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Genetic variability in the iron homeostasis pathway and patient outcomes after aneurysmal subarachnoid hemorrhage

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Abstract

Background/objective: Iron can be detrimental to most tissues both in excess and in deficiency. The brain in particular is highly susceptible to the consequences of excessive iron, especially during blood brain barrier disruption after injury. Preliminary evidence suggests that iron homeostasis is important during recovery after neurologic injury, therefore, the exploration of genetic variability in genes involved in iron homeostasis is an important area of patient outcomes research. The purpose of this study was to examine the relationship between tagging SNPs in candidate genes related to iron homeostasis and acute and long-term patient outcomes after aneurysmal subarachnoid hemorrhage (aSAH).

Methods: This study was a longitudinal, observational, candidate gene association study of participants with aSAH that used a two tier design including tier 1 (discovery, n=197) and tier 2 (replication, n=277). Participants were followed during the acute outcome phase for development of cerebral vasospasm (CV) and delayed cerebral ischemia (DCI) and during the long-term outcome phase for death and gross functional outcome using the Glasgow Outcome Scale (GOS; poor = 1–3). Genetic association analyses were performed using a logistic regression model adjusted for age, sex, and Fisher grade. Approximate Bayes Factors (ABF) and Bayesian False Discovery Probabilities (BFDP) were used to prioritize and interpret results.

Results: In tier 1, 235 tagging SNPs in 28 candidate genes were available for analysis and 26 associations (20 unique SNPs in 12 genes) were nominated for replication in tier 2. In tier 2, we observed an increase in evidence of association for three associations in the ceruloplasmin (*CP*) and cubilin (*CUBN*) genes. We observed an association between rs17838831 (*CP*) with GOS at 3 months (tier 2 results, Odds Ratio [OR] = 2.10, 95% Confidence Interval [CI] = 1.14 – 3.86, p = 0.018, ABF = 0.52, and BFDP = 70.8%) and GOS at 12 months (tier 2 results, OR = 1.86, 95% CI = 0.98 – 3.52, p = 0.058, ABF = 0.72, and BFDP = 77.3%) as well as between rs10904850 (*CUBN*) with DCI (tier 2 results, OR = 0.70, 95% CI = 0.48 – 1.02, p = 0.064, ABF = 0.59, and BFDP = 71.8%).

Conclusions: Among the genes examined, our findings support a role for *CP* and *CUBN* in patient outcomes after aSAH. In an effort to translate these findings into clinical utility and improve outcomes after aSAH, additional research is needed to examine the functional roles of these genes after aSAH.

Keywords

Polymorphism; Iron; Subarachnoid Hemorrhage; Patient Outcome Assessment; Bayes Theorem

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Aneurysmal subarachnoid hemorrhage (aSAH) is a type of hemorrhagic stroke most commonly resulting from a ruptured aneurysm.[1] Although it doesn't account for a large percentage of strokes, it does account for a substantial percentage of stroke-related death and

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disability. Specifically, aSAH is responsible for between 5% and 10% of strokes, as many as 25% of all stroke-related deaths, and has a 30 day mortality of at least 30%.[1–3] Moreover, greater than 50% of aSAH survivors have long-term functional deficits.[1–3] Important gaps in our knowledge as healthcare providers includes a lack of information surrounding the biology contributing to poor outcomes in this population and the inability to comprehensively predict which patients will do poorly after aSAH. This gap in knowledge limits the potential for targeted, early interventions from the physicians, nurses, physical, occupational, and speech therapists, and other members of the stroke recovery and rehabilitation team.[4]

In an effort to identify potential biomarkers of poor outcomes after aSAH, we have focused on the iron homeostasis pathway which has been shown to be important in recovery following subarachnoid hemorrhage in both a preclinical study and a small pilot study in humans.[5,6] The scientific premise of this study was described in detail as part of our pilot work.[7] In brief, the brain is highly susceptible to damage from excessive iron, especially after neurologic injury and disruption of the blood brain barrier. In healthy humans, iron is typically bound to carrier proteins as ferric iron. However, after aSAH, catabolized blood from the subarachnoid space breaks down into several products including non-protein-bound ferrous iron (*i.e.*, free iron) that can be toxic to nearby tissues.[8–10] Because of the toxicity of free iron, we posited that genetic variability within the iron homeostasis pathway may impact iron management after a large influx of free iron into the subarachnoid space following aSAH, subsequently accounting for a potentially important portion of variability in patient outcomes in this population. Exploration of the potential relationship between genetic variability in the iron homeostasis pathway and acute and long-term patient outcomes after aSAH may support interventions to improve outcomes and reduce the burden of this substantial public health problem. The purpose of this study was to examine genetic variability of a large number of candidate genes within the iron homeostasis pathway and patient outcomes after aSAH in humans.

