

Genome-wide and gene-centric analyses of circulating myeloperoxidase levels in the charge and care consortia

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Increased systemic levels of myeloperoxidase (MPO) are associated with the risk of coronary artery disease (CAD). To identify the genetic factors that are associated with circulating MPO levels, we carried out a genome-wide association study (GWAS) and a gene-centric analysis in subjects of European ancestry and African Americans (AAs). A locus on chromosome 1q31.1 containing the complement factor H (*CFH*) gene was strongly associated with serum MPO levels in 9305 subjects of European ancestry (lead SNP rs800292; $P = 4.89 \times 10^{-41}$) and in 1690 AA subjects (rs505102; $P = 1.05 \times 10^{-8}$). Gene-centric analyses in 8335 subjects of European ancestry additionally identified two rare *MPO* coding sequence variants that were associated with serum MPO levels (rs28730837, $P = 5.21 \times 10^{-12}$; rs35897051, $P = 3.32 \times 10^{-8}$). A GWAS for plasma MPO levels in 9260 European ancestry subjects identified a chromosome 17q22 region near *MPO* that was significantly associated (lead SNP rs6503905; $P = 2.94 \times 10^{-12}$), but the *CFH* locus did not exhibit evidence of association with plasma MPO levels. Functional analyses revealed that rs800292 was associated with levels of complement proteins in serum. Variants at chromosome 17q22 also had pleiotropic *cis* effects on gene expression. In a case-control analysis of ~80 000 subjects from CARDIoGRAM, none of the identified single-nucleotide polymorphisms (SNPs) were associated with CAD. These results suggest that distinct genetic factors regulate serum and plasma MPO levels, which may have relevance for various acute and chronic inflammatory disorders. The clinical implications for CAD and a better understanding of the functional basis for the association of *CFH* and *MPO* variants with circulating MPO levels require further study.

INTRODUCTION

Myeloperoxidase (MPO) is a lysosomal enzyme stored within the azurophilic granules of circulating neutrophils, monocytes and tissue macrophages (1). It is released upon leukocyte (both neutrophils and monocytes) activation and generates various reactive oxidants and free radicals that play important roles in killing invading parasites and pathogens. The same MPO-derived oxidants have also been implicated in the formation of atherogenic low-density lipoprotein particles, the development of dysfunctional high-density lipoprotein (HDL) particles, catalytic consumption of nitric oxide, vascular endothelial injury, and development of atherosclerotic plaque and its clinical sequelae (2–4). Furthermore, high circulating levels of MPO, as measured in serum, plasma or leukocytes, predict major adverse cardiac events in healthy individuals, and in patients with coronary artery disease (CAD) or heart failure (5–8). In addition, MPO has been shown to be positively correlated with traditional and inflammatory CAD risk factors such as age, sex, blood pressure, body mass index, cigarette smoking, glucose, white blood cell count and C-reactive protein levels (9–11).

Heritability estimates for serum MPO range from 25 to 30% (9,12), suggesting that variation in serum MPO levels has a significant genetic component. Loss-of-function mutations in the *MPO* gene that result in total or partial MPO deficiency are rare in the population (~1 in 2000) (13,14) and have been associated with increased susceptibility to infection as well as protection from CAD (15). Candidate gene studies have also identified common variants of *MPO* that have been associated with CAD as well as circulating MPO levels (16–19). However, a more complete understanding of the genetic factors controlling circulating MPO levels is still lacking. Therefore, the aim of the present study was to use large-scale unbiased genome-wide and targeted

gene-centric analyses to identify loci controlling serum and plasma MPO levels and to determine whether MPO-associated variants influence the risk of CAD. To our knowledge, these analyses would represent the first genome-wide association study (GWAS) for circulating MPO levels.

RESULTS

GWAS for serum MPO levels

We first carried out a meta-analysis of GWAS data for serum MPO levels in 9305 subjects of European ancestry. The characteristics of the Cleveland Clinic GeneBank (GeneBank), the Coronary Artery Risk Development in Young Adults (CARDIA) Study, Cardiovascular Health Study (CHS), and Framingham Heart Study (FHS) cohorts and datasets used for these analyses are summarized in Table 1. The observed variability in MPO levels, which could have also been influenced by either sample storage-related effects or acute minor infections at the time of blood collection, made it difficult to harmonize MPO assays across cohorts. Therefore, single-nucleotide polymorphism (SNP) association results were combined using an effective sample-weighted Z-score meta-analysis method. The quantile–quantile (Q–Q) and Manhattan plots for serum MPO levels are presented in Figure 1A and B. The observed genomic control factors in the four discovery cohorts were near unity (GeneBank = 0.999; CHS = 1.015; FHS = 1.004; CARDIA = 1.004), suggesting that the GWAS results were not strongly confounded by underlying population stratification. SNPs at three loci on chromosomes 1q31.3, 6p21.32 and 20p13 exceeded the pre-specified threshold for genome-wide significance (5.0×10^{-8}). The characteristics of the lead SNPs at these three loci and their association with serum MPO levels are summarized

Table 1. Description of cohorts and datasets used in this study

Cohort	Ethnicity	N	Age (year)	M/F	MPO levels (pmol/l)	Source (Assay)	Genotyping platform	Number of SNPs
GeneBank	European	2189	62.5 ± 11.2	1554/635	760.1 ± 719.5	Serum (CardioMPO)	Affymetrix 6.0 (Imputed)	2 421 770
	European	2191	62.5 ± 11.2	1555/636	225.5 ± 293.9	Plasma (CardioMPO)	Affymetrix 6.0 (Imputed)	2 421 770
CHS	European	2667	71.9 ± 5.0	1028/1639	345.6 ± 280.1	Serum (CardioMPO)	Illumina CNV370 (Imputed)	2 397 181
	European	3085	72.2 ± 5.2	1327/1758	353.1 ± 318.6	Serum (CardioMPO)	IBCv2 SNP array	46 423
FHS	AAs	643	72.8 ± 5.6	238/405	382.1 ± 330.7	Serum (CardioMPO)	IBCv2 SNP array	47 046
	European	2940	61.0 ± 9.5	1359/1581	334.3 ± 218.8	Serum (OXIS)	Affymetrix 500 K (Imputed)	2 486 777
CARDIA	European	2660	61.0 ± 9.5	1218/1442	335.4 ± 220.1	Serum (OXIS)	IBCv2 SNP array	46 930
	European	1509	25.6 ± 3.3	701/808	137.0 ± 97.0	Serum (CardioMPO)	Affymetrix 6.0 (Imputed)	2 409 479
MONICA/KORA	European	1262	40.7 ± 3.3	580/682	139.1 ± 98.2	Serum (CardioMPO)	IBCv2 SNP array	46 506
	AAs	1047	39.5 ± 3.8	440/607	108.4 ± 85.6	Serum (CardioMPO)	IBCv2 SNP array	46 346
GHS I	European	1328	52.7 ± 10.6	700/628	134.2 ± 67.5	Serum (MercoDIA)	IBCv2 SNP array	44 142
	European	2996	55.8 ± 10.9	1453/1543	327.7 ± 189.6	Plasma (CardioMPO)	Affymetrix 6.0 (Imputed)	2 614 503
GHS II	European	1178	55.1 ± 10.9	589/589	290.9 ± 132.7	Plasma (CardioMPO)	Affymetrix 6.0 (Imputed)	2 612 433
LURIC1	European	794	59.9 ± 12.0	523/271	241.2 ± 71.0	Plasma (Immudiagnostik)	Affymetrix 500 K (Imputed)	5 979 070
LURIC2	European	2100	63.5 ± 10.0	1488/610	174.0 ± 61.6	Plasma (Immudiagnostik)	Affymetrix 6.0 (Imputed)	6 420 716

