

Variable Association of Reactive Intermediate Genes with Systemic Lupus Erythematosus in Populations with Different African Ancestry

Paula S. Ramos, James C. Oates, Diane L. Kamen, Adrienne H. Williams, Patrick M. Gaffney, Jennifer A. Kelly, Kenneth M. Kaufman, Robert P. Kimberly, Timothy B. Niewold, Chaim O. Jacob, Betty P. Tsao, Graciela S. Alarcón, Elizabeth E. Brown, Jeffrey C. Edberg, Michelle A. Petri, Rosalind Ramsey-Goldman, John D. Reveille, Luis M. Vilá, Judith A. James, Joel M. Guthridge, Joan T. Merrill, Susan A. Boackle, Barry I. Freedman, R. Hal Scofield, Anne M. Stevens, Timothy J. Vyse, Lindsey A. Criswell, Kathy L. Moser, Marta E. Alarcón-Riquelme, Carl D. Langefeld, John B. Harley, and Gary S. Gilkeson

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. We analyzed variation in reactive intermediate genes for association with SLE in 2 populations with African ancestry.

Methods. A total of 244 single-nucleotide polymorphisms (SNP) 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). Single-marker, haplotype, and 2-locus interaction tests were computed for these populations.

Results. The glutathione reductase gene *GSR* (rs2253409; $p = 0.0014$, OR 1.26, 95% CI 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 2.10, 95% CI 1.23–3.59) and NO synthase gene *NOS1* (rs561712; $p = 0.0072$, OR 0.62, 95% CI 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 1.26, 95% CI 1.10–1.44). Haplotype and 2-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. (First Release May 1 2013; *J Rheumatol* 2013;40:842–9; doi:10.3899/jrheum.120989)

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From the Department of Medicine, Medical University of South Carolina, Charleston, South Carolina, USA; Wake Forest School of Medicine and Center for Public Health Genomics, Winston-Salem, North Carolina, USA; Arthritis and Clinical Immunology Research Program, and Clinical Pharmacology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA; Division of Rheumatology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA; Department of Medicine, School of Medicine, and Department of Epidemiology, School of Public Health, University of Alabama at Birmingham, Birmingham, Alabama, USA; Section of Rheumatology and Gwen Knapp Center for Lupus and Immunology Research, University of Chicago, Chicago, Illinois, USA; Keck School of Medicine, University of Southern California, Los Angeles, California, USA; David Geffen School of Medicine, University of California, Los Angeles, California, USA; Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA; Division of Rheumatology, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA; Rheumatology and Clinical Immunogenetics, University of Texas Health

Science Center at Houston, Houston, Texas, USA; Department of Medicine, Division of Rheumatology, University of Puerto Rico Medical Sciences Campus, San Juan, Puerto Rico; Department of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, USA; Division of Rheumatology, University of Colorado Denver, Aurora, Colorado, USA; Department of Internal Medicine/Nephrology, Wake Forest School of Medicine, Winston-Salem, North Carolina, USA; US Department of Veterans Affairs Medical Center, Oklahoma City, Oklahoma, USA; Division of Rheumatology, Department of Pediatrics, University of Washington, Seattle, WA, USA; King's College London, Divisions of Genetics and Molecular Medicine and Immunology, Infection and Inflammatory Disease, London, UK; Rosalind Russell Medical Research Center for Arthritis, University of California, San Francisco, California, USA; and Centro de Genómica e Investigaciones Oncológicas, Pfizer-Universidad de Granada-Junta de Andalucía, Granada, Spain.

