Shared Genetic Links Between Amyotrophic Lateral Sclerosis and Obesity-Related Traits: A Genome-Wide Association Study

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Abstract

Background: Epidemiological and clinical studies have suggested comorbidity between amyotrophic lateral sclerosis (ALS) and obesity-related traits. However, little is known about their shared genetic architecture.

Objective: To examine whether there exist genetic enrichment between ALS and eleven obesity-related traits, including body mass index (BMI), waist hip ratio, body fat percentage (BFP), birth weight, triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), type 2 diabetes (T2D), fasting glucose, fasting insulin, and identify shared loci among them.

Methods: Using the conditional false discovery rate (FDR) statistical framework, we analyzed genome wide association summary statistics for ALS (n=80610) and obesity-related traits, and further conducted functional enrichment analysis.

Results: Robust genetic enrichment was observed for ALS conditional on BMI, BFP, HDL-C, LDL-C and T2D, but minimal enrichment on the other traits. 9 shared genetic loci with conjunctional FDR < 0.05 was identified, among which 6 were replicated in a second ALS cohort, including rs3849942 (*C9orf72*), rs170663 (*G2E3*), rs8018993 (*SCFD1*), rs978220 (*ATXN3*), rs62333164 (*CLCN3*) and rs12603276 (*GGNBP2*). We further identified *GGNBP2* as a novel potential ALS risk gene, by integrating cis-expression quantitative trait loci analysis in human brain tissue and summary-data-based Mendelian randomization analysis. Functional analysis indicated the shared risk genes were involved in pathways membrane trafficking and vesicle-mediated transport.

Conclusions: Our findings demonstrate selective genetic overlap between ALS and obesity-related traits, and identified new shared risk loci, including novel potential ALS risk gene *GGNBP2*. These results provide better understanding for the pleiotropy of ALS and have implications for future therapeutic trials.

Background

Amyotrophic lateral sclerosis (ALS) is a fetal neurodegenerative disorder with an average survival period around 3–5 years since the symptom onset, which brings a substantial impact on the quality of life for patients and their families, and huge socioeconomic burdens[1, 2]. With the advent of next-generation sequencing, emerging evidence suggests a substantial genetic component underlying ALS[3, 4]. Large genome-wide association studies (GWAS) have identified a growing number of susceptibility loci related to ALS and provided novel insight into the pathogenesis of ALS[5, 6]. However, due to the polygenic architecture of ALS, risk loci with weak associations cannot be identified with current GWAS sample size[7]. There is still a large "missing heritability" to be discovered, with an estimated heritability by twin data as high as 65%[8], while just 21% by current risk loci[9]. Moreover, some of the nominated risk loci have not been implicated in disease pathogenesis and are awaiting further exploration.

The association between ALS and obesity-related traits has long been observed. ALS patients often encounter a loss of weight or a decreased body mass index (BMI) and body fat along with disease progression[10, 11]. Dyslipidemia is also a quite common characteristic of ALS patients, and higher levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), total triglycerides (TG), and LDL-C/high-density lipoprotein cholesterol (HDL-C) ratio have been shown to be more prevalent in ALS patients than in controls, although subsequent results are not consistent[12–14]. The polygenic risk scores based on LDL-C and TC risk alleles are also shown to be associated with ALS risk[15]. Besides, a protective role of type 2 diabetes (T2D) on ALS has been found in observational studies[16–18]. These clinical and epidemiological findings suggest the obesity-related traits may potentially possess shared genetic background with ALS[19], but to what extent they overlap has not been investigated yet.

Recently, a novel statistical method to investigate overlapping between polygenic traits using GWAS summary statistics has been developed, and utilized in several human traits and diseases[20–23]. By incorporating GWAS results from multiple disorders and phenotypes, this method could provide insights into genetic pleiotropy (defined as a single gene or variant being associated with more than one distinct phenotype) and increase statistical power to discover significant associations[20–22]. Applying this approach, we systematically evaluated shared genetic risk between ALS and eleven obesity-related traits, including obesity traits like BMI, waist hip ratio (WHR), body fat percentage (BFP) and birth weight (BW), obesity-related lipid traits like LDL-C, HDL-C, TC and TG, and obesity related glucose traits like T2D, fasting glucose (FG) and fasting insulin (FI). We further replicated the shared risk loci in a second ALS cohort, and conducted functional enrichment analysis with the identified shared risk genes.

Methods

GWAS summary statistics

We investigated the shared genetic architecture between ALS[6] and obesity-related traits including BMI[24], WHR[25], BFP[26], BW[27], HDL-C, LDL-C, TC, TG[28], FI, FG[29] and T2D[30] based on GWAS summary statistics. Details of the summary data from all GWAS used in the current study were listed in **SupplementaryTable 1**, and the study design including collection of samples, quality control procedures, imputation methods etc. for each GWAS have been described in each publication. To confirm the findings in the discovery phase, we further assessed the P values of the identified pleiotropic single nucleotide polymorphisms (SNP) in a second ALS cohort of mixed ancestries[31] (n=41395). If the identified pleiotropic SNP was not in the replication GWAS, the nearest proxy SNP in strong linkage disequilibrium (LD) (R^2>0.8, D'>0.8) was chosen instead. The relevant institutional review boards or ethics committees approved the research protocol of each GWAS used in the current analysis, and all human participants gave written informed consent.

