (GWAS) where, among other findings, the authors found evidence that an increased body mass index causally increases triglyceride levels [21].

MR was recently used to test for an effect of serum iron on Parkinson's disease (PD) risk, using three genetic variants influencing iron levels (*HFE* rs1800562, *HFE* rs1799945, and *TMPRSS6* rs855791) [22]. The combined MR estimate showed a statistically significant protective effect of increased serum iron in PD, suggesting that over the course of a life time, alteration in tissue iron homeostasis reflected by a decrease in serum iron levels is on the causal pathway in the pathogenesis of PD. Twelve iron associated SNPs identified though GWAS were also used to investigate the role of iron in atherosclerosis, and identified a potential causal role in women [23].

Single genetic variants that influence serum iron levels have not been shown to have a large effect on AD risk. The transferrin genetic variant C2 has been investigated and shown to have a small but significant association (OR = 1.11, 95% CI 1.05 to 1.17, in a meta-analysis of 19 studies [24]). Several studies previously reported an increased frequency of the *HFE* H63D (rs1799945) mutation in AD patients [25], but these findings have not been replicated in the largest AD GWAS meta-analysis [26]. There is evidence of interaction effects, which would not be apparent in GWAS meta-analyses, involving H63D and *APOE* ε4 alleles where the combination appears to affect age of onset and, to a lesser extent, risk [27–29].

Since several genes are well characterized for their impact on peripheral iron variation, we sought to determine their combined causal effect on AD risk. We test the effect of a large number of genetic variants affecting the iron-related measures of serum iron concentration, transferrin (the major iron transporter), ferritin (which reflects iron storage in bone marrow), and transferrin saturation (ratio between serum iron and total iron binding capacity) on AD risk, in combination using a genetic profile score (GPS) approach. Variants are selected from an iron GWAS meta-analysis discovery cohort [30] ($n = 23{,}986$) and tested in large independent target AD case-control datasets ($n = 9{,}251$). In addition, we test for the converse scenario, whether those at a high genetic risk for AD have higher peripheral iron levels throughout life, using SNPs identified by the AD GWAS meta-analysis discovery cohort [26] (from the International Genomics of Alzheimer's Project, IGAP $n = 54{,}162$) in an independent population-based target sample with available iron measures ($n = 8{,}893$). Previously an AD polygenic score analysis has shown that disease prediction accuracy is greatest including SNPs with p value <0.5. Including the full polygenic score significantly improved prediction over use of APOE alone where including both APOE and PRS gave AUC = 78.2% [31]. Examples of the AD PRS based on the IGAP discovery analysis demonstrating genetic overlap with other traits include neuroimaging measures of subcortical brain volumes, plasma C-reactive protein, and lipids [32, 33]. Finally, to confirm our findings using an alternative method, we used SNP effect concordance analysis (SECA) with only the discovery datasets, to examine whether SNPs found to be associated with the serum iron measures are enriched within associated SNPs with AD risk, and vice versa.

MATERIAL AND METHODS

Subjects

The AD case-control cohort comprises the datasets shown in Table 1. All individuals were of European

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Table 1
Alzheimer's disease case-control cohort data sets. The AD cohorts which contributed data to the assessment of the effect of iron genetic profile scores to risk of AD. The *APOE* ε4 frequency is shown for the individuals where *APOE* genotype data was available, with the sample size in brackets. AD, Alzheimer's disease; CN, controls

Cohorts	N AD cases	N Controls	Mean Age (range, SD)	% Female	*APOE* ε4 Frequency
Genetic and Environmental Risk for Alzheimer's disease (GERAD1) [43]	2,361	942	79.0 (60–108, 7.7)	64.6	AD = 0.33 (n = 2,183) CN = 0.13 (n = 906)
Innovative Medicines in Europe (AddNeuroMed) [44]	223	280	77.5 (60–98, 6.9)	59.8	AD = 0.33 (n = 217) CN = 0.15 (n = 143)
Kings Health Partners- Dementia Case Register (KPH-DCR) [45]	64	85	79.5 (61–93, 6.8)	59.7	AD = 0.38 (n = 52) CN = 0.14 (n = 65)
Alzheimer's Disease Neuroimaging Initiative (ADNI) [46]	165	205	76.3 (60–91, 6.0)	44.9	AD = 0.42 (n = 165) CN = 0.14 (n = 204)
Wellcome Trust Case Control Consortium 1958 British Birth Cohort (WTCCC2) [47]	0	4,926	54 (all 54)	49.7	CN = 0.16 (n = 4,862)

descent and all AD case-control cohort individuals were age ≥60 years. Controls were screened for dementia using either MMSE or ADAS-cog and were determined to be free from characteristic AD plaques at neuropathological examination or had a Braak score ≤2.5. Individuals with AD met criteria for either probable (NINCDS-ADRDA, DSM-IV) or definite (CERAD) AD. Individuals classed as MCI were excluded. The WTCCC2 1958 BC samples are population samples aged 54 years at collection and are included as unscreened controls in this analysis.

The population-based sample set comprises (a) adult twins, their spouses, and first degree relatives who volunteered for studies on risk factors or biomarkers for physical or psychiatric conditions (n = 8,380); (b) people with self-reported endometriosis and unaffected relatives (n = 830) [34, 35]. The mean age is 47 years (ranged 15–92 years) with 62% female. Biochemical markers of iron status were measured using standard clinical methods on Roche/Hitachi 917 or Modular P analyzers [30]. Serum iron was measured by colorimetry with Ferrozine reagent, serum transferrin by immunoturbidimetry, and ferritin by latex particle immunoturbidimetry. Transferrin saturation was calculated from the iron and transferrin results. The values for ferritin were log transformed to produce a normal distribution.