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P.S. Ramos, PhD; J.C. Oates, MD; D.L. Kamen, MD; G.S. Gilkeson, MD, Division of Rheumatology and Immunology, Department of Medicine, Medical University of South Carolina; A.H. Williams, MA; C.D. Langefeld, PhD, Wake Forest School of Medicine and Center for Public Health Genomics; P.M. Gaffney, MD; J.A. Kelly, MPH; J.M. Guthridge, PhD; K.L. Moser, PhD, Arthritis and Clinical Immunology Research Program, Oklahoma Medical Research Foundation; K.M. Kaufman, PhD; J.B. Harley, MD, PhD, Division of Rheumatology, Cincinnati Children's Hospital Medical Center; R.P. Kimberly, MD; J.C. Edberg, PhD, Department of Medicine, University of Alabama at Birmingham; T.B. Niewold, MD, Section of Rheumatology and Gwen Knapp Center for Lupus and Immunology Research, University of Chicago; C.O. Jacob, MD, PhD, Keck School of Medicine, University of Southern California at Los Angeles; B.P. Tsao, PhD, David Geffen School of Medicine, University of California Los Angeles; G.S. Alarcón, MD, MPH, Departments of Medicine and Epidemiology, Schools of Medicine and Public Health, University of Alabama at Birmingham; E.E. Brown, PhD, MPH, Department of Epidemiology, University of Alabama at Birmingham; M.A. Petri, MD, MPH, Department of Medicine, Johns Hopkins University School of Medicine; R. Ramsey-Goldman, MD, DrPH, Division of Rheumatology, Northwestern University Feinberg School of Medicine; J.D. Reveille, MD, Rheumatology and Clinical Immunogenetics, University of Texas Health Science Center at Houston; L.M. Vilá, MD, Department of Medicine, Division of Rheumatology, University of Puerto Rico Medical Sciences Campus; J.A. James, MD, PhD, Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation, and Department of Medicine, University of Oklahoma Health Sciences Center; J.T. Merrill, MD, Clinical Pharmacology Research Program, Oklahoma Medical Research Foundation; S.A. Boackle, MD, Division of Rheumatology, University of Colorado Denver; B.I. Freedman, MD, Department of Internal Medicine/Nephrology, Wake Forest School of Medicine; R.H. Scofield, MD, Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation, Department of Medicine, University of Oklahoma Health Sciences Center, and US Department of Veterans Affairs Medical Center; A.M. Stevens, MD, PhD, Division of Rheumatology, Department of Pediatrics, University of Washington Seattle; T.J. Vyse, MA, MBBS, MRCP, PhD, King's College London; L.A. Criswell, MD, MPH, DSc, Rosalind Russell Medical Research Center for Arthritis, University of California, San Francisco; M.E. Alarcón-Riquelme, MD, PhD, Oklahoma Medical Research Foundation, and Centro de Genómica e Investigaciones Oncológicas, Pfizer-Universidad de Granada-Junta de Andalucía.

Address correspondence to Dr. P.S. Ramos, Division of Rheumatology and Immunology, Medical University of South Carolina, 96 Jonathan Lucas Street, Suite 912, Charleston, SC 29425, USA. E-mail: ramosp@musc.edu
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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 (AA), i.e., 0.009% of white men, 0.066% of white women, 0.038% of AA men, and 0.282% of AA women¹. A genetic contribution to SLE is unequivocal; recent genome-wide association studies in whites and Asians have identified nearly 40 validated susceptibility loci and implicated a broad array of biological pathways². 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^{5,6,7,8,9}.

Overproduction of nitric oxide (NO) and reactive oxygen intermediates is implicated in disease pathogenesis. Markers of systemic NO production and reactive oxygen species (ROS) are higher in patients with SLE than in 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^{10,11,12}. 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 United States. 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 coasts¹³. Until recent times, the estimated 100,000 to 300,000 Gullah remained relatively isolated. While continental AA average about 80% West African and 20% European ancestry¹⁴, the white admixture in the Gullah is < 3.5%, and the Gullah are the most homogeneous AA population described^{15,16,17}. Interestingly, there is the perception that SLE is rare in Africa, suggesting that comparative studies of related cohorts from the 2 continents may provide insight into the genetic etiology of SLE¹⁸. A higher than predicted prevalence of SLE multiplex families and a high prevalence of seropositivity in SLE first-degree relatives was observed in the Gullah, suggesting a major genetic influence in this population¹⁹. In parallel, studies report a higher prevalence of certain common complex traits in the Gullah when compared to other AA^{19,20,21}. 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, we attempted to identify specific genetic variants in genes involved in reactive intermediate production and scavenging predisposing to SLE in the Gullah population and admixed AA.

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 AA (1432 cases and 1687 controls). 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 SLE²². 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 descent¹⁹.