Genomic control

Due to population stratification or cryptic relatedness or overcorrection of test statistics[32], the empirical null distribution in GWAS is sometimes inflated or deflated. To correct for such bias, we computed the

genomic inflation factor λ_{GC} using intergenic SNPs, and adjusted the summary statistics by λ_{GC} for each GWAS respectively. Briefly, we annotated each SNP with ANNOVAR[33] and filtered intergenic SNPs only, since they provide a robust estimate of the true null effects[34]. Then we estimated λ_{GC} as the median Z score squared divided by the expected median of a chi-square distribution with one degree of freedom. After genomic control adjustment, we pruned the SNPs by removing SNPs in LD ($r^2 > 0.2$ within 250kb) based on 1000 Genomes Project LD structure.

Genetic enrichment

Quantile-quantile plots compare a nominal probability distribution against an empirical distribution, and leftward deflection of the observed distribution reflect enrichment of low P values. To assess the pleiotropic enrichment, we plotted conditional quantile-quantile plot for a primary phenotype by creating subsets of SNPs based on their association with a second phenotype. Specifically, we computed the empirical cumulative distribution of nominal P values of ALS for all SNPs and for subsets of SNPs with significance level below the indicated cutoffs on another trait ($-\log_{10}(P) \geq 0$, $-\log_{10}(P) \geq 1$, $-\log_{10}(P) \geq 2$, $-\log_{10}(P) \geq 3$, corresponding to $P \leq 1$, $P \leq 0.1$, $P \leq 0.01$, $P \leq 0.001$)[20, 34].

Furthermore, to assess the level of enrichment, we constructed fold-enrichment plots of nominal $-\log_{10}(P)$ values of ALS for all SNPs and subsets of SNPs determined by the significance of their association with each obesity-related trait. The presence of enrichment is reflected as an upward deflection of the curve for the primary phenotype, and the degree of deflection depends on the degree of association with the second phenotype. Enrichment can be directly interpreted in terms of the true discovery rate, which is equal to 1 - false discovery rate (FDR). To assess polygenic effects, we focused on SNPs with nominal $-\log_{10}(P) < 7.3$ (corresponding to $P > 5 \times 10^{-8}$)[20, 34].

Identification of risk loci

To identify risk loci associated with ALS conditional on each obesity-related trait, we computed the conditional FDR statistics using the stratified FDR approach[20-23]. The FDR framework is based on Bayesian statistics, which incorporates information from GWAS summary results of a second phenotype to adjust its original significance level. The conditional FDR is the probability of the SNP being null given its P value is as small as or smaller than observed. To remove false positives, a significance threshold of FDR < 0.01 was utilized. Conditional Manhattan plot was created to illustrate the genetic markers associated with ALS conditional on each trait[20, 34].

Furthermore, to identify shared risk loci associated with ALS and each obesity-related trait, we computed the conjunctional FDR statistics. The conjunctional FDR is an extension of the conditional FDR and is defined as the maximum of the two conditional FDR statistics for a specific SNP. Conjunctional FDR estimates the posterior probability that a SNP is null for both traits simultaneously, given that the P values for both phenotypes are smaller than observed respectively. A significance threshold of FDR < 0.05 was utilized, corresponding to five false positives per 100 reported associations. Conjunctional

Manhattan plot was created to illustrate the shared genetic markers associated with ALS and each trait[20, 34].

Functional evaluation of shared risk loci

To assess whether the shared risk loci modify gene expression, we evaluated cis-expression quantitative trait loci (eQTL) in Braineac, a public dataset of normal control brains for investigating the genes and SNPs associated with neurological disorders[35]. We analyzed eQTL for the mean P value derived across these brain regions: the cerebellum, frontal cortex, hippocampus, medulla, occipital cortex, putamen, substantia nigra, temporal cortex, thalamus, and white matter. To minimize false positives, a P value below 1.5E-03 was considered significant after Bonferroni correction.

To identify enrichments in gene ontologic (GO) features associated with ALS and obesity-related traits, we used ConsensusPathDB[36] for functional interaction analysis, which compare GO terms between background and candidate gene sets using the hypergeometric test and generates P values corrected for multiple testing. The shared risk genes identified with conjunctional FDR method and eQTL analysis were utilized with default parameters and default background gene set. Biological, cellular, and molecular GO terms were analyzed.

Results

Estimation of pleiotropic enrichment

In the stratified quantile-quantile plots for ALS conditional on association P values with each obesity-related trait, successive enrichment was found for BMI, BFP, HDL-C, LDL-C and T2D (**Figure 1**), indicating the proportion of non-null SNPs in ALS increase with higher levels of association with these traits. In contrast, minimal or no enrichment was found for WHR, BW, TC, TG, FG and FI. For progressively stringent P value thresholds, we could observe approximately 13-fold enrichment conditional on BMI, 13-fold enrichment on BFP, 10-fold enrichment on LDL-C, 8-fold enrichment on HDL-C and 8-fold enrichment on T2D, while minimal enrichment on the other traits, suggesting selective genetic overlap between ALS and obesity-related traits (**Figure 2**).

