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### Permalink

<https://escholarship.org/uc/item/3qr7j4p3>

### Journal

The Journal of Rheumatology, 40(6)

### ISSN

0315-162X

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### Publication Date

2013-06-01

### DOI

10.3899/jrheum.120989

Peer reviewed

Published in final edited form as:

*J Rheumatol.* 2013 June ; 40(6): 842–849. doi:10.3899/jrheum.120989.

## Variable association of reactive intermediate genes with systemic lupus erythematosus (SLE) in populations with different African ancestry

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## Abstract

**Objective**—Little is known about the genetic etiology of systemic lupus erythematosus (SLE) in individuals of African ancestry, despite its higher prevalence and greater disease severity. Overproduction of nitric oxide (NO) and reactive oxygen species are implicated in the pathogenesis and severity of SLE, making NO synthases and other reactive intermediate related genes biological candidates for disease susceptibility. This study analyzed variation in reactive intermediate genes for association with SLE in two populations with African ancestry.

**Methods**—A total of 244 SNPs from 53 regions were analyzed in non-Gullah African Americans (AA; 1432 cases and 1687 controls) and the genetically more homogeneous Gullah of the Sea Islands of South Carolina (133 cases and 112 controls) and. Single-marker, haplotype, and two-locus interaction tests were computed for these populations.

**Results**—The glutathione reductase gene *GSR* (rs2253409,  $P=0.0014$ , OR [95% CI]=1.26 [1.09–1.44]) was the most significant single-SNP association in AA. In the Gullah, the NADH dehydrogenase *NDUFS4* (rs381575,  $P=0.0065$ , OR [95%CI]=2.10 [1.23–3.59]) and nitric oxide synthase gene *NOS1* (rs561712,  $P=0.0072$ , OR [95%CI]=0.62 [0.44–0.88]) were most strongly associated with SLE. When both populations were analyzed together, *GSR* remained the most significant effect (rs2253409,  $P=0.00072$ , OR [95%CI]=1.26 [1.10–1.44]). Haplotype and two-locus interaction analyses also uncovered different loci in each population.

**Conclusion**—These results suggest distinct patterns of association with SLE in African-derived populations; specific loci may be more strongly associated within select population groups.

## Key Indexing Terms

systemic lupus erythematosus; African Americans; genetic association studies; oxygen compounds; single nucleotide polymorphism

## INTRODUCTION

Systemic lupus erythematosus (SLE [MIM 152700]) is a chronic, often severe, systemic autoimmune disease characterized by the production of high titers of autoantibodies directed against native DNA and other cellular antigens. SLE disproportionately affects women and African Americans (AAs; 0.009% of white men, 0.066% of white women, 0.038% of AA men, and 0.282% of AA women) (1). A genetic contribution to SLE is unequivocal; recent genome-wide association studies (GWAS) in Caucasians and Asians have identified nearly 40 validated susceptibility loci and implicated a broad array of biological pathways (2). Despite a higher prevalence, incidence and disease severity (3, 4), little is known about the genetic etiology of SLE in individuals of African ancestry. Recently, large candidate gene studies have uncovered associations of specific loci in AA (5–9).

Overproduction of nitric oxide (NO) and reactive oxygen intermediates is implicated in disease pathogenesis. Markers of systemic NO production and reactive oxygen species are higher in lupus patients than controls; these markers correlate with disease activity, and early studies suggest that failure to suppress these markers associates with lack of clinical response to therapy for lupus nephritis (10–12). These combined observations make NO synthases and other reactive intermediate producing and scavenging genes biological candidates for disease susceptibility.

The Gullah are a unique population of African ancestry in the U.S. Their ancestors were forcibly brought from the Sierra Leone and Ivory Coast area in West Africa, and were kept in the geographically isolated Sea Islands along the South Carolina and Georgia coasts (13). Until recent times, the estimated 100,000 to 300,000 Gullah remained relatively isolated. While continental AAs average approximately 80% West African and 20% European ancestry (14), the Caucasian admixture in the Gullah is less than 3.5%, and the Gullah are the most homogeneous AA population described (15–17). Interestingly, there is the perception that SLE is rare in Africa, suggesting that comparative studies of related cohorts from the two continents may provide insight into the genetic etiology of SLE (18). A higher than predicted prevalence of SLE multiplex families and a high prevalence of seropositivity in lupus first-degree relatives was observed in the Gullah, suggesting a major genetic impact in this population (19). In parallel, studies report a higher prevalence of certain common complex traits in the Gullah when compared to other AAs (19–21). Because of their genetic and environmental homogeneity, low European admixture, and increased prevalence and familial clustering of certain diseases, the Gullah are a unique population for deciphering the African heritability in these diseases. The power to detect associations may be higher in more genetically homogeneous populations, such as the Gullah. Given these advantages of an homogeneous population, this study attempted to identify specific genetic variants in genes involved in reactive intermediate production and scavenging predisposing to SLE in the Gullah population and admixed AAs.