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

Statistical analyses. Only SNP meeting the quality control criteria of < 10% overall missing data, no evidence of differential missingness between cases and controls ($p > 0.05$), 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 sex inconsistencies and heterozygosity outliers excluded. Potential confounding substructure or admixture was controlled for using 306 ancestry informative markers to compute admixture proportions as implemented in the Admixmap program; principal component analysis using all SNP confirmed the results. After adjustment for population substructure, the inflation factor using all SNP was $\lambda = 0.98$ in the Gullah and $\lambda = 1.19$ in the AA. Principal component analysis plots of the AA and Gullah samples were conducted (data available from the author on request). Although the higher inflation factor would be expected given the selection of SNP in candidate genes, a genomic control-adjusted p value was also computed in AA. In contrast, the λ 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 (Division of Public Health Sciences, Wake Forest School of Medicine; 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 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 metaanalysis was computed as implemented in METAL software (Center for Statistical Genetics, University of Michigan; www.sph.umich.edu/csg/abecasis/metal), with weights being the square root of the sample size for each dataset; thus, the metaanalysis incorporates direction, magnitude of association, and sample size.

To uncover potential haplotype associations, a sliding window haplotype analysis of 3 to 8 SNP was performed in each region. A logistic regression model was employed, adjusting for population structure as implemented in PLINK software (Harvard University; pngu.mgh.harvard.edu/~purcell/plink)²³. Haplotypes with frequency < 10% were excluded.

To test for 2-locus interactions among SNP, all reported results met the quality criteria defined above. Specifically, SAS software (SAS Institute Inc.) 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 SNP under the additive model. To reduce false-positive

interactions due to low MAF, we rejected all pairs for which the expected number of individuals in the dataset was < 5 for minor allele homozygotes. In addition, all SNP pairs with a linkage disequilibrium (LD) measure of $r^2 > 0.2$ in YRI (a West African ancestry population) were excluded. Interactions were adjusted for population substructure. For chromosome X, only females were included.

A power analysis was computed with Quanto (University of Southern California; hydra.usc.edu/gxe) using a prevalence of 0.1% and $\alpha = 0.01$. LD between SNP was assessed with SNAP software using data from the 1000 Genomes Project in YRI (Broad Institute; www.broadinstitute.org/mpg/snap/index.php). SNP functionality was evaluated with the University of California at Santa Cruz (UCSC) genome browser (genome.ucsc.edu).

RESULTS

Genes with known association with production of or regulation of reactive oxygen and nitrogen intermediates were selected (data available from author on request). SNP mapping to 53 regions harboring reactive intermediate related genes were identified (data available from author on request). Association was assessed between these 244 SNP 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. To minimize potentially spurious associations, effects that were not supported by associations at neighboring SNP were excluded (Figure 1). In admixed AA, 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, 18 kDa (NADH-coenzyme Q reductase; *NDUFS4*), and NO synthase 1 (*NOS1*) genes. It should be noted that the variants reported here as associated with SLE met quality control thresholds in both AA and Gullah populations, but revealed association in only one of the populations (Figure 2).

The strongest signal in AA was observed at an intronic variant in a DNaseI hypersensitive cluster in the *GSR* gene (rs2253409; $p = 1.43 \times 10^{-03}$, OR 1.26, 95% CI 1.09–1.44). Although modest, other associations with neighboring SNP 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 2.1, 95% CI 1.23–3.59). Interestingly, this SNP locates in the transcription factor binding site for the RE1-silencing transcription factor 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 0.62, 95% CI 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 SNP (Figure 1). These variants

Table 1. SNP with the most significant association with SLE.