ALS-associated loci identified with conditional FDR

To discover genetic variants associated with ALS, we performed the conditional FDR statistical analysis. A total of 20 risk loci were identified with conditional FDR < 0.01 (**Supplementary Table 2**), including 14 novel loci which were not significant (P<5E-08) in the original ALS GWAS[6]. In these 20 loci, 11 were suggestively significant (P<1E-04) in the replication GWAS results, namely rs10463311 (*GPX3,TNIP1*), rs7813314 (*LOC101927815*), rs7864502 (*C9orf72*), rs58854276 (*ACSL5*), rs12810996 (*TBK1*), rs447614 (*G2E3*), rs229150 (*SCFD1*), rs978220 (*ATXN3*), rs35714695 (*SARM1*), rs2285642 (*GGNBP2*) and rs12608932 (*UNC13A*) (**Supplementary Table 2**). Among these replicated genes, *GPX3*, *TNIP1*, *C9orf72*,

TBK1, *SCFD1*, *ATXN3* and *UNC13A* have been described as risk genes for ALS by earlier GWAS, while the others were novel risk genes, including *LOC101927815*, *ACSL5*, *G2E3*, *GGNBP2* and *SARM1*.

Loci shared between ALS and obesity-related traits

To identify shared risk loci between ALS and obesity-related traits, we further performed conjunctional FDR analysis. A total of 9 shared risk loci were identified with conjunctional FDR < 0.05, including rs62333164 (*CLCN3*), rs170663 (*G2E3*), rs8018993 (*SCFD1*), rs11160036 (*TRIP11*), rs978220 (*ATXN3*) and rs12603276 (*GGNBP2*) between ALS and obesity traits BMI/WHR/BFP, rs3849942 (*C9orf72*) and rs1976704 (*PGS1*) between ALS and HDL-C/LDL-C, and rs68069258 (*DENND6B*) between ALS and T2D (**Table 1**). In these 9 risk loci, 6 were suggestively significant (P<1E-04) in the replication GWAS results, namely rs62333164 (P=3.84E-06), rs3849942 (P=1.20E-22), rs170663 (P=2.22E-06), rs8018993 (P=8.00E-06), rs978220 (P=2.61E-05) and rs12603276 (P=9.21E-06) (**Table 1**). Among these replicated genes, *CLCN3*, *G2E3*, and *GGNBP2* were newly discovered risk genes for ALS, while the others have been described as risk genes for ALS by previous GWAS. No shared risk loci were found for ALS conditional on TC, TG, FG, FI and BW, which was consistent with the stratified quantile-quantile plots and fold-enrichment plots with no apparent enrichment observed. Notably, in the 6 shared risk loci identified between ALS and BMI, 5 were in reverse effect direction for ALS and BMI in the original GWAS. This is in line with recent Mendelian randomization results[37] that premorbid higher BMI contributes to decreased ALS risk. In contrast, the effect direction of the two shared risk SNPs between ALS and HDL-C were not consistent.

Functional interpretation of shared risk loci

To determine the functional effects of the shared risk loci, we evaluated cis-eQTL in human brains free of neuropathologic characteristics. The pleiotropic risk SNPs in *GGNBP2* were associated with expression of MYO19 (P=3.10E-04) and GGNBP2 (P=8.40E-12), both of which were risk genes associated with BMI. Pleiotropic SNPs in *C9orf72* affect expression of TEK (P=3.40E-05), which is also associated with blood protein level. Pleiotropic SNPs in *ATXN3* were related to expression of SLC24A4 (P=1.10E-03) and ATXN3 (P=1.10E-06), particularly in cerebellum. Interestingly, *SLC24A4* is risk gene for Alzheimer disease and *ATXN3* is risk gene for ALS, suggesting this region might have some common effect in neurodegenerative diseases. In addition, pleiotropic SNPs in *PGS1* affect expression of PGS1 (P=6.60E-11) and USP36 (P=2.00E-04) (**Table 2**).

To determine the biological pathways represented by shared risk genes and the genes identified with eQTL analysis, we conducted pathway overrepresentation analysis. Two pathways were enriched, namely membrane trafficking (P=0.004) and vesicle-mediated transport (P=0.005), both of which have been identified as involved in the pathogenesis of ALS. Membrane trafficking has been implicated in virtually every aspect of neuronal function and, in particular, neuronal maintenance and degeneration[38], and intracellular membrane trafficking defects affecting key neuronal functions may be an early determinant of motor neuron loss in ALS[39]. Meanwhile, the molecular regulation of intracellular and extracellular vesicle trafficking is an important pathway in ALS pathogenesis[40], and mutation in the vesicle-

trafficking protein has been implicated to cause late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. Additionally, 7 GO sets were identified, most of which are related to the membrane trafficking and cytoskeleton (**Supplementary Table 3**).