in Table 2 and Supplementary Material, Table S2. The lead SNP at 1q31.3, rs800292 ($P = 4.89 \times 10^{-41}$), is a non-synonymous Val62Ile (GTA>ATA) substitution in the complement factor H gene (*CFH*) with a minor allele frequency (MAF) of 0.23 in Europeans. The *CFH-CFH3-CFH1* genomic region on chromosome 1q31.3 contains several other variants that were also significantly associated with serum MPO levels, which show varying levels of linkage disequilibrium (LD) with rs800292 (Fig. 2A). Of note, rs800292 is in low LD with other *CFH* variants, such as rs1061170 (Tyr402His), previously identified as susceptibility alleles for other disease phenotypes (Supplementary Material, Table S3). To determine whether the other SNPs in this region represent independent association signals, we also ran analyses taking into the account the effect of the lead SNP rs800292. These conditional analyses revealed that the strength of the association for the other SNPs in this region was attenuated and did not exceed the genome-wide threshold for significance (Supplementary Material, Table S3). Using the GeneBank cohort, we also analyzed the loci associated with serum MPO levels with further adjustment for CAD, history of hypertension, lipid levels, or white blood cell count. However, these additional adjustments did not alter the effect estimates or P -values compared with those adjusted for only age and sex (data not shown).

The locus on chromosome 6p21.32 contains several immune system related genes, including complement component 2 (*C2*) and *HLA* (Fig. 2B). The lead SNP (rs3134931; $P = 1.49 \times 10^{-8}$) is located within intron 2 of *NOTCH4* and is not in strong LD ($r^2 < 0.4$) with any of the other 130 variants in this region that demonstrate suggestive ($P < 5.0 \times 10^{-6}$) association with serum MPO levels (Fig. 2B). One SNP at chromosome 20p13, which is not in LD ($r^2 < 0.2$) with other variants in this region, was also significantly associated with serum MPO levels (rs6042507; $P = 4.30 \times 10^{-8}$) (Fig. 2C). This SNP encodes a non-synonymous Ala215Glu substitution (GCG>GAG) in exon 3 of the signal-regulatory protein beta 2 gene (*SIRPB2*), which belongs to the family of genes expressed predominantly in neutrophils and monocytes. Suggestive evidence of association with serum MPO levels was also observed on chromosome 1q42.13 (rs2144300; $P = 2.52 \times 10^{-6}$)

containing *N*-acetylgalactosaminyltransferase 2 (*GALNT2*) and a region on 8p21.3 (rs1390943; $P = 9.38 \times 10^{-7}$) containing the vacuolar proton ATPase subunit B (*ATP6V1B*) and leucine zipper putative tumor suppressor 1 (*LZTS1*) genes (Fig. 1B; Table 2; Supplementary Material, Table S2).

Gene-centric analyses for serum MPO levels

Using CARE data from the CARDIA ($n = 1262$), CHS ($n = 3085$) and FHS ($n = 2660$) cohorts, as well as 1328 additional subjects of European ancestry from the Monitoring of Trends and Determinants in Cardiovascular Disease Cooperative Health Research in the Region of Augsburg (MONICA/KORA) cohort, we carried out gene-centric analyses using the IBC 50K custom SNP array. This chip has dense SNP coverage for ~2100 CAD candidate genes, including *CFH*, *C2* and *GALNT2* (but not *NOTCH4* or *SIRBP2*) and contains specific rare amino acid substitutions that would otherwise not be captured by imputed GWAS datasets. As a result, the gene-centric analyses allowed us to validate and fine map a subset of the loci identified in the GWAS meta-analysis, as well as potentially identify other variants associated with serum MPO levels.

As shown in Table 2 and Supplementary Material, Table S4, a strong association of serum MPO levels was observed with variants in *CFH* where the lead SNPs yielded P -values of 6.65×10^{-43} (rs6680396) and 4.74×10^{-42} (rs505102). The rs6680396 and rs505102 variants are in high LD ($r^2 = 0.87$) with each other in subjects of European descent (based on the 1000 Genome Project CEU data), and also with the lead SNP identified in the GWAS for serum MPO levels (rs800292). A combined analysis of the *CFH* locus using all available unique individuals in the GWAS and IBC datasets, including the GeneBank cohort (combined $n = 10 524$), further strengthened the association of rs6680396 with serum MPO levels ($P = 9.34 \times 10^{-45}$). The gene-centric analyses also identified a SNP on chromosome 6p21.32 (rs9332739) in *C2* that was strongly associated with serum MPO levels ($P = 4.83 \times 10^{-10}$) (Table 2; Supplementary Material, Table S4). Rs9332739 (MAF = 0.044) encodes a non-synonymous Glu318Asp (GAG>GAC) substitution in *C2* and is located ~287 kb proximal to the lead SNP (rs3134931) in

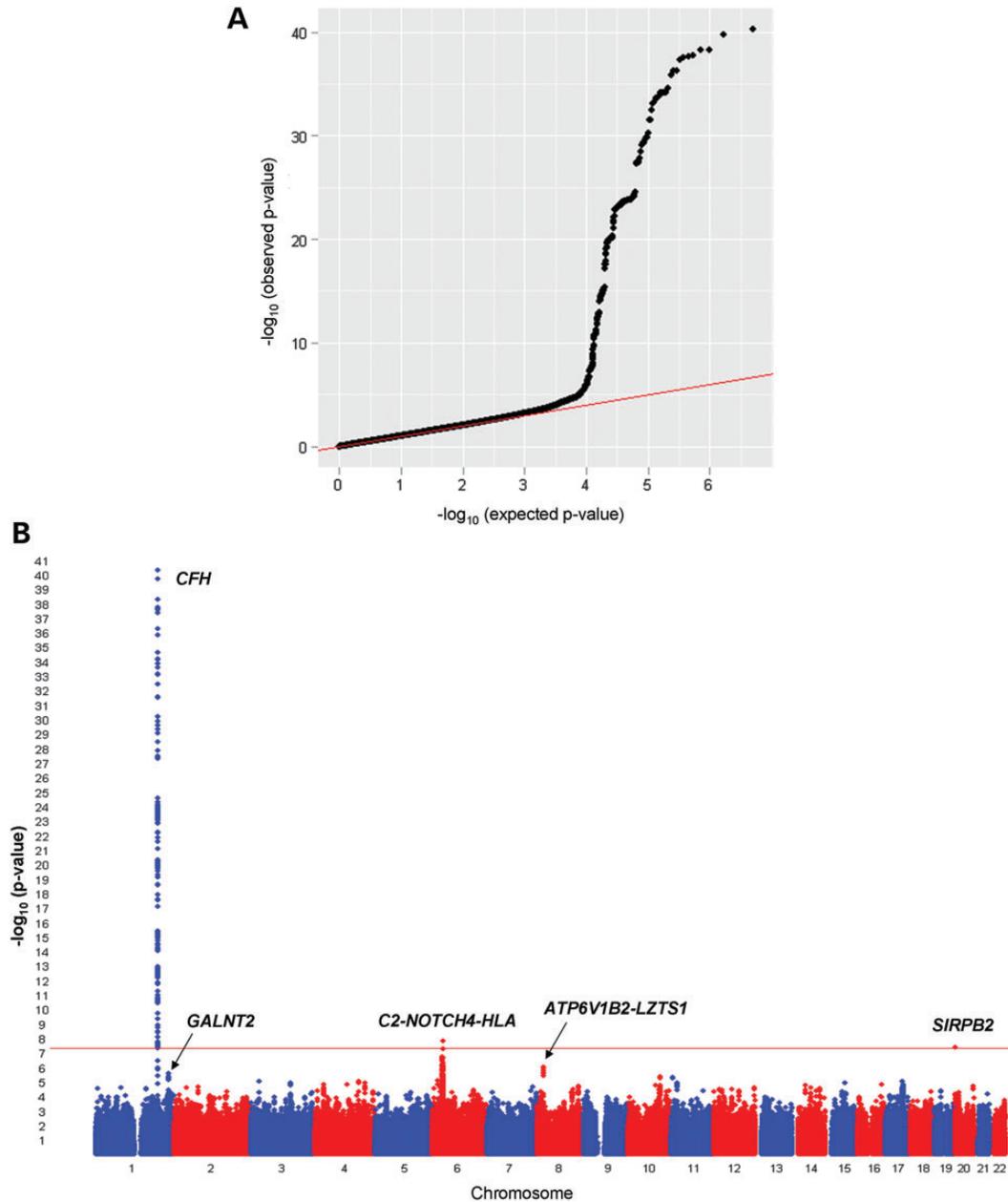


Figure 1. Results of the GWAS for serum MPO levels in subjects of European ancestry. The Q–Q (A) and Manhattan (B) plots are shown for the meta-analysis of 9305 subjects from the GeneBank, CHS, FHS and CARDIA cohorts.