## MATERIALS AND METHODS

### Patients and genotyping

The study population consisted of 1565 SLE cases and 1799 controls of African ancestry from the collaborative Large Lupus Association Study 2 (LLAS2), including Gullah (133 cases and 112 controls) and non-Gullah AAs (1432 cases and 1687 controls) (Supplemental Table 1). All study participants provided written informed consent that was approved by institutional review boards at each institution. Cases met the 1997 American College of Rheumatology (ACR) criteria for SLE (22). Race was self reported. Gullah ancestry was self identified as AA Gullah from the Sea Islands region of South Carolina and Georgia, with all known grandparents being of Gullah descent (19).

Genotyping was performed with the LLAS2 project, which involved multiple investigators and >32,000 single nucleotide polymorphisms (SNPs). A total of 244 SNPs from 53 regions harboring genes selected for their role in producing NO, reactive oxygen species, or scavenging reactive oxygen and nitrogen species were analyzed (Supplemental Table 2). SNPs were chosen for their position and function in each gene, with priority given to those with potentially stronger phenotypic risks (Supplemental Table 3). SNPs were genotyped on a customized Illumina Infinium II platform.

### Statistical analyses

Only SNPs meeting the quality control criteria of less than 10% overall missing data, no evidence of differential missingness between cases and controls ( $P$ -value>0.05), consistency

with Hardy-Weinberg Equilibrium genotype frequency expectations ( $P < 0.01$  controls,  $P < 0.0001$  cases), and minor allele frequency (MAF)  $> 5\%$  were included. Related and duplicate individuals were removed and gender inconsistencies and heterozygosity outliers excluded. Potential confounding substructure or admixture was controlled for using 306 Ancestry Informative Markers (AIMs) to compute admixture proportions as implemented in Admixmap; Principal Component analysis using all SNPs confirmed the results. After adjusting for population substructure, the inflation factor using all SNPs was  $\lambda = 0.98$  in the Gullah and  $\lambda = 1.19$  in the AAs. Principal component analysis plots of the AA and Gullah samples are shown in Supplemental Figure 1. Although the higher inflation factor would be expected given the selection of SNPs in candidate genes, a genomic control-adjusted P-value was also computed in AAs. In contrast, the  $\lambda$  value close to 1 in the Gullah ensured that false positive associations due to population stratification were excluded.

The computer program SNP-GWA was used for the association analysis ([www.phs.wfubmc.edu/public/bios/gene/home.cfm](http://www.phs.wfubmc.edu/public/bios/gene/home.cfm)). The additive genetic model is reported unless the lack-of-fit test for the additive model reached significance ( $P < 0.05$ ). In that case, the minimum P-value from the additive, recessive and dominant genetic models is reported. Tests can be affected by low genotype counts; therefore, a minimum of 30 homozygotes and 10 homozygotes for the minor allele were required in order to consider the recessive or additive models, respectively. Genetic models were defined relative to the minor allele, and reported results were adjusted for population substructure. In addition to the joint-analysis of AA and Gullah samples, a weighted Z-score meta-analysis was computed as implemented in METAL ([www.sph.umich.edu/csg/abecasis/metal](http://www.sph.umich.edu/csg/abecasis/metal)), with weights being the square root of the sample size for each dataset; thus, the meta-analysis incorporates direction, magnitude of association and sample size.

To uncover potential haplotype associations, a sliding window haplotype analysis of three to eight SNPs was performed in each region. A logistic regression model was employed, adjusting for population structure as implemented in PLINK ([pngu.mgh.harvard.edu/~purcell/plink](http://pngu.mgh.harvard.edu/~purcell/plink)) (23). Haplotypes with frequency  $< 10\%$  were excluded.

