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ARTICLE

Genome-wide association study of homocysteine in African Americans from the Jackson Heart Study, the Multi-Ethnic Study of Atherosclerosis, and the Coronary Artery Risk in Young Adults study

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Abstract

Homocysteine (Hcy) is a heritable biomarker for CVD, peripheral artery disease, stroke, and dementia. Little is known about genetic associations with Hcy in individuals of African ancestry. We performed a genome-wide association study for Hcy in 4927 AAs from the Jackson Heart Study (JHS), the Multi-Ethnic Study of Atherosclerosis (MESA), and the Coronary Artery Risk in Young Adults (CARDIA) study. Analyses were stratified by sex and results were meta-analyzed within and across sex. In the sex-combined meta-analysis, we observed genome-wide significant evidence ($p < 5.0 \times 10^{-8}$) for the *NOX4* locus (lead variant rs2289125, $\beta = -0.15$, $p = 5.3 \times 10^{11}$). While the *NOX4* locus was previously reported as associated with Hcy in European-American populations, rs2289125 remained genome-wide significant when conditioned on the previously reported lead variants. Previously reported genome-wide significant associations at *NOX4*, *MTR*, *CBS*, and *MMACHC* were also nominally ($p < 0.050$) replicated in AAs. Associations at the *CPS1* locus, previously reported in females only, also was replicated specifically in females in this analysis, supporting sex-specific effects for this locus. These results suggest that there may be a combination of cross-population and population-specific genetic effects, as well as differences in genetic effects between males and females, in the regulation of Hcy levels.

Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States, accounting for approximately a third of deaths. African Americans (AAs) have elevated rates of CVD and CVD mortality; approximately 47% of AAs over 19 years of age have CVD [1]. Plasma homocysteine (Hcy) is an established marker for CVD, and elevated Hcy concentrations are associated with risk of venous thrombosis, coronary heart disease, stroke, peripheral artery disease, and atherosclerosis [2–5]. Due to the influence of multiple environmental factors, the role of Hcy in CVD is complex and remains incompletely understood. Age, sex, renal function, vitamin intake, cigarette smoking, and menopause are among the main environmental determinants of Hcy levels [6]. Elevated Hcy has also been associated with increased risk of dementia and Alzheimer's disease [7]. Hcy has an important genetic component, as heritability is estimated as 47–70%, depending on the population [8–11], and genetic mutations in enzymes for Hcy metabolism have long been known to be associated with Hcy level.

Hcy is synthesized by the demethylation of methionine. There are at least two functional variants (677C > T and 1298A > C) in the gene for *methylenetetrahydrofolate reductase* (*MTHFR*), which catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate used for the remethylation of Hcy to methionine [12]. Homozygous functional variants in *cystathionine-β-synthase* (*CBS*), which catalyzes the transsulfuration reaction converting Hcy to cystathionine, are known to markedly increase Hcy level and may increase risk of stroke and

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venous thrombosis [13]. Folic acid and vitamins B6 and B12 are involved in Hcy metabolism, and supplementation with these factors can lower Hcy concentrations [14].

Genome-wide association studies (GWAS) have been conducted for Hcy in populations of European ancestry [15–19] and Filipinos [20]. All of these GWAS observed evidence for association with Hcy for common variants in the *MTHFR* locus. The largest GWAS meta-analysis yet conducted for Hcy included 44,147 subjects of European ancestry and discovered 13 loci associated with Hcy, with a risk score of these variants explaining around 5% of variation in Hcy levels. While this study by van Meurs et al. [19] did not examine differential associations by sex, some studies have observed sex-specific genetic effects on Hcy; a GWAS of 13,974 EA females from the Women's Genome Health Study (WGHS) identified and replicated the *carbamoyl-phosphate synthase 1* (*CPS1*) locus, with *CPS1* replicated only in females in the confirmatory sample [15]. The *CPS1* locus was also associated with Hcy in a GWAS of 1786 Filipino women; additional analysis in the adult offspring of the original sample confirmed that the association is only observed in females [20]. Evidence for modification of genotype effects by sex has also been seen for *MTHFR* in the Filipino population; the effect magnitude of the 677C > T polymorphism was stronger in males than females [20]. Some studies have also found sex-specific associations of Hcy with clinical endpoints like peripheral artery disease [4]. Associations of Hcy-related polymorphisms with CVD events have also been assessed; evidence for association with coronary artery disease (CAD) is mixed [19, 21], casting doubt on Hcy's causal role in CAD, but some recent studies have found Hcy-related polymorphisms were associated with stroke risk [22, 23] and peripheral artery disease [24]. Results from interventional trials of folic acid supplementation have found more promising results for stroke than CAD as well [25]. Some studies have also found associations of Hcy-associated polymorphisms with Alzheimer's disease case/control status [26, 27].