SNP	Chr	Pos (Mb)	Region	Risk Allele	MAF		Best-P*	P-GC	OR (95% CI)*
					Case	Control			
African (AA + Gullah)									
rs2551715	8	30,666	GSR	A	0.30	0.27	4.57E-03 ^d	6.43E-03	1.21 (1.06–1.38)
rs2253409	8	30,667	GSR	G	0.33	0.30	7.21E-04 ^d	1.16E-03	1.26 (1.10–1.44)
AA									
rs2253409	8	30,667	GSR	G	0.33	0.30	1.43E-03 ^d	3.43E-03	1.26 (1.09–1.44)
Gullah									
rs381575	5	52,949	NDUFS4	C	0.62	0.55	6.51E-03 ^r	—	2.10 (1.23–3.59)
rs561712	12	116,236	NOS1	A	0.38	0.51	7.18E-03 ^a	—	0.62 (0.44–0.88)
rs3730013	17	23,150	NOS2A	A	0.22	0.33	1.74E-03 ^d	—	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; MAF: minor allele frequency; P-GC: best-P after a genomic control adjustment; SNP: single-nucleotide polymorphism; AA: African American; GSR: glutathione reductase; NDUFS4: nicotinamide adenin dinucleotide hydrogen; NOS: nitric oxide synthase; SLE: systemic lupus erythematosus.