Genetic profile scores

GPS for serum iron, transferrin, transferrin saturation, and ferritin (log) were calculated in target AD case-control cohorts, using stage 1 summary data from the discovery sample of a GWAS meta-analysis combining 11 population-based studies of biochemical markers of iron status, with a sample size of 23,986 [30] using the method previously described ([36] and Supplementary Methods). In brief, linkage disequilibrium-based clumping was used to select SNPs in the discovery data, providing the most significantly associated SNP available in the target data set. The total score is calculated by the number of risk alleles weighted by the standardized per-allele effects for p value thresholds of 1×10^{-6}, 1×10^{-4}, 1×10^{-3}, 0.01, 0.05, 0.1, 0.5, and 1 (all SNPs) (Supplementary Table 1).

The AD GPS was generated in the target population-based cohort using summary data from the AD GWAS meta-analysis from the IGAP discovery sample consisting of 17,008 AD cases and 37,154 controls [26]. GPS were calculated as described above, with the number of risk alleles weighted by the effect on AD risk (log odds ratio). All *APOE* associated signal was removed and APOE genotype assessed separately.

APOE genotype

In the AD cohorts, a subset of samples have available *APOE* genotypes (Table 1) inferred from rs429358 and rs7412 SNPs genotyped using TaqMan SNP genotyping assays. In the Australian dataset, *APOE* genotype was estimated from imputed rs429358 and rs7412 SNP genotypes (Supplementary Methods).

GPS association analysis

In the AD cohort data sets, we tested for an association between iron, transferrin, transferrin saturation, and ferritin GPS at each p value threshold

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with AD case-control status using logistic regression (performed in STATA v11) controlling for age, sex, and four ancestry principal components. Results for each dataset were combined in a meta-analysis allowing a test for between study heterogeneity (STATA METAN specifying a random effects model). Finally, all datasets were combined in a mega analysis also controlling for study. In addition, we separately assessed the effect of the three iron level influencing variants that have previously been shown to associate with PD risk [22]. We tested for an association with the following SNPs: HFE rs1800562, HFE rs1799945, and TMPRSS6 rs855791 using logistic regression under an additive model and then combined the three variants in a GPS. To investigate any potential interaction effect of *APOE* ε4 genotype, we also repeated these analyses controlling for *APOE* ε4 carrier status and also in *APOE* ε4 positive and *APOE* ε4 negative groups.

In the population-based dataset, we tested for an association of AD GPS and number of *APOE* ε4 alleles with peripheral iron measures (iron, transferrin, transferrin saturation, and ferritin) using Genome-wide Efficient Mixed Model Association algorithm (GEMMA) software [37]. The sample contains related individuals including monozygotic and dizygotic twin pairs, and other first degree relatives. We used linear mixed model regression using the likelihood ratio test, including sex, age, and four ancestry principal components as covariates and controlling for family structure using a genetic relatedness matrix estimated from genome-wide genotypes.

SNP effect concordance analysis

We carried out SECA analysis of large scale GWAS meta-analysis summary statistics to examine the genetic overlap between AD and each iron measure using the default approach [38]. SECA allows a larger sample size to be examined without the need for individual level genotype data. The GWAS meta-analysis results for AD (meta-analysis $n = 74{,}046$) [26] and iron measures (iron, transferrin, transferrin saturation, and ferritin; meta-analysis $n = 23{,}986$) [30] were used to test for an excess of SNPs associated in the AD and iron phenotype data sets, and whether the SNP effect directions are concordant. SNP effects across the two GWAS summary results were aligned (AD and iron) to the same effect allele and independent SNPs were extracted via LD clumping identifying a subset of independent SNPs with the most significant p-values in the AD dataset. Restricting to SNPs associated with $p_1 \leq 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9$, and 1.0 in the AD dataset, exact binomial statistical tests determine whether there is an excess of SNPs associated in both datasets for the subset of SNPs associated with $p_2 \leq 0.01$, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 in the iron dataset. Fisher's exact test is then used to determine whether there is an excess of SNPs where the effect directions are concordant across the datasets for each p value subset.

Due to the larger sample size the AD GWAS summary statistics were initially used as dataset 1, and each of the iron measures as dataset 2, providing the greatest possible power. Because the analysis is restricted to those SNPs which are most highly associated in dataset 1, we also repeated the analysis with the iron GWAS summary statistics as dataset 1 (in case of a scenario where SNPs strongly affecting iron phenotypes had an effect on AD risk, but SNPs strongly affecting AD risk did not affect iron phenotypes).

RESULTS

GPS analysis

The discovery GWAS meta-analysis datasets used in the study contain large sample sizes (in total 54,162 for AD and 23,986 for serum iron status) and show both AD and serum iron measures to have a strong polygenic components [27, 31]. For serum iron measures using replication cohorts, the lead SNPs at the 11 significant loci explained 3.4, 7.2, 6.7, and 0.9% of the phenotypic variance for iron, transferrin, saturation, and (log-transformed) ferritin, respectively [30]. There is large deviation from the expected distribution of association test statistics compared to observed values, with association signals observed far below the level of genome-wide significance (Fig. 1). Therefore, using SNPs below genome-wide significance will increase power to detect an association.

Association analysis conducted in each AD disease case-control data set identified no effect of any serum iron status GPS (serum iron, transferrin, ferritin, and transferrin saturation) on AD risk, and the meta-analysis identified no significant between study heterogeneity (Supplementary Figure 1). When combined in a mega analysis no effect of any serum iron status GPS (serum iron, transferrin, ferritin, and transferrin saturation) on AD risk was identified with a sample size of 6,381 controls and 2,870