Discussion

Using summary statistics form large GWAS and conditional FDR statistical framework, we investigated genetic enrichment between ALS and 11 obesity-related traits. Genetic enrichment was observed for ALS conditional on BMI, BFP, HDL-C, LDL-C and T2D, while not on the others. Totally, we identified 9 shared risk loci between ALS and these traits in the discovery phase, with 6 validated in the replication GWAS, and rs12603276 (*GGNBP2*) was further annotated as related to gene expression in human brain tissue by eQTL analysis. This is the first time that pleiotropic enrichment was systematically assessed between ALS and obesity-related traits. Our findings suggest the polygenic component in ALS is enriched in obesity-related traits, and the enrichment varies between different traits.

We observed obvious enrichment for ALS as a function of BMI and BFP, but not BW, which was consistent with previous epidemiologic evidence[41, 42]. For example, lower BMI or quick BMI decrease has been observed associated with short ALS survival[41, 42]. A prospective study on 518,108 individuals found that increased pre-diagnostic body fat is associated with a decreased risk of ALS mortality. In contrast, little evidence was found for the association between ALS and BW, in line with the notion that ALS is a neurodegenerative disease, rather than neurodevelopmental disease. Our findings demonstrated correlation between ALS and obesity traits from a genetic perspective. In the shared risk genes identified using obesity traits, *SCFD1* and *ATXN3* have been reported associated with ALS by GWAS[6]. It is noteworthy that both of these two genes were involved in regulation of protein processing, transport and metabolism, and abnormal protein metabolism has been observed in both ALS and obesity[43]. Therefore, these two genes might act as genetic links between ALS and obesity through these cellular processes.

Moreover, we identified a novel risk locus rs12603276 (*GGNBP2*) for ALS conditional on BMI, WHR and BFP, and validated this locus as cis-eQTL for GGNBP2 in human brain tissue. Previous studies have also suggested that *GGNBP2* might be associated with ALS through gene-based association analysis and summary statistics-based Mendelian randomization (SMR) analysis[31, 44]. Our findings demonstrate that *GGNBP2* may be a link between ALS and obesity. Meanwhile, we noticed that *GGNBP2* is an important tumor suppresser involved in several kinds of cancers[45]. Cancer and neurodegenerative diseases are often hailed as the two sides of a coin[46]. As is observed in epidemiological studies, the overall risk of cancer was significantly reduced in cases with ALS[47]. The pathways that cause neuronal apoptosis, like mitogen-activated protein kinase (MAPK) signaling, can cause uncontrolled neuronal growth as well[48]. *GGNBP2* might act as potential genetic links between cancer and ALS. Based on joint evidence from genetic enrichment, eQTL and SMR analyses, *GGNBP2* might be risk gene for ALS and is worth further functional exploration.

In clinical practice, dyslipidemia has been suggested to be associated with ALS, and hypercholesterolemia was initially associated with lower risk of ALS[49]. However, contradictory evidence has also been presented, and several studies have shown conflicting results on whether the levels of HDL-C, LDL-C or TC were different between ALS patients and controls[50, 51]. In our study, enrichment was found for ALS conditional on HDL-C and LDL-C, but minimal or no enrichment on TC and TG. In addition, *C9orf72* was identified as a shared risk gene for ALS and HDL-C/LDL-C. *C9orf72* repeat expansion was a common cause for ALS, especially in the Caucasian population. The pathogenesis of *C9orf72* is still debated, with proposed mechanisms including repeated RNA-mediated toxicity, dipeptide protein toxicity or haplodeficiency[52]. Recently, decreased total serum HDL-C concentration level was observed in *C9orf72* repeat expansion carriers, suggesting that the pathogenic mechanism of *C9orf72* repeated expansion mutation may be related to abnormal lipid metabolism[53]. Our findings further confirmed that *C9orf72* is an important gene coupling ALS and lipid metabolism from a genetic perspective. The underlying mechanism of *C9orf72* in the lipid metabolism changes and ALS pathogenesis was worth further exploration.

Previous studies have suggested that T2D has a protective effect for ALS in case-control studies[54, 55]. In the current study, we found enrichment for ALS conditional on T2D, but not FI and FG. Lack of enrichment on FI and FG suggests the origin of glucose homeostasis abnormalities in ALS may be multifactorial, and other pathways might explain the enrichment for T2D. For example, our previous study found that higher levels of HbA1c, but not fasting blood glucose concentrations, were significantly associated with higher risks of ALS mortality[56]. Additionally, we identified one shared risk gene *DENND6B* between ALS and T2D. *DENND6B* belongs to Differentially Expressed in Normal and Neoplasia (DENN) like families, which is a GDP/GTP exchange factor (GEF) that activates Rab-GTPases[57]. It has been established that *DENND6B* is involved in the Rab guanyl-nucleotide exchange factor activity and vesicle-mediated transport, which was suggested to play an important role in ALS[58]. Interestingly, structural prediction engines suggested DENN as a possible homologue of *C9orf72*, prompting a more complex potential relationship between the DENN family and ALS[59].