To test for two-locus interactions among SNPs, all reported results met the quality criteria defined above. Specifically, SAS ([www.sas.com](http://www.sas.com)) was used to compute a logistic regression model with each SNP modeled under an additive genetic model and the interaction as the centered crossproduct of the SNPs under the additive model. To reduce false-positive interactions due to low MAFs, we rejected all pairs for which the expected number of individuals in the data set was  $< 5$  for minor allele homozygotes. In addition, all SNP pairs with a linkage disequilibrium (LD) measure of  $r^2 > 0.2$  in YRI were excluded. Interactions were adjusted for population substructure. For chromosome X, only females were included.

A power analysis was computed with Quanto ([hydra.usc.edu/gxe](http://hydra.usc.edu/gxe)) using a prevalence of 0.1% and  $\alpha = 0.01$ . LD between SNPs was assessed with SNAP using data from the 1000 Genomes Project in YRI ([www.broadinstitute.org/mpg/snap/index.php](http://www.broadinstitute.org/mpg/snap/index.php)). SNP functionality was evaluated with the UCSC genome browser ([genome.ucsc.edu](http://genome.ucsc.edu)).

## RESULTS

Genes with known association with production of or regulation of reactive oxygen and nitrogen intermediates were selected (Supplemental Table 2). SNPs mapping to 53 regions harboring reactive intermediate related genes were identified (Supplemental Table 3). Association was assessed between these 244 SNPs and SLE in the Gullah (133 cases and 112 controls) and AA populations (1432 cases and 1687 controls).

The most significant single-marker associations are shown in Table 1. In order to minimize potentially spurious associations, effects that were not supported by associations at neighboring SNPs were excluded (Figure 1). In admixed AAs the most significant association was identified in the glutathione reductase (*GSR*) gene. In the Gullah the most significant associations mapped to the NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase) (*NDUFS4*) and nitric oxide synthase 1 (*NOS1*) genes. It should be noted that the variants reported herein as associated with SLE met quality control thresholds in both AA and Gullah populations, but only revealed association in one of the populations (Figure 2).

The strongest signal in AAs was observed at an intronic variant in a DNaseI hypersensitive cluster in the *GSR* gene (rs2253409,  $P=1.43 \times 10^{-03}$ , OR [95%CI]=1.26 [1.09–1.44]). Although modest, other associations with neighboring SNPs corroborated the association observed at rs2253409, suggesting it is unlikely to be spurious (Figure 1). Samples had 74% power to detect this effect. Despite meeting quality control thresholds, rs2253409 was not associated in the Gullah (Figure 2).

In the Gullah the most significant association was an intronic risk variant in the *NDUFS4* gene (rs381575,  $P=6.51 \times 10^{-03}$ , OR [95%CI]=2.1 [1.23–3.59]). Interestingly, this SNP locates in the transcription factor binding site for the RE1-silencing transcription factor (*REST*) and *GATA1* and *GATA3* proteins. An intronic variant with a protective effect in *NOS1* was also identified in the Gullah (rs561712,  $P=7.18 \times 10^{-03}$ , OR [95%CI]=0.62 [0.44–0.88]). Despite modest power (58% and 45% power to detect the effects reported for *NDUFS4* and *NOS1*, respectively), it is noteworthy that both the *NDUFS4* and *NOS1* associations were corroborated by associations at neighboring SNPs (Figure 1). These variants also met quality control thresholds in AAs, but were only associated with SLE in the Gullah (Figure 2).

Combining all samples of African ancestry (AA and Gullah) in a joint-analysis, the signals identified in the AAs predominated, as expected given their larger sample size. Table 1 reveals that in the African population, the aforementioned variant in *GSR* (rs2253409,  $P=7.21 \times 10^{-04}$ , OR [95%CI]=1.26 [1.10–1.44]) and another intronic variant in *GSR* (rs2551715,  $P=4.57 \times 10^{-03}$ , OR [95%CI]=1.21 [1.06–1.38]) not in LD with the former ( $r^2=0.07$  in YRI), were among the top associations. Very similar results were obtained when a meta-analysis of the AA and Gullah results was computed (rs2253409,  $P=6.85 \times 10^{-04}$ , rs2551715,  $P=4.49 \times 10^{-03}$ ). The samples had 57% and 77% power to detect the effects reported for the first and second variants in *GSR*, respectively. Figure 2 shows how the patterns of association for the reported genes vary among the AA, Gullah, and combined African populations.