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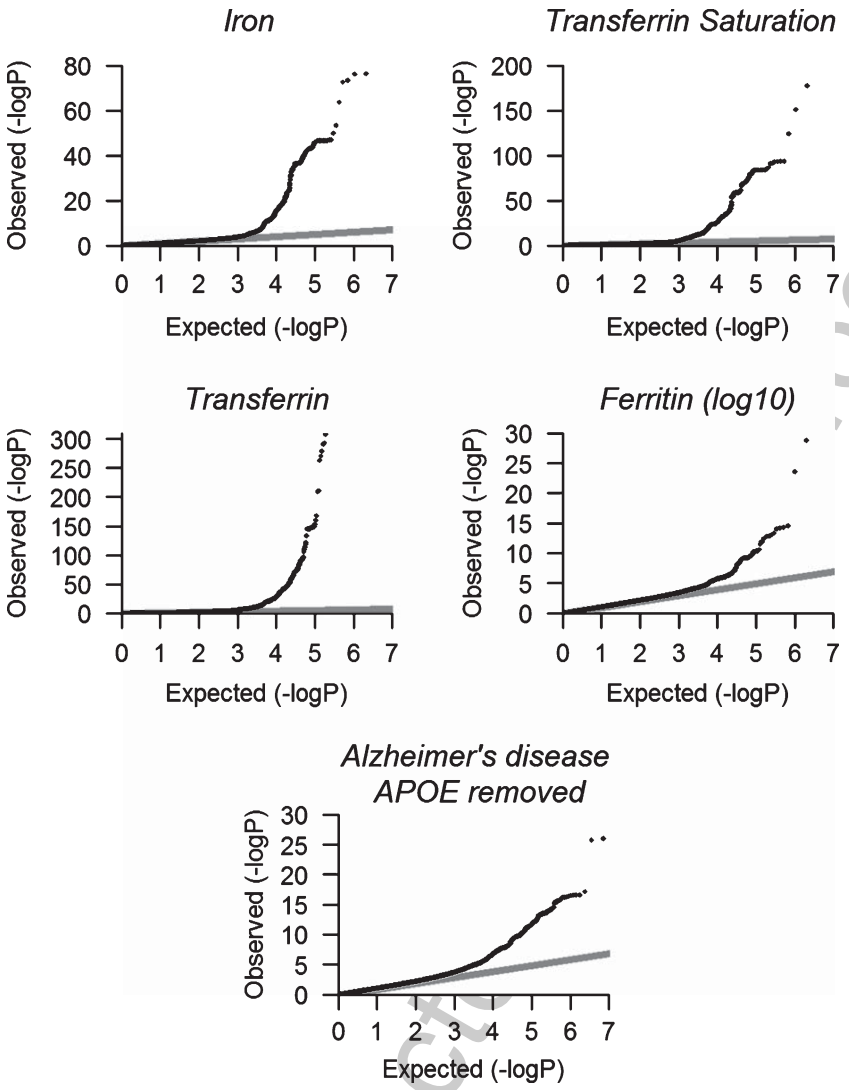

Fig. 1. Q-Q plots of the association *p*-values from the discovery GWAS meta-analyses. Including the GWAS meta-analysis of biochemical markers of iron status [30] and the International Genomics of Alzheimer's Project [26]. SNPs in the *APOE* region (within 500 kb either side of *APOE* locus) are excluded from the AD plot. The red line is the line of equivalence, observed = expected.

AD cases (Table 3). Controlling for *APOE* genotype did not significantly affect the results, and no significant association was identified in separate *APOE* ε4 carrier and non-carrier groups (data not shown). Previously three iron level influencing genetic variants (HFE rs1800562, HFE rs1799945, and TMPRSS6 rs855791) have been shown to be associated with PD risk [22]. There was no association of these SNPs with AD status in our dataset and no interaction identified with *APOE* ε4 status (Supplementary Table 2). In addition, the GPS constructed from these three SNPs did not have an effect on AD risk (Supplementary Table 2).

Table 2
Serum iron measures in the Australian data set

Serum measure	N	Mean	Range	SD
Iron (μmol/L)	8,751	19.54	0.10–50.50	6.74
Transferrin Saturation (%)	8,800	28.71	0.12–95.3	10.80
Transferrin (g/L)	8,891	2.82	1.40–5.19	0.44
Ferritin (log10) (μg/L)	8,892	2.00	0.00–3.26	0.50

There was no association of AD GPS or *APOE* ε4 with any peripheral iron measure (Table 4).

SNP effect concordance analysis

In agreement with the GPS analysis, we did not identify any significant pleiotropy between datasets

Table 3
The association of serum iron measure genetic profile score (GPS) at different p value thresholds with AD risk. The association analysis was carried out using logistic regression controlling for sex, age, four ancestry principal components, and study. β, standardized Beta

GPS		Association with AD risk (n = 9,251)		
		β	SE	p
Iron	$p \leq 1$	0.04	0.03	0.278
	$p \leq 0.5$	0.03	0.03	0.365
	$p \leq 0.1$	0.01	0.03	0.868
	$p \leq 0.05$	0.02	0.03	0.638
	$p \leq 0.01$	–0.01	0.03	0.695
	$p \leq 0.001$	–0.01	0.03	0.839
	$p \leq 0.0001$	0.02	0.03	0.624
	$p \leq 0.000001$	0.02	0.33	0.632
Transferrin Saturation	$p \leq 1$	0.03	0.03	0.291
	$p \leq 0.5$	0.03	0.03	0.330
	$p \leq 0.1$	0.03	0.03	0.381
	$p \leq 0.05$	0.02	0.03	0.584
	$p \leq 0.01$	0.02	0.03	0.510
	$p \leq 0.001$	0.02	0.03	0.590
	$p \leq 0.0001$	0.02	0.03	0.628
	$p \leq 0.000001$	0.03	0.03	0.408
Transferrin	$p \leq 1$	0.00	0.03	0.933
	$p \leq 0.5$	0.00	0.03	0.950
	$p \leq 0.1$	0.02	0.03	0.589
	$p \leq 0.05$	0.01	0.03	0.797
	$p \leq 0.01$	–0.02	0.03	0.517
	$p \leq 0.001$	–0.03	0.03	0.299
	$p \leq 0.0001$	–0.03	0.03	0.404
	$p \leq 0.000001$	–0.02	0.03	0.467
Ferritin	$p \leq 1$	0.02	0.03	0.577
	$p \leq 0.5$	0.03	0.04	0.465
	$p \leq 0.1$	0.03	0.04	0.465
	$p \leq 0.05$	0.05	0.04	0.196
	$p \leq 0.01$	0.03	0.03	0.347
	$p \leq 0.001$	0.03	0.03	0.355
	$p \leq 0.0001$	0.03	0.03	0.377
	$p \leq 0.000001$	0.04	0.03	0.170