METHODS

Study Design

This study was a longitudinal, observational, candidate gene association study that assessed the relationship between genetic variability and patient outcomes acutely (between 0 and 14 days post-aSAH) and in the long-term (at 3 and 12 months following aSAH) using a two tier design (discovery and replication). In tier 1 (discovery), we used existing genome-wide genotype and patient outcome data collected from a larger study of aSAH participants[11] and analyzed the relationship between tagging single nucleotide polymorphisms (SNPs) in candidate genes involved in iron homeostasis and acute and long-term patient outcomes. In tier 2 (replication), we used existing patient outcome data and stored biosamples from an independent (*i.e.*, non-overlapping) test sample of participants from the same cohort to replicate findings for the top hits identified in tier 1. A depiction of the study workflow is presented in Figure 1.

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Setting and Sample

This study was approved by the Institutional Review Board of the University of Pittsburgh and informed consent was obtained from all research participants. Participants included in this study if they were prospectively recruited from UPMC Presbyterian neurovascular intensive care unit in Pittsburgh, Pennsylvania between 2000 and 2013. Our inclusion and exclusion criteria, as well as standard treatment for patients, was previously described.[7] In brief, participants were eligible for this study if they were over the age of 18 years and diagnosed with aSAH from aneurysm rupture using cerebral angiogram. Participants were ineligible for this study if their subarachnoid hemorrhage was caused by a source other than an aneurysm or if they had a history of a significant neurological disorder. Because minor allele frequencies can differ based on ancestry, and this population substructure can confound statistical analyses, the current analyses were limited to participants who self-reported their race as White.

Genotype Data Collection

Tier 1 (Discovery)—For tier 1, this study used existing genome-wide genotype data collected previously on a subset of available participants (n=244) using the Affymetrix Gene Chip Assay SNP 6.0 (Affymetrix, Santa Clara, CA, USA) as described elsewhere.[11] Our initial tier 1 analyses made the assumption that the genome-wide data had undergone thorough quality control (QC). We then realized that the QC pipeline was out of date. To ensure our findings were rigorous, we completed the tier 1 genome-wide data QC and then repeated the genetic association analyses. Genome-wide data QC was completed using PLINK Version 1.9,[12,13] R statistical software,[14] the *plinkQC* package,[15] and established QC thresholds.[16] The Affymetrix array yielded data for 904,087 variants for 244 participants. As part of our QC pipeline, 47 participants and 303,429 SNPs not meeting QC standards were removed from the genome-wide data. Details of our data QC pipeline is presented (Supplemental Material, Section I). The final genome-wide dataset consisted of 197 participants and 600,658 SNPs for extraction of tier 1 data.

Building on the pilot work for this project,[7] candidate genes (n=38) for this study were selected based on their known biological roles in iron homeostasis (Supplemental Material, Section II). For each candidate gene, tagging SNPs within the gene region ± 1 kb were identified using the UCSC Genome Browser[18] and the National Cancer Institute Division of Cancer Genetics and Epidemiology SNPchip and SNPclip tools[19] using a linkage disequilibrium threshold of r^2=0.8. Following genome-wide data QC, tagging SNP data were extracted from the genome-wide data for tier 1 analysis. None of the selected tagging SNPs located in the calreticulin (*CALR)*, ferritin light *(FTL)*, or heme carrying protein 1 (*SLC46A1)* transcript regions were available in the raw genome-wide data. Following data QC, none of the selected tagging SNPs located in the ferritin heavy *(FTH1)*, glutathione S transferase *(GSTP1)*, hemojuvelin BMP co-receptor *(HJV)*, haptoglobin *(HP)*, poly(RC) binding protein 1 *(PCBP1)*, transferrin receptor 2 (*TFR2)*, or tumor necrosis factor *(TNF)* transcript regions were available (Supplemental Material, Section II). Final tier 1 data for analysis were available for 235 tagging SNPs in 28 candidate genes (Table 1).

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Tier 2 (Replication)—For tier 2, DNA was extracted for an independent test sample of aSAH participants (n=288) from buffy coat using a simple salting out procedure.[20] Participants were genotyped for top hits identified from the earlier pre-QC tier 1 analysis using iPLEX on the MassARRAY Typer 4.0 (Agena Bioscience) platform and software[21] according to the manufacturer's standard protocols at the University of Pittsburgh Genomics Research Core. Duplicates were included on the plate with no inconsistencies identified and all genotypes were called by two blinded individuals. The assay design with primer and probe sequences are presented (Supplemental Material, Section III). Our raw genotype data included 13 SNPs for 288 participants. As part of our QC pipeline, 11 participants and 0 SNPs not meeting QC standards were removed. Details of our genotype data collection methods and QC pipeline is presented (Supplemental Material, Section III). The final tier 2 dataset consisted of 277 participants and 13 SNPs. Note that tier 2 replication SNPs were chosen, and the genotype data generated, before implementation of the updated QC pipeline in tier 1. Therefore, in the analyses presented here (*i.e.*, post-QC), some of our top associations in the post-QC tier 1 are missing tier 2 replication data because they were not present in the pre-QC tier 1 results used to select SNPs for tier 2. This limitation is discussed in more detail in the discussion section. A depiction of the study workflow is presented in Figure 1.