There are some limitations of this study. First, the GWASs used in current study were mostly performed on participants of European ancestry, thus the findings of shared genetic architecture might be biased and not applicable to other populations. Future studies in other non-European populations will provide more comprehensive understandings. Second, there were potential sample overlap between ALS and each obesity-related trait in the original GWAS. Such overlap might bring some bias to the statistical analysis, although the bias will be minimal. Third, how the identified pleiotropic SNPs were involved in the pathogenesis of ALS and obesity-related traits cannot be determined. Further functional explorations will provide better understandings.

Conclusions

By integrating GWAS summary data and conditional FDR statistical framework, we identified selective pleiotropy and novel shared loci between ALS and 11 obesity-related traits. Moreover, we identified a

novel ALS risk gene *GGNBP2* by combining eQTL analysis results. These findings may provide novel insights into the shared genetic background between ALS and obesity-related traits, and help better understand the etiology of ALS and have an impact on the clinical treatment.

Abbreviations

ALS
amyotrophic lateral sclerosis; BMI = body mass index; WHR = waist hip ratio; BFP = body fat percentage; BW = birth weight; TG = triglycerides; TC = total cholesterol; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; T2D = type 2 diabetes; FG = fasting glucose; FI = fasting insulin; FDR = false discovery rate; GWAS = genome-wide association studies; SNP = single nucleotide polymorphisms; LD = linkage disequilibrium; eQTL = cis-expression quantitative trait loci; GO = gene ontology; SMR = summary statistics-based Mendelian randomization

Declarations

Competing interests

The authors declare that they have no competing interests.

Availability of data and material

The GWAS summary statistics used to perform the analyses described in the study were obtained from publicly available published data. All data generated or analyzed in the study were included in the article and supplementary material.

Supplemental Data include three tables.

Authors' contributions

LCY and SHF conceived the study. LCY performed the statistical analyses and prepared the drafted manuscript. ORW and GXJ assisted in the statistical analysis. LCY, ORW, GXJ, WQQ and H.S. contributed to writing and editing of the manuscript. All authors reviewed and approved the final manuscript

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Tables

Table 1
Shared risk loci between ALS and obesity-related traits.

SNP	Genomic position (GRCh37)	Closest gene	A1	Associated phenotype	FDR value	Original ALS P value	Replication ALS P value
rs62333164	4:170583157	*CLCN3*	A	BMI	0.0386	5.24E-06	3.84E-06
rs3849942	9:27543281	*C9orf72*	T	HDL-C,LDL-C	0.0329	5.11E-30	1.20E-22
rs170663	14:31049409	*G2E3*	T	BMI	0.0269	5.64E-07	2.22E-06
rs8018993	14:31138219	*SCFD1*	A	BMI	0.0223	2.02E-06	8.00E-06
rs11160036	14:92481047	*TRIP11*	A	BMI	0.0238	1.38E-06	1.50E-04
rs978220	14:92558135	*ATXN3*	T	BMI	0.0231	1.66E-06	2.61E-05
rs12603276	17:34946547	*GGNBP2*	T	BMI,WHR,BFP	0.0259	6.06E-05	9.21E-06
rs1976704	17:76401318	*PGS1*	T	HDL-C	0.0384	1.50E-04	2.38E-04
rs68069258	22:50748930	*DENND6B*	C	T2D	0.0454	6.00E-06	2.89E-02

n.a., not available; SNP, single nucleotide polymorphism; A1, effect allele; FDR, false discovery rate.

Table 2
eQTL revealing functional effects of shared risk SNPs in human brain tissue.

Genomic position (GRCh37)	SNP	Closest gene	eQTL	
			Gene	P value
9:27511593	rs1845699	*C9orf72*	*TEK*	3.40E-05
14:92525145	rs1047795	*ATXN3*	*SLC24A4*	1.10E-03
14:92558135	rs978220	*ATXN3*	*ATXN3*	1.10E-06
17:34917608	rs6607326	*GGNBP2*	*MYO19*	3.10E-04
17:34946547	rs12603276	*GGNBP2*	*GGNBP2*	8.40E-12
17:76395421	rs2292643	*PGS1*	*PGS1*	6.60E-11
17:76395421	rs2292643	*PGS1*	*USP36*	2.00E-04