or concordant effects using SECA. We tested for an excess of SNPs associated with AD also associating with iron phenotypes. Using a binomial test, we compared the AD dataset with each of the iron phenotype datasets in turn examining 144 SNP subsets (testing twelve p value threshold combinations). No SNP sets were found to have nominally significant pleiotropy (Fig. 2). Using Fisher's test, we also tested for an excess of SNPs where the effect directions (BETA) are concordant between SNP subsets in each dataset. Again, we identified no significant concordance (Supplementary Figure 2). Additionally, no significant pleiotropy or concordant effects were seen when switching the primary dataset, i.e., testing for an excess of SNPs associated with each iron phenotype also associating with AD.

Table 4
The association of AD GPS at different p value thresholds (excluding *APOE*) and number of *APOE* ε4 alleles with iron phenotypes. The association analysis was carried out using linear mixed models implemented in GEMMA (genome-wide efficient mixed-model association) [37] using the likelihood ratio test. Family relationships were controlled for using a genetic relatedness matrix estimated from genotypes. Sex, age, and four ancestry principal components were also included as covariates. β, standardized Beta

Serum Iron Measure	AD GPS	N	β	*SE*	p
Iron	$p \leq 1$	8,751	0.02	0.01	0.153
	$p \leq 0.5$	8,751	0.02	0.01	0.148
	p ≤ 0.1	8,751	0.01	0.01	0.349
	$p \leq 0.05$	8,751	0.01	0.01	0.594
	$p \leq 0.01$	8,751	0.00	0.01	0.747
	$p \leq 0.001$	8,751	0.01	0.01	0.405
	$p \leq 0.0001$	8,751	0.01	0.01	0.615
	$p \leq 0.000001$	8,751	0.02	0.01	0.119
	APOE ε4	8,494	0.00	0.01	0.843
Transferrin Saturation	$p \leq 1$	8,800	371.45	224.20	0.097
	$p \leq 0.5$	8,800	201.12	136.43	0.140
	$p \leq 0.1$	8,800	46.40	54.11	0.391
	$p \leq 0.05$	8,800	13.37	38.99	0.732
	$p \leq 0.01$	8,800	2.82	18.46	0.878
	$p \leq 0.001$	8,800	0.76	6.58	0.908
	$p \leq 0.0001$	8,800	0.25	2.15	0.908
	$p \leq 0.000001$	8,800	3.19	1.27	0.012
	APOE ε4	8,531	0.02	0.02	0.477
Transferrin	$p \leq 1$	8,891	–218.75	225.19	0.331
	$p \leq 0.5$	8,891	–78.29	137.03	0.568
	$p \leq 0.1$	8,891	9.86	54.36	0.856
	$p \leq 0.05$	8,891	23.12	39.16	0.555
	$p \leq 0.01$	8,891	5.87	18.52	0.751
	$p \leq 0.001$	8,891	16.29	6.58	0.013
	$p \leq 0.0001$	8,891	4.97	2.15	0.021
	$p \leq 0.000001$	8,891	–1.77	1.28	0.166
	APOE ε4	8,619	–0.02	0.02	0.466
Ferritin	$p \leq 1$	8,892	156.22	192.51	0.417
	$p \leq 0.5$	8,892	81.98	117.14	0.484
	$p \leq 0.1$	8,892	35.61	46.42	0.442
	$p \leq 0.05$	8,892	7.49	33.47	0.822
	$p \leq 0.01$	8,892	11.05	15.85	0.485
	$p \leq 0.001$	8,892	2.53	5.64	0.654
	$p \leq 0.0001$	8,892	–0.64	1.84	0.728
	$p \leq 0.000001$	8,892	0.85	1.09	0.435
	APOE ε4	8,621	0.01	0.02	0.486

DISCUSSION

It is becoming increasingly clear from investigations of iron homeostasis and recent advances in iron imaging methods that iron dysregulation is an important feature of AD, and therefore lowering of iron content in the brain is a potential therapeutic target [39]. But there is limited understanding of the importance of peripheral iron levels in AD risk, and whether prolonged increased or decreased iron levels may be a risk factor for AD. We investigated whether there is a shared genetic basis between AD and

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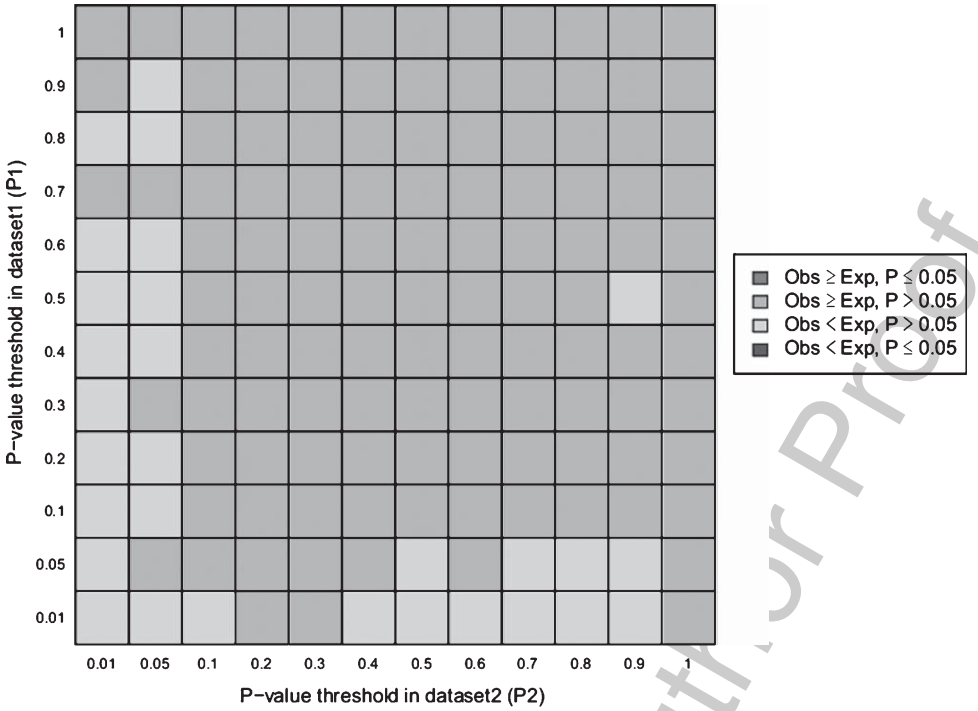