Patient Outcomes

This study used demographic (e.g., age, sex, race), social (e.g., marital status), clinical (e.g., severity of injury measured using the clinical grading scales Fisher grade and World Federation of Neurosurgical Societies [WFNS] grade), and treatment (e.g., intervention [clip versus coil], medications administered) data extracted from the medical record as well as patient outcome data collected during the acute and long-term phases.

Acute outcome measures used in this study were cerebral vasospasm (CV) and delayed cerebral ischemia (DCI) within 14 days of aSAH. Both outcomes are important acute complications that can occur during the recovery phase following aSAH and are indicators of potentially poor recovery in the long-term.[4] CV was defined as cerebral vessel narrowing of ≥25% during cerebral angiogram performed and measured by a neurosurgeon.[7] DCI was defined as the co-occurrence of (1) non-ischemic neurological deterioration such as an increase of ≥2 points on the National Institutes of Health Stroke Scale or a new and persistent (present for > 1 hour) neurological deficit and (2) abnormal cerebral blood flow measured using cerebral angiogram or transcranial Doppler.[7]

In the long-term outcome phase, trained study staff performed patient interviews in person or via telephone at 3 and 12 months following aSAH. To measure global functional status, the Glasgow Outcomes Scale (GOS), which has established validity in people with neurological injury, was used as a quantitative measure of participants' ability to function on a scale of 1 (death) to 5 (good recovery).[22] If participants were unable to participate in the interview, their caregiver or proxy was interviewed. Death data were obtained from the medical record, caregiver/family report, or the Social Security Death Index. All study staff involved in patient recruitment and patient outcome data collection were blinded to participant genotype.

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Statistical Analysis

Descriptive and preliminary analyses—Statistical analyses were conducted using R statistical software[14] and PLINK.[12,13] The patient outcomes of CV, DCI, and death were treated as binary (occurrence versus no occurrence) and GOS scores were dichotomized as good (4 to 5) or poor (1 to 3). Standard descriptive statistics were computed in R for all variables and data were screened for assumptions of logistic regression and examined for outliers. Preliminary analyses were conducted to identify potential confounders/covariates. HWE was evaluated for all SNPs as part of our QC pipelines described above.

Genetic association analyses—Genetic association analyses were performed in PLINK[12,13] using a logistic regression model adjusted for age, sex, and Fisher grade. Only additive models (treating SNPs as ordinal based on variant allele dosage) were considered. The Approximate Bayes Factor (ABF) was used to compute Bayesian False Discovery Probabilities (BFDP) for each SNP-phenotype association which was subsequently used to prioritize and 'flag' SNPs for replication in tier 2.[23,24] The ABF is an approximation to the Bayes Factor where an ABF <1 indicates an increase in evidence for association and an ABF >1 indicates a decrease in evidence for association.[23,24] The ABF is used to compute the BFDP which can be interpreted as a probability of false discovery regardless of power, sample size, or how many other SNPs were tested ultimately preventing the need for correction for multiple testing.[23,24]

For the purpose of transparency, replication, and future application of the study methodology in other populations, we present an expanded and detailed explanation of the genetic association analyses performed here including calculation of the ABF and the BFDP as well as the follow-up flagging approach, including formulae (Supplemental Material, Section IV). Because the direction of effect of associations observed in tier 1 are not integrated in tier 2 replication calculations, we also performed a mega-analysis combining tier 1 and tier 2 data to aid in interpretation. Lastly, in genetic association studies, ancestry can be an important confounder of results given that minor allele frequencies often differ by ancestry and race. In order to explore the influence of ancestry (versus self-reported race) and aid in interpretation of results, we performed an ancestry sensitivity analysis as described in detail (Supplemental Material, Section IV).

RESULTS

Sample Characteristics

Sample sizes for each association test varied between tier 1 and tier 2 as well as between associations depending on genotyping success rate and outcome data availability; results detailing specific samples sizes for all associations are presented (Supplemental Material, Section V). An overview of the available sample sizes and associated demographic and clinical characteristics is presented (Table 2). For tier 1, we had an overall sample size of 197 participants. Our tier 1 sample had a mean age of 54.4 (±11.3) years, was 69% female, and Fisher grades of 2, 3, or 4 accounted for 29.4%, 53.3%, and 17.3%, respectively. For tier 2, we had an overall samples size of 277 participants. Our tier 2 sample had a mean age of 54.1 (± 11.1) years, was 74.7% female, and Fisher grades of 2, 3, or 4 accounted for 52%,

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33.9%, and 14.1%, respectively. Age, WFNS scores, and marital status were similar between the groups as shown but we observed differences in treatment between the groups with 40.1% in tier 1 receiving treatment via clipping compared with only 30.3% in tier 2. In a pooled analysis of tier 1 and tier 2 participants, older age and higher Fisher grade were associated with poor outcomes at all time points. Sex was not associated with outcomes in our sample but was included as a covariate because of the importance of estrogen response elements in iron homeostasis.[25]

Genetic Association Analyses

In our tier 1 data, 235 tagging SNPs passing QC procedures were available from 28 candidate genes listed in Table 1. Sample sizes ranged from 106 to 189 and 239 to 273 in tiers 1 and 2, respectively, depending on the SNP and patient outcome of interest. Expanded details for all SNPs examined are presented (Supplemental Material, Section V).