Figures

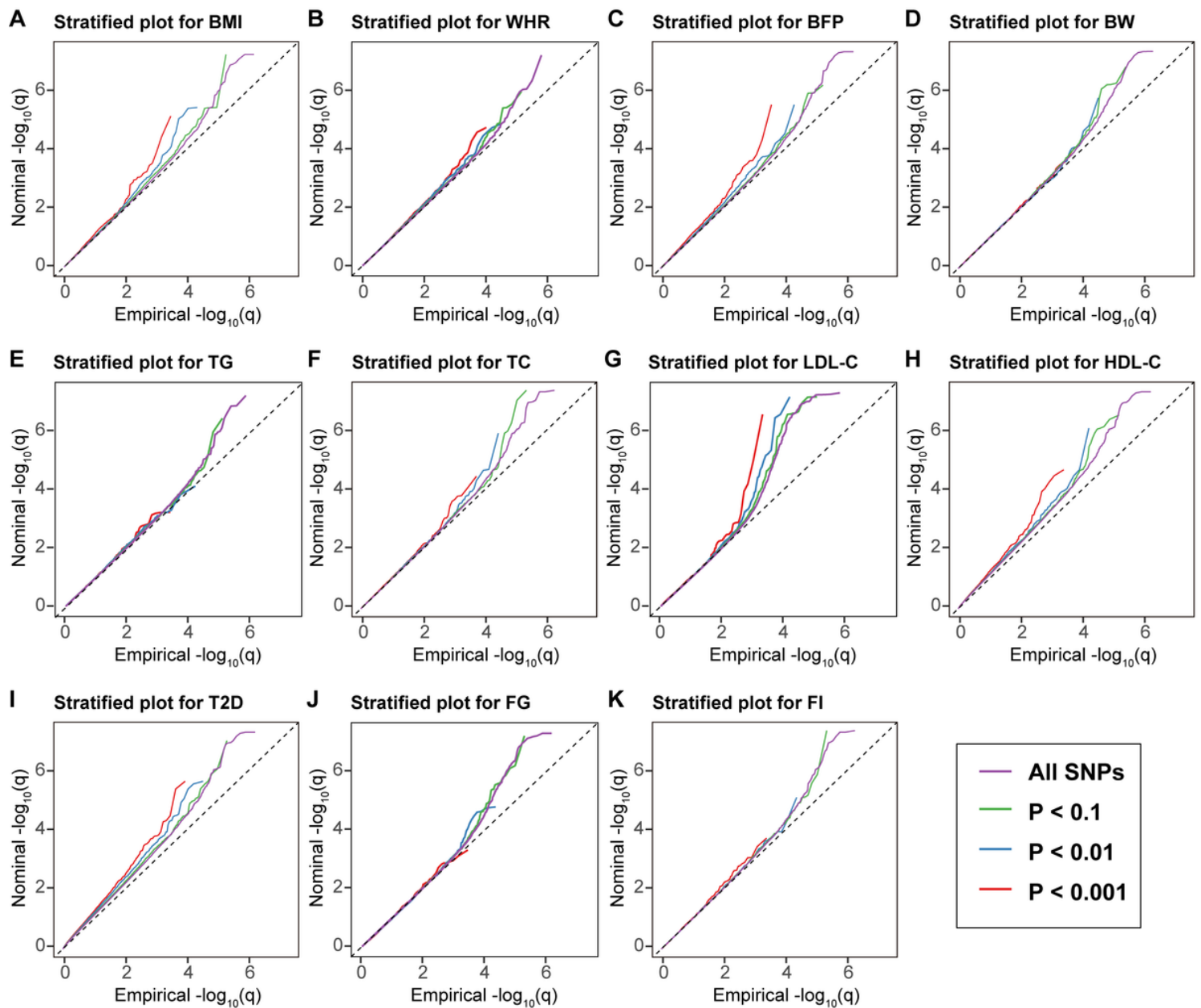**Figure 1**

Enrichment plots. Conditional quantile-quantile plots of nominal versus empirical -log10(P) of ALS as a function of significance of association with obesity-related traits. Dashed lines indicate the null hypothesis.