Fig. 2. Genetic overlap between dataset 1 (AD) and dataset 2 (Serum iron). In the SECA analysis, exact binomial statistical tests are performed to determine whether there is an excess of SNPs associated in both datasets for 144 SNP subsets from 12×12 p-value threshold combinations. A binomial test 'heatmap' plot is generated to graphically summarize the proportion of SNP subsets with an excess [observed(obs)≥expected (exp)] or deficit (obs<exp) number of associated SNPs, and empirical p-values (adjusted for testing all 144 subsets) are calculated via permutation.

peripheral iron levels using a PRS approach. We identified no effect of genetic variants affecting peripheral iron biomarkers (including iron, transferrin, transferrin saturation, and ferritin) on AD risk. Nor did we find increased serum iron levels in those who are at increased genetic risk of developing AD, including both *APOE* ε4 carriers and those with a higher load of other common risk variants. In addition, in an investigation of the genetic overlap between AD and each iron measure, we do not find any significant overlap of genetic loci from the results of large-scale GWAS meta-analysis studies.

Taken together, our results suggest that the causes of variation in brain iron that might contribute to AD are distinct from those causing variations in circulating iron (serum iron) or in iron stores in bone marrow or other organs (serum ferritin). Iron retention is complex in different organs, and our current data on peripheral iron measurement cannot exclude causation by other genes that affect iron levels in the brain that are not reflected by serum values. In addition, the peripheral iron measurements used are standard clinical pathology measures. Non-standard and possibly more direct measures (such as transferrin saturation using size exclusion chromatography-inductively coupled plasma-mass spectrometry) have been shown to be more sensitive to differences in the blood between AD patients and controls [15].

It is also possible that, even if iron is not a primary cause of increase in AD risk, it accumulates after the initiation of cell damage by other mechanisms, and exacerbates it. Evidence for this comes from recent work showing that once Aβ forms aggregates they induce iron accumulation [40]. Iron-related therapies could still be relevant for patients who are in the early stages of AD.

Iron accumulation in tissues is a feature of many diseases, and may prove to be causal for some. Our current results for AD are in contrast to previous evidence of a causal association of increased peripheral iron measures with a decreased risk of PD [22]. The authors hypothesized that low peripheral iron may decrease neuronal iron storage though a reduction in ferritin, resulting in free iron accumulation in the brain. To investigate whether a similar effect exists for AD, we tested a larger number

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of iron-affecting variants against the most recent GWAS meta-analysis on AD risk. These explain a larger proportion of the variance and therefore we would expect them to have more power to detect any effect.

However, our analysis has limitations that need to be considered. Firstly, the multi-SNP GPS includes a large number of genetic variants of unknown effect or multiple effects; therefore we cannot rule out that as well as affecting iron levels, some also affect AD risk though other pathways and could potentially do so in opposite directions. To attempt to address this issue, we also tested for an effect of three genetic variants (in *HFE* and *TMPRSS6*) known to have a direct role in peripheral iron levels and previously shown to have an effect on PD risk [22], where we also did not find an effect. In addition, we cannot rule out the possibility that other genomic variations, such as epigenetic dysregulation, affect iron levels which are then causal for AD.

Secondly, as in other complex diseases and phenotypes, discovered genetic variants only represent a small proportion of the variance in both iron levels and AD risk. This study utilizes summary data from the two largest GWAS meta-analysis discovery cohorts for both AD and biochemical markers of iron status (total sample sizes of 54,162 and 23,986, respectively [26, 30]) to compute comprehensive GPS. In addition, the GPS were applied to large samples with individual level genotype and phenotype data (For AD cases-control: 2,813 AD cases, and 6,438 controls (of which 4,926 are unscreened for AD, aged 54), and $\geq$8,751 for iron measures). Even so, we cannot rule out a small effect that is not detectable with this sample size.

Thirdly, effects on iron in relevant brain areas may differ from effects on circulating iron or iron in other organs. Previous studies identified an association between iron accumulation in the basal ganglia of elderly men and peripheral iron measures [13]. However, only 9% of the variance of CSF ferritin can be explained by plasma ferritin [9], highlighting the separation between these compartments. It is also possible that there are genetic loci more relevant to iron-homeostasis in elderly people, as the sample used to construct the iron phenotypes GPS have a mean age of 47.

Our results suggest that there is not a causal connection between lifetime peripheral iron measures and increased risk of AD. We did not replicate the previous finding of an effect of *HFE* SNPs on risk of AD and an epistatic interaction for risk with *APOE* ε4 genotype, but we cannot yet rule out an association of *HFE* SNPs with AD age of onset or phenotypic interactions [25, 27, 28].

It has been suggested that public recommendations for AD risk reduction should caution the use of iron supplementation for those whom it is not required [18, 41, 42]. Dietary patterns such as a Mediterranean diet and reduced red meat consumption that associate with lower AD risk do tend to have a low iron intake, but also have other unrelated health benefits for example high intake of vegetables and low saturated fat. Consistent with our genetic findings, there is no clear evidence that dietary intervention affecting iron intake alters the risk of AD [18]. More work is needed to assess the effect of iron on the progression (as opposed to the initiation) and age of onset of AD.