Among the SLE cases, 42% of the 1527 total AA and 46% of the 152 total Gullah cases show renal involvement. Keeping in mind the smaller sample sizes and reduced power to detect associations, testing for association of these SNPs with LN showed very modest associations: *GSR* in African (rs2253409,  $P=0.01$ ) and AA (rs2253409,  $P=0.01$ ) and *NOS1* in African (rs10850803,  $P=0.01$ ) and AA (rs10850803,  $P=0.007$ ).

Haplotype-association methods may have more power and accuracy than single markers to detect disease effects. As shown in Table 2, the most significant haplotype association detected in AAs and combined African samples was a 3-SNP haplotype in an intronic *NOS1* region (rs3741476, rs1875140 and rs1077490;  $P=2.87 \times 10^{-04}$ , frequency=0.21, OR=1.32 in AAs; and  $P=7.36 \times 10^{-04}$ , frequency=0.21, OR=1.28 in all samples with African-ancestry). A 4-SNP protective haplotype in an intronic glutathione synthetase (*GSS*) region was also uncovered (rs6087651, rs2236270, rs17092180 and rs2273684,  $P=2.65 \times 10^{-04}$ , frequency

=0.14, OR=0.73 in AA; and  $P=1.71 \times 10^{-04}$ , frequency=0.15, OR=0.73 in African). No significant haplotypes were identified in the Gullah population.

Table 3 shows the most significant two-locus interaction analysis results. Nearly all interactions were specific to one ethnic group or the other. Only an interaction between a *NDUFS2* variant (rs4656993) with a *minichromosome maintenance complex component 5 (MCM5)* SNP (rs4645794) was observed in the combined African samples ( $P=4.00 \times 10^{-04}$ , OR [95%CI]=1.34 [1.27–1.40]) and AAs ( $P=9.74 \times 10^{-05}$ , OR [95%CI]=1.40 [1.32–1.48]).

## DISCUSSION

This is the first comprehensive analysis of reactive intermediate genes for their association with SLE in populations of African ancestry. Despite relatively modest power, strict quality criteria filters were applied to reduce the likelihood of false positive associations. In the Gullah, the virtually perfect inflation factor ensures that false positive associations due to population stratification can be conclusively excluded. Despite the small sample size of the Gullah being a limitation of this study, all the associations herein reported were corroborated by associations at SNPs in linkage disequilibrium with the top associated SNPs, underscoring that these associations are not genotyping artifacts.

The genes chosen were of interest because they all had the potential to affect reactive intermediate production or clearance/scavenging. Reactive oxygen species can oxidatively modify proteins to affect their activity. Transcription factors AP1 (cJun), NF $\kappa$ B, HIF1 $\alpha$ , and p53 are all redox-regulated (24). For instance, H<sub>2</sub>O<sub>2</sub>-generated by endothelial cell NOX leads to NF $\kappa$ B-mediated transcription of ICAM and VCAM (25), both of which associate with lupus nephritis disease activity (26, 27) and atherosclerosis (28, 29). Two inflammatory cytokines, IL6 and MCP1 (CCL2), important to the pathogenesis of lupus nephritis (30–35) and atherosclerosis (36, 37), have in common redox-regulated NF $\kappa$ B response elements (UCSC Genome browser). Both MCP1 and IL6 secretion by activated EC is dependent on ROS production and can be inhibited by reactive oxygen species scavengers (38).

The functional relevance of the described SNPs in reactive intermediate genes is not known. However, there is a rational basis for surmising that reductions in the activity or expression of functionally protective genes could predispose to SLE, SLE disease activity, or target organ damage. SLE is associated with increased markers of oxidative stress, particularly among AAs (10, 39, 40). The consequences of this increased oxidative stress may be increased antigenicity of self-antigens and pathogenic redox signaling.