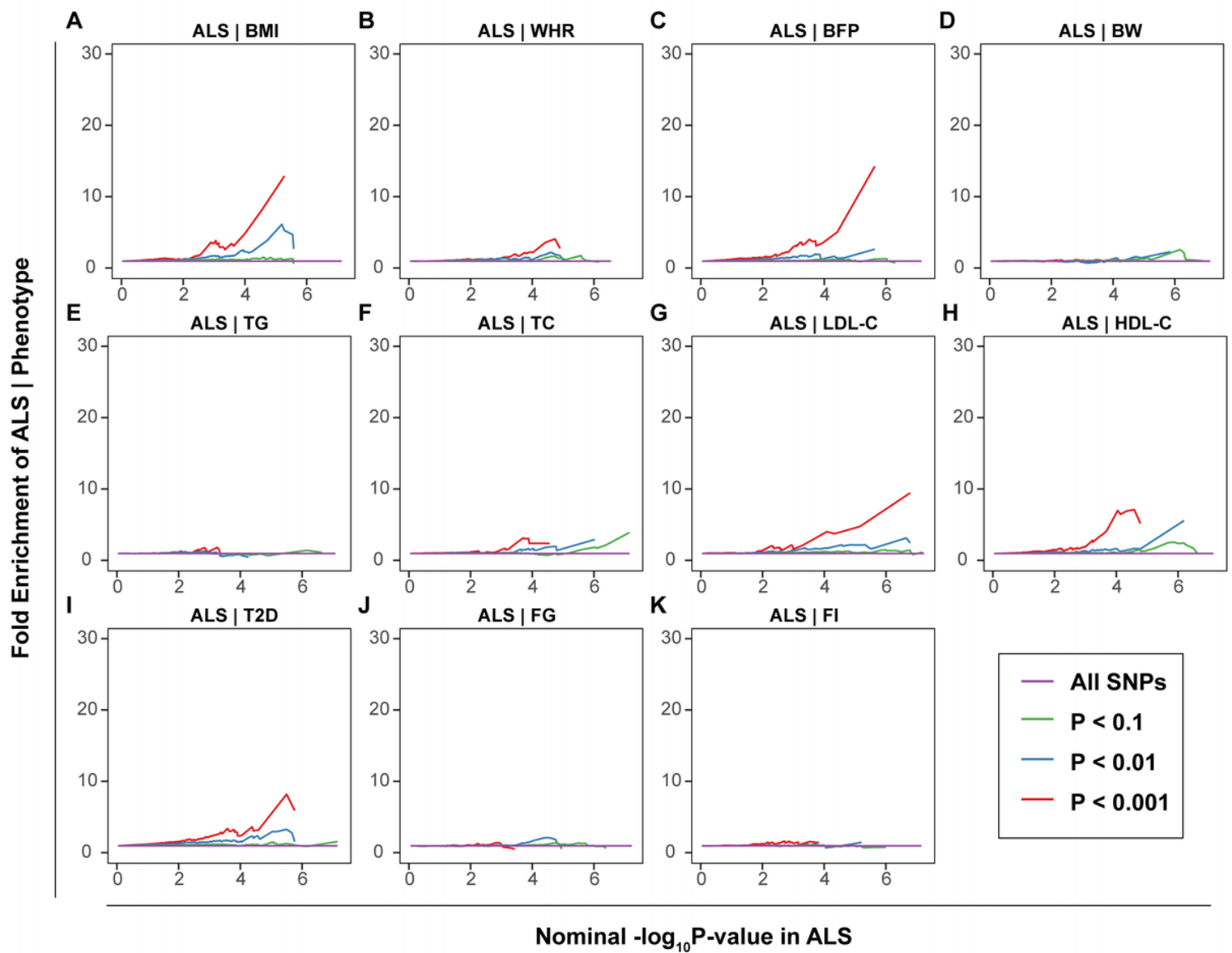**Figure 2**

Fold-enrichment plot. Fold-enrichment plot of nominal -log10(P) of ALS as a function of significance of association with obesity-related traits.
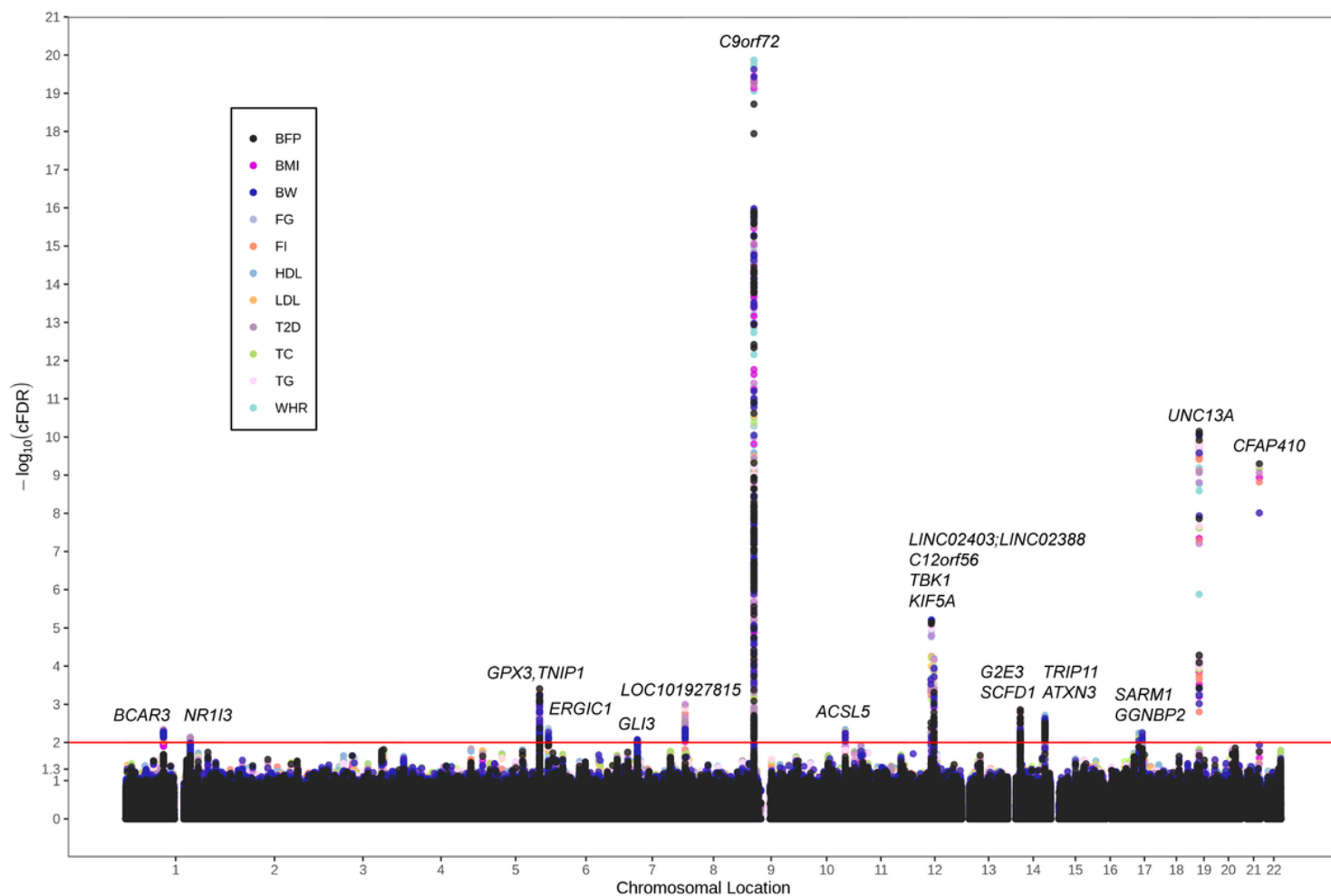
Figure 3