In conclusion, although iron deposition is an important feature of AD brain tissues, these results suggest that there is not a significant causal relationship between lifetime peripheral iron levels and AD.

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SUPPLEMENTARY METHODS

GWAS Data and Imputation Methods

All AD cohorts were genotyped on the Illumina 610-Quad or Illumina 666W-Quad chip. All GWAS data were imputed to the 1000G phase 1 integrated reference panel (April 2012 National Center for Biotechnology Information [NCBI] build 37). As genotype data was used from multiple sources stringent quality control filters were applied. GWAS data quality control, merging and imputation steps have been described in detail previously [1].

The population-based sample set was genotyped on several different genome wide platforms (Illumina Human317K, HumanCNV370v1, HumanCNV370-Quadv3, Human610-Quadv1). Sample QC included omitting ethnic outliers, duplicate samples, and samples with unresolved sex, identity, or pedigree issues (if not correctable after investigation). Mendelian error genotypes per marker were removed across families. Exclusion criteria for markers were MAF<1%, call rate <0.99, p HWE<10-6, mean GenCall score <0.7. Approximately 281,000 markers are observed in all genotyping projects. Imputation of approximately 12,000,000 SNPs was carried out using the 1000 Genomes reference panel (August 4, 2010 release with European haplotypes) using minimac. After imputation 7,262,077 markers passed QC (R2≥0.3).
In the Australian dataset *APOE* genotype was estimated from imputed rs429358 and rs7412 SNP genotypes, which are not perfectly imputed (R^2 values are 0.68 and 0.63, respectively). We found the concordance between the imputed and genotyped *APOE* ε4 was 93%. This was calculated by comparing genotyped and imputed *APOE* (from the Queensland Twin Imaging (QTIM) cohort, which had available directly genotyped APOE and was included in the same imputation dataset) in a sample size of 3879 [2].

Genetic Profile Scores

SNPs with MAF≤0.02, genotyping rate≤0.99 and HWP<$1x10^{-6}$ in the target sample were excluded. Linkage disequilibrium (LD)-based clumping was carried out on all SNPs in the discovery data, providing the most significantly associated SNP available in the target data set, in each region of LD (using PLINK clumping command with a pairwise r^2 threshold of 0.2 and a physical distance threshold of 300 kb). SNPs were checked for flip strands between the discovery and target sample. The total score is calculated by the number of risk alleles weighted by the standardized per-allele effects, beta using PLINK score function. The risk score was calculated for p value thresholds of $1x10^{-6}$, $1x10^{-4}$, $1x10^{-3}$, 0.01, 0.05, 0.1, 0.5, and 1 (all SNPs). The iron GPS were calculated separately in three imputed AD case-control datasets (as described in detailed imputation methods [1]; set 1 consists of GERAD1 and WTCCC2, set 2 of ADNI and part of AddNeuroMed, and set 3 the remaining Addneuromed and KPH-DCR). SNPs within 500kb either side of the *APOE* locus were excluded from the GPS to ensure all *APOE* associated signal was removed. The *APOE* effect is not well represented within a GRS owing to the ε4 allele being a diplotype acting under a co-dominant genetic model, and with a much larger effect size than the other common AD risk variants [3].

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Alzheimer's Disease				
p value threshold	**Australian**			
p≤1	833,350			
p≤0.5	483,466			
p≤0.1	127,186			
p≤0.05	69,634			
p≤0.01	17,566			
p≤0.001	2,108			
p≤0.0001	514			
p≤0.000001	42			
Iron				
p value threshold	**GERAD1-WTCCC**	**AddNeuroMed_1**	**AddNeuroMed2-DCR**	**ADNI**
p≤1	252,456	244,606	253,672	240,836
p≤0.5	179,022	172,554	178,822	170,344
p≤0.1	53,802	51,316	53,124	50,772
p≤0.05	30,488	28,972	29,954	28,656
p≤0.01	7,990	7,576	7,854	7,504
p≤0.001	1,222	1,172	1,218	1,158
p≤0.0001	276	264	284	256
p≤0.000001	92	88	98	82
Transferrin Saturation				
p value threshold	**GERAD1- WTCCC**	**AddNeuroMed_1**	**AddNeuroMed2-DCR**	**ADNI**
p≤1	253590	240732	240918	236990
p≤0.5	179912	170458	170380	168114
p≤0.1	54280	51132	51100	50692
p≤0.05	30986	28980	28996	28742
p≤0.01	8190	7670	7664	7594
p≤0.001	1302	1214	1202	1214
p≤0.0001	352	338	324	330
p≤0.000001	164	158	148	148
Transferrin				
p value threshold	**GERAD1- WTCCC**	**AddNeuroMed_1**	**AddNeuroMed2-DCR**	**ADNI**
p≤1	254286	242506	242854	238678
p≤0.5	182046	173622	174096	171426
p≤0.1	57478	54324	54370	53534
p≤0.05	33606	31760	31766	31414
p≤0.01	9236	8732	8754	8654
p≤0.001	1620	1534	1536	1530
p≤0.0001	420	400	398	388
p≤0.000001	162	148	158	150
Ferritin				
p value threshold	**GERAD1- WTCCC**	**AddNeuroMed_1**	**AddNeuroMed2-DCR**	**ADNI**
p≤1	242692	232648	232986	228938
p≤0.5	173016	165518	165664	163236