Glutathione reductase (GSR) catalyzes the reduction of glutathione disulfide to glutathione (GSH), an important antioxidant molecule (41). Glutathione synthetase catalyzes the production of glutathione itself. Reduced levels of GSH can lead to increased oxidant stress. Of significance to SLE, lower levels of reduced GSH were observed in T-cells from patients with SLE, in association with mitochondrial hyperpolarization and ATP depletion (42), a process that can predispose cells to necrosis. The current study does not address whether the reduced levels of GSH observed in SLE patients is due to increased production of reactive oxygen species, reduced enzyme activity, or both.

The proteins encoded by *NDUFS2* and *NDUFS4* are subunits of the mitochondrial membrane respiratory chain NADH dehydrogenase (complex I). Reports are conflicting; however, defects in *NDUFS2* can lead to increased reactive intermediate production (43), while described mutations in *NDUFS4* do not appear to lead to increased oxidative stress (44).

Low levels of NO produced by NOS3, also known as endothelial nitric oxide synthase (eNOS), are protective in vascular disease and inflammation. Expression of *NOS3* is reduced in proliferative lupus nephritis (45), and the effects of low level NO production are functionally reduced in the vasculature of patients with lupus (46). Similarly, NOS1 appears to prevent leukocyte adhesion in mice lacking *NOS3* (47). This finding is also seen in disease, since in both humans and mice with cirrhosis *NOS1* (normally expressed in vascular smooth muscle cells) (48) is upregulated in eNOS (*NOS3*) deficiency, suggesting that NOS1 can compensate for reduced eNOS activity (49). Thus, NOS1 deficiency could exacerbate NOS3 dysfunction or deficiency, leading to inflammation and vascular dysfunction. There is supporting evidence for this notion, as *NOS1* is associated with end-stage renal disease in African-Americans (50).

In summary, this study uncovered several novel associations of reactive intermediate-related genes with SLE in patients with African ancestry. We show that many of the loci associated with SLE differ in Gullah and AAs, suggesting that specific loci may be more strongly associated in specific populations with African ancestry. This is not a surprising finding given the great genetic diversity present on the African continent (51, 52). These results suggest that patterns of disease association for SLE may be distinct and specific loci may be more strongly lupus-associated in select African ancestry populations.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We would like to express our gratitude to the SLE patients and the controls that participated in this study. We are thankful to the research assistants, coordinators and physicians that helped in the recruitment of participants. We would like to thank Dr. Peter K. Gregersen for contributing control samples genotyped in this study.

This project was supported by the South Carolina Clinical & Translational Research (SCTR) Institute, with an academic home at the Medical University of South Carolina, through the National Institutes of Health (NIH) Grant Numbers UL1 RR029882 and UL1 TR000062; by NIH grant numbers P60 AR062755 (PSR, JCO, DLK, GSG), R01 AR45476 (JCO), U54 RR026107 (JCO), R01 AI063274 (PMG), P01 AR49084 (RPK, CDL, JBH, EEB, JCE, JDR, RRG, MAP, LMV), R01 AR043274 (CDL, KLM), R01 AR33062 (RPK), R01 AR445650 (COJ), R01 AR043814 (BPT), P01 AR49084 (GSA), P30 AR055385 (EEB), 5UL1 RR025777 (JCE), AR 43727 (MAP), K24 AR 002138 (RRG), P60 2 AR 30692 (RRG), UL 1 RR 025741 (RRG), AI082714 (JAJ, JMG), AI101934 (JAJ), AR053843 (JAJ, JMG), AR058554 (JAJ, JMG), RR031152 (JAJ, JMG), R01 AR44804 (LAC), K24 AR02175 (LAC), P60 AR053308 (LAC), 5 M01 RR-00079 (LAC), RR020143 (KLM, MAR), AR042460 (JBH), AI024717 (JBH), AR062277 (JBH); and by the US Department of Veterans Affairs (JBH), the Mary Kirkland Scholarship (JBH, LAC), the Instituto de Salud Carlos III (PS09/00129 to MAR), FEDER funds of the European Union (MAR), the Lupus Foundation of Minnesota (KLM), arc project grant 17761 (TJV), Wellcome Trust programme grant 085492 (TJV), the Lupus Research Institute (BPT), Wake Forest School of Medicine Center for Public Health Genomics, and the Alliance for Lupus Research (COJ, LAC).