NOTCH4 that was identified in the GWAS for serum MPO levels (Fig. 2B). However, in an analysis that included GeneBank (combined $n = 10\,524$) the association of rs9332739 with serum MPO levels was less significant than in the IBC analyses ($P = 1.76 \times 10^{-7}$), as this variant was not associated with serum MPO levels in GeneBank ($P = 0.39$). The gene-centric analyses also identified two rare variants (MAF $\sim 1\%$) of *MPO* (the structural gene for the enzyme) on chromosome 17q22 that were significantly associated with serum MPO levels (Table 2; Supplementary Material, Table S4). One SNP encodes an Ala332Val substitution (rs28730837; GCG>GTG; $P = 5.21 \times 10^{-12}$) and the other interrupts the first position (AG>CG) of the 3' splice junction of intron 11 (rs35897051; $P = 3.32 \times 10^{-8}$). Since these rare

variants were specifically included on the IBC array and not present in any of the imputed GWAS datasets, we were not able to carry out a combined analysis with all subjects. In addition to these rare variants, another SNP (rs8081967, MAF = 0.36) located ~ 724 kb telomeric to *MPO* in intron 23 of *TRIM37* exhibited suggestive evidence of association ($P = 2.13 \times 10^{-6}$) with serum MPO levels (Table 2; Supplementary Material, Table S4). Of note, rs8081967 also demonstrated suggestive evidence in the GWAS analyses of serum MPO levels ($P = 6.59 \times 10^{-5}$), which reached significance ($P = 1.44 \times 10^{-8}$) in an analysis using all unique subjects in the GWAS and IBC datasets (combined $n = 10\,524$). In addition, we also observed suggestive evidence of association with rs2144300 in *GALNT2* ($P = 2.52 \times$

Table 2. Significant and suggestive loci associated with decreased circulating MPO levels in the GWAS and gene-centric analyses

Locus [nearest Gene(s)]	Lead SNP	Position (bp) ^a	Effect/Other allele ^b	EAF	Beta (SE)	P-value	MPO Levels	Ethnicity	Analysis	I ² -statistic
1q31.3 (<i>CFH</i>)	rs800292	194 908 856	A/G	0.23	-0.15 (0.01)	4.89 × 10 ⁻⁴¹	Serum	European	GWAS	89.7
6p21.32 (<i>NOTCH4-C2-HLA</i>)	rs3134931	32 298 598	T/C	0.73	-0.05 (0.01)	1.49 × 10 ⁻⁰⁸	Serum	European	GWAS	0
20p13 (<i>SIRPB2</i>)	rs6042507	1 407 060	A/C	0.11	-0.09 (0.02)	4.30 × 10 ⁻⁰⁸	Serum	European	GWAS	38.5
1q42.13 (<i>GALNT2</i>)	rs2144300	228 361 539	C/T	0.39	-0.05 (0.01)	2.52 × 10 ⁻⁶	Serum	European	GWAS	34.5
8p21.3 (<i>ATP6V1B2-LZTS1</i>)	rs1390943	20 126 170	G/T	0.33	-0.05 (0.01)	9.38 × 10 ⁻⁷	Serum	European	GWAS	30.9
1q31.3 (<i>CFH</i>)	rs6680396	194 899 093	G/A	0.22	-0.13 (0.01)	6.65 × 10 ⁻⁴³	Serum	European	IBC	88.5
6p21.32 (<i>C2</i>)	rs9332739	32 011 783	C/G	0.044	-0.12 (0.02)	4.83 × 10 ⁻¹⁰	Serum	European	IBC	0
17q22 (<i>MPO</i>)	rs28730837	53 710 396	T/C	0.014	-0.27 (0.04)	5.21 × 10 ⁻¹²	Serum	European	IBC	21.6
17q22 (<i>MPO</i>)	rs35897051	53 703 225	C/A	0.007	-0.31 (0.06)	3.32 × 10 ⁻⁸	Serum	European	IBC	63.9
17q22 (<i>TRIM37</i>)	rs8081967	54 427 483	T/C	0.36	-0.05 (0.01)	2.13 × 10 ⁻⁶	Serum	European	IBC	37.5
1q42.13 (<i>GALNT2</i>)	rs2144300	228 361 539	C/T	0.39	-0.04 (0.01)	2.52 × 10 ⁻⁷	Serum	European	IBC	0
1q31.3 (<i>CFH</i>)	rs505102	194 886 125	G/A	0.71	-0.15 (0.03)	1.05 × 10 ⁻⁸	Serum	AAs	IBC	0
1q23.3 (<i>DARC</i>)	rs2814778	157 441 307	G/A	0.78	-0.21 (0.04)	6.48 × 10 ⁻⁸	Serum	AAs	IBC	44.3
17q22 (<i>C17orf71</i>)	rs6503905	54 642 236	A/G	0.37	-0.06 (0.01)	2.94 × 10 ⁻¹²	Plasma	European	GWAS	39.7
17q22 (<i>RNF43</i>)	rs2680701	53 793 300	G/A	0.81	-0.06 (0.01)	4.98 × 10 ⁻¹⁰	Plasma	European	GWAS	42.4
17q22 (<i>PPM1E</i>)	rs9911753	54 337 956	G/A	0.39	-0.05 (0.01)	1.51 × 10 ⁻⁹	Plasma	European	GWAS	44.7
17q22 (<i>MPO</i>)	rs12940923	53 724 848	A/T	0.84	-0.07 (0.01)	3.85 × 10 ⁻⁹	Plasma	European	GWAS	44.5
1q42.13 (<i>ABCB10-TAF5L-URB2-GALNT2</i>)	rs12049351	227 784 624	C/G	0.79	-0.05 (0.01)	1.08 × 10 ⁻⁶	Plasma	European	GWAS	0

^aSNP base pair (bp) positions are given according to NCBI build 36.1 of the reference human genome sequence.

^bThe allele that lowers MPO levels is referred to as the effect allele. Units for betas are natural log-transformed circulating MPO levels in pmol/l.

10⁻⁷) in the IBC analyses (Table 2; Supplementary Material, Table S4), but this association did not achieve genome-wide significance ($P = 2.68 \times 10^{-7}$) in a combined analysis with all unique subjects in the GWAS and IBC datasets ($n = 10\,524$).