Based on our calibration approach described above, 26 associations from tier 1 were flagged as noteworthy of investigation and replication in tier 2 (Table 3). Models presented in Table 3 include age, sex, and Fisher grade as covariates. The 26 flagged associations were from 20 unique SNPs positioned in 12 of our candidate genes including aconitase 1 (*ACO1*), amyloid precursor protein (*APP*), ceruloplasmin (*CP*), cubilin (*CUBN*), cytochrome B reductase 1 (*CYBRD1*), ferrochelatase (*FECH*), ferritin mitochondrial (*FTMT*), frataxin (*FXN*), glutaredoxin 5 (*GLRX5*), hemopexin (*HPX*), LDL receptor related protein 1 (*LRP1*), and transferrin (*TF*). An expanded results section has been included detailing all tier 1 results (Supplemental Material, Section V).

The tier 2 replication results are presented and are ranked by the tier 2 BFDP (Table 3). Six associations were flagged as increasing evidence of association from tier 1 to tier 2 based on an ABF <1 and a decrease in the BFDP. However, it should be noted that three of our top six associations had odds ratios (OR) in the opposite direction in tier 2 compared with tier 1. Specifically, rs11087985 (*APP)* with death at 3 and 12 months and rs13302577 (*ACO1)* with death at 12 months had OR <1 in tier 2, suggesting the minor allele confers protection compared with an OR >1 in tier 1, suggesting the minor allele confers risk. As a result, the signals for these associations were canceled out in the mega-analysis (Supplemental Material, Section V). The opposite effect directions observed in these associations reduces our confidence that these are actually notable signals of interest. Three of the six associations, however, were important in tier 1, tier 2, and a mega-analysis combining tier 1 and tier 2 data. Specifically, rs17838831 (*CP*) was associated with GOS at 3 months in tier 1 (OR= 2.83, 95% Confidence Interval [CI] = 1.33 – 5.99, $p = 0.007$, ABF = 0.52, BFDP = 82.2%), tier 2 (OR = 2.10, 95% CI = 1.14 – 3.86, $p = 0.018$, ABF = 0.52, BFDP = 70.8%), and a mega-analysis (OR = 2.32, 95% CI = 1.45 – 3.70, $p = 0.0004$, ABF = 0.10, BFDP = 47.8%). Similarly, we observed an association between the same SNP and poor GOS at 12 months in tier 1 (OR = 3.09, 95% CI = 1.39 – 6.87, $p = 0.006$, ABF = 0.53, BFDP = 82.7%), tier 2 (OR = 1.86, 95% CI = 0.98 – 3.52, $p = 0.058$, ABF = 0.72, BFDP = 77.3%), and a mega-analysis (OR = 2.22, 95% CI = 1.36 – 3.63, $p = 0.001$, ABF = 0.18, BFDP = 61.6%). Finally, we also observed an association between rs10904850 in *CUBN* and DCI in tier 1 (OR = 0.57, 95% CI = 0.35 – 0.93, p = 0.024, ABF = 0.48, BFDP = 81.2%), tier 2 (OR =

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0.70, 95% CI = 0.48 – 1.02, p= 0.064, ABF = 0.59, BFDP = 71.8%), and a mega-analysis (OR = 0.65, 95% CI = 0.48 – 0.87, p = 0.004, ABF = 0.11, BFDP = 50.1%). For the remaining 12 associations, we observed a tier 2 ABF >1 and an increase in the BFDP compared with tier 1 which suggests that the tier 2 data decreased the evidence for association. As with tier 1, details of tier 2 and the mega-analysis results are presented (Supplemental Material, Section V). A depiction of the study workflow, including an overview of the main results, is presented in Figure 1.

In an ancestry sensitivity analysis of tier 1 data, our top hits (rs17838831 [*CP*] with GOS at 3 and 12 months; and rs10904850 [*CUBN*] with DCI) remained top hits in the sensitivity analysis. Overall, we observed strong correlation between the results with an 82.5% concordance between the top 40 hits from the original analysis compared with the sensitivity analysis (*i.e.*, 33 of the top 40 associations were common between the two analyses). The details of this sensitivity analysis and results are presented (Supplemental Material, Section VI).