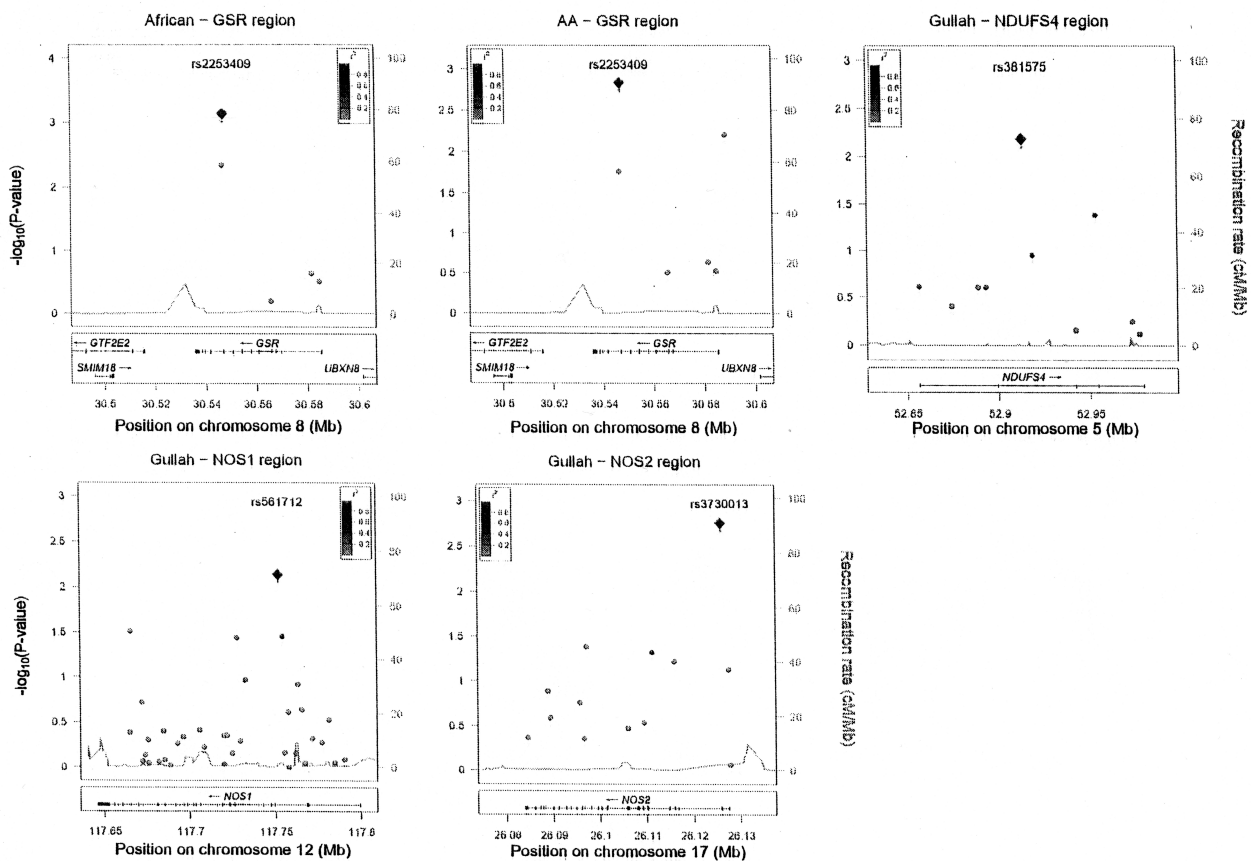


Figure 1. Regional plots of reactive intermediate gene loci in African, African American (AA), and Gullah populations. Genotyped SNP are plotted with their p values [as $-\log_{10}(p \text{ values})$] as a function of genomic position (Human Genome Build 18) within a region surrounding the most significant SNP (black diamond). Recombination rates from the 1000 Genomes AFR (11/2010) are plotted in gray to reflect the regional linkage disequilibrium (LD) structure. In each region the index SNP is represented by a large black diamond, and the shade of all other SNP (circles) indicates LD with the index SNP based on pairwise r^2 values from the 1000 Genomes AFR study. Known human genes in the University of California at Santa Cruz Genome Browser are at the bottom. GSR: glutathione reductase gene; NOS: nitric oxide synthase.

also met quality control thresholds in AA, but were associated only 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 AA predominated, as expected given their larger sample size. Table 1 reveals that in the African population, among the top

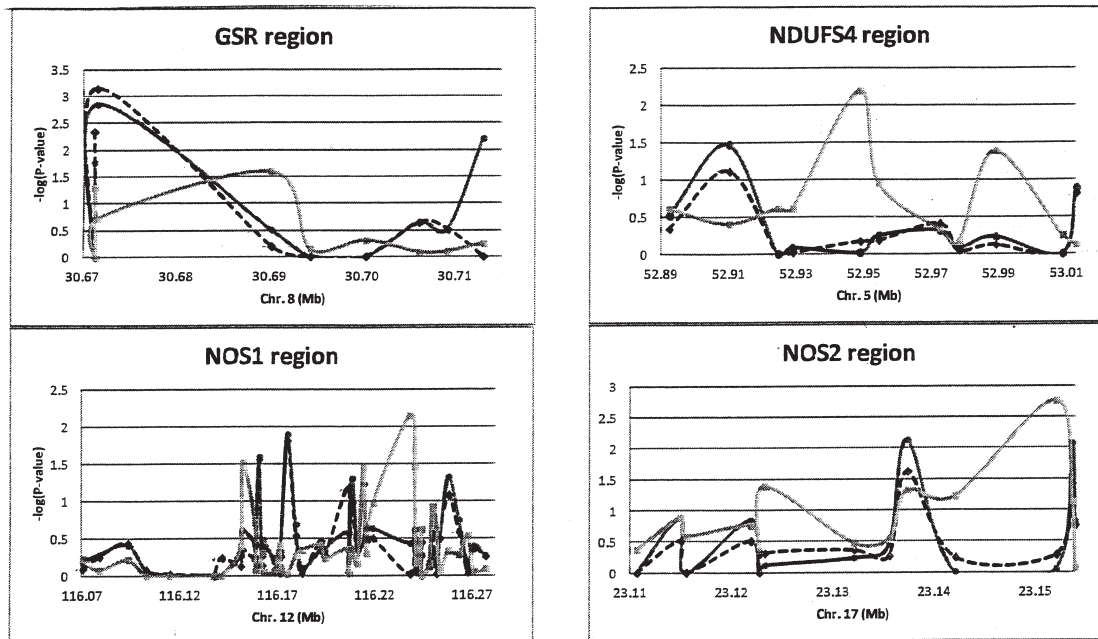


Figure 2. Patterns of association results of reactive intermediate gene loci in African, African American, and Gullah populations. Association results are shown for the Gullah (gray solid line), African American (black solid line), and combined African (dashed black line). Genotyped SNP (symbols) are plotted with their p values [as $-\log_{10}(p)$ values] as a function of genomic position (Mb) in each gene's region. *GSR*: glutathione reductase gene; *NOS*: nitric oxide synthase.

associations were the aforementioned variant in *GSR* (rs2253409; $p = 7.21 \times 10^{-04}$, OR 1.26, 95% CI 1.10–1.44) and another intronic variant in *GSR* (rs2551715; $p = 4.57 \times 10^{-03}$, OR 1.21, 95% CI 1.06–1.38) not in LD with the former ($r^2 = 0.07$ in YRI). Very similar results were obtained when a metaanalysis 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 cases of SLE, 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 SNP with lupus nephritis 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 AA 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 AA; 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 2-locus interaction analysis results. Nearly all interactions were specific to one ethnic group or the other. Only an interaction between an *NDUFS2* variant (rs4656993) with a *minichromosome maintenance complex component 5* SNP (rs4645794) was observed in the combined African samples ($p = 4.00 \times 10^{-04}$, OR 1.34, 95% CI 1.27–1.40) and AA ($p = 9.74 \times 10^{-05}$, OR 1.40, 95% CI 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 our study, all the associations reported were corroborated by associations at SNP in LD with the top

Table 2. Haplotypes with the most significant association with SLE.