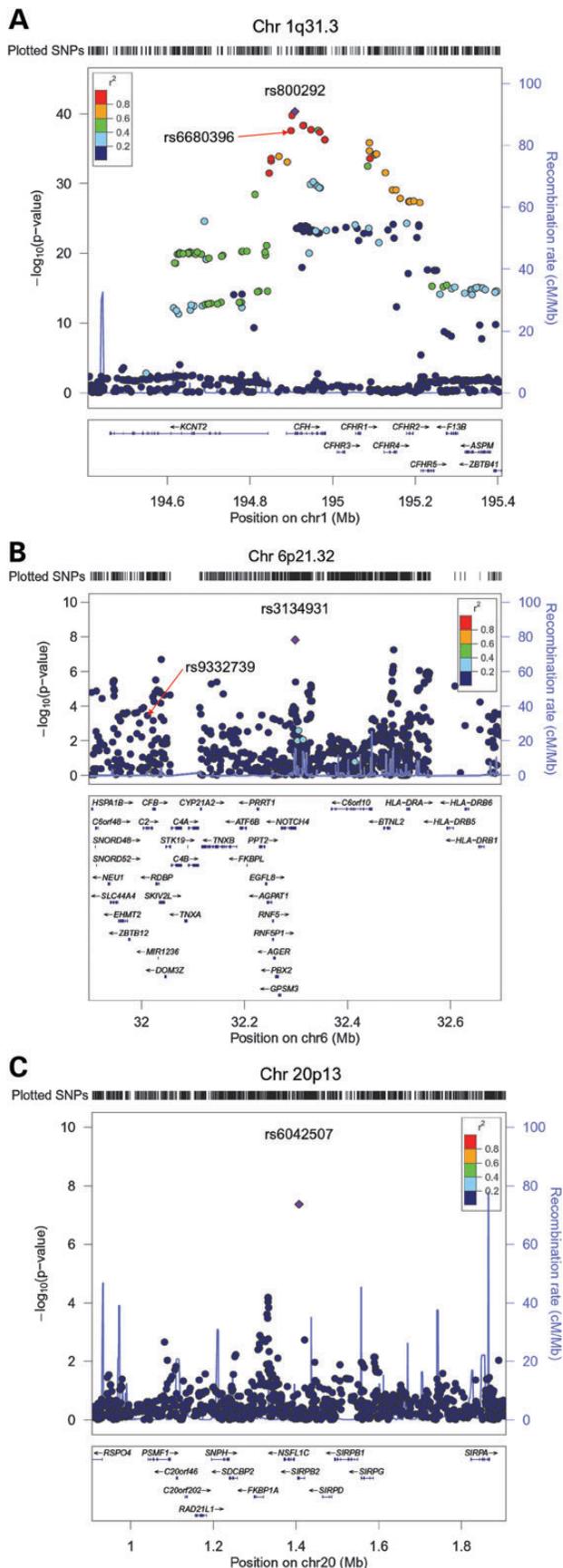
Replication of loci for serum MPO levels in African Americans (AAs)

To replicate the association findings observed with serum MPO levels and determine whether the loci have similar effects on other ethnicities, we used IBC SNP array data available in 1690 African Americans (AAs) from the CARDIA ($n = 1047$) and CHS ($n = 643$) cohorts. Consistent with the results on subjects of European ancestry, rs505102 in *CFH* was significantly associated ($P = 1.05 \times 10^{-8}$) with serum MPO levels in AAs (Table 2; Supplementary Material, Table S5), thus providing independent validation of this locus in a different ethnic group. The IBC analyses in AAs also yielded suggestive evidence for association of serum MPO levels with rs2814778 ($P = 6.48 \times 10^{-8}$) (Table 2; Supplementary Material, Table S5), which is located in the 5' UTR of the Duffy blood group antigen gene (*DARC*) on chromosome 1q23.3. Because rs2814778 is an important determinant of circulating neutrophil count in AAs, we further adjusted this analysis for white blood count. The association was strongly attenuated ($P = 0.02$), suggesting that the association of rs2814778 with serum MPO in AAs is largely mediated through the number of circulating neutrophils. In addition to validating the association of *CFH* with serum MPO levels observed in subjects of European ancestry, there was supportive evidence in AAs for association of serum MPO levels with rs9332739 in *C2* ($P = 0.0045$) and the rare Ala332Val (rs28730837) substitution in *MPO* ($P = 0.061$) (Supplementary Material, Table S5),

but not with the other variant in *MPO* (rs35897051; $P = 0.93$) or with rs2144300 in *GALNT2* ($P = 0.77$).

GWAS for plasma MPO levels

We next carried out a GWAS meta-analysis for plasma MPO levels in subjects of European ancestry from the GeneBank, Gutenberg Health Study (GHS), and Ludwigshafen Risk and Cardiovascular Health (LURIC) cohorts (total $n = 9260$). The Q-Q and Manhattan plots for the plasma MPO analyses are shown in Figure 3A and B, and the genomic control factors were 0.999, 1.008, 0.980, 0.970 and 0.991 for the GeneBank ($n = 2191$), GHS I ($n = 2997$), GHS II ($n = 1178$), LURIC1 ($n = 794$) and LURIC2 cohorts ($n = 2100$), respectively. The most significant locus for plasma MPO levels mapped to chromosome 17q22 near *MPO* with the lead SNP (rs6503905) yielding a P -value of 2.94×10^{-12} (Fig. 3B, Table 2; Supplementary Material, Table S6). The variant, rs6503905 (MAF = 0.37), encodes a putative synonymous substitution (GCG>GCA; Ala14Ala) in the predicted gene *C17orf71*, which is located ~930kb telomeric of *MPO*. As shown in Figure 3C and Supplementary Material, Table S6, several other SNPs spanning an ~1 Mb interval in this region and within different LD blocks were also significantly associated with plasma MPO levels, including rs9911753 ($P = 1.51 \times 10^{-9}$), rs2680701 ($P = 4.98 \times 10^{-10}$), and rs12940923 ($P = 3.85 \times 10^{-9}$). However, rs6503905 is only in moderate LD with rs9911753 ($r^2 = 0.28$) and completely unlinked to rs2680701 and rs12940923 ($r^2 < 0.1$ for each SNP). In addition, rs9911753 is in strong LD ($r^2 = 0.87$) with rs8081967, which was identified in our combined GWAS/IBC analyses for serum MPO levels (Table 2). We next re-performed the analyses conditioned on rs6503905, rs9911753, rs2680701 or rs12940923. The



association of these SNPs with plasma MPO was attenuated when conditioned on each other, with only rs6503905 exceeding the genome-wide threshold for significance ($P = 9.07 \times 10^{-9}$) when the analyses were conditioned on rs12940923 (Supplementary Material, Table S7).

In addition to the chromosome 17q22 locus, we also observed suggestive evidence for association of plasma MPO levels with the region surrounding *GALNT2* (Fig. 3B; Table 2; Supplementary Material, Table S6), although the lead SNP (rs12049351; $P = 1.08 \times 10^{-6}$) differs from that identified for serum MPO levels in the GWAS/IBC analyses (rs2144300). Rs12049351 is not in LD with rs2144300 and is located ~ 485 kb proximal to *GALNT2* in between *ABCB10* and *TAF5L*. We also evaluated the reciprocal association of all identified SNPs that were available across all genotyping platforms/datasets with both plasma and serum MPO levels. SNPs identified in our GWAS for plasma MPO levels also demonstrated varying degrees of association with serum MPO levels, whereas reciprocal associations were not observed with plasma MPO levels (Supplementary Material, Table S8). Since the SNPs on chromosome 17q22 and rs12049351 at the *ABCB10-TAF5L-GALNT2* locus demonstrated association with both serum and plasma MPO levels (Supplementary Material, Table S8), we also carried out a meta-analysis across all independent subjects (combined $n = 16\,376$). These analyses yielded P -values of 1.5×10^{-11} and 5.4×10^{-9} for association of rs6503905 and rs12049351, respectively, with circulating MPO levels.

Functional effects of MPO-associated variants

To determine whether any of the identified SNPs were functional, we carried out biochemical and expression quantitative trait locus (eQTL) analyses. For the rs800292 variant in *CFH*, we measured serum levels of C3a-desArg, a downstream cleavage product of complement activation, in a subset of 171 subjects from the GeneBank cohort. These GeneBank subjects were selected to represent equal numbers of the GG, AG, and AA genotypes for rs800292 ($n = 57$ each) and matched for age, sex and CAD status. As shown in Figure 4, serum C3a-desArg levels were significantly lower in carriers of the A allele compared with GG homozygotes in a dose-dependent manner ($P = 0.04$). To further evaluate the functional effects of SNPs associated with circulating MPO levels, we used previously generated microarray data in monocytes from 1467 subjects in the GHS I and II cohorts (20). Significant and directionally consistent *cis* eQTLs were observed with SNPs located on chromosome 17q22 and mRNA levels of *MPO* as well as several other genes in this region, including *RAD51C*, *SEPT4* and *TRIM37* (Table 3). For *MPO* and *RAD51C*, these associations remained significant even after conditioning on the lead eQTL SNPs, which suggest that this region may have pleiotropic effects on the expression of multiple genes. No significant eQTL associations were detected with SNPs at other loci that were associated with serum or plasma MPO levels, including the *CFH* locus on chromosome 1.

Figure 2. Regional plots for loci demonstrating a significant association with serum MPO levels on chromosomes 1q31.3 (A), 6p21.32 (B), and 20p13 (C). For each locus, a 1 Mb region is shown, centered on the lead SNP (purple diamond). Genes in the selected intervals are indicated in the bottom panel.

Effects of MPO-associated variants on the risk of CAD

To further investigate the significance of the loci for circulating MPO levels, we evaluated the association of the identified SNPs with prevalent CAD using the CARDIoGRAM consortium. This consortium represents a GWAS meta-analysis of subjects with history of CAD and is comprised of ~22 000 cases and ~65 000 controls. As shown in Table 4, none of the SNPs for which CARDIoGRAM data were available showed significant evidence of association with prevalent CAD.

DISCUSSION

Using meta-analyses of both GWAS and gene-centric data, we identified distinct loci that were associated with serum and plasma MPO levels. The most significant locus for serum MPO levels in subjects of European ancestry was observed with *CFH* on chromosome 1, which was replicated in AA subjects. Of the three highly associated and linked *CFH* SNPs in subjects of European ancestry (rs505102, rs800292 and rs6680396), only rs505102 and rs800292 are in strong LD in AAs. As a result of this different LD pattern, candidate causal SNPs responsible for the association with lower serum MPO levels could include rs505102, rs800292 or another SNP in LD with these variants. Of these, rs800292 encodes a Val62Ile substitution in *CFH* and has been previously associated with decreased risk of age-related macular degeneration (AMD) in a Japanese population (21). Other *CFH* variants have also been associated with AMD (22–25), as well as meningococcal susceptibility (26), IgA-induced nephropathy (27), atypical hemolytic uremic syndrome (HUS) (28), membranoproliferative glomerulonephritis type II (29) and systemic lupus erythematosus (SLE) (30). However, it is not known whether MPO plays a biological role in these inflammatory diseases. In addition, the association of the other disease-associated *CFH* variants with serum MPO levels was attenuated when the analyses were conditioned on rs800292, even though these SNPs are in weak LD ($r^2 < 0.30$) with rs800292 in subjects of European ancestry. Thus, while several independent alleles at the *CFH* locus influence multiple inflammatory-related disease phenotypes, the strong association signal at this locus with serum MPO levels is primarily due to rs800292 or other tightly linked variants.

Complement fixation is well known as a potent trigger of leukocyte activation and degranulation, and serum generation is associated with activation of protease cascades, including complement proteins, such as C3a and C3b (31–33). Thus, an association between a genetic variant for a complement protein and serum MPO levels, but not plasma MPO levels, can be mechanistically rationalized and could be related to leukocyte activation and partial degranulation during blood coagulation. A role for the complement system in influencing serum MPO levels is also supported by the results of our gene-centric analyses, which identified an amino acid substitution in *C2* (rs9332739) for serum MPO levels that has also been previously associated with AMD (34). The minor alleles of rs800292 in *CFH* and rs9332739 in *C2* protect against the development of AMD and are associated with decreased serum MPO levels. The disease protective Ile62 *CFH* variant (rs800292), which is located within the SCR2 domain, has been shown to increase binding of *CFH* to C3b and lead to greater inactivation of fluid-phase

and surface-bound C3b (35). Presumably, this would reduce complement activation and leukocyte activation during serum separation, thereby leading to decreased MPO release from neutrophils and/or monocytes. This notion is consistent with our functional data, showing that the A allele of rs800292, which is associated with decreased MPO levels, is associated with lower serum levels of C3a-desArg, a cleavage product generated by complement activation. In addition, clotting factors and proteases that activate complement proteins and trigger degranulation of MPO-rich phagocytes, such as neutrophils, are present in serum but are mostly depleted in plasma. Therefore, *CFH* and *C2* variants that decrease complement activation, either through increased *CFH* or reduced *C2* activity, could be one potential mechanism through which they lead to decreased MPO levels in serum. By analogy, a common Asp42Gly variant of *DARC* has been associated with serum, but not plasma, concentrations of several pro-inflammatory chemokines (36). The mechanism appears to be due to the release of these chemokines during blood coagulation as a result of differential binding to *DARC* (36).

Whether the association of complement pathway variants with AMD (and other inflammatory diseases) may be causally related to enhanced MPO-related inflammatory processes remains to be determined. *MPO* variants, including those identified herein, have not been reported to be associated with AMD, meningococcal susceptibility, IgA-induced nephropathy, HUS, membranoproliferative glomerulonephritis type II or SLE, suggesting that circulating MPO levels may not play a causal role in the pathogenesis of such inflammatory diseases. On the other hand, MPO-induced oxidative damage to lipoproteins and the vascular wall have been implicated in the development of atherosclerosis and its clinical sequelae, and systemic levels of MPO predict cardiovascular risk. Oxidative stress and lipid peroxidation are also involved in other chronic inflammatory diseases, including AMD. In addition to regulating the complement system, *CFH* is a major binding protein of malondialdehyde, a common lipid peroxidation product (37). Taken together, these observations may mechanistically link stress-related oxidative tissue injury to localized regulation of complement activity in a variety of chronic inflammatory disorders.

In addition to loci containing genes of the complement system, the region containing *SIRPB2* also demonstrated genome-wide significant association with serum MPO levels in subjects of European ancestry. *SIRPB2* is a member of the signal-regulatory protein (SIRPs) family of transmembrane glycoproteins that are expressed predominantly on myeloid cells and involved in the regulation of innate immunity and complement receptor-mediated phagocytosis (38). The role of *SIRPB2* in antigen-specific proliferation and activation of *T*-cells (39) may provide an additional biological link for its association with serum MPO levels. By comparison, the gene-centric analyses in AA subjects identified a promoter variant of *DARC* (rs2814778) that achieved near genome-wide significance for association with serum MPO levels. Interestingly, the G allele of this variant is highly prevalent in subjects of African descent and results in the loss of the Duffy antigen on red blood cells, which has been associated with resistance to malaria infection (40) and low neutrophil count (41). Thus, this is consistent with our observations that the A allele of rs2814778 is associated with increased serum MPO levels since MPO is most abundantly present in neutrophils.

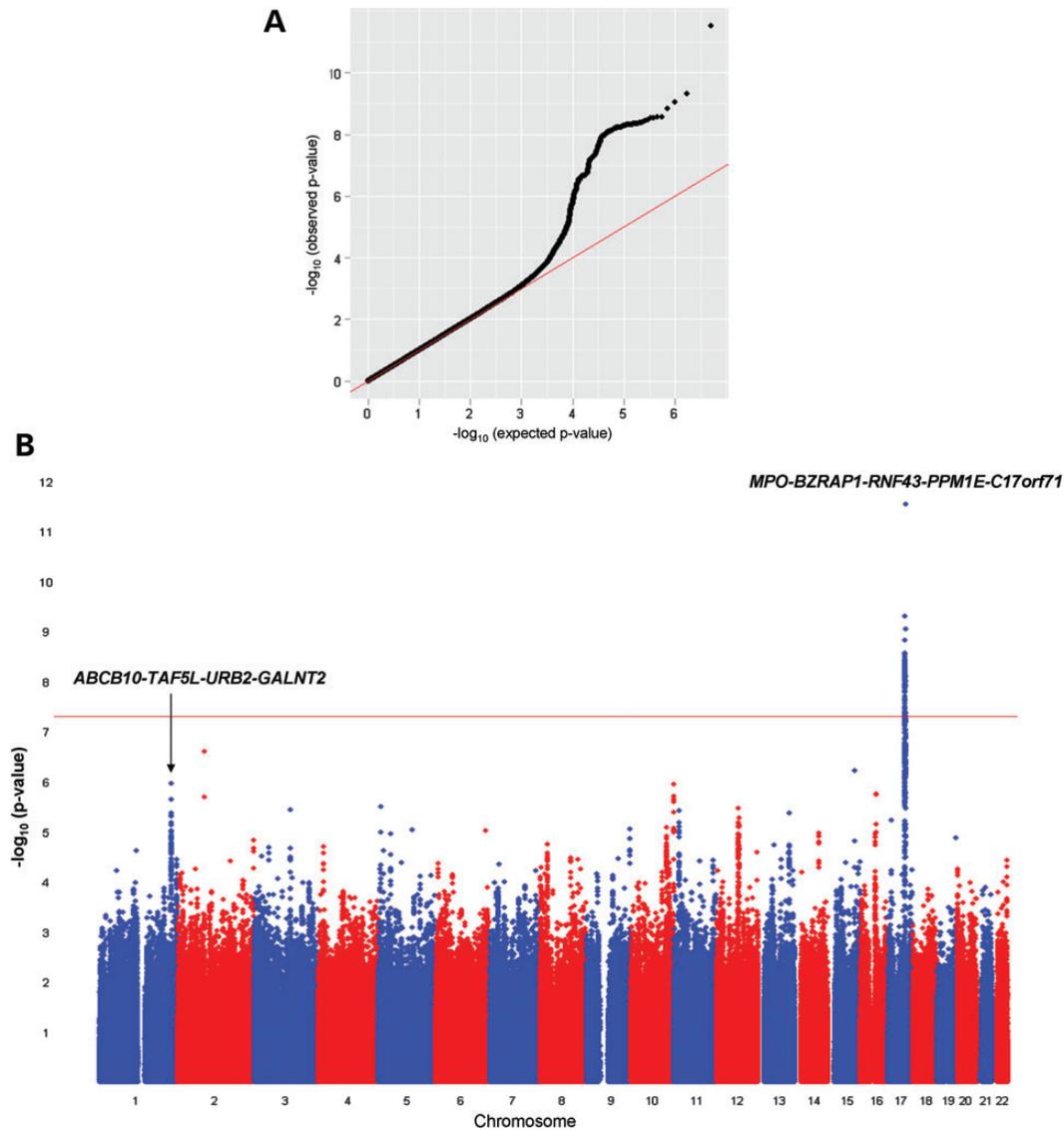


Figure 3. Results of the GWAS for plasma MPO levels in subjects of European ancestry. The Q–Q (A) and Manhattan (B) plots are shown for the meta-analysis of 9260 subjects from the GeneBank, GHS I, GHS II, LURIC1 and LURIC2 cohorts. (C) Regional plot of a 1 Mb interval on chromosome 17q22 demonstrates several independent SNPs that exceed the genome-wide threshold for significance. The bottom panel shows the LD pattern across this region using CEU data from HAPMAP.

As a comparative analysis, we also carried out a GWAS for plasma MPO levels in subjects of European descent. These results identified multiple SNPs in a large ~ 1 Mb interval encompassing *MPO* on chromosome 17q22 that demonstrated association with both plasma and serum MPO levels. These results are further supported by our gene-centric analyses, which identified rare *MPO* SNPs that were also associated with serum MPO levels, including the previously reported rs28730837 Ala332Val substitution (9). In contrast, the *CFH* locus only demonstrated association with MPO levels in serum. Based on the LD structure in the chromosome 17q22 region, the four SNPs that we identified are not tightly linked to each other. However, with the exception of rs6503905, which remained strongly associated with plasma MPO levels after taking into account the effect of rs12940923, the association signals with the remaining SNPs were attenuated in the

conditional analyses. Of note, rs12940923 is in relatively strong LD ($r^2 = 0.67$) with a -463 G>A promoter polymorphism (rs2333227) in *MPO* that has previously been associated with plasma MPO levels (42). The association of rs12940923 and rs6503905 with circulating MPO levels is also supported by the directionally consistent and strong *cis* eQTLs these variants exhibit with *MPO* expression, even after conditioning on the lead eQTL SNP. Similar *cis* genetic effects were observed on the expression of other genes at this locus (i.e. *RAD51C*) as well. Taken together, these data indicate that several SNPs at chromosome 17q22 contribute independently to circulating MPO levels and suggest that this locus may contain a regulatory region(s) with pleiotropic effects on gene expression.

Another interesting observation from our analyses of plasma MPO levels is the suggestive association between circulating MPO levels and variants in the vicinity of *GALNT2*. For

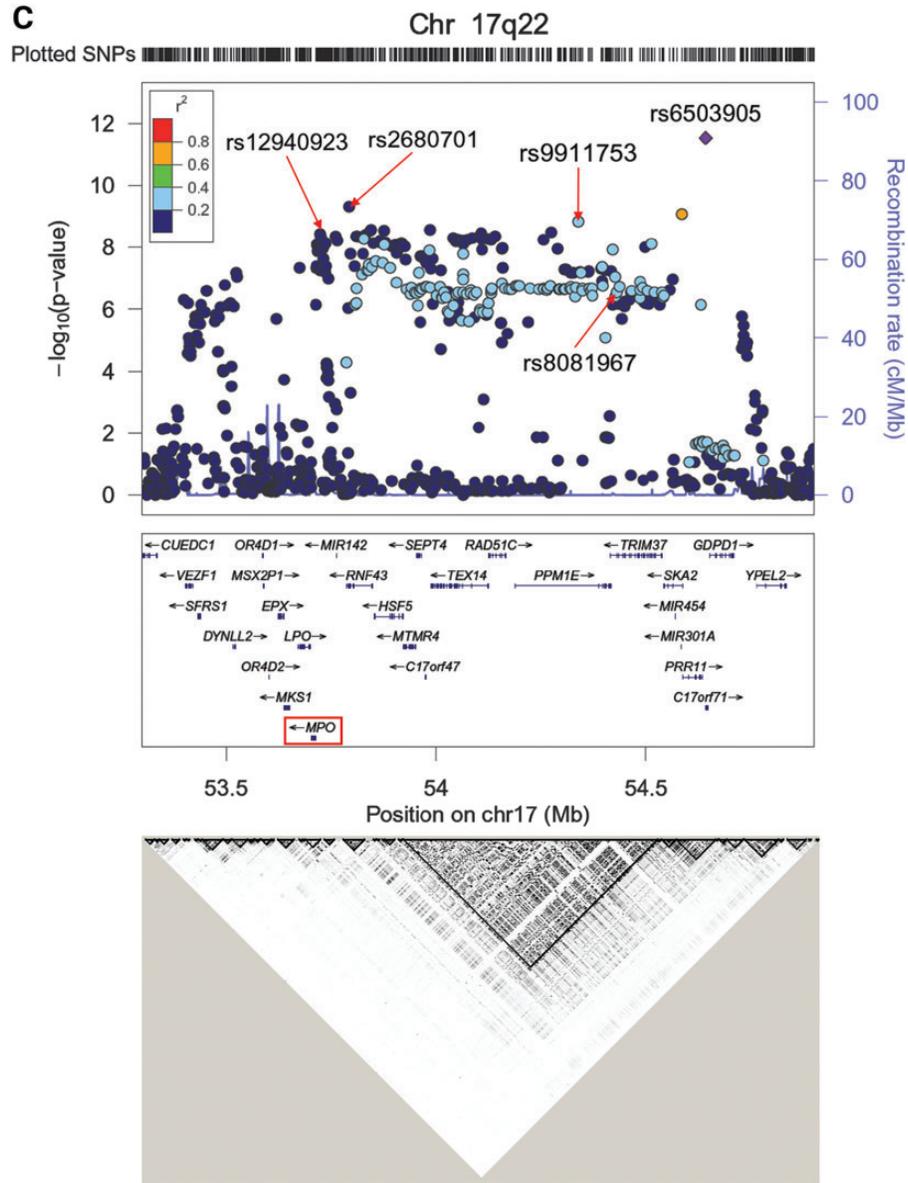


Fig. 3 Continued

example, an intronic variant (rs2144300) of *GALNT2*, which we identified in both GWAS and gene-centric analyses for serum MPO levels, has previously been associated with lower HDL levels (43). *GALNT2* is involved in protein O-linked glycosylation and, while its role in regulating MPO levels is not immediately evident, it is interesting to note that MPO binds to HDL within atherosclerotic lesions via apolipoprotein A-1 and catalyzes HDL oxidation (44–46), thereby impairing its cardioprotective properties. By comparison, the GWAS for plasma MPO levels identified another SNP (rs12049351) located ~485 kb proximal to *GALNT2* in between *ABCB10* and *TAF5L* that was suggestively associated with plasma MPO levels. Rs12049351 was also associated with serum MPO levels whereas rs2144300 was not associated with plasma MPO levels. These results suggest that specific alleles in this region may control both serum and plasma MPO levels

whereas other independent variants are associated with only levels in serum, possibly through a mechanism related to HDL metabolism.

While the results of our analyses have revealed potentially interesting loci that control circulating MPO levels, several limitations of our study should be noted. First, circulating MPO levels could be affected by environmental and/or transient factors, such as physical activity or acute infections, which our analyses did not take into account. Given the apparent effect of serum generation on complement and leukocyte activation, differences in handling and/or processing of blood samples in the various cohorts may have also increased experimental variability and led to additional confounders. Furthermore, GeneBank recruited consecutive patients undergoing elective diagnostic coronary angiography, which would enrich for subjects with CAD, and lead to MPO levels that are on average higher

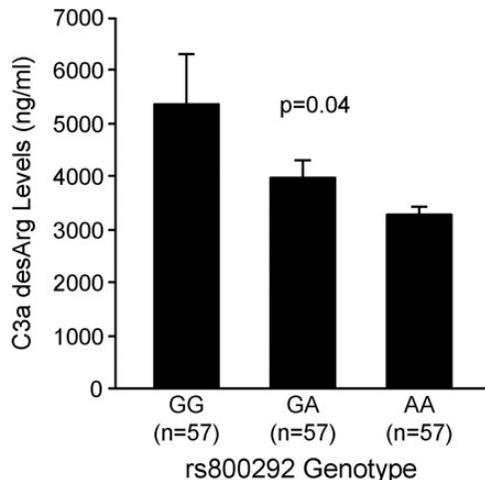


Figure 4. Serum C3a-desArg levels as a function of *CFH* rs800292 genotype. Serum levels of C3a-desArg are significantly lower in carriers of the A allele compared with GG homozygotes in a dose-dependent manner. Data were measured in a subset of age-, sex- and CAD status-matched GeneBank subjects and shown as untransformed mean \pm SE. The *P*-value was obtained using linear regression analyses with natural-log transformation.

compared with the other population-based studies. This could explain, in part, the variation seen in circulating MPO levels across cohorts even when using the same assay, and the observed heterogeneity in the association results for some of the identified loci. However, these limitations are somewhat mitigated by our use of a sample-weighted *Z*-score meta-analysis method and the high level of significance obtained from the analyses.

In summary, our comprehensive genetic studies on two ethnicities identified several unique loci that are associated with either serum or plasma MPO levels. These results indicate a potentially prominent role for the complement system in influencing serum MPO levels, presumably via leukocyte activation. By comparison, independent variants at the *MPO* locus were strongly associated with plasma MPO levels but modestly associated with serum levels. Despite the clinical association of both plasma and serum MPO levels with both prevalent cardiovascular phenotypes and incident risk for major adverse cardiac events, genetic variants associated with circulating MPO levels do not show association with history of CAD in \sim 80 000 subjects from the CARDIoGRAM consortium. Additional studies will be needed to gain a better understanding of the functional basis for the association between circulating MPO levels and the identified variants in *CFH* and *MPO*, as well as to determine the clinical implications for inflammatory diseases that could be mediated in part through MPO-related activity.

MATERIALS AND METHODS

Study populations

The populations with serum and/or plasma MPO levels used in the present study were the GeneBank, CARDIA, CHS, FHS, MONICA/KORA, GHS and LURIC cohorts. Details of these study populations are provided in the online Supplemental Materials section. The CARE Consortium is a National Heart Lung and Blood Institute supported resource for analyses of the

association of genotypes with heart, lung and blood phenotypes (47). All participants in each study provided a written informed consent prior to being enrolled, and the studies were approved by the Institutional Review Boards of the participating institutions.

Circulating measurements

Serum MPO levels were measured in the GeneBank, CARDIA, CHS, FHS and MONICA/KORA cohorts, whereas plasma MPO was measured in the GeneBank, GHS I and GHS II and LURIC studies. The various assays used for these measurements in each cohort are shown in Table 1. The CardioMPO assay is FDA and EU cleared, and appropriate as an *in vitro* diagnostic test for use in patient care. Serum levels of C3a-desArg, a downstream cleave product of complement activation, were measured using an OptEIA ELISA kit from BD Biosciences (San Diego, CA, USA).

Genome-wide genotyping and imputation

The cohorts included in this meta-analysis of GWAS data used different genotyping platforms: the Affymetrix Human SNP Array 6.0 in the GeneBank, CARDIA, GHS I and II and LURIC2 cohorts; the Affymetrix 500K Array Set for the FHS and LURIC1 cohorts; and the Illumina HumanCNV370-Duo for the CHS cohort. As a standard approach for facilitating meta-analyses, all studies used their genotype data to impute unmeasured, autosomal SNPs using either the CEU data from HapMap (release 22 or 24, build 36) or the 1000 Genomes project. Imputation was performed using either the MACH 1.0.16, Beagle 3.2, or BAMBAM 0.99 programs. Imputation results were filtered at an r^2 threshold of 0.5 and a MAF threshold of 0.01. For imputed genotypes, we used dosage information (i.e. a value between 0.0 and 2.0 calculated using the probability of each of the three possible genotypes) in the regression model implemented in PLINK. Details of the genotyping platforms and imputation methods for the GWAS are provided in Table 1 and Supplementary Material, Table S1.

For gene-centric analyses, subjects with serum MPO measurements from the CHS, FHS, CARDIA, and MONICA/KORA cohorts were genotyped using the custom IBCv2 genotyping array that contains high SNP marker density and LD coverage for \sim 2100 genes related to cardiovascular, inflammatory, hemostasis/coagulation, and metabolic phenotypes (48). A total of 49 320 SNPs are present on the IBC array, including \sim 15 000 SNPs in candidate genes that were not present in HapMap. Additional details regarding SNP selection and the tagging approach that was used have been described previously (48). IBC genotyping was not available in participants from the three cohorts with plasma MPO measurements.

Expression quantitative trait locus (eQTL) analysis

The functional effects of the identified SNPs on gene expression in *cis* were determined using microarray data from monocytes of 1467 individuals in the GHS I and II cohorts, as described previously (20). Briefly, total RNA was isolated from purified monocytes using RNeasy Mini kits (Qiagen, Hilden, Germany) and hybridized to Illumina HT-12 v3 BeadChips (www.illumina.com). Quality control filtering resulted in 22 305 genes that

Table 3. Pleiotropic associations of chromosome 17q22 SNPs with gene expression in monocytes

Gene	SNP	Effect/Other Allele	Beta (SE)	P-value	^a Conditioned Beta (SE)	^a Conditioned P-value	MPO levels
<i>MPO</i>	rs12940923	A/T	-0.25 (0.02)	8.49×10^{-35}	-	-	Plasma
	rs2680701	G/A	-0.19 (0.02)	7.42×10^{-25}	-0.04 (0.03)	0.191	Plasma
	rs6503905	A/G	-0.08 (0.02)	4.32×10^{-7}	-0.04 (0.02)	0.007	Plasma
	rs9911753	G/A	-0.07 (0.01)	3.36×10^{-7}	-0.02 (0.01)	0.089	Plasma
<i>SEPT4</i>	rs8081967	T/C	-0.07 (0.01)	3.57×10^{-6}	-0.02 (0.01)	0.115	Serum
	rs9911753	G/A	-0.09 (0.02)	7.43×10^{-7}	-	-	Plasma
<i>RAD51C</i>	rs8081967	T/C	-0.08 (0.02)	8.64×10^{-6}	-0.01 (0.05)	0.769	Serum
	rs9911753	G/A	-0.09 (0.01)	1.92×10^{-39}	-	-	Plasma
	rs8081967	T/C	-0.09 (0.01)	2.16×10^{-35}	-0.02 (0.02)	0.369	Serum
	rs6503905	A/G	-0.09 (0.01)	5.18×10^{-30}	-0.03 (0.01)	0.003	Plasma
<i>TRIM37</i>	rs2680701	G/A	-0.05 (0.01)	3.65×10^{-7}	-0.10 (0.01)	4.84×10^{-28}	Plasma
	rs12940923	A/T	-0.05 (0.01)	3.29×10^{-6}	-0.10 (0.01)	1.69×10^{-21}	Plasma
	rs8081967	T/C	-0.05 (0.01)	1.67×10^{-14}	-	-	Serum
	rs9911753	G/A	-0.04 (0.01)	2.12×10^{-11}	-0.02 (0.02)	0.341	Plasma
	rs6503905	A/G	-0.04 (0.01)	7.36×10^{-8}	-0.001 (0.01)	0.909	Plasma

^aConditioned on lead eQTL SNP for each gene. Units for betas are log₂-transformed signal intensities obtained from Illumina HT-12 v3 microarrays. eQTL results are only shown for SNPs identified in the GWAS for plasma levels and for which data were available in the monocyte dataset.

Table 4. MPO-associated SNPs and risk of CAD in the CARDIoGRAM consortium

MPO Levels (Analysis)	Locus (Nearest Gene(s))	SNP	Allele	Frequency	OR (95% CI)	P-value	n
Serum (GWAS)	1q31.3 (<i>CFH</i>)	rs800292	G	0.74	0.99 (0.96–1.03)	0.71	78 841
Serum (GWAS)	1q42.13 (<i>GALNT2</i>)	rs2144300	C	0.40	1.03 (1.0–1.06)	0.06	83 756
Serum (GWAS)	8p21.3 (<i>ATP6V1B2-LZTS1</i>)	rs1390943	G	0.33	0.99 (0.96–1.02)	0.57	78 176
Serum (IBC)	1q31.3 (<i>CFH</i>)	rs6680396	G	0.23	1.01 (0.98–1.05)	0.43	81 028
Serum (IBC)	6p21.32 (<i>C2</i>)	rs9332739	C	0.08	1.00 (0.94–1.07)	0.98	79 862
Serum (IBC)	17q22 (<i>TRIM37</i>)	rs8081967	C	0.65	1.01 (0.98–1.03)	0.75	80 337
Plasma (GWAS)	17q22 (<i>C17orf71</i>)	rs7502947 ^a	G	0.34	0.99 (0.96–1.02)	0.37	71 939
Plasma (GWAS)	17q22 (<i>RNF43</i>)	rs2680701	G	0.80	1.00 (0.96–1.03)	0.91	76 793
Plasma (GWAS)	17q22 (<i>PPM1E</i>)	rs9911753	G	0.39	1.00 (0.97–1.03)	0.91	82 162
Plasma (GWAS)	17q22 (<i>MPO</i>)	rs12940923	T	0.15	1.03 (0.99–1.07)	0.16	82 301
Plasma (GWAS)	1q42.13 (<i>ABCB10-TAF5L-URB2-GALNT2</i>)	rs12049351	C	0.80	0.97 (0.94–1.01)	0.12	83 470

Results are only shown for index SNPs or proxy variants that were available in the CARDIoGRAM Consortium.

^aUsed as proxy for rs6503905 ($r^2 = 0.70$).

were considered to be significantly expressed in monocytes and tested for association using the available genotype data. When the numbers of homozygotes for the minor allele of a SNP was <30, they were grouped with heterozygotes. Association of gene expression levels with SNPs was tested using the analysis of variance models (20).

Statistical methods

SNPs were evaluated for association with natural log-transformed circulating MPO levels using linear regression analyses, with adjustment for age and sex. Due to the difficulty of harmonizing MPO assays across cohorts, SNP association results for each ethnic group were combined using an effective sample-weighted Z-score meta-analysis method, as implemented in the software METAL (49), including a test for heterogeneity. Effect estimates within and across all cohorts are expressed as beta coefficients with standard errors. GWAS and gene-centric meta-analyses for serum and plasma MPO levels (from the relevant cohorts) were carried out separately. Differences in serum C3a-desArg levels as a function of genotype were carried out with linear regression using natural log-transformed values.

Associations with coronary artery disease (CAD)

The CAD Genome-wide Replication And Meta-Analysis (CARDIoGRAM) Consortium represents a GWAS meta-analysis of CAD comprising a discovery set of ~22 000 cases and ~65 000 controls (50). For each cohort in CARDIoGRAM, logistic regression was first used to test for association with CAD assuming an additive genetic model with adjustment for age and sex and taking into account the uncertainty of possibly imputed genotypes. Subsequently, a meta-analysis was performed separately for every SNP from each study that passed the quality control criteria using a fixed-effects model with inverse variance weighting (50). The results of this default meta-analysis were used to determine whether SNPs affecting circulating MPO levels were also associated with CAD.

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See the online-only Data Supplement.

Conflict of Interest statement. Dr Hazen (SLH) is named as a co-inventor on pending and issued patents held by the Cleveland

Clinic relating to cardiovascular diagnostics. Dr Hazen reports that he has been working as a consultant or speaker for the following companies: Cleveland Heart Lab, Inc., Esperion, Liposcience Inc., Merck & Co., Inc. and Pfizer Inc. He reports that he has received research funds from Abbott, Cleveland Heart Lab, Esperion and Liposcience, Inc. He has the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics from Abbott Laboratories, Cleveland Heart Lab, Inc., Frantz Biomarkers, LLC and Siemens.

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