

Nitric Oxide Synthase 2 Promoter Polymorphisms and Systemic Lupus Erythematosus in African-Americans

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ABSTRACT. *Objective.* Systemic lupus erythematosus (SLE) is an autoimmune disease in which morbidity and mortality are higher in African-Americans. The etiology of this racial disparity is unknown. A genetic predisposition to enhanced nitric oxide (NO) production may predispose African-Americans to develop SLE and may increase disease severity. We have demonstrated a correlation between NO production and disease activity in SLE. Two polymorphisms in the inducible NO synthase (NOS2) promoter region (G-954C and CCTTT microsatellite repeat polymorphisms) are associated with improved outcome in some African patients with malaria. This study was designed to determine if these polymorphisms are associated with SLE.

Methods. We assessed the frequency of both the G-954C and CCTTT microsatellite repeat NOS2 promoter polymorphisms in a cohort of patients with SLE and age, sex, and race matched controls in North Carolina and South Carolina.

Results. Both polymorphisms were more frequent among African-American female SLE patients when compared with controls ($p = 0.04$ for the G-954C polymorphism and $p = 0.03$ for the CCTTT-8 repeat polymorphism). Further, the G-954C and CCTTT-8 repeat polymorphisms were in linkage disequilibrium ($D' = 0.89$, $p = 0.0001$) among African-American female SLE patients.

Conclusion. Altered genetic control of NOS2 transcription may be a risk factor for SLE among African-American females. The extent of linkage disequilibrium between the G-954C and CCTTT-8 repeat NOS2 promoter polymorphisms suggests that they were co-inherited. (J Rheumatol 2003;30:60–7)

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Systemic lupus erythematosus (SLE) is a systemic autoimmune syndrome that has a higher prevalence and causes more morbidity and mortality among African-Americans than Caucasians^{1,2}. African-American females experience the highest burden of disease. The cause of the disparity between African-American and Caucasian SLE disease severity is widely debated but likely involves a combination of socioeconomic, environmental, and genetic factors¹⁻³. It has been suggested that among those of African origin, SLE is more prevalent in the United States than in Western Africa. This hypothesis, known as the “prevalence gradient hypothesis,” also suggests that genetic traits beneficial to individuals living in Africa may be detrimental (perhaps due to environmental factors) to individuals living in North America⁴.

Nitric oxide (NO) is a short-lived effector molecule, with both protective and pathologic properties, that is produced by 3 different isoforms of nitric oxide synthase (NOS)⁵. Endothelial NOS (NOS3, expressed primarily by endothelial cells) and neuronal NOS (NOS1, expressed primarily in neuronal tissue) constitutively produce low levels of NO that acts to dilate blood vessels, prevent thrombosis, and transmit neuronal signals⁵. Inducible NOS (NOS2, produced mostly by activated cells) produces much larger amounts of NO in the presence of inflammatory stimuli⁵. NO has beneficial antitumor and antimicrobial effects, but can also mediate pathologic sequelae⁵.

A genetic predisposition to enhanced production of NO may predispose African-Americans to develop SLE and may increase disease severity as compared to Caucasian patients with SLE. Several laboratories have described increased systemic measures of NO production among subjects of multiple racial backgrounds with SLE, particularly during disease activity⁶⁻¹². Our laboratory demonstrated that (1) sera 3-nitrotyrosine (3NT) levels (a surrogate measure of systemic NO production) were elevated in SLE subjects with active disease, (2) sera 3NT levels were correlated with disease activity among African-Americans, but not Caucasians, with SLE, and (3) African-American SLE and control subjects had elevated measures of NO compared to Caucasian SLE and control subjects¹³. The mechanism for this heightened NO production among African-Americans is unclear.

Studies of African populations with malaria demonstrated a relationship between increased NO production and milder disease severity^{14,15}. A polymorphism in the 5' untranslated region (5'UTR) of the NOS2 gene has been described at position -954 (G-954C)¹⁶. This polymorphism was associated with protection from severe forms of malaria in Gabon¹⁶. Similarly, the number of CCTTT repeats in a microsatellite located 2.5 kb 5' to the NOS2 transcription start site was related to the risk of fatal cerebral malaria among Africans in Gambia¹⁷. Taken together, these studies suggest that the CCTTT repeat and/or G-954C NOS2 promoter polymorphisms may be associated with increased NOS2 transcription and protective responses during malaria infection.

