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ORIGINAL ARTICLE

Evidence for the role of *EPHX2* gene variants in anorexia nervosa

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Anorexia nervosa (AN) and related eating disorders are complex, multifactorial neuropsychiatric conditions with likely rare and common genetic and environmental determinants. To identify genetic variants associated with AN, we pursued a series of sequencing and genotyping studies focusing on the coding regions and upstream sequence of 152 candidate genes in a total of 1205 AN cases and 1948 controls. We identified individual variant associations in the Estrogen Receptor- β (*ESR2*) gene, as well as a set of rare and common variants in the Epoxide Hydrolase 2 (*EPHX2*) gene, in an initial sequencing study of 261 early-onset severe AN cases and 73 controls ($P=0.0004$). The association of *EPHX2* variants was further delineated in: (1) a pooling-based replication study involving an additional 500 AN patients and 500 controls (replication set $P=0.00000016$); (2) single-locus studies in a cohort of 386 previously genotyped broadly defined AN cases and 295 female population controls from the Bogalusa Heart Study (BHS) and a cohort of 58 individuals with self-reported eating disturbances and 851 controls (combined smallest single locus $P<0.01$). As *EPHX2* is known to influence cholesterol metabolism, and AN is often associated with elevated cholesterol levels, we also investigated the association of *EPHX2* variants and longitudinal body mass index (BMI) and cholesterol in BHS female and male subjects ($N=229$) and found evidence for a modifying effect of a subset of variants on the relationship between cholesterol and BMI ($P<0.01$). These findings suggest a novel association of gene variants within *EPHX2* to susceptibility to AN and provide a foundation for future study of this important yet poorly understood condition.

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INTRODUCTION

A multitude of environmental, behavioral and genetic factors have been shown to be associated with anorexia nervosa (AN) and AN predisposition.^{1,2} However, although AN and its psychological correlates have been shown to be quite heritable (for example, 46–78%;^{3–6}) and have a very high sibling recurrence risk (~10-fold;^{7,8}), candidate gene and genome-wide searches for common single nucleotide variants (SNVs) and copy number variants (CNVs) that influence AN susceptibility have not yielded statistically compelling and replicable findings to date^{9–11} except possibly in the case of AN recovery.¹²

Research focused on the discovery of genes and genetic variants associated with common neuropsychiatric conditions has been greatly aided by the rapid development of genetic technologies, including efficient high-throughput genotyping, CNV detection and

next-generation sequencing. For example, recent applications of high-throughput genetic assays have identified unequivocal associations involving rare CNVs with autism and related developmental disorders,^{13,14} as well as schizophrenia.^{15–18} However, no genetic studies of other common neuropsychiatric conditions have identified strong associations with either more frequent CNVs or other more abundant forms of genetic variation, such as SNVs and small insertion-deletion variants (indels). This may be attributable to a number of factors including the following: (1) the fact that neuropsychiatric diseases are oligogenic or polygenic in nature, making it difficult to identify each and every variant contributing to the diseases through single-locus analyses; (2) complex diseases such as AN may be influenced by collections of rare SNVs and indels—in addition to more common variant effects—which are hard to detect without large sample sizes, next-generation

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sequencing technologies and sophisticated data analytic strategies; and (3) in the absence of deep phenotyping, the broad AN diagnosis may include a very heterogeneous set of patients with unique genetic profiles, confounding the detection of any one gene or set of genes. In light of these issues, the identification of groups of rare variants that collectively contribute to AN that may affect different physiologic pathways or mechanisms will thus require well-characterized and -phenotyped patients, large sample sizes, sophisticated DNA-sequencing strategies or all of these.

We exploited a multistage, large-scale sequencing strategy interrogating the coding regions and neighboring sequence of 152 candidate genes hypothesized to be involved in feeding behaviors, dopamine function, GABA and serotonin signaling, as well as previously identified candidate genes and regions from genome-wide association studies, including *OPRD1*, *CHD9* and *EPHX2* (Epoxide Hydrolase 2),^{9,12,19,20} to identify genetic variants that contribute to AN. We carried out the sequencing on an initial Discovery cohort (261 AN cases; 73 controls) and a DNA-pooling-based Replication cohort (500 AN cases and 500 controls) to identify associations involving both individual SNVs and indels, as well as collections of rare SNVs and indels, that are associated with AN. In this way, we sought to identify variants that are not likely to be detectable with the current genome-wide association study (GWAS) strategies that focus on a select number of common SNVs that explain some of the heritable components of AN and neuropsychiatric disorders in general.^{21–23} We further replicated a subset of the associated variants arising from the two sequencing studies in an additional set of previously genotyped AN and eating disordered (ED) cases ($N=444$) and controls ($N=1146$) using imputation-based methods. Finally, we assessed the impact of these variants on the longitudinal body mass index (BMI) and cholesterol profiles of participants in the Bogalusa Heart Study (BHS),²⁴ tested for association between *EPHX2* variants and relevant psychometric variables, and considered the expression profiles of the associated genes in the human brain via the Allen Brain Atlas.^{25,26}