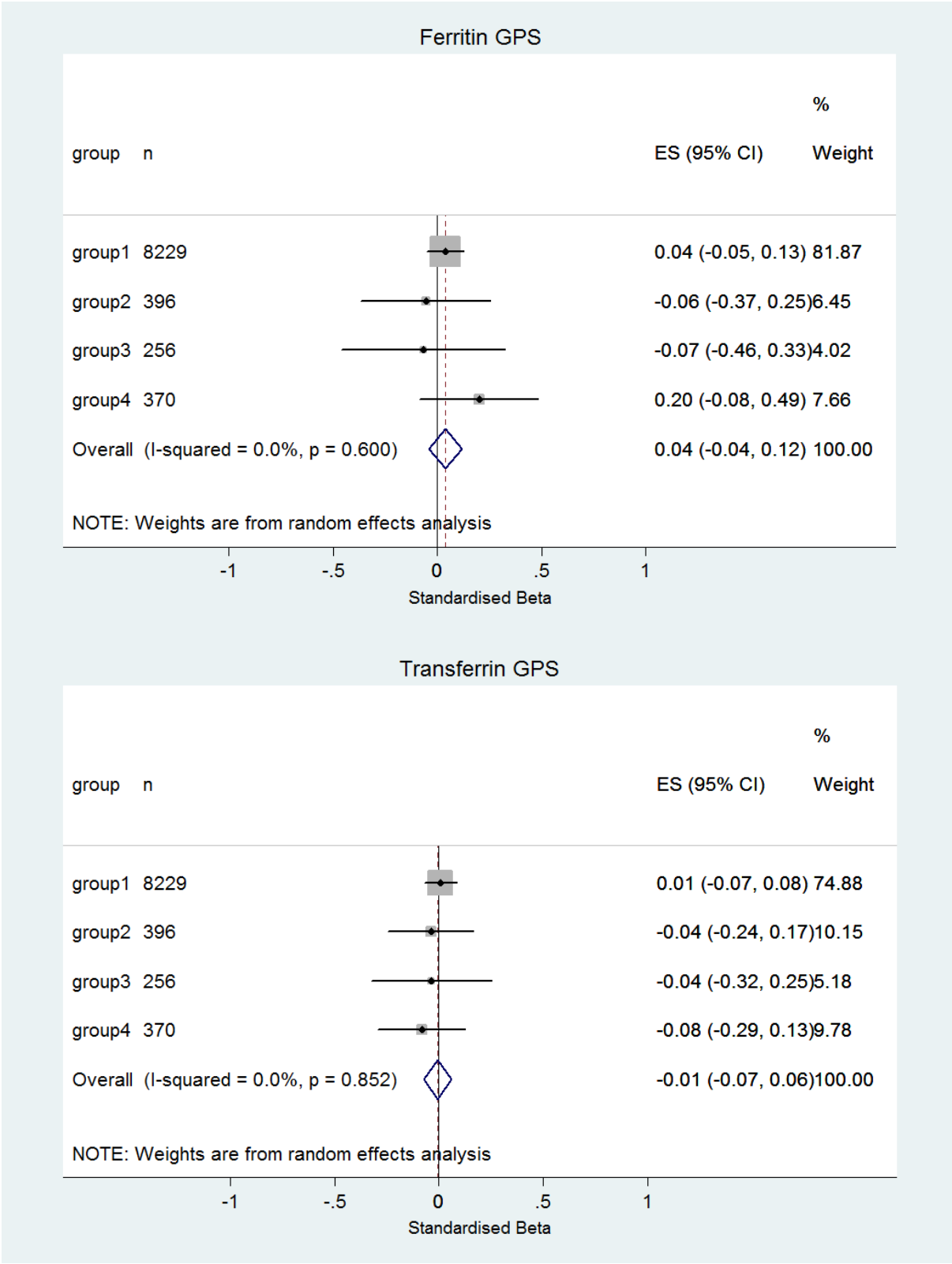
p≤0.1	53188	50292	50072	49560
p≤0.05	30394	28840	28736	28380
p≤0.01	8140	7702	7690	7622
p≤0.001	1204	1138	1138	1126
p≤0.0001	212	198	202	198
p≤0.000001	38	40	36	38

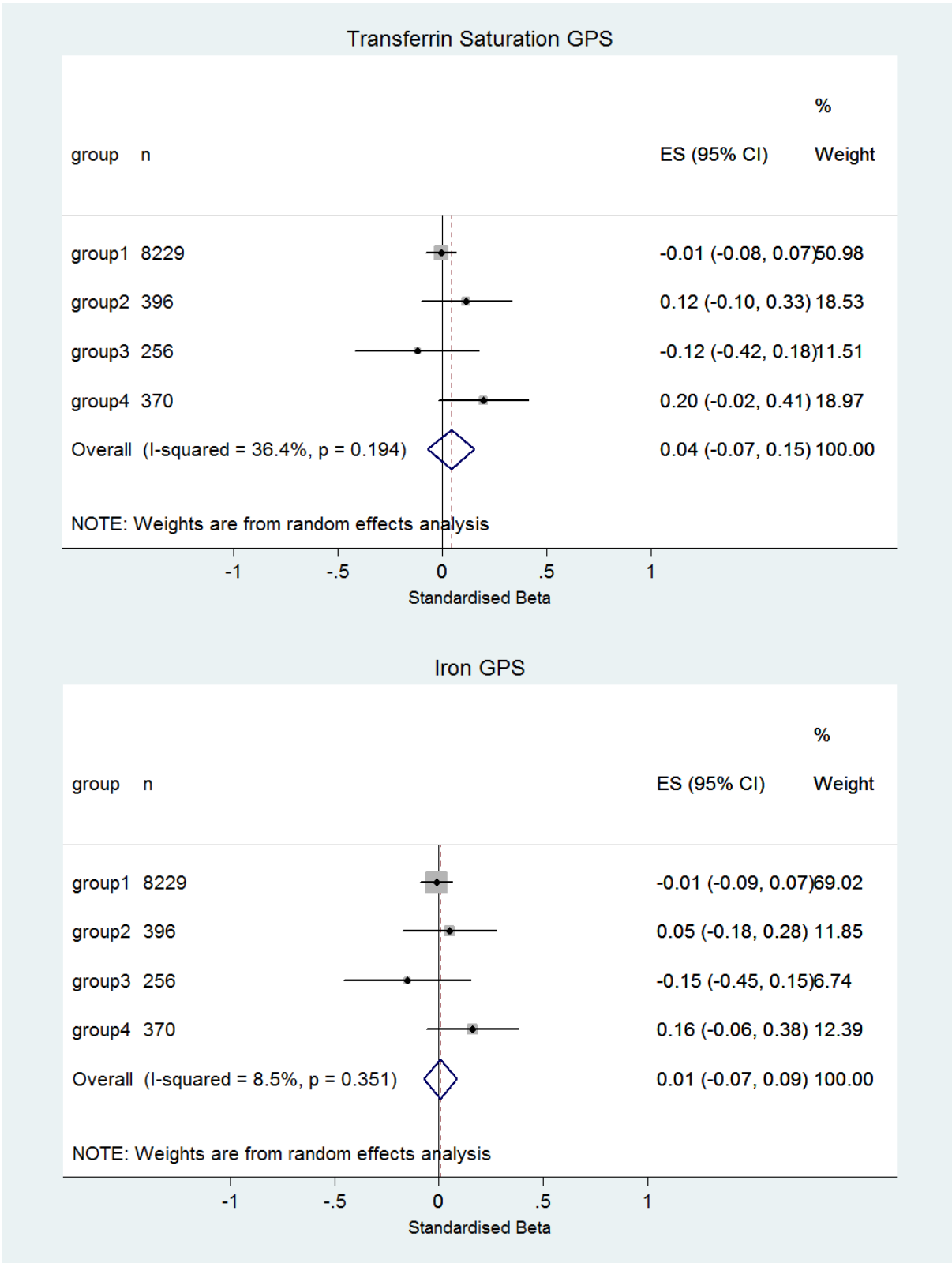
Supplementary Table 1. Number of SNPs included in each genetic profile score for each imputation dataset.