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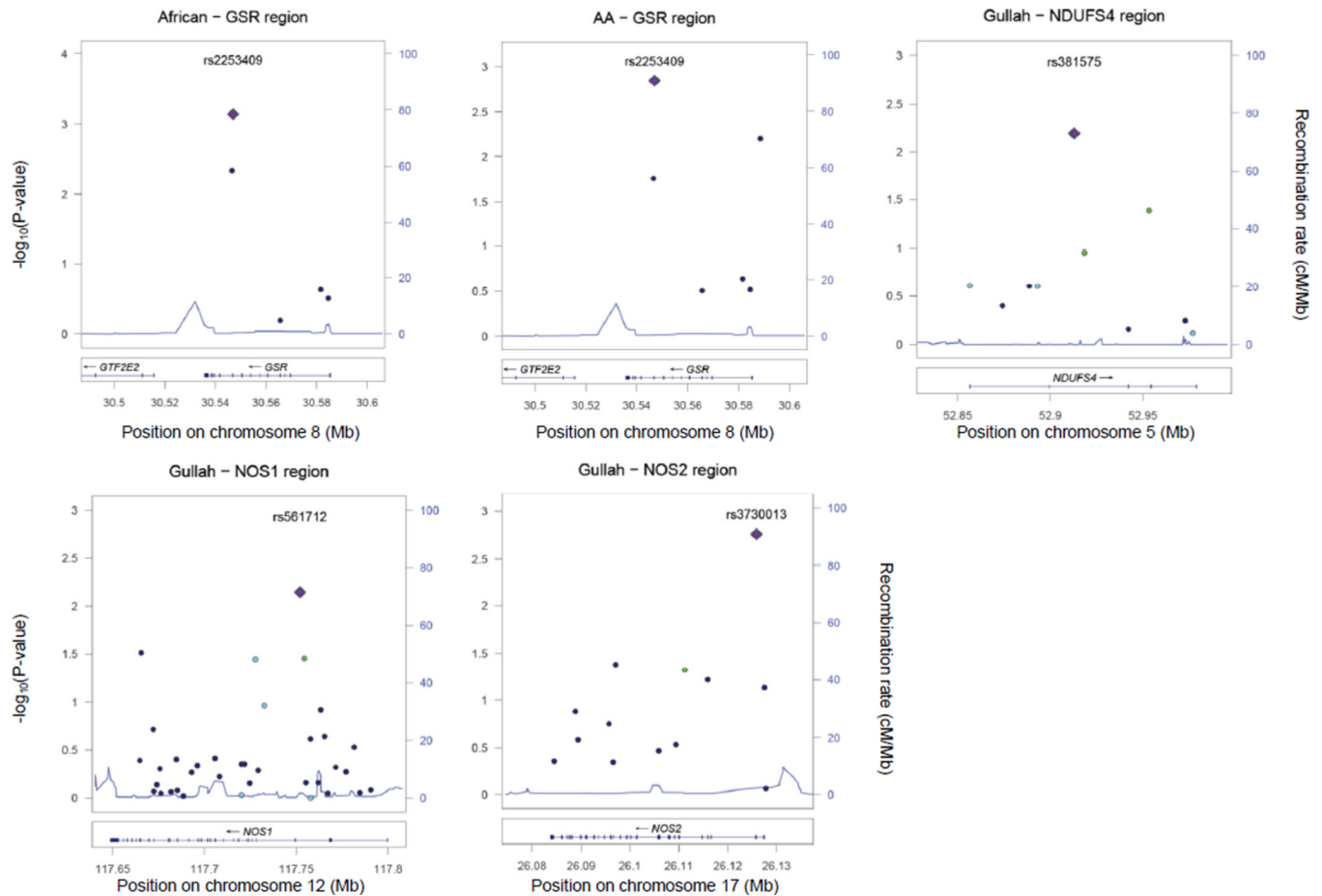
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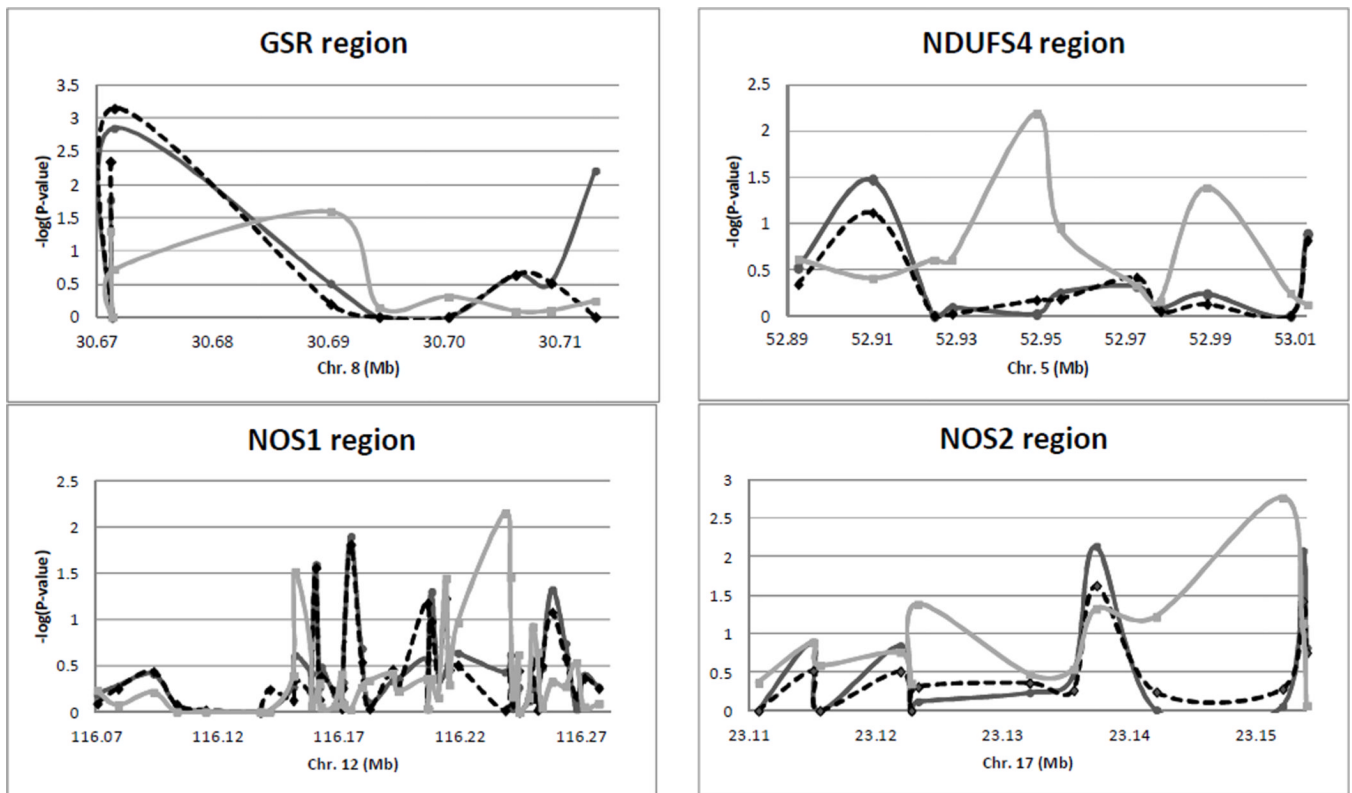
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**Figure 1. Regional plots of reactive intermediate gene loci in African, AA and Gullah populations**