DISCUSSION

We selected the candidate genes for this study based on attributes that are relevant to our phenotypes of interest during recovery from aSAH. Of our candidate genes, the associations from the *CP* and *CUBN* genes stand out in our results suggesting potential importance to aSAH recovery. Specifically, one SNP in *CP*, rs17838831 (located on chromosome 3 at position 148939861 [GRCh37/hg19]), was important in the tier 1 discovery sample, tier 2 replication sample, and mega-analysis for GOS at 3 and 12 months. Ceruloplasmin, the protein encoded for by *CP*, is a multicopper oxidase that accounts for a majority of serum copper and is heavily involved in ferroxidase activity, mitochondrial function, and antioxidant and anti-inflammatory mechanisms.[26,27] Existing literature demonstrates the importance of ceruloplasmin in iron homeostasis. Specifically, even when total body iron stores are normal, low plasma levels of ceruloplasmin cause hypoferremia.[28] Moreover, aceruloplasminemia, a disorder of impaired iron homeostasis and classical example of the clinical features of ceruloplasmin dysfunction, is characterized by iron accumulation in microglia and neurons and increased reactive oxygen species which are important disrupted mechanisms after aSAH.[26] Further, ceruloplasmin knockout mice show evidence of increased lipid peroxidation and iron accumulation, functions important during dysregulated iron and lipid metabolism and ferroptosis after aSAH.[26,29] Outside of its role in iron homeostasis, ceruloplasmin is thought to help deliver copper to damaged areas of infection, inflammation, or trauma.[27] Although there has been less mechanistic work investigating the plausible role of ceruloplasmin after aSAH specifically, interestingly, lower ceruloplasmin levels in the cerebrospinal fluid of aSAH patients have been associated with development of deep cerebral infarcts in a small pilot study.[6] Moreover, rs17838831 has been shown to be strongly associated with plasma levels of ceruloplasmin though the allele and direction of association were not reported.[30] In tier 1, tier 2, and the mega-analysis of this current study, with each dose of the rs17838831 variant allele, participants had between a 2.10 and 2.83 times higher odds of poor GOS at 3 months. Similarly, we observed an association between the same SNP and poor GOS at 12 months in tier 1 with OR between 1.86 and 3.09. Given our findings, this SNP warrants further investigation in patient outcomes after aSAH.

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In addition, a SNP in *CUBN*, rs10904850 (located on chromosome 10 at position 16997707 [GRCh37/hg19]), was important for DCI in tier 1, tier 2, and the mega-analysis. Cubilin, the protein encoded for by *CUBN*, plays an important role in iron homeostasis by facilitating uptake of transferrin iron and clearance of hemoglobin at the kidneys.[31,32] Interestingly, in a multi-ethnic study of iron disorders, rs10904850 was associated with serum iron in African Americans but not in other ethnic groups;[33] unfortunately, similar to above, the allele and direction of this association are not clear.[33] The influence of *CUBN* in chronic kidney disease has been shown[32] but has not been investigated after aSAH specifically. After aSAH, acute kidney injury occurs in upwards of 25% of patients and even subtle decreases in creatine clearance have been associated with poor outcomes after aSAH.[34] Based on this research, it is possible that this SNP may impact iron homeostasis by influencing efficiency of reabsorption of iron in the kidney. In tier 1, tier 2, and a mega-analysis, with each dose of the rs17838831 variant allele, participants had between 0.57 and 0.70 times lower odds of DCI. Given our findings, this SNP warrants further investigation in patient outcomes after aSAH.

Of note, there are some important differences between the tier 1 and tier 2 samples. Although we controlled for age, sex, and severity of injury as measured by Fisher grade, between sample differences may be important to the interpretation of study findings. Specifically, our samples were 69% and 74.7% female for tier 1 and tier 2, respectively. Though research suggests that the overall differences in outcomes between men and women are null and we controlled for sex in our analyses,[35] estrogen is known to play a role in iron homeostasis and may be an important consideration here.[25] Moreover, an unexpected observation between the cohorts included discordant Fisher grade distributions. Specifically, Fisher grades of 2 and 3 were 29.4% and 53.3%, respectively for tier 1 and 52% and 33.9%, respectively for tier 2. Severity of injury is an important predictor of patient outcomes and we had several measures available in our cohort that were not only significantly associated with patient outcomes after aSAH, but also more similar between tier 1 and tier 2 (e.g., WFNS). Ultimately, we chose to control for Fisher grade as opposed to WFNS because it is a more direct measure of the amount of blood within the subarachnoid space, more closely associated with iron homeostasis within the body, and more relevant to the scientific premise of this study. In an attempt to explore the role of our choice of measure for severity of injury in our analyses, we repeated the tier 1 genetic association analysis controlling for WFNS rather than Fisher grade. Importantly, our associations between rs17838831 (*CP*) and GOS at 3 and 12 months and rs10904850 (*CUBN*) and DCI remained in the top hits in our sensitivity analysis. Overall, we identified an 80% concordance between the top 40 associations in tier 1 (Supplemental Material, Section VII).