Conditional Manhattan plots. Conditional Manhattan plots showing risk loci for ALS conditional on each obesity-related trait. The dotted horizontal line represents the significant threshold (conditional FDR = 0.01).
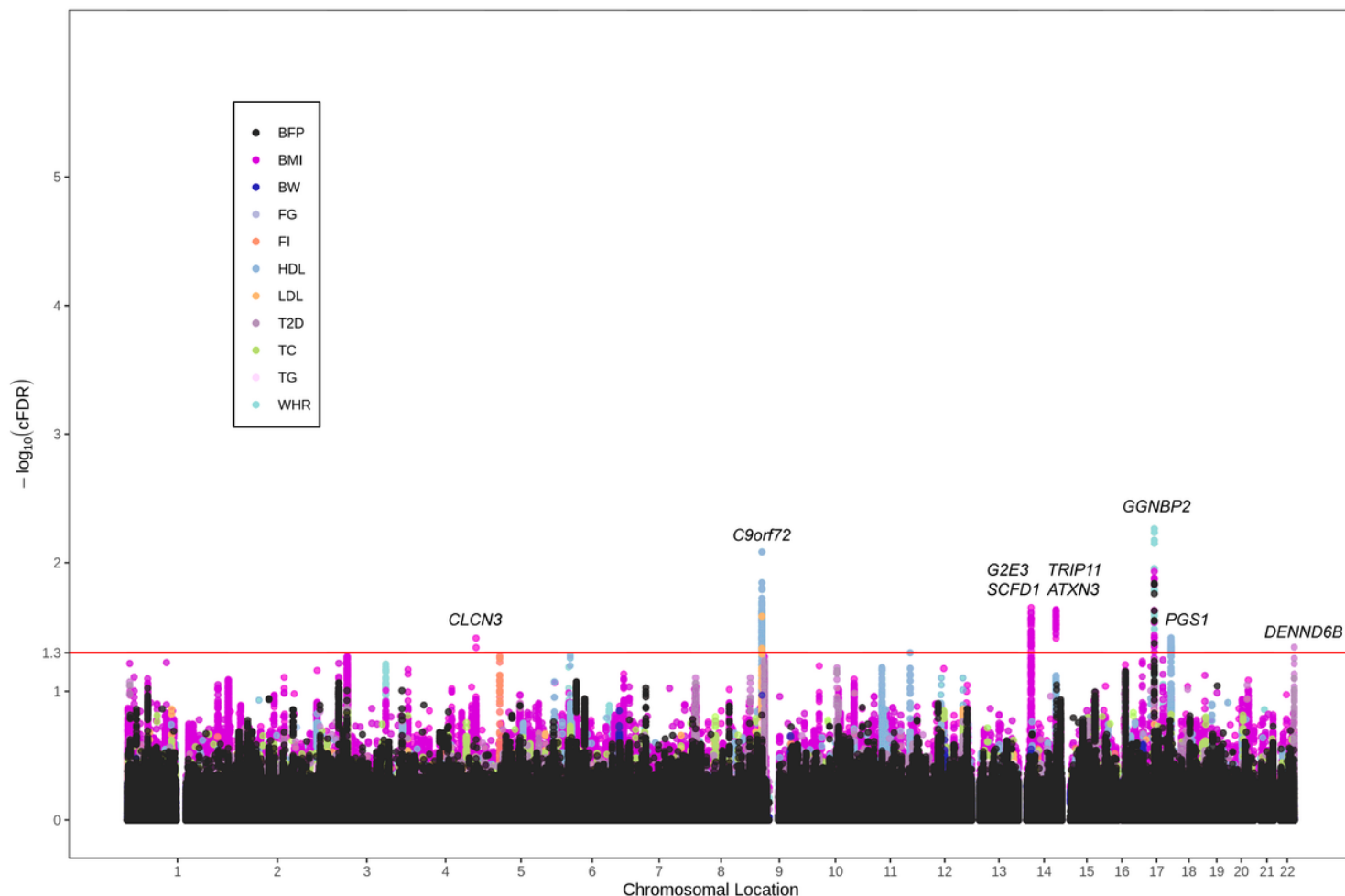
Figure 4

Conjunctional Manhattan plots. Conjunctional Manhattan plots showing shared genetic loci between ALS and each obesity-related trait. The dotted horizontal line represents the significant threshold (conjunctional FDR = 0.05).

Supplementary Files

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- SupplementaryMaterial.pdf