MATERIALS AND METHODS

Samples

We used DNA samples from 262 individuals diagnosed with AN and 80 controls with extensive phenotype information from the Price Foundation (PF) sample repository for the initial sequencing study.⁹ We initially selected a sample of 300 women, self-reported Caucasian, with a clinically diagnosed history of Restricting-type Anorexia or Restricting-type with Purging Anorexia (average age of symptom onset 14 years; see Supplementary Table 1), a lifetime BMI of 15 or less and an assessment age of 19 years or greater as an evidence that they had a chronic disease course (65% of selected patients reported symptoms within the previous 12 months). Women with a history of regular bingeing behaviors were initially excluded (bulimia nervosa, binge ED and so on), with the aim of creating a more homogeneous sample to increase power for gene discovery. A total of 100 control women with no history of AN who also had current BMI measures between 18 and 29 were selected and matched by age, self-reported ethnicity and study enrollment site. Individuals with AN and controls had been previously phenotyped on a wide variety of psychometric scales, including the Temperament and Character Inventory;²⁷ Beck Depression Inventory (BDI),²⁸ State-Trait Anxiety Inventory;²⁹ Yale-Brown-Cornell Eating Disorders Scale;³⁰ and Structured Inventory of Anorexia Nervosa and Bulimic Syndromes.³¹ Of the 300 women with AN and 100 control women, 262 AN cases and 80 controls had sufficient high-quality DNA for sequencing. We also used DNA samples from 500 individuals with AN and 500 controls from the PF repository that were independent of the 262 AN and 80 control women used in the initial sequencing study. In addition, we took advantage of additional cohorts for replication studies, leveraging an independent set of AN cases from a previous GWAS of PF AN cases.⁹ Figure 1 provides a schematic detailing the samples and analyses.

Ancestry assessment

In order to determine the ancestry of the individuals from the PF repository to be used in the sequencing studies, we exploited a database of 1115 individuals with known ancestries from 11 global populations for whom genotype data are available from the third phase of the International HapMap Project.³² We used multidimensional scaling analyses on the genotype data with the PF samples along with the individuals with known ancestry. Only individuals who exhibited clear clustering with individuals of European descent were included in the sequencing analysis. In the Discovery stage, one case was excluded because of evidence of cryptic admixture with a Hispanic population, and in the Imputation-based cohort replication, one BHS female and six control females from the Scripps Genomic Health Initiative (SGHI) were excluded because of cryptic admixture.

Target capture

We used the solution-based hybridization target capture technology developed by Agilent³³ to target ~775 kilobases (kb) of unique DNA sequences covering the exons plus 1 kb upstream from exon 1 for 152 candidate genes involved in feeding behaviors, dopamine, GABA and serotonin signaling, as well as previously identified candidate genes and regions.^{9,12,19} Supplementary Table 5 lists all the genes that we studied.

Initial study sequencing and variant calling procedures

After the target capture assays, we performed sequencing with the Illumina GAIIX using indexing across four runs with 12 barcodes per lane. We sequenced all 262 cases and 80 controls. Average coverage for each individual for the targeted regions was 125X, with at least 10X coverage for 93.4% of the targeted regions. To call variants from the sequence data, reads were first aligned to the Human Genome Reference (HG18) using the BFAST alignment program,³⁴ and the variants were called using CRISP with the default parameters³⁵ for all targeted regions plus 100 flanking base pairs.

Quality controls

Quality-control steps included exclusion of individuals with greater than 10% missing genotype calls (seven Discovery-stage controls), and exclusion of variants with greater than 10% missing genotype calls and Hardy-Weinberg equilibrium test $P < 10^{-4}$. To assess the quality of the variant calls, we included technical control samples (HuRef) on multiple sequencing runs and comparing genotype assignments at the loci for which genotype information was available from a previous sequencing study.³⁶ These comparisons indicated a high level of fidelity in our sequencing and variant calling pipelines. We achieved concordance of 95.85% with Sanger sequencing data for the HuRef technical control. Our average between-run concordance for 15 technical replicate samples (inclusive of HuRef) sequenced on multiple runs was 93%. A significant fraction of discordant variants across samples was indels.