Less is known about the genetic determinants of Hcy levels in AAs. While fewer GWASs have been conducted in AAs for many quantitative traits, populations of African ancestry are particularly well suited to both discovery of novel loci and fine-mapping of loci discovered in European populations, due to generally smaller regions of linkage disequilibrium and differing allele frequencies [28]. Exclusively European-ancestry GWAS results can also not be assumed to transfer to other populations with uniform effects [29]. A recent small GWAS ($n = 1858$) in AAs and Yoruba Nigerians confirmed the association of variants in *CBS* with Hcy in a candidate gene analysis and identified a novel genome-wide association with rs6940729, an intronic variant in the gene for *CD2-associated protein* (*CD2AP*) [30]. Candidate gene analyses have also been performed. The Coronary Artery Risk Development in Young Adults (CARDIA) study performed a candidate gene analysis of variants in *MTHFR*, *CBS*, and two additional genes for Hcy enzymes before and after folic acid fortification, which was mandated by the US Food and Drug Administration in 1996. Significant associations were detected in both European Americans (EAs) and AAs in the pre-folic acid fortification era when jointly considering all tested loci; post-folic acid fortification, the independent association of the *MTHFR* 677C > T variant with Hcy was attenuated to nonsignificance in AAs [31]. In an analysis of 476 AA women from the WGHS, no association between the *MTHFR* 677C > T variant and Hcy was observed [32].

To better elucidate the genetic determinants of Hcy levels in AAs, we assessed the heritability of Hcy levels in related individuals from the Jackson Heart Study (JHS) cohort and performed a GWAS meta-analysis of AA participants from three cohorts: JHS, the Multi-Ethnic Study of Atherosclerosis (MESA), and CARDIA. We also evaluated previously reported variants associated with Hcy to assess replication in this AA population.

Materials and methods

Study populations and Hcy measurements

Jackson Heart Study

JHS is a longitudinal population-based study designed to assess the causes of the high prevalence of CVD among AAs in the Jackson, Mississippi metropolitan area. During the baseline examination period (2000–2004), 5301 self-identified AAs were recruited through random sampling from a commercial listing, participation in the Atherosclerosis Risk in Communities (ARIC) study, a structured volunteer sample designed to mirror the eligible population, and a nested family cohort. Participants were between 35 and 84 years old with the exception of the family cohort, where those ≥ 21 years old were eligible [33]. Fasting blood samples from the baseline visit were assayed for Hcy. Hcy was measured at the University of Minnesota, in conjunction with the University of Minnesota Medical Center, Fairview. Plasma Hcy was assayed by the fluorescence polarization immunoassay method, supplied by Abbott Diagnostics (Abbott Park, IL). A total of 2961 JHS participants with genotype and phenotype data were included in this study.

Multi-Ethnic Study of Atherosclerosis

MESA is a longitudinal population-based study of the prevalence, determinants, and progression of subclinical CVD. MESA includes 6814 men and women aged 45–84 without prevalent CVD at baseline (2002–2002); the

cohort is approximately 38% EA, 28% AA, 22% Hispanic American, and 12% Asian American (primarily Chinese). Participants were recruited from six field centers (Winston-Salem, NC, St. Paul, MN, Chicago, IL, Los Angeles, CA, New York, NY, and Baltimore, MD) [34, 35]. Blood samples collected at baseline were processed with the use of a standardized protocol and stored at −80 °C until analyzed. Participants were asked to fast for 12 h, avoid smoking on the morning of the exam, and avoid heavy exercise 12 h before the exam. Total plasma Hcy was measured using high-performance liquid chromatography with fluorometric detection. A total of 1628 AA MESA participants with genotype and phenotype data were included in this study.

Coronary Artery Risk in Young Adults

CARDIA is a longitudinal study of cardiovascular disease risk initiated in 1985–1986 in 5115 AA and EA men and women, then aged 18–30 years. The CARDIA sample was recruited at four sites: Birmingham, AL, Chicago, IL, Minneapolis, MN, and Oakland, CA [36, 37]. Six repeat examinations were performed; fasting serum samples that were collected in 2000 (year 15 visit, 74% retention) and stored at −70 °C were used for Hcy measurement. Hcy was measured in serum by a fluorescence polarization immunoassay (IMx Homocysteine Assay, Axis Biochemicals ASA, Oslo, Norway) using the IMx Analyzer (Abbott Diagnostics, Abbott Park, IL). A total of 338 AA CARDIA participants with genotype and phenotype data were included in this study.