Variant	All (n=9,251)			*APOE* ε4 +ve (n=3,676)			*APOE* ε4 –ve (n=5,575)		
	β	**SE**	**p**	**β**	**SE**	**P**	**β**	**SE**	**p**
HFE rs1799945	-0.009	0.062	0.885	-	0.095	0.320	0.023	0.090	0.803
HFE	0.098	0.090	0.279	0.105	0.138	0.444	0.081	0.133	0.540
TMPRSS6 rs855791	-0.048	0.046	0.295	-	0.069	0.962	-	0.067	0.164
Three SNP GPS	-0.002	0.032	0.960	-	0.049	0.900	-	0.048	0.691

Supplementary Table 2. The association of iron influencing mutations with AD risk. Analysis was carried out using logistic regression controlling for sex, age, four ancestry principal components, and study. Genotypes were tested under an additive model with the risk allele being that associated with increased iron levels. The genetic profile score (GRS) is generated from the three genotypes. Standardized Betas (β) are shown.

Supplementary Figure 1. The meta-analysis used effect size estimates and standard errors with a random effects model. ES represents the effect size which is the combined β value. I^2 is a measure of between study heterogeneity. Results shown for $p \leq 0.5$ threshold only, but no significant association or heterogeneity between datasets was observed at any p value threshold. Group 1 is GERAD1 together with WTCCC21958 British Birth Cohort, Group 2 is AddNeuroMed (second batch) with DCR, Group 3 is AddNeuroMed (first batch), and Group 4 is ADNI.


Ferritin GPS
group n
ES (95% CI)
%
Weight
group1 8229
0.04 (-0.05, 0.13) 81.87
group2 396
-0.06 (-0.37, 0.25) 6.45
group3 256
-0.07 (-0.46, 0.33) 4.02
group4 370
0.20 (-0.08, 0.49) 7.66
Overall (I-squared = 0.0%, p = 0.600)
0.04 (-0.04, 0.12) 100.00
NOTE: Weights are from random effects analysis
-1
-.5
0
.5
1
Standardised Beta
Transferrin GPS
group n
ES (95% CI)
%
Weight
group1 8229
0.01 (-0.07, 0.08) 74.88
group2 396
-0.04 (-0.24, 0.17) 10.15
group3 256
-0.04 (-0.32, 0.25) 5.18
group4 370
-0.08 (-0.29, 0.13) 9.78
Overall (I-squared = 0.0%, p = 0.852)
-0.01 (-0.07, 0.06) 100.00
NOTE: Weights are from random effects analysis
-1
-.5
0
.5
1
Standardised Beta


Transferrin Saturation GPS
group n
ES (95% CI)
% Weight
group1 8229
-0.01 (-0.08, 0.07) 50.98
group2 396
0.12 (-0.10, 0.33) 18.53
group3 256
-0.12 (-0.42, 0.18) 11.51
group4 370
0.20 (-0.02, 0.41) 18.97
Overall (I-squared = 36.4%, p = 0.194)
0.04 (-0.07, 0.15) 100.00
NOTE: Weights are from random effects analysis
-1
-.5
0
.5
1
Standardised Beta
Iron GPS
group n
ES (95% CI)
% Weight
group1 8229
-0.01 (-0.09, 0.07) 69.02
group2 396
0.05 (-0.18, 0.28) 11.85
group3 256
-0.15 (-0.45, 0.15) 6.74
group4 370
0.16 (-0.06, 0.38) 12.39
Overall (I-squared = 8.5%, p = 0.351)
0.01 (-0.07, 0.09) 100.00
NOTE: Weights are from random effects analysis
-1
-.5
0
.5
1
Standardised Beta

Supplementary Figure 2. SNP effect direction between dataset 1 (AD) and dataset 2 (Serum iron). In the SECA analysis, Fisher's exact statistical tests are performed to determine whether there is an excess of SNPs where the effect directions (BETA) are concordant across dataset1 and dataset2 for 144 SNP subsets from 12x12 p-value threshold combinations. A Fisher's test 'heatmap' plot is generated to graphically summarize the proportion of SNP subsets with concordant (Fisher's test odds ratio, $OR_{FT} \geq 1$) and discordant ($OR_{FT} < 1$) SNP effects, and an empirical p-value (p_{FTsig}-permuted) is calculated via permutation for the observed number of subsets (n_{FTsig}) with nominally significant concordance ($OR_{FT} \geq 1$and $p_{FT} \leq 0.05$).