Pooling-based replication study sequencing and variant calling procedures

We used the pooling-based sequencing protocol and analysis strategy outlined by Bansal *et al.*³⁷ We created 50 total pools, each containing DNA from 20 individuals (25 pools with DNA from 20 AN subjects and 25 pools with DNA from 20 control subjects). In addition, we constructed one pool made up of individuals who had been sequenced previously in the Discovery phase. This allowed us to compare allele frequencies derived from the sequencing of the pool against allele frequencies based on genotype counts from the individual genotype data (see Supplementary Materials) to test the fidelity of the pooling and sequencing processes. A total of 2087 variants were called in this pool. A total of 4 variants were excluded as tri-allelic, 57 variants were missed when compared with the previous genotype information, 17 were called with no support for alternate allele and 15 were from one individual. Allele frequencies estimated from the pooled sequencing for the remaining genotypes showed a very strong correlation with the previously determined genotype-based allele frequencies on the basis of explicit genotype counting (overall $R^2 = 0.988$; Supplementary Figure 2). The equimolar pools were sequenced on the Illumina HiSeq. Each pool of AN subjects was sequenced to ~908X coverage, or ~45X per individual in the pool and each pool of control subjects was sequenced to ~895X per pool, or 45X per individual. Reads from the pools were aligned to the human reference

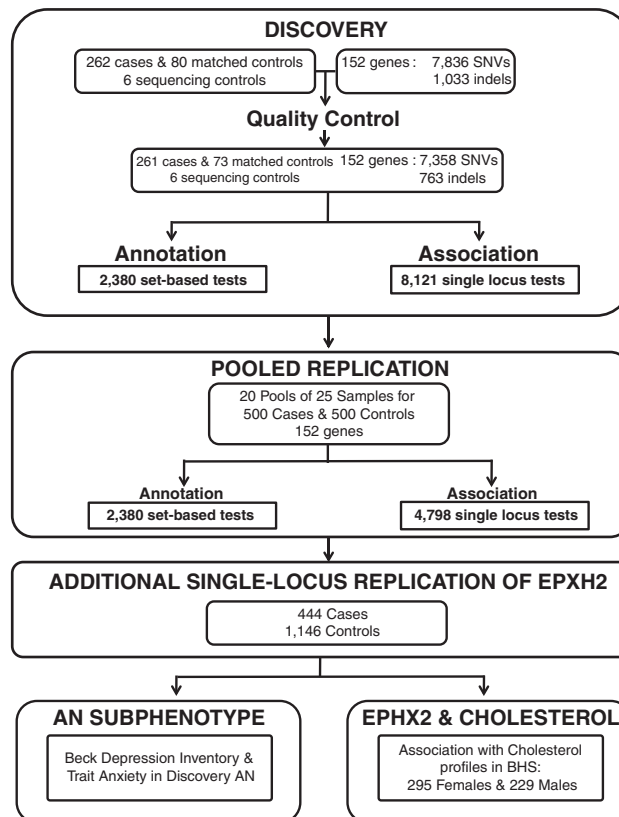


Figure 1. Diagrammatic representation of the study design and workflow for the initial and replication sequencing studies including the number of variants identified and analyzed at each stage. In the Discovery phase, 262 cases with anorexia nervosa (AN) and 80 matched controls were selected for sequencing. Exons and upstream promoter regions for 152 candidate genes were captured for targeted sequencing. A total of 8121 variants were tested for association with AN using both single-locus tests and set-based methods (2380 sets). Independent replication was pursued in 500 AN cases and 500 controls in the same set of 152 candidate genes (4798 variants; 2380 sets). Additional study cohorts from previous genome-wide association studies (GWAS) were used in a second independent replication analysis among 444 cases and 1146 controls and subphenotype-association tests.

genome with the program BWA³⁸ and variant calling was performed with the program CRISP.³⁵ After quality control, 4798 variants remained for further analysis.

Variant annotations

All variants passing quality-control filters were annotated using a suite of computational and bioinformatics techniques including Polyphen2³⁹ and SIFT.⁴⁰ In addition, identified variants were compared with variants in single nucleotide polymorphism database⁴¹ and the 1000 Genomes Database⁴² to determine their novelty. The results of the functional annotations were used to inform the association analyses as described below.

Statistical analysis in the initial sequencing study

Variants passing quality filters were tested for association with AN using simple single-locus analyses based on the regression model-based tests in the software package PLINK.⁴³ In addition, a ridge regression-based collapsed set association test was used to test the hypothesis that collections of variants, informed by likely functionally significant annotations, distinguish AN patients from controls.^{44,45} Although we did pursue other collapsed set analyses, ridge regression-based tests were chosen as the primary analysis methods because of their flexibility in accommodating covariates, their ability to accommodate correlations between predictors attributable to, for example, linkage disequilibrium, their ability to accommodate weighting, and their ability to simultaneously assess common variant effects, individual rare variant effects and collapsed sets of rare variant effects.^{44,45} A total of 2380 sets made up of collections of rare variants included variants in each exon that were likely damaging to the encoded protein based on Polyphen2 score,³⁹ variants in each exon,

variants in each gene and variants in known complexes of genes and pathways. To obtain an accurate estimate of the *P*-value for the set-based tests, 10000 permutations of AN/control status were generated, the test was recomputed and the frequency with which a permutation-based test resulted in a value more outlying than the original test statistic was recorded to establish an empirical *P*-value.

Statistical analysis in the pooled DNA-replication study

Variants passing quality-control measures were tested for frequency differences between the pools composed of DNA from AN patients and pools composed of DNA from controls. Allele frequency estimates were obtained by summing DNA-sequencing reads harboring the variant and dividing by the total number of DNA-sequencing reads across the pools mapping to a genomic location covering the variant position. Single-locus tests for frequency differences were pursued with Fisher's exact test. Set-based tests using the same set derived in the initial sequencing study analysis were conducted by computing the collective frequency of the variants estimated from the sequencing reads in the AN and control pools and comparing these frequencies with Fisher's exact test after transforming the read counts for the two alleles at each variant to allele counts.³⁷

Imputation-based replication cohorts

For replication, we exploited two previously genotyped groups of cases obtained from different GWAS studies, one that focused on AN specifically (referred to as the 'GWAS' cases⁹) and one with self-reported ED histories assessed in the context of a study evaluating the impact of learning about disease risk with genetic information (referred to as the SGHI subjects⁴⁶). We then compared genotypes among these cases with two sets of control

individuals having comparable ancestral backgrounds (that is, European) based on genotype profile. One set was derived from the Bogalusa Heart Study²⁴ and one derived from the SGHI.⁴⁶ This comparison included genotyped as well as imputed markers based on 1000 Genomes Phase-I-integrated variant set reference haplotypes. We used IMPUTE2 software v2.2.2 (software provided by the Department of Statistics, Oxford University) to impute a subset of associated variants arising from the sequencing studies in these subjects.^{47,48} We used an information threshold of 0.5. For samples that were sequenced and had prior GWAS data, we performed the same imputation to assess concordance for the imputed SNPs. For 128 samples on which we had sequencing and GWAS data, the concordance between sequence-based genotypes and GWAS or imputation-based genotypes for SNPs within *EPHX2* was 97.7%. The AN cases used for this second replication had extensive psychometric profiling, and secondary analyses of these phenotypes were pursued.

Longitudinal analyses

As a further set of replications and to explore the phenotypic impact of variants identified from our sequencing studies as associated with AN, we assessed associations between these variants and longitudinal data on BMI and other metabolic phenotypes (for example, cholesterol levels) in the BHS data.²⁴ The analyses were pursued to investigate the impact of associated variants on the relationship between weight gain (that is, change in BMI over time) and changes in total cholesterol level. A linear mixed model that considered total cholesterol level as the dependent variable and age, degree of European ancestry, BMI, genotype and a BMI × genotype interaction term as independent variables was used for these analyses. Separate analyses for male and female subjects were pursued.

Brain gene-expression analysis

We leveraged the Allen Brain Atlas to explore the expression patterns of genes found to be associated with AN from our sequencing studies.^{25,26} Data for this analysis included *EPHX2* expression data from three different donors (H0351.2001, H0351.1009 and H0351.2002) represented by two probes. Ten regions in which both the *EPHX2* probes had an absolute Z-score of ≥2.5 and were consistent across more than one donor were noted as over/under expressing the gene. Five of the 13 brain regions are biologically relevant structures and are highlighted in Supplementary Table 4.

RESULTS

Initial sample characteristics

Sample sizes for each stage of data analysis are shown in Table 1, and Supplementary Table 1 further provides information on the phenotypic and clinical characteristics of these individuals. As expected, the Discovery and Replication AN cases are significantly different from the controls with respect to current and previous BMI, anxiety levels and other measures of psychological health (Supplementary Table 1).

Ancestry determination

Multidimensional Scaling analysis with individuals of known ancestries was used to assess homogeneity of our initial sample based on genotype information (see Materials and Methods). Supplementary Figure 1 depicts the multidimensional Scaling results, after excluding one AN individual who demonstrated evidence of admixture, and indicates that the final sample of AN case and control subjects for the Discovery-stage sequencing study cluster with individuals of known European ancestry, suggesting that there is little potential for population stratification and genetic background heterogeneity to influence the association analyses.

Initial sequencing association analysis results

After quality control (see Materials and methods), 7358 SNVs and 763 indels remained for further analysis. We performed single-locus-based association analyses using the logistic regression test implemented in the genetic analysis software package PLINK.⁴³ No single variant reached genome-wide significance ($P < 10^{-8}$). The

Sample	N
<i>Price foundation phase 1</i>	
Cases	261
Controls	73
<i>Price foundation phase 2</i>	
Cases	500
Controls	500
<i>Price foundation GWAS</i>	
Cases	386
<i>Scripps Genomic Health Initiative</i>	
Cases	58
Controls	851
<i>Bogalusa Heart Study</i>	
Controls—female	295
Controls—male	229
Total cases	1205
Total controls	1948

two variants with the lowest *P*-values resided in the Estrogen Receptor Beta gene (*ESR2*; 7.1×10^{-4}) and had been previously observed and reported in single nucleotide polymorphism database⁴¹ (as rs1256066 and rs944050). We also pursued collapsed set variant analyses using ridge regression methods as described in the Materials and Methods section.^{44,45} A total of 2380 collapsed variant sets were tested for association. Supplementary Table 2 provides a ranked list (by *P*-value from the ridge regression analysis) of the top collapsed sets of variants that fall within the targeted exon regions, the nearest gene and the nature of the collapsing used to define the set (that is, all variants within a targeted exon, predicted functional variants within a region, predicted functional variants within a gene category and so on). The top two sets comprised 35 variants in *ITPR3* and 14 variants in *EPHX2*.