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

* Haplotype defined by SNP: ^a rs3741476-rs1875140-rs10774909; ^b rs6087651-rs2236270-rs17092180-rs2273684. SLE: systemic lupus erythematosus; AA: African American; NSNP: number of single-nucleotide polymorphisms in haplotype; size: size of the haplotype; SNP1: first SNP on haplotype; Chr: chromosome; Pos: position of the first SNP in haplotype; F: frequency of haplotype; GSS: intronic glutathione synthetase; NOS: nitric oxide synthase.

Table 3. Most significant 2-loci interaction analysis results. Interactions due to linkage disequilibrium 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).

SNP1	Chr1	Pos1 (bp)	Region1	SNP2	Chr2	Pos2 (bp)	Region 2	p	OR (95% CI)
African									
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)
AA									
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)
Gullah									
rs256094	5	53008681	NDUFS4	rs133415	22	34136238	HMOX1, MCM5	2.00E-04	0.31 (0.15–0.64)

Chr: chromosome; Pos: position; SNP: single-nucleotide polymorphism; AA: African American; NDUFS: nicotinamide adenin dinucleotide hydrogen; NOS: nitric oxide synthase; CYP: cytochrome; SOD: superoxide dismutase; HMOX: heme oxygenase; MCM: minichromosome maintenance complex; CYB5B: cytochrome b5 type B.

associated SNP, 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. ROS can oxidatively modify proteins to influence their activity. Transcription factors AP1 (cJun), nuclear factor- κ B (NF- κ B), hypoxia-inducible factor-1 α , and p53 are all redox-regulated²⁴. For instance, H₂O₂ generated by endothelial cell (EC) NOX leads to NF- κ B-mediated transcription of intercellular adhesion molecule and vascular cell adhesion molecule²⁵, both of which associate with lupus nephritis disease activity^{26,27} and atherosclerosis^{28,29}. Two inflammatory cytokines, interleukin 6 (IL-6) and monocyte chemoattractant proteins 1 (MCP-1; CCL2), important to the pathogenesis of lupus nephritis^{30,31,32,33,34,35} and atherosclerosis^{36,37}, have in common redox-regulated NF- κ B response elements (UCSC Genome browser). Both MCP-1 and IL-6 secretion by activated EC is dependent on ROS production and can be inhibited by ROS scavengers³⁸.

The functional relevance of the described SNP in reactive intermediate genes is not known. However, 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 AA^{10,39,40}. The consequences of this increased oxidative stress may be increased antigenicity of self-antigens and pathogenic redox signaling.

GSR catalyzes the reduction of glutathione disulfide to glutathione (GSH), an important antioxidant molecule⁴¹. 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 depletion⁴², a process that can predispose cells to necrosis. Our study does not address whether the reduced levels of GSH observed in patients with SLE is due to increased production of ROS, 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 production⁴³, while described mutations in *NDUFS4* do not appear to lead to increased oxidative stress⁴⁴.

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 nephritis⁴⁵, and the effects of low-level NO production are functionally reduced in the vasculature of patients with SLE⁴⁶. Similarly, NOS1 appears to prevent leukocyte adhesion in mice lacking *NOS3*⁴⁷. This finding is also seen in disease, because 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 activity⁴⁹. Thus, NOS1 deficiency could exacerbate NOS3 dysfunction or deficiency, leading to inflammation and vascular dysfunction. There is evidence for this notion, because *NOS1* is associated with endstage renal disease in AA⁵⁰.

We 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 AA, 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 SLE-associated in select African ancestry populations.

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Variable Association of Reactive Intermediate Genes with Systemic Lupus Erythematosus in Populations with Different African Ancestry

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