Genotyped SNPs are plotted with their P-values (as  $-\log_{10}(\text{P-values})$ ) as a function of genomic position (Human Genome Build 18) within a region surrounding the most significant SNP (purple diamond). Recombination rates from the 1000 Genomes AFR (11/2010) (for the autosomal regions) or HapMap Phase II YRI (for the X chromosome region) are plotted in blue to reflect the regional LD structure. In each region the index SNP is represented by a large purple diamond, and the color of all other SNPs (circles) indicates LD with the index SNP based on pairwise  $r^2$  values from 1000 Genomes AFR/HapMap YRI (red,  $r^2 > 0.8$ ; orange,  $r^2 = 0.6-0.8$ ; green,  $r^2 = 0.4-0.6$ ; light blue,  $r^2 = 0.2-0.4$ ; dark blue,  $r^2 < 0.2$ ). Known human genes in the UCSC Genome Browser are in the bottom.



**Figure 2. Patterns of association results of reactive intermediate gene loci in African, AA and Gullah populations**

Association results are shown for the Gullah (light grey solid line with square symbols), AA (dark grey solid line with circle symbols) and combined African (dashed black line with diamond symbols). Genotyped SNPs (symbols) are plotted with their P-values (as  $-\log_{10}(P\text{-values})$ ) as a function of genomic position (Mb) in each gene's region.