Pooling-based replication analysis results

All variants arising from the pooling-based replication sequencing study were subjected to single-locus and set-based allele frequency difference tests between AN and control pools (see Materials and methods). Of the top ranked sets from the Discovery-stage analysis, only one set of variants in the *EPHX2* gene was significantly associated in the pooling-based replication study and thus suggested replication. Eight of the 14 variants initially observed in the *EPHX2* gene set (chr8:27456902–27458639) at the Discovery stage were identified in the same set in the Replication stage and are denoted by an asterisk (*) in Table 2. The columns labeled 'Pools' in Table 3 provide the frequencies for the *EPHX2* gene variants within the replicated set in the pooled replication cohort. We also found evidence for additional replication of the *ESR2* gene variants in our other cohorts of previously studied (GWAS) AN cases and self-reported (SGHI) ED women, consistent with the previous evidence for a role for estrogen in AN^{49,50} and prior association with this gene.²⁰ Note that sets of variants identified in the initial sequencing were tested for association in the pooling-based replication study using combined frequency estimates of the variants defining the sets. In addition, in conducting these tests, only variants that were present in the replication sequencing data were used to form the set (for example, some variants identified in the initial sequencing

Table 2. Significant *EPHX2* variants from exon-based set test, discovery and replication

Replication	Chr	Position	VarType	Ref	Alt	Location	Coding impact	rsID
<i>EPHX2</i>	8	27454730	snp	G	A	Intron_15/14	—	rs59039594
*	8	27457059	snp	G	A	Intron_16/15	—	rs2291635
	8	27457077	del	ACA	—	Intron_16/15	—	—
*	8	27457179	snp	C	T	Intron_16/15	—	—
	8	27457194	snp	G	A	Exon_17/16	Synonymous	—
	8	27457304	snp	G	A	Intron_17/16	—	—
	8	27457623	snp	C	A	Exon_18/17	Nonsynonymous	—
*	8	27457709	snp	G	T	Intron_18/17	—	—
*	8	27457881	snp	A	C	Exon_19/18	Synonymous	rs1126452
*	8	27457991	snp	A	G	3UTR	—	rs1042032
*	8	27458049	snp	T	C	3UTR	—	rs1042064
*	8	27458411	snp	C	T	Downstream	—	rs4149259
*	8	27458470	snp	A	G	Downstream	—	—
	8	27458512	snp	G	A	Downstream	—	—
	8	27458581	snp	G	A	Downstream	—	—

Abbreviation: *EPHX2*, Epoxide Hydrolase 2.**Table 3.** Single-locus minor allele frequencies for all cohorts

Gene	Chr	Position	rsID	Cases				Controls				Pools P	Combined P
				Discovery	Pools	GWAS	SGHI	Discovery	Pools	BHS	SGHI		
<i>EPHX2</i>	8	27457059	rs2291635	0.0785	0.0859	0.0925	0.0603	0.1319	0.1061	0.1058	0.1122	7.45E-02	9.18E-03
	8	27457179	—	0.0019	0.001	NA	NA	0	0	NA	NA	NA	NA
	8	27457709	—	0	0	NA	NA	0.0068	0.002	NA	NA	NA	NA
	8	27457881	rs1126452	0.228	0.1967	0.2487	0.2456	0.274	0.2473	0.3034	0.2581	5.03E-03	7.70E-03
	8	27457991	rs1042032	0.228	0.1962	0.2487	0.2456	0.2708	0.2445	0.3034	0.2578	6.67E-02	6.20E-02
	8	27458049	rs1042064	0.228	0.2021	0.2552	0.2411	0.2945	0.2514	0.3102	0.2579	2.38E-02	1.59E-02
	8	27458411	rs4149259	0.1494	0.1201	0.1554	0.1842	0.137	0.16	0.1932	0.1410	5.93E-03	9.11E-02
	8	27458470	rs189089713	0.0019	0	0	0	0	0.001	0	0	NA	NA
<i>ESR2</i>	14	63768644	rs1256066	0.0383	0.0294	0.04275	0.0175	0.1096	0.0412	0.0356	0.0294	9.02E-02	1.44E-03
	14	63769798	rs944050	0.0383	0.0318	0.04275	0.0175	0.1096	0.0454	0.0373	0.0294	8.14E-02	1.54E-03
	14	63793804	rs1256049	0.0383	0.0274	0.04036	0.0259	0.1027	0.0425	0.0339	0.0287	3.36E-02	1.43E-03

Abbreviations: BHS, Bogalusa Heart Study; *EPHX2*, Epoxide Hydrolase 2; *ESR2*, Estrogen Receptor- β ; GWAS, genome-wide association study.

study—especially novel variants—were not identified in the replication sequencing study). It can be seen from Supplementary Table 2 that a set of variants in the *EPHX2* gene exhibited association in both the initial (14 variant sets; ridge regression $P=0.0004$) and the replication (8 variant sets; Fisher's exact test adjusted P -value after control for multiple comparisons via 10 000 permutations = 0.0062) sequencing studies. The *EPHX2* gene variants in the set included coding and non-coding variants with the variants that replicated residing primarily in a linkage disequilibrium block that covers the last three exons of the gene, including part of the 3'-untranslated region (UTR) (Table 2).