Although there are many strengths to this study including embedded replication and the use of Bayesian statistical methods to aid in interpretation of results, there are some important limitations that should be acknowledged. First, while our data QC pipeline resulted in rigorous analyses of more accurate data, it did reduce our tier 1 sample size and power significantly (though our overall sample size remains quite large compared with similar patient outcomes work in the aSAH population). Specifically, the small sample size in tier 1 (*i.e.*, post-QC) may have prevented us from detecting signals of association for SNPs with small effect sizes which were therefore not carried forward for tier 2 replication. Similarly,

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because we were limited to examining SNPs available in the tier 1 genome-wide genotype data, we were not able to comprehensively examine all SNPs within our candidate genes. It is possible that SNPs located in our candidate genes, but not examined as part of this study, may be associated with patient outcomes after aSAH. For example, we had no data available for the haptoglobin gene which has received a great deal of recent attention and been shown both experimentally and clinically to be important in outcomes after aSAH.[36,37] Additionally, we lacked tier 2 replication data for some associations flagged as noteworthy in tier 1 (*i.e.*, SNPs in *HPX*, *FECH*, *LRP1*, and *GLRX5*) so we were unable to determine if all tier 1 SNP-phenotype associations could be replicated or not. Therefore, while *CP* and *CUBN* rose to the top in our analyses, we cannot necessarily eliminate the remaining list of candidate genes as plausible future targets of investigation in aSAH recovery research. Future areas of investigation should include attempting replication of associations observed in tier 1 that we were lacking tier 2 data for and exploring genetic variability of genes with inadequate tier 1 data for inclusion (Supplemental Material, Section II).

Next, this study was limited only to genetic variation of the iron homeostasis pathway. A future area of research should be to examine other omic mechanisms including levels of gene products in serum or cerebrospinal fluid and subsequent associations with patient outcomes. Finally, given that minor allele frequencies often differ based on race and ancestry, this study was limited to only participants who self-reported their race as White which restricts the generalizability of findings. A strength of this study, however, was the ability to perform a tier 1 sensitivity analysis controlling for ancestry using principal components computed from the genome-wide data. Identifying a concordance of 82.5% between the main analysis and ancestry sensitivity analysis offers some evidence of the utility of self-reported race for the Pittsburgh, Pennsylvania population when lacking genome-wide data. However, these results also underscore the importance that ancestry can play in genetic association studies as well as the critical need to replicate of findings.

CONCLUSION

Patient outcomes after aSAH vary widely and reliable and stable biomarkers to identify patients who may do poorly are needed to improve supportive care. In this study, SNPs in the *CP* and *CUBN* genes were flagged as important for future investigation. Specifically, we observed associations between rs17838831 (*CP*) and GOS at 3 months and 12 months and rs10904850 (*CUBN*) and DCI after aSAH in a discovery and replication sample, and in a mega-analysis. In order to translate this work to clinical practice in the future, functional investigation of *CP* and *CUBN* after aSAH is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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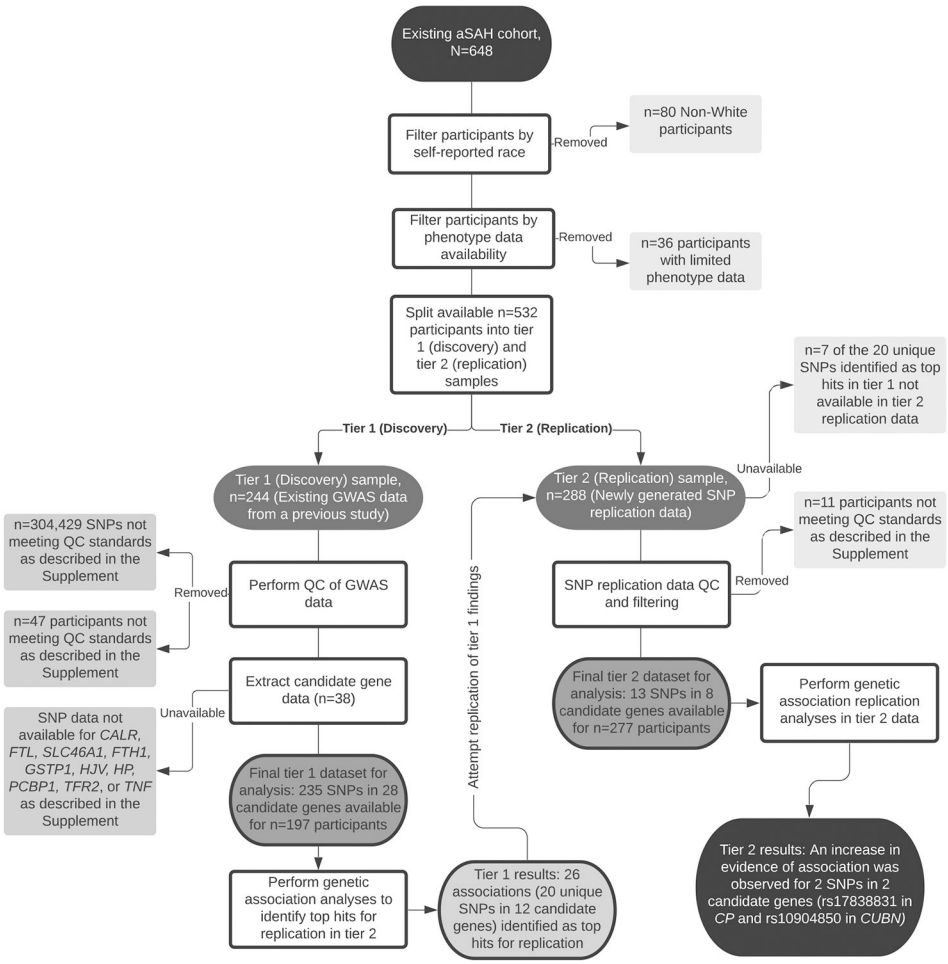

Figure 1.

Overview of study workflow and findings

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Table 1.

Iron homeostasis candidate genes examined

Gene	Name	Gene	Name
ACO1	Aconitase 1	*HMOX1*	Heme-oxygenase 1
ACO2	Aconitase 2	*HMOX2*	Heme-oxygenase 2
APP	Amyloid precursor protein	*HPX*	Hemopexin
CD163	Hemoglobin scavenger receptor	*IREB2*	Iron responsive element binding protein 2
CP	Ceruloplasmin	*LRP1*	LDL receptor related protein
CUBN	Cubulin	*PGRMC1*	Progesterone receptor membrane
CYBRD1	Duodenal cytochrome b	*SLC11A1*	Solute carrier family 11 member 1
FECH	Ferrochelatase	*SLC11A2*	Divalent metal transporter 1
FLVCR1	Feline leukemia virus subgroup C receptor	*SLC25A37*	Solute carrier family 25 member 37 (Mitoferrin 1)
FTMT	Mitochondrial ferritin	*SLC40A1*	Solute carrier family 40 member 1 (Ferroportin)
FXN	Frataxin	*SLC48A1*	Solute carrier family 48 member 1
GLRX5	Glutaredoxin 5	*STEAP3*	STEAP3 metalloreductase
HEPH	Hephaestin	*TF*	Transferrin
HFE	Human hemochromatosis protein	*TFRC*	Transferrin receptor 1

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Table 2.

Demographic and clinical characteristics for tier 1 and tier 2

Variable	Tier 1 (Discovery), n=197	Tier 2 (Replication), n=277
Age, mean years (SD)	54.4 (11.3)	54.1 (11.1)
Sex, female (n, %)	136 (69.0)	207 (74.7)
Treatment, clip (n, %)	79 (40.1)	84 (30.3)
Fisher grade (n, %)		
2	58 (29.4)	144 (52.0)
3	105 (53.3)	94 (33.9)
4	34 (17.3)	39 (14.1)
WFNS grade (n, %)		
1	104 (52.8)	143 (51.6)
2	37 (18.8)	44 (15.9)
3	10 (5.1)	25 (9.0)
4	26 (13.2)	37 (13.4)
5	20 (10.2)	28 (10.1)
Married, yes (n, %)	131 (66.8)[a]	174 (62.8)

[a]Marital status of one participant was unknown in the tier 1 sample; percentage calculated from n=196 of known marital status;

SD, standard deviation; WFNS, World Federation of Neurological Societies

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Table 3.

Results of binary logistic regression exploring associations of candidate tagging SNPs with patient outcomes while controlling for age, sex, and Fisher grade

			Tier 1 (Discovery)								Tier 2 (Replication)							
SNP	**Gene**	**Outcome**	**n, case**	**n, control**	**MAF**	**OR**	**95% CI**	***p***	**ABF**	**BFDP**	**n, case**	**n, control**	**MAF**	**OR**	**95% CI**	***p***	**ABF**	**BF**
rs17838831	*CP*	GOS3	42	120	0.15	2.83	1.33 – 5.99	0.007	0.52	82.2	69	184	0.14	2.10	1.14 – 3.86	0.018	0.52	70
rs10904850	*CUBN*	DCI	71	118	0.31	0.57	0.35 – 0.93	0.024	0.48	81.2	108	165	0.34	0.70	0.48 – 1.02	0.064	0.59	71
rs11087985	*APP*	MORT3	27	150	0.37	2.79	1.39 – 5.63	0.004	0.43	79.4	42	223	0.34	0.63	0.36 – 1.11	0.113	0.81	75
rs13302577	*ACO1*	MORT12	28	130	0.35	2.17	1.14 – 4.13	0.019	0.55	83.3	45	194	0.33	0.53	0.28 – 0.99	0.046	0.67	77
rs17838831	*CP*	GO12	32	128	0.15	3.09	1.39 – 6.87	0.006	0.53	82.7	59	180	0.14	1.86	0.98 – 3.52	0.058	0.72	77
rs1411675	*FXN*	GOS3	42	119	0.40	0.40	0.22 – 0.75	0.004	0.36	76.2	69	184	0.46	1.01	0.64 – 1.60	0.975	1.31	8
rs8177248	*TF*	DCI	71	118	0.36	1.56	1.04 – 2.41	0.031	0.47	80.8	107	165	0.37	0.81	0.56 – 1.17	0.261	1.04	8
rs11087985	*APP*	MORT12	29	131	0.37	2.21	1.15 – 4.26	0.018	0.56	83.3	45	194	0.34	0.69	0.40 – 1.19	0.182	0.91	82
rs7870295	*FXN*	GOS3	41	119	0.38	0.41	0.22 – 0.76	0.005	0.39	77.7	69	184	0.42	0.98	0.62 – 1.55	0.915	1.31	8
rs3991	*APP*	GOS3	42	119	0.24	2.14	1.19 – 3.85	0.011	0.44	80	69	184	0.22	1.09	0.63 – 1.88	0.763	1.21	8
rs3847364	*CUBN*	MORT3	27	147	0.47	0.36	0.17 – 0.76	0.007	0.52	82.3	42	222	0.45	0.83	0.50 – 1.40	0.485	1.15	8
rs3847364	*CUBN*	MORT12	29	128	0.47	0.37	0.17 – 0.78	0.009	0.54	83	45	194	0.45	0.82	0.49 – 1.38	0.453	1.13	8
rs8177224	*TF*	GOS3	42	120	0.34	1.89	1.09 – 3.28	0.024	0.52	82.5	69	183	0.32	1.03	0.63 – 1.70	0.898	1.27	8
rs12476341	*CYBRD1*	DCI	70	118	0.23	1.97	1.13 – 3.43	0.018	0.48	81.2	108	165	0.25	1.01	0.68 – 1.52	0.952	1.39	8
rs8177224	*TF*	MORT3	27	150	0.34	2.12	1.13 – 3.98	0.020	0.55	83.3	42	222	0.32	0.95	0.54 – 1.67	0.849	1.21	8
rs7870295	*FXN*	GOS12	31	126	0.38	0.41	0.21 – 0.82	0.012	0.54	82.8	59	180	0.42	0.94	0.58 – 1.52	0.794	1.27	8
rs10435797	*ACO1*	DCI	70	118	0.34	0.62	0.39 – 0.98	0.039	0.53	82.8	105	164	0.30	1.06	0.72 – 1.55	0.783	1.340	8

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			Tier 1 (Discovery)								Tier 2 (Replication)							
SNP	**Gene**	**Outcome**	**n, case**	**n, control**	**MAF**	**OR**	**95% CI**	***p***	**ABF**	**BFDP**	**n, case**	**n, control**	**MAF**	**OR**	**95% CI**	***p***	**ABF**	**BI**
rs1560550	*FTMT*	DCI	71	117	0.50	0.62	0.39 – 0.97	0.038	0.53	82.5	107	165	0.45	1.03	0.73 – 1.44	0.883	1.53	8
rs2035675	*HPX*	MORT12	28	126	0.23	2.08	1.16 – 3.75	0.015	0.48	81.3					NA			
rs533952	*FECH*	DCI	70	118	0.30	0.55	0.33 – 0.92	0.022	0.48	81.3					NA			
rs2035675	*HPX*	MORT3	26	145	0.23	2.08	1.15 – 3.77	0.015	0.49	81.6					NA			
rs10876966	*LRP1*	DCI	71	118	0.26	1.77	1.06 – 2.97	0.030	0.53	82.7					NA			
rs2306692	*LRP1*	DCI	71	115	0.17	0.44	0.23 – 0.85	0.014	0.53	82.7					NA			
rs1736439	*FECH*	DCI	67	118	0.39	0.60	0.38 – 0.97	0.038	0.54	83					NA			
rs716315	*GLRX5*	GOS12	32	128	0.45	2.27	1.17 – 4.40	0.016	0.55	83.1					NA			
rs1028932	*ACO1*	MORT3	27	150	0.49	2.11	1.12 – 3.97	0.020	0.56	83.3					NA			

SNP, single nucleotide polymorphism; DCI, delayed cerebral ischemia; GOS3, Glasgow Outcome Scale at 3 months; GOS12, Glasgow Outcome Scale at 12 months; MORT3, death at 3 months; MORT12, death at 12 months; n case, count for affected individuals (cases); n control, count for unaffected individuals (controls); OR, odds ratio; CI, confidence interval; *p, p* value based on alpha of 0.05; ABF, Approximate Bayes Factor; BFDP, Bayesian False Discovery Probability; NA, Not applicable as no tier 2 replication data existed;

[a] Increase in evidence of association in Tier 2 based on ABF <1 and drop in BFDP as well as directionally consistent OR and significant results in mega-analysis;

[b] Increase in evidence of association in Tier 2 based on ABF <1 and drop in BFDP, but discordant OR directions and no evidence of association in mega-analysis. Note: All tier 1 associations presented had a C value of <5.