Because the highest prevalence of SLE is found among African-American females, we hypothesized that the G-954C and certain repeat lengths of the CCTTT NOS2 promoter polymorphisms would be observed more frequently among female African-American SLE subjects as compared to age, sex, race, and geographically matched control subjects. To test this hypothesis, we analyzed DNA samples from SLE cases and control subjects living in North Carolina and South Carolina [Carolina Lupus (CLU) Study] for the G-954C and CCTTT repeat NOS2 promoter polymorphisms.

MATERIALS AND METHODS

Subjects. Subjects were enrolled in the CLU Study, a retrospective case control study of genetic and environmental factors predisposing to SLE. All subjects signed an informed consent approved by local institutional review boards. The CLU Study included 265 individuals with SLE living in eastern North Carolina and South Carolina. The SLE patients met the 1997 revised American College of Rheumatology classification criteria¹⁸ and were diagnosed between January 1, 1995, and July 31, 1999. As part of the CLU Study protocol, SLE entry criteria were recorded, and blood was obtained by phlebotomy for analysis of sera for antibodies to the SSA/Ro, SSB/La, Smith, and RNP antigens. Sixty percent of the SLE patients were African-American, and 90% were female. Four university based and 30 community based rheumatology practices participated in the study. University based rheumatologists referred about 50% of patients. The CLU Study also included 355 population based controls who were randomly selected from state driver's license registries and who were frequency-matched to the age (in 5 year groups), sex, race, and state distribution of the SLE cases. Data collection included a 60 min standardized in-person interview and a medical record review. At the

time of the study interview, a blood sample was obtained from 244 (92%) SLE cases and 301 (85%) controls.

NOS2 promoter G-954C polymorphism analysis. Restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) analysis of DNA from 453 CLU Study subjects was performed to determine if the G-954C polymorphism was present in either allele of NOS2. All available DNA samples from African-American CLU subjects were analyzed. Because of the very low prevalence of the G-954C polymorphism in a pilot study in our Caucasian subjects (0.5%), we analyzed only a random selection of samples (63%) from Caucasian subjects. Briefly, 573 bp of DNA between residues 2727 and 3299, as defined by Spitsen, *et al*¹⁹, was amplified by PCR and digested with the restriction enzyme BsaI as described²⁰ and electrophoresed on an agarose gel with ethidium bromide staining²⁰. The presence of the G-954C polymorphism abolishes the BsaI restriction enzyme site, leaving a larger PCR product than cleaved, wild-type DNA samples (Figure 1). Therefore, known wild-type non-polymorphic and G-954C polymorphic samples were analyzed with each assay to confirm the effectiveness of the amplification and endonuclease reaction. In addition, randomly selected samples were analyzed on more than one occasion to determine the accuracy of the assay. Samples that were difficult to characterize were analyzed on 2 additional occasions to allow complete characterization of all 453 samples.

CCTTT microsatellite repeat NOS2 promoter polymorphism analysis. DNA from 338 CLU Study subjects was analyzed for the CCTTT microsatellite repeat NOS2 promoter polymorphism (the sample size differed between this and the G-954C polymorphism analysis because DNA was not available on all subjects for both analyses). Briefly, the number of CCTTT pentanucleotide repeats on each allele was determined by analysis of DNA between residues 1076 and 1272¹⁹. This fragment, ~2.5 kb 5' to the NOS2 start site, was amplified by PCR and the number of CCTTT repeats determined from the length of the PCR product as described²⁰. In total 337 (99%) samples were reliably characterized for both alleles.

Sera 3-nitrotyrosine (3NT) analysis. Sera samples were available for analysis from 14 SLE subjects with the G-954C polymorphism, 108 wild-type SLE subjects, 4 control subjects with the G-954C polymorphism, and 60 wild-type controls. Sera were analyzed for 3-nitrotyrosine (3NT) by immuno-dot blot. Quantitation of 3NT is a validated measure of oxidative stress induced by peroxynitrite or other potential nitrating agents²¹⁻²³. The protein concentration of each serum sample was determined by employing the BCA assay (Pierce; Rockford, IL, USA). 100 µg of serum protein in 50 µl of phosphate buffered saline (PBS) was bound to a polyvinylidene difluoride (PVDF) membrane (Fisher Scientific; Suwanee, GA, USA) using low suction through a 96 sample enzyme linked immunofilter assay Easy-TiterTM suction manifold (Pierce). The membranes were dried and reactivated with methanol before blocking with 5% powdered milk in PBS/Tween overnight at 4°C. A mouse monoclonal anti-3NT antibody (Upstate Biotechnology; Lake Placid, NY, USA; 1:2500 in PBS/Tween 5% milk) was used as the primary antibody, while a goat anti-mouse alkaline phosphatase conjugate (Southern