Imputation-based single-locus replication

To further test the association between AN and *ESR2* and *EPHX2* gene variants, we imputed the subset of *EPHX2* and *ESR2* variants that were implicated from the previous analysis stages in the AN GWAS, BHS and SGHI replication cohorts (see Materials and methods). There was some evidence of association with AN status at the single-locus level via a combined analysis, as indicated in Table 3. To further characterize the impact of the associated *EPHX2* gene variants, we investigated associations with these variants with measures of depression and anxiety collected on the

Discovery AN cases ($N=261$) (see Supplementary Table 3). Table 4 provides the results of these analyses and suggests that these variants may exhibit association with AN-relevant psychometric traits of Trait Anxiety (TA) and Depression (BDI). Interestingly, we observed an association not only between variants in *EPHX2* and BDI and TA but also a significant interaction between *EPHX2* gene variants and BMI and BDI scores. As shown in Supplementary Figure 4, women with AN who carry AN-associated *EPHX2* variants (rs1126452, rs1042032 and rs1042064) show increasing BDI depression scores with decreasing BMI. No BMI \times SNP interaction models for TA were significant, and thus only the main effects are reported.

Longitudinal analyses

Given that the *EPHX2* gene is known to influence cholesterol function (as discussed in greater depth in the Discussion), we considered the influence of the subset of associated variants that could be imputed in the BHS data set on longitudinal BMI and cholesterol levels. We found evidence that one *EPHX2* gene variant, rs2291635, had an impact on the relationship between weight gain (that is, increase in BMI over time) and cholesterol profile measures (total cholesterol, triglycerides, high-density

lipoprotein cholesterol and low-density lipoprotein cholesterol) based on an interaction model (see Materials and methods and Table 5) among both female (Figure 2) and, interestingly, male subjects from the BHS study. No other *EPHX2* gene variants showed significant association with cholesterol profiles.

Gene-expression analysis

Given that *EPHX2* has been implicated primarily in cholesterol metabolism with documented expression in tissues such as liver and kidney,⁵¹ we performed a *post hoc* exploration of *EPHX2* gene expression using publically available data from a recent meta-analysis by Liang and colleagues (2013)⁵² and the Allen Brain Atlas (see Materials and methods). Data from a recent exploration of 14 177 expression quantitative trait loci (eQTL) accessed at www.hsph.harvard.edu/liming-liang/software/eqtl/ reveal that one of the replicated SNPs from our study, rs4149259, acts as an eQTL for gene expression of *EPHX2* ($P = 5.17E-20$). Significant *EPHX2* gene-expression patterns identified from the Allen Brain Atlas are provided in Supplementary Table 4 and suggest that the *EPHX2* gene is expressed in neural tissues of relevance to feeding behaviors, anxiety and other AN-associated phenomena. Namely, elevated expression of *EPHX2* observed in the paraventricular nucleus (PVN) of the thalamus is interesting in light of studies linking PVN function to food and water intake,⁵³ weight gain in rats⁵⁴ and stress response.⁵⁵ Additionally, abnormal expression in sexually dimorphic regions such as the corpus callosum and the hippocampus may also indicate a sex-specific effect of *EPHX2* in the manifestation of AN. Finally, high levels of *EPHX2* expression were noted in the subcallosal gyrus, which has been implicated in depression and is functionally connected to the thalamus and limbic system.⁵⁶

DISCUSSION

AN is likely influenced by complex interactions between genetic variants, environmental and social factors. Whereas some genetic variants that contribute to these interactions are likely common, results of the only GWAS analyses to date did not show strong associations between common variants and AN,⁹ although it was limited by small sample size. We therefore leveraged DNA-sequencing studies in a multistage design to identify and replicate both common and rare variants in biologically relevant AN candidate genes that would not necessarily have been found in common variant-based candidate gene and GWAS analyses. Our initial sequencing study focused on a well-characterized cohort of 261 early-onset AN-affected individuals (mean 14 years) and 73 controls followed by a pooling-based replication study involving a much larger set of ($n = 500$) AN cases and ($n = 500$) controls (mean onset 16 years). We searched for both single-locus associations and collapsed rare variant set-based associations in

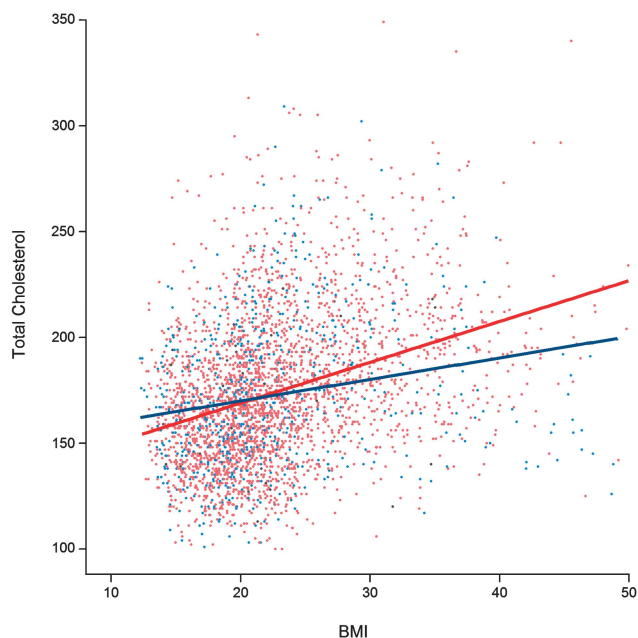


Figure 2. Scatter plot depicting the relationship between longitudinal BMI trajectories against longitudinal total cholesterol trajectories as a function of rs2291635 genotype for Bogalusa female subjects. Points in red represent the common homozygote genotype and those in blue represents the heterozygote. The rare homozygote is omitted.

Table 4. *EPHX2* psychometric subphenotype analysis in AN

Variant	BDI main effect ^a		BDI BMI × SNP ^a		Trait anxiety main effect ^b	
	β	P	β	P	β	P
rs2291635	0.266	0.458	-0.162	0.650	0.131	0.030*
rs1126452	1.280	0.002**	-1.180	0.004**	0.125	0.039*
rs1042032	1.280	0.002**	-1.180	0.004**	0.125	0.039*
rs1042064	1.280	0.002**	-1.180	0.004**	0.125	0.039*
rs4149259	1.156	0.005	-1.115	0.007	0.046	0.449

Abbreviations: BDI, Beck Depression Inventory; *EPHX2*, Epoxide Hydrolase 2. * $P < 0.05$; ** $P < 0.005$; ^a $N = 257$; ^b $N = 254$.

Table 5. *EPHX2* rs2291635 associations with cholesterol profile

	Females						Males					
	BMI		SNP		BMI × SNP		BMI		SNP		BMI × SNP	
	β	P	β	P	β	P	β	P	β	P	β	P
Total Cholesterol	1.03	8.5×10^{-12}	12.69	0.045*	-0.636	0.009**	1.22	1.43×10^{-8}	-13.28	0.107	0.577	0.044*
Triglycerides	3.36	4.15×10^{-33}	20.06	0.068	-1.11	0.014*	5.80	5.90×10^{-28}	-20.31	0.290	0.920	0.208
HDL Cholesterol	-0.054	3.08×10^{-10}	7.39	0.031*	-0.188	0.179	-0.97	7.60×10^{-19}	4.42	0.273	-0.228	0.138
LDL Cholesterol	0.946	4.18×10^{-12}	0.750	0.896	-0.204	0.352	1.31	2.62×10^{-12}	-15.13	0.034	0.650	0.009**

Abbreviations: BMI, body mass index; *EPHX2*, Epoxide Hydrolase 2; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SNP, single-nucleotide length polymorphism. * $P < 0.05$; ** $P < 0.01$.

both initial and replication stages. We found some evidence that *ESR2* may harbor variants associated with AN, which is interesting in light of the fact that AN is observed in female subjects to a much higher degree than in male subjects, and given its typical onset in adolescence.⁵⁷ This finding is also consistent with previous studies suggesting that estrogen and estrogen receptors may have a role in mediating the disease.^{49,58}

Importantly, we found more statistically compelling evidence for set-based associations involving a collection of rare variants in the *EPHX2* gene and were able to replicate a subset of these variants in additional case cohorts and controls. *EPHX2* was included in the gene list for targeted sequencing because of its presence in a list of top associated genes from a preliminary GWAS analysis.⁹ Still, the role of *EPHX2* in AN was not obvious, so we explored potential links between *EPHX2* function and AN. The *EPHX2* gene is expressed in vascular and non-vascular neural tissues of relevance to AN and is known to influence cholesterol function,^{51,59} suggesting multiple potential roles in the etiology or maintenance of AN. Interestingly, it has been well documented that a substantial proportion of malnourished individuals with AN have high serum cholesterol,⁶⁰ and this may be due to genetic variation.⁶¹ In fact, the *EPHX2* gene variants were also found to be associated with total cholesterol levels measured on one of our replication cohorts (BHS) as well as comorbid symptoms that are common in AN for a large proportion of our study subjects.

The *EPHX2* gene codes for the soluble epoxide hydrolase (sEH) protein, which is widely expressed in a variety of tissues, including the liver, lungs, kidney, heart, brain, adrenals, spleen, intestines, urinary bladder, placenta, skin, mammary gland, testis, leukocytes, vascular endothelium and smooth muscle but is most highly expressed in the liver and kidney.^{51,62,63} sEH catalyzes the hydrolysis of epoxides to their corresponding diols, easily metabolizes both saturated and unsaturated epoxy fatty acids and is highly conserved with nearly all functioning polymorphisms having been defined.⁵¹ The specific activity of sEH varies 500-fold in the human liver, suggesting a wide range of potential regulatory mechanisms,⁶⁴ and sEH gene transcription may also be induced by sex hormones and regulated by the hypothalamic–pituitary–gonadal axis.⁵¹ Through its complex epoxide hydrolysis of epoxy eicosatrienoic acids (EETs) into diols (DHETs), sEH reduces the number of EETs ready for release by phospholipases and may stop the biological activity of these lipids.⁶² Together, they influence the regulation of inflammation, blood pressure, lipid and carbohydrate metabolism.⁵¹

Relevant to this study are previous animal studies that reveal a dynamic change in sEH activity in response to diet. Mice fed high-carbohydrate high-fat (HCHF) diets show typical sequelae of metabolic syndrome, including increased body weight, abdominal fat and plasma lipid abnormalities, among others. In these obese animals, sEH activity in liver was 18% higher than mice fed a standard diet.⁶⁵ However, when rats fed a HCHF diet were given an sEH inhibitor, metabolic, liver and cardiovascular abnormalities were attenuated, suggesting a direct role for sEH in the etiology of dyslipidemia in response to diet. Total sEH activity was also found to be higher in adipose tissue of rats fed a HCHF diet.⁶⁶ These studies, together with the findings of this study, may suggest a role for *EPHX2* and the gene product sEH in lipid regulation in response to diet.

The observation that AN patients often display hypercholesterolemia in addition to their condition is counterintuitive, given the under-nutrition and low body weight of affected individuals. Fortunately, recovery from anorexia nearly always leads to recovery from hypercholesterolemia, even in very severe cases.⁶⁷ It has been hypothesized that low levels of cholesterol may decrease the activity of serotonin receptors and transporters and that significantly lower cholesterol levels are associated with depressive symptoms, impulsive/self-harmful behavior (cutting and/or burning) and suicide thoughts/attempts in anorexia

patients.⁶⁷ Moreover, lower cholesterol levels have been associated with increased suicidality more broadly, including ideation and attempts, in depressed patients.⁶⁸ Notably, from this research, it has been suggested that among depressed patients, BMI and total cholesterol had a negative correlation, and patients with higher cholesterol levels were observed to be significantly less depressed, impulsive and suicidal. We found evidence that a subset of *EPHX2* gene variants associated with AN also influence the relationship between increases in BMI and total cholesterol. These data may suggest a role for *EPHX2* gene variants in mediating AN-associated physiological changes consistent with previous studies showing an association between genetic variants within *EPHX2* and association with lipid profiles and cardiovascular disease.^{69,70}

The observed association between AN and variants in *EPHX2* is particularly interesting in light of the *EPHX2* gene-expression pattern observed in the brain. Data from the Allen Brain Atlas indicate *EPHX2* enrichment in tissues associated with feeding behaviors, depression and stress response, including the paraventricular nucleus and subcallosal gyrus. Although our investigation of *EPHX2* gene expression in the brain in this study was exploratory, future studies leveraging functional or structural brain imaging may clarify neural correlates of *EPHX2* genetic variants and contribution to AN-related phenotypes. For example, previous neuroimaging genetic studies have identified associations between genetic variants in the serotonin transporter, anxiety and neural tissues of relevance such as amygdala and cingulate,⁷¹ and correlated *CNTNAP2* gene-expression patterns in brain to frontostriatal functional connectivity patterns associated with variants in the *CNTNAP2* gene.⁷²

It will be important to verify the significance of the variants we have identified in a number of ways.⁷³ First, replication of the variants we have identified in larger data sets is crucial. Second, identifying additional phenotypes that may be influenced by the variants we found to be associated with AN would help put in perspective the physiological and neural pathways these genetic variants influence to impact AN susceptibility. For example, it would be interesting to evaluate associations between cholesterol-related phenotypes and the *EPHX2* gene in a sample of individuals with AN. Third, it would be important to assess the functional significance of the variants in order to characterize the molecular pathways that they influence, specifically whether they are associated with lower basal sEH activity. Fourth, imaging-genetics studies that evaluate possible links between *EPHX2* and structural and functional measures of the brain regions implicated in AN may provide further insight into the relationship between this gene and AN susceptibility. Taken together, our study represents the largest sequencing study of AN to date, and we hope that it will set the stage for further work into this debilitating and life-threatening disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)