Genotyping and imputation

Genome-wide genotyping on all cohorts was performed using the Affymetrix 6.0 SNP Array (Affymetrix, Santa Clara, CA). SNPs were excluded if they were genotyped successfully in less than 90% of samples; subjects were removed if less than 95% of SNPs were genotyped successfully. No SNPs were removed due to deviation from Hardy–Weinberg equilibrium (HWE) expectations because the AA population is an admixed population, which may result in departures from HWE expectations even under ideal conditions. After quality control, genotype data were available on ~800,000 autosomal single-nucleotide polymorphisms (SNPs). Imputation to the 1000 Genomes project reference panel (Phase I, Version 3, March 2012 release) was performed using MACH 1.0 and minimac [38]. Imputed variants with a minor allele frequency <1% or an imputation quality < 0.3 were excluded from all analyses. Data were available across cohorts for ~16.8 million SNPs on autosomes and chromosome X in 4927 AA subjects with Hcy measures. All participants gave written informed consent prior to their inclusion in the studies, and all studies were overseen by the relevant Institutional Review Boards.

Data analysis

Hcy levels were highly skewed and simple transformations (e.g., using natural logarithm) did not remedy numerous outliers. Hence, we used inverse normalized residuals, obtained from modeling Hcy on established nongenetic risk factors: age, body mass index (BMI), and current smoking status. These residuals were used as our outcome measure to meet linear model assumptions of normally distributed residuals and to minimize influence of extreme values when modeling Hcy on genotype. Residuals were calculated separately by cohort and by sex. For heritability analyses, residuals were calculated for JHS participants in males and females together, adjusting for sex, to maximize sample size and avoid dividing families. Heritability estimation was performed using SOLAR 6.6.2 [6] using variance component-based methods. We assessed associations between Hcy (using normalized residuals) and individual SNPs under the additive model, with covariate adjustment for the first 10 principal components calculated using the program EIGENSTRAT [39] to control for potential population substructure. The association analyses were performed separately by cohort and by sex. Association analyses were performed using linear models implemented in MACH2QTL v.1.13 for MESA and CARDIA; [38] a mixed model approach (EMMAX [40]) with adjustment for a kinship matrix implemented in Efficient and Parallelizable Association Container Toolbox (EPACTS) 3.2.6 was used for JHS due to the inclusion of related individuals in both males-only and females-only analyses.

After obtaining cohort-specific results, we performed fixed-effect, inverse variance-weighted meta-analyses across cohorts using the METAL software [41], first separately by sex and then combining samples from both males and females. We used a conventional GWAS significance threshold ($p = 5.0 \times 10^{-8}$) to maintain an overall experimental type I error rate of ~5%. Finally, conditional analyses, where association analyses are repeated with additional covariate adjustment for the top SNP in the region, were performed for the *NOX4* region in order to assess evidence for multiple independent signals.

Replication of variants identified in previous GWAS was also attempted. Only the lead variant at each locus was included for each study. We did not have good-quality imputation data for the *FGF21* intronic variant rs838133 ($r^2 < 0.3$ in all cohorts), previously reported as the *FUT2* locus [19], and hence this variant is not included in Table 3. While not genome-wide significant, we also included rs28635199 ($\beta = -0.030$, $p = 5.7 \times 10^{-6}$), the top variant at the *CBS* locus in a previous GWAS in individuals of

Table 1 Demographic characteristics of participants stratified by study

	CARDIA		JHS		MESA	
	Males	Females	Males	Females	Males	Females
N	116	222	1134	1827	738	890
Current smoker, %	30	17	18	11	20	17
Age, years, mean ± SD	40 ± 3.7	40 ± 3.9	54 ± 13	55 ± 13	63 ± 10	62 ± 10
BMI, kg/m^2, mean ± SD	29 ± 4.8	32 ± 7.6	30 ± 6.3	33 ± 7.8	29 ± 4.7	31 ± 6.5
Homocysteine, μmol/L, mean ± SD	10 ± 2.6	8.2 ± 2.1	10 ± 3.3	8.9 ± 4.0	11 ± 5.0	9.0 ± 3.2
Homocysteine, μmol/L, median (Q1, Q3)	9.4 (8.3, 11)	7.8 (6.8, 8.9)	9.5 (8.1, 11)	8.2 (6.9, 9.9)	9.7 (8.1, 12)	8.4 (7.1, 10)

Studies include the Coronary Artery Risk Development in Young Adults (CARDIA) study, Jackson Heart Study (JHS), and the Multi-Ethnic Study of Atherosclerosis (MESA)

SD standard deviation, *Q1* quartile 1, *Q3* quartile 3

African descent [30]. One published GWAS identified no genome-wide significant loci and thus was not included here [18]. If there were different lead variants in different studies at the same locus, both were included; several of the variants listed are in modest linkage disequilibrium (LD; $r^2 > 0.2$ in 1000 Genomes Project data) in Europeans, the population in which the majority of variants were reported (rs9369898 and rs4267943 at *MUT*, rs154657 and rs1126464 at *DPEP1*, and rs6586282 with both rs28635199 and rs234709 at *CBS*). However, only the two reported *NOX4* variants (rs7130284 and rs11018628) are in high LD in Europeans ($r^2 > 0.7$), though they are not in significant LD in African populations ($r^2 < 0.2$). Only the *MUT* variants and two of the *CBS* variants (rs6586282 and rs28635199) are also in modest LD ($r^2 > 0.2$) in African populations.

Results

Demographic characteristics of the 4927 AA study participants are presented in Table 1. Consistent with prior reports, Hcy levels were higher in males. A higher proportion of males currently smoked while females had, on average, higher BMI. The average age of participants was near 40 years in CARDIA, near 50 years in JHS, and near 60 years in MESA. We assessed the heritability of Hcy in related males and females from JHS, which included a nested family study component. Inverse normalized residuals adjusted for age, sex, BMI, and current smoking status were analyzed in 1355 related individuals. Heritability was estimated as 28% (standard error 7.2%, $p = 1.7 \times 10^{-5}$) for Hcy; this significant heritability indicates that common genetic variants may contribute to variation in Hcy levels in AAs, which we assessed through a GWAS.

Three meta-analyses (combined-sex, males-only, and females-only) of genome-wide association results for inverse normalized Hcy levels were conducted. Lead variants are displayed in Table 2, and association statistics for these variants for each individual cohort are presented in Supplementary Table 1. Considering males and females separately, no significant heterogeneity between cohorts was observed for any of the lead variants ($I^2 \leq 18\%$, $p \geq 0.30$). All nominally significant variants from each meta-analysis ($p < 1.0 \times 10^{-6}$) are displayed in Supplementary Table 2. Manhattan plots and quantile–quantile plots for each meta-analysis are displayed in Supplementary Figs. 1 and 2. In our combined-sex meta-analysis, genome-wide significant evidence ($p < 5.0 \times 10^{-8}$) for association with Hcy was observed for chromosome 11 SNPs at the *NADPH oxidase 4 (NOX4)* locus (Supplementary Fig. 3a). The lead variant at this locus was rs2289125 ($\beta = -0.15$, $p = 5.3 \times 10^{-11}$), which is either an intronic or a 5' untranslated region variant depending on the *NOX4* isoform. This variant is not in strong linkage disequilibrium (LD) ($r^2 < 0.2$ in European and African populations) with a *NOX4* variant (rs7130284) previously reported to be associated with Hcy in an EA meta-analysis of males and females [19] or in LD with another variant (rs11018628) reported in a genome-wide analysis of EA females from the WGHS [15]. These two previously reported lead *NOX4* variants are in strong LD ($r^2 = 0.7$, D' = 0.9) in Europeans from the 1000 Genomes project but are not in strong LD in African participants ($r^2 < 0.2$). As noted in Table 3, rs7130284 does not show compelling evidence for association with Hcy in the AA participants included in our meta-analysis ($\beta = -0.069$, $p = 0.023$) but the rs11018628 variant reported in WGHS approached borderline significance ($\beta = -0.14$, $p = 2.0 \times 10^{-6}$). Conditioning on rs7130284 only modestly attenuated the associations of rs2289125 ($\beta = -0.15$, $p = 2.8 \times 10^{-10}$) and rs11018628 ($\beta = 0.12$, $p = 1.1 \times 10^{-4}$) with Hcy. Similarly, when conditioned on rs11018628, rs2289125 was still genome-wide significant ($\beta = -0.14$, $p = 1.1 \times$

Table 2 Lead variants associated with homocysteine (Hcy) levels for sex-combined, males-only, and females-only meta-analysis across three cohorts

Chr	SNP	Base pair	Nearest genes	Annotation	Effect allele	Alternate allele	Effect allele frequency	Both males and females		Males		Females	
								β (SE)	*P*-value	β (SE)	*P*-value	β (SE)	*P*-value
11	rs2289125	89224453	*NOX4*	Intronic	A	C	0.69	−0.15 (0.023)	5.3×10^{-11}	−0.16 (0.037)	1.3×10^{-5}	−0.15 (0.030)	9.2×10^{-7}
8	rs144009661	20267580	*LZTS1*	Intergenic	A	G	0.98	0.29 (0.076)	1.3×10^{-4}	0.65 (0.12)	6.3×10^{-8}	0.052 (0.098)	0.59
11	rs116810794	89352758	*NOX4*	Intergenic	A	G	0.93	−0.24 (0.044)	3.8×10^{-8}	−0.18 (0.071)	0.010	−0.28 (0.057)	5.9×10^{-7}

SE standard error

10^{-9}), and the association of rs7130284 with Hcy was nonsignificant ($\beta = 0.0017$, $p = 0.96$).

Conditioning on rs2289125 reduced the remaining signal at *NOX4* below genome-wide significance (Supplementary Fig. 3b), though some residual signals remained. The lead variant at *NOX4* after conditioning on rs2289125 was rs145661799 ($\beta = -0.34$, $p = 3.1 \times 10^{-6}$); after conditioning on both rs2289125 and rs145661799, the lead variant was rs10765210 ($\beta = 0.14$, $p = 5.4 \times 10^{-7}$). Variant rs10765210 was less significant when conditioned on rs2289125 alone ($\beta = 0.11$, $p = 8.4 \times 10^{-5}$) and in the original analysis ($\beta = 0.13$, $p = 2.3 \times 10^{-6}$). These results may indicate the presence of multiple distinct signals at the locus.

In males-only analyses, we observed no genome-wide significant associations. A near genome-wide significant signal was observed for intergenic variant rs144009661 on chromosome 8 ($\beta = 0.65$, $p = 6.3 \times 10^{-8}$, Supplementary Fig. 4); the nearest gene is *leucine zipper, putative tumor suppressor 1* (*LZTS1*). No significant evidence for association was observed at this locus in females ($\beta = 0.052$, $p = 0.59$). In females-only analyses, there were also no genome-wide significant variants. The lead variant was rs116810794 ($\beta = -0.28$, $p = 5.9 \times 10^{-7}$, Supplementary Fig. 5a), near *NOX4*. Adjustment for the lead *NOX4* variant in sex-combined analyses, rs2289125, attenuated this association in females ($\beta = -0.22$, $p = 1.5 \times 10^{-4}$, Supplementary Fig. 5b); we would also note that rs2289125 has better imputation quality across cohorts (0.82–0.87) than rs116810794 (0.67–0.70), increasing our confidence in the rs2289125 association.

Comparison with other previously identified GWAS loci

Association statistics for 20 previously identified genome-wide significant variants for Hcy from six published studies [15–17, 19, 20, 30] are listed in Table 3; meta-analysis results from combined males and females, males-only, and females-only are displayed. As the Hcy phenotype transformations differed between studies, β values cannot be directly compared. However, out of 20 previously reported variants, 18 in our meta-analysis had the same direction of effect compared to previous studies of both males and females; 16 had the same direction of effect in the males-only analyses and 15 in females-only analyses (Table 3). Of these variants, 8 were at least nominally ($p < 0.050$) associated in the meta-analysis of both males and females, with 4 of these same variants nominally associated in the males-only meta-analysis and 7 associated in the females-only meta-analysis. Along with the two *NOX4* polymorphisms previously discussed, all 3 previously reported lead variants at the *CBS* locus (rs28635199, rs6586282, and rs234709)

Table 3 Evidence of association for inverse normalized homocysteine (Hcy) levels at previously reported genome-wide association study loci

Author	Chr	Nearest gene	SNP	Original study				Current study						
								EAF	Both males and females		Males		Females	
				Effect allele	EAF	β	Population		β (SE)	*P*-value	β (SE)	*P*-value	β (SE)	*P*-value
Lange, et al. (F)	1	*MTHFR*	rs1801133	A	0.22	0.083	Fil							
Tanaka, et al. (MF)	1	*MTHFR*	rs1801133	A	0.47	1.3	Eur	0.14	0.052 (0.031)	0.094	0.039 (0.049)	0.42	0.061 (0.040)	0.13
van Meurs, et al. (MF)	1	*MTHFR*	rs1801133	A	0.34	0.16	Eur							
Pare, et al. (F)	1	*MTHFR*	rs1801133	A	0.33	0.048	Eur							
Hazra, et al. (MF)	1	*MTHFR*	rs12085006	A	0.43	0.04	Eur	0.32	0.0090 (0.023)	0.69	0.022 (0.035)	0.53	−0.0003 (0.030)	0.99
van Meurs, et al. (MF)	1	*MMACHC*	rs4660306	T	0.33	0.044	Eur	0.19	0.053 (0.026)	**0.043**	0.14 (0.042)	**6.0×10^{-4}**	−0.0038 (0.033)	0.91
van Meurs, et al. (MF)	1	*MTR*	rs2275565	T	0.21	−0.054	Eur	0.46	−0.060 (0.020)	**2.7×10^{-3}**	−0.043 (0.032)	0.18	−0.072 (0.026)	**5.7×10^{-3}**
Lange, LA, et al. (F)	2	*CPS1*	rs7422339 (now rs1047891)	A	0.24	0.076	Fil							
Pare, G, et al. (F)	2	*CPS1*	rs7422339 (now rs1047891)	A	0.31	0.027	Eur	0.36	0.037 (0.022)	0.092	0.0018 (0.035)	0.96	0.059 (0.028)	**0.034**
van Meurs, et al. (MF)	2	*CPS1*	rs7422339 (now rs1047891)	A	0.33	0.086	Eur							
van Meurs, et al. (MF)	6	*SLC17A3*	rs548987	C	0.13	0.060	Eur	0.39	0.037 (0.021)	0.078	0.056 (0.032)	0.081	0.023 (0.027)	0.40
Kim, S, et al. (MF)	6	*CD2AP*	rs6940729	T	0.48	−0.027	AA	0.46	−0.0064 (0.021)	0.75	0.018 (0.033)	0.59	−0.022 (0.026)	0.40
van Meurs, et al. (MF)	6	*MUT*	rs9369898	A	0.62	0.045	Eur	0.59	0.023 (0.021)	0.26	0.015 (0.032)	0.65	0.029 (0.027)	0.27
Pare, G, et al. (F)	6	*MUT*	rs4267943	A	0.36	0.024	Eur	0.39	0.025 (0.021)	0.23	−0.0037 (0.033)	0.91	0.044 (0.027)	0.10
van Meurs, et al. (MF)	7	*GTPBP10*	rs42648	A	0.40	−0.040	Eur	0.58	0.048 (0.021)	**0.019**	0.055 (0.032)	0.086	0.043 (0.027)	0.11
Hazra, et al. (MF)	9	*GABBR2*	rs10986018	C	0.22	−0.06	Eur	0.12	−0.0085 (0.031)	0.79	−0.0058 (0.049)	0.91	−0.010 (0.040)	0.80
van Meurs, et al. (MF)	10	*CUBN*	rs1801222	A	0.34	0.045	Eur	0.20	0.033 (0.025)	0.19	−0.0058 (0.040)	0.89	0.059 (0.033)	0.070
van Meurs, et al. (MF)	11	*NOX4*	rs7130284	T	0.07	−0.12	Eur	0.14	−0.069 (0.030)	**0.023**	−0.045 (0.047)	0.34	−0.084 (0.039)	**0.030**
Pare, G, et al. (F)	11	*NOX4*	rs11018628	G	0.07	−0.050	Eur	0.15	−0.14 (0.029)	**2.0×10^{-6}**	−0.11 (0.045)	**0.014**	−0.15 (0.037)	**3.8×10^{-5}**
van Meurs, et al. (MF)	12	*HNF1A*	rs2251468	A	0.65	−0.051	Eur	0.89	−0.016 (0.032)	0.62	−0.065 (0.051)	0.20	0.017 (0.041)	0.68
Pare, G, et al. (F)	16	*DPEP1*	rs1126464	C	0.24	−0.031	Eur	0.17	−0.035 (0.035)	0.32	−0.028 (0.055)	0.62	−0.039 (0.045)	0.38
	16	*DPEP1*	rs154657	A	0.47	0.096	Eur	0.11	−0.016 (0.033)	0.62	0.029 (0.052)	0.57	−0.046 (0.042)	0.27

Table 3 (continued)

Author	Chr	Nearest gene	SNP	Original study			Current study						
								EAF	Both males and females		Males		Females	
				Effect allele	EAF	Population			β (SE)	*P*-value	β (SE)	*P*-value	β (SE)	*P*-value
van Meurs, et al. (MF)														
Kim, S, et al. (MF)	21	*CBS*	rs28635199	C	0.17	−0.030 AA		0.17	−0.10 (0.028)	$\mathbf{2.6 \times 10^{-4}}$	−0.13 (0.044)	$\mathbf{4.0 \times 10^{-3}}$	−0.084 (0.036)	**0.018**
Pare, G, et al. (F)	21	*CBS*	rs6586282	A	0.18	−0.030 Eur		0.23	−0.098 (0.024)	$\mathbf{6.1 \times 10^{-5}}$	−0.097 (0.039)	**0.013**	−0.098 (0.031)	$\mathbf{1.7 \times 10^{-3}}$
van Meurs, et al. (MF)	21	*CBS*	rs234709	T	0.45	−0.072 Eur		0.79	−0.071 (0.026)	$\mathbf{6.5 \times 10^{-3}}$	−0.050 (0.040)	0.21	−0.086 (0.034)	**0.012**

(F) indicates the prior association was reported in females only; (MF) indicates the association was reported in both males and females

Nominally significant associations ($p < 0.050$) are highlighted in bold

AA African American, *Eur* European, *Fil* Filipino, *EAF* effect allele frequency, *SE* standard error

[15, 19, 30] were associated ($p \leq 6.5 \times 10^{-3}$) with Hcy in the sex-combined meta-analysis. The *5-methyltetrahydrofolate-homocysteine methyltransferase* (*MTR*) variant rs2275565 was also associated with Hcy levels in males and females ($p = 2.7 \times 10^{-3}$).

In addition to the variants which replicated in the meta-analysis of both males and females, we observed sex-specific associations for some of these variants. There was an association between *CPS1* missense variant rs7422339 (now renamed rs1047891) and Hcy in females ($p = 0.034$), but no evidence for this association was observed in males ($p = 0.96$), consistent with earlier reports [15, 20]. The *methylmalonic aciduria (cobalamin deficiency) cblC type, with homocystinuria* (*MMACHC*) locus (rs4660306) nominally associated with Hcy in the sex-combined meta-analysis ($\beta = 0.053$, $p = 0.043$); this effect was entirely driven by males ($\beta = 0.14$, $p = 6.0 \times 10^{-4}$) and absent in females ($\beta = -0.0038$, $p = 0.91$).

One variant, rs42648 near *GTP binding protein 10* (*GTPBP10*), was nominally significant in the sex-combined meta-analysis ($p = 0.019$) but had the opposite direction of effect as that previously reported in a meta-analysis in European populations [19]. This variant differs in allele frequency between European and African populations (59% for European populations vs. 39% for African populations for the G allele in 1000 Genomes) and has more variants in strong LD ($r^2 > 0.8$) in European ($n = 21$) as opposed to African ($n = 4$) populations in 1000 Genomes project data; differing causal variants might explain this opposite direction of effect.

While the estimated direction of effect was the same, the well-studied non-synonymous polymorphism, rs1801133, in *MTHFR* at chromosome 1 did not reach significance ($p = 0.094$) in our analysis; this may in part be due to the lower effect allele frequency (14%) observed in these AA cohorts as opposed to the European (33–47%) [15, 16, 19] and Filipino cohorts (22%) [20].

Discussion

To our knowledge, this study in 4927 AAs from three community-based cohorts represents the largest GWAS for Hcy concentration in individuals of African descent yet conducted. We observed a significant heritable component for Hcy levels in JHS (estimated heritability 28%); this is somewhat lower than previous heritability estimates of 47–70%, depending on the population [8–11], but still supports the importance of assessing common variants for their contribution to Hcy levels. We observed genome-wide significant evidence for association at *NOX4*, a gene previously identified in a GWAS in EA females from the WGHS [15] and in a meta-analysis of EA males and

females [19]. Previously identified variants near *NOX4*, *MTR*, *CBS*, and *MMACHC* were replicated in the sex-combined meta-analysis, with *CPS1* missense variant rs1047891 additionally replicated in females.

The most strongly associated SNP in the current study (rs2289125) is in *NOX4*; the *NOX4* locus has been previously associated with Hcy in two analyses in European-ancestry populations, including a meta-analysis of males and females [19] and an analysis of females only in the WGHS [15]. Associations between Hcy and *NOX4* have not been reported previously in an AA population. The NADPH oxidases are a family of enzymes that serve as a significant source of cellular reactive oxygen species (ROS), which play roles in both homeostasis and pathogenesis. Low to moderate levels of ROS contribute to important functions such as cell signaling, apoptosis, cell differentiation, fibrosis, and cellular migration; elevated levels of ROS are associated with cell-mediated inflammation and a variety of diseases including CVD [42, 43]. The *NOX4* gene is expressed in a wide array of tissue and cell types including cardiomyocytes, endothelial cells, and vascular smooth muscle cells [43]. NOX4 expression may be upregulated by oxidized lipids, endoplasmic reticulum stress, injury to arterial walls, and ischemia, among other factors, with elevated expression reported in patients with atherosclerosis, hypertension, heart failure, stroke, and diabetes [44]. Upregulation of NOX2 and NOX4 may also contribute to Hcy-mediated apoptosis of endothelial cells [45]. Neither rs2289125 nor other variants in moderate LD ($r^2 > 0.6$) in African populations are listed as significant expression quantitative trait loci for NOX4 or any other genes in any tissues tested in HaploReg v4.1 [46]. However, rs2289125 is located in open chromatin in most tissues as tested by DNAse hypersensitivity assays and does have evidence for protein binding in ENCODE chromatin immunoprecipitation sequencing experiments by the transcription factors TATA-binding protein (TBP) and yin-yang-1 (YY1) and by TATA-box binding protein associated factor (TAF7), a subunit of the complex required for RNA polymerase II transcription. rs2289125 has also recently been found to associate with pulse pressure in European populations and increased NOX4 expression in endothelial cells [47]. In our analyses the signal at rs2289125 appeared distinct from the *NOX4* associations previously reported in Europeans for homocysteine, as it remained genome-wide significant when conditioning on either of the European *NOX4* variants (rs7130284 and rs11018628).

We also analyzed previously reported Hcy-associated variants in our AA meta-analysis to assess replication in this population (Table 3). Two of the listed variants, rs6940729 in *CD2AP* and rs28635199 near *CBS*, were identified in an African-ancestry population GWAS [30]. This *CBS* variant, as well as the lead *CBS* variants in two European population studies [15, 19], replicated in our meta-analysis, but the novel variant in *CD2AP* reported by Kim et al. [30] did not replicate here ($p > 0.05$), although the direction of effect was concordant. In the sex-combined meta-analysis, we also observed evidence of association at *MTR* (rs2275565), which is a key enzyme for methionine biosynthesis. Nominal evidence of association was also observed at the less well-studied locus (*MMACHC*, rs4660306); this gene is associated with a disorder of vitamin B12 metabolism characterized by accumulation of Hcy in serum [48].

The well-known *MTHFR* 677C > T polymorphism was not associated with Hcy in our meta-analysis. Similarly, this polymorphism was not associated with Hcy levels in a previous candidate gene analysis in CARDIA post-folic acid fortification [31] nor in an analysis from African-ancestry participants in the WGHS [32]; our study had a greatly increased sample size compared with these studies, which each had < 1000 participants. We hypothesize that the *MTHFR* variant's effect is attenuated in our cohorts due to high folic acid consumption, as all tested Hcy levels after the introduction of widespread folic acid fortification of cereal grains (implemented in 1998) [49]. This is in contrast to the largest available European meta-analysis, which included cohorts with Hcy assessment both before and after this time period. The *MTHFR* polymorphism has been found in a previous comparison of European and Asian-ancestry populations to have a lower effect size for both homocysteine and stroke risk in populations with higher average folate levels [50]. Greater reductions in stroke risk with folic acid supplementation interventional trials is observed in individuals with lower initial plasma folate levels [25]. Also, the minor allele frequency for this polymorphism is lower in our meta-analysis (14%) and in African populations in the 1000 Genomes project data (11%) than is generally reported in Europeans (35% in 1000 Genomes data), reducing power to detect effects of this *MTHFR* variant and making it likely a less important contributor to variation in Hcy levels in AAs as compared to European populations.

Sex-specific differences in circulating Hcy have been reported, but lower concentrations in females may be characteristic of the premenopausal years only [6, 51]. Higher levels in males may be partially explained by differences in muscle mass, as reflected by serum creatinine concentrations, and estrogen levels [51]. Reduced plasma Hcy concentrations in pregnant women and older women using estrogen supplementation supports the role of estrogen in these sex-specific differences in Hcy [6, 51]. Such differences may contribute to the sex-specific effects previously observed for genes involved in the regulation of Hcy metabolism; based on these data, we conducted sex-stratified analyses. While no genome-wide significant

variants were identified in analyses of males or females only, some evidence of sex-specific effects was observed for previously reported variants near *MMACHC* and in *CPS1*. The *MMACHC* variant was significant in males only, with the observed sex-combined association driven entirely by male participants (in females the effect was non-significant and in the opposite direction). Sex-stratified results were not presented in the European meta-analysis by van Meurs et al [19]., and hence it is unclear if this differential effect has been observed previously. As had been previously reported in European and Filipino populations [15, 20], no evidence for the *CPS1* locus was observed in males, but the locus did replicate in females in our AA meta-analysis. Other variants at *CPS1* have been reported to impact coronary artery disease risk in females only [52].

The strengths of this analysis include the largest population of AA subjects in a genetic analysis of Hcy [30–32], though sample size is limited compared to European-focused GWAS meta-analyses [19]. Sex-stratified analyses allowed assessment of sex-specific associations with Hcy; sex-stratified analyses are not available in previous genetic analyses in AAs [30–32] or the largest European GWAS meta-analysis [19]. We also assessed the relevance of Hcy associations discovered in other ancestries to an AA population. Finally, all populations included in our analysis collected Hcy data post widespread folic acid fortification in the United States, which is known to affect a population's blood folate and homocysteine levels and impact the effect size of the *MTHFR* polymorphism [50]. More than 50 countries have adopted folic acid fortification for commercially available foods such as wheat flour [49], and hence this provides a more current view of the genetics of homocysteine. Limitations of our analysis include a lack of assessment of interactions with clinical factors other than sex relevant to homocysteine levels, such as menopause status, use of hormone replacement therapy, kidney function, and intake of folate and vitamins B6 and B12 [6]. We also did not have data to perform Mendelian randomization analyses with Hcy-associated SNPs for factors such as stroke and Alzheimer's disease in AAs. Current data also suggest that modest elevations in Hcy are not causally related to coronary artery disease [19, 25], limiting the relevance of our results to this outcome.

In conclusion, our findings provide additional insight regarding the genes that may be involved across and within populations in the regulation of Hcy levels. While the *NOX4* locus affects Hcy levels in both AAs and EAs, the lead variants are distinct, suggesting multiple signals at this locus in AAs. Other previously identified loci, including *MTR*, *CBS*, *MMACHC*, and *CPS1* in females, also impact Hcy levels in AA populations. The effects of a well-established *MTHFR* variant appear to be considerably smaller in AAs. Our findings further highlight the need to conduct GWAS in non-European populations and to assess potential sex-specific genetic association signals for biomarkers that differ significantly between males and females.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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