**Table 1**

SNPs with the most significant association with SLE.

SNP	Chr	Pos (Mb)	Region	RA	MAF		Best-P*	P-GC	OR [95%CI]*
					Case	Control			
<i>African (AA + Gullah)</i>									
rs2551715	8	30,666	GSR	A	0.30	0.27	4.57E-03 <sup>d</sup>	6.43E-03	1.21 [1.06–1.38]
rs2253409	8	30,667	GSR	G	0.33	0.30	7.21E-04 <sup>d</sup>	1.16E-03	1.26 [1.10–1.44]
<i>AA</i>									
rs2253409	8	30,667	GSR	G	0.33	0.30	1.43E-03 <sup>d</sup>	3.43E-03	1.26 [1.09–1.44]
<i>Gullah</i>									
rs381575	5	52,949	NDUFS4	C	0.62	0.55	6.51E-03 <sup>r</sup>	-	2.10 [1.23–3.59]
rs561712	12	116,236	NOS1	A	0.38	0.51	7.18E-03 <sup>a</sup>	-	0.62 [0.44–0.88]
rs3730013	17	23,150	NOS2A	A	0.22	0.33	1.74E-03 <sup>d</sup>	-	0.46 [0.28–0.75]

\* Best-P, OR and CI reported under the following genetic models: a- additive, d- dominant, r- recessive.

Chr: chromosome; Pos: Position, RA: risk allele; MAF: minor allele frequency; P-GC: Best-P after a genomic control adjustment; OR: odds ratio; CI: confidence interval.

**Table 2**

Haplotypes with the most significant association with SLE.

NSNP	Size (kb)	SNP1	Chr	Pos	Haplotype*	F	OR	P-value	Region
<b>African (AA+Gullah)</b>									
3	2.0	rs3741476	12	116156518	GAG <sup>a</sup>	0.21	1.28	7.36E-04	NOS1
4	11.4	rs6087651	20	32982014	GCGC <sup>b</sup>	0.15	0.73	1.71E-04	GSS
<b>AA</b>									
3	2.0	rs3741476	12	116156518	GAG <sup>a</sup>	0.21	1.32	2.87E-04	NOS1
4	11.4	rs6087651	20	32982014	GCGC <sup>b</sup>	0.14	0.73	2.65E-04	GSS

NSNP: number of SNPs in haplotype; Size: size of the haplotype; SNP1: first SNP in haplotype; Chr: chromosome; Pos: position of the first SNP in haplotype; F: frequency of haplotype.

\* Haplotype defined by SNPs: a) rs3741476-rs1875140-rs10774909; b) rs6087651-rs2236270-rs17092180-rs2273684.

**Table 3**

Most significant two-loci interaction analysis results.

SNP1	Chr1	Pos1 (bp)	Region1	SNP2	Chr2	Pos2 (bp)	Region2	P-value	OR [95%CI]
<b>African</b>									
rs4656993	1	159442761	NDUFS2	rs4645794	22	34142051	HMOX1,MCM5	4.00E-04	1.34 [1.27–1.40]
rs10789501	1	47382076	CYP4A22	rs1142530	19	1339538	NDUFS7	6.00E-04	1.77 [1.47–2.14]
<b>AA</b>									
rs4656993	1	159442761	NDUFS2	rs4645794	22	34142051	HMOX1,MCM5	9.74E-05	1.40 [1.32–1.48]
rs561712	12	116236452	NOS1	rs728546	16	68013029	CYB5B	5.00E-04	0.67 [0.61–0.73]
rs2758346	6	160035411	SOD2	rs7797834	7	91581086	CYP51A1	5.00E-04	0.74 [0.71–0.78]
rs3741480	12	116190157	NOS1	rs3180279	16	87238334	CYBA	5.00E-04	1.35 [1.28–1.42]
<b>Gullah</b>									
rs256094	5	53008681	NDUFS4	rs133415	22	34136238	HMOX1,MCM5	2.00E-04	0.31 [0.15–0.64]

Interactions due to LD were excluded, as well as interactions where the expected number of individuals homozygous for both minor alleles was <5 in both cases and controls (to avoid potentially spurious interactions).