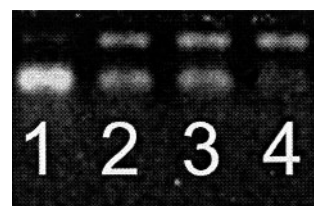


Figure 1. Representative gel illustrating obliteration of endonuclease site with G-954C polymorphism: 573 bp of DNA containing the -954 region of the NOS2 promoter was amplified by PCR and digested with the restriction enzyme BsaI. Samples were electrophoresed onto an agarose gel. The presence of the G-954C polymorphism abolishes the BsaI restriction enzyme site. Lane 1 is a homozygous wild-type sample. Lanes 2-4 are heterozygous samples, and lane 4 is a homozygous G-954C polymorphism sample.

Biotechnology; Birmingham, AL, USA, in 1:20,000 PBS/Tween) was used as the secondary antibody. Membranes were developed using ECF (Amersham; Arlington Heights, IL, USA) and read using a Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics; Sunnyvale, CA, USA). For calibration purposes, 2 standard curves of 50% nitrated BSA and 50% nitrated IgG (Sigma) by weight were blotted on each membrane. A single batch of nitrated bovine serum albumin (BSA) and IgG standards were prepared as described^{24,25}. Results were reported as a percentage of the signal intensity from 100 µg of standard nitrated BSA/IgG. Samples were performed in triplicate.

Autoantibody analysis. Sera collected as part of the CLU study protocol were used to measure autoantibodies. A standardized protocol at a single laboratory was used for all patients as described²⁶. Included in the analysis were determinations for antinuclear antibodies and antibodies to SSA/Ro, SSB/La, Smith, and RNP antigens.

Linkage disequilibrium analysis. Disequilibrium between the G-954C and CCTTT repeat polymorphism was calculated by using a maximum likelihood algorithm to estimate the haplotype frequencies²⁷. Disequilibrium (D') was then calculated using a modification of the Lewontin methods as described²⁸. **Statistical analysis.** Because G-954C homozygotes were rare, the heterozygotes and homozygotes were analyzed together and compared to the wild-type subjects. The frequencies of the G-954C genotypes were compared between SLE cases and controls using contingency tables stratified by race and sex. P values were assessed using Fisher's exact test. The odds ratio (OR) and 95% confidence interval were calculated as a measure of association. The number of male subjects was too small to warrant statistical testing in this subgroup. Similar statistical methods were used for comparison of the CCTTT repeat alleles between SLE cases and controls. We first determined heterogeneity of all of the CCTTT repeat polymorphisms (7 to 16 repeats) between SLE cases and controls using contingency tables. We analyzed for differences in the distribution of alleles between female African-American cases and controls as well as differences between female African-American and female Caucasian controls using the likelihood ratio test. Differences in individual alleles (e.g., CCTTT-8 or CCTTT-14) were then determined when there was evidence of differences in allele frequency using Fisher's exact test. A similar analytic strategy was used to determine the association, among patients, between individual elements of the SLE clinical presentation at the time of subject enrollment (this includes all of the elements of the 1997 revised American College of Rheumatology classification criteria and the presence of antibodies to SSA/Ro, SSB/La, Smith, and RNP antigens) and either the G-954C allele or any of the CCTTT repeat NOS2 polymorphisms. Sera 3NT levels were compared for case and control subjects with and without the G-954C polymorphism using one or 2-way analysis of variance (ANOVA) with -954 amino acid (G vs C) and case/control status as variables.

RESULTS

Frequency of G-954C NOS2 promoter polymorphism in SLE cases and control subjects. Among the African-American women, the G-954C polymorphism occurred significantly more frequently among SLE subjects (OR 3.4, $p = 0.042$; Table 1). The number of African-American males was small (9 cases, 7 controls), and no cases and 3 controls had the G-954C allele. Among Caucasian-Americans, none of the 63 SLE cases and one of the 138 controls tested had a G-954C allele. Given the low incidence of the G-954C polymorphism in the CLU Study Caucasian population, we did not analyze the Caucasian G-954C polymorphism data further. We also did not perform a statistical analysis of data from the 14 cases and 22 controls that were neither Caucasian nor African-American because of the low frequency of the G-954C polymorphism in this population (1 case, 0 controls). Analysis of

Table 1. Frequency of the G-954C NOS2 promoter polymorphism in African-American SLE cases ($n = 144$) and controls ($n = 73$)^a.

	Cases, n (%)	Controls, n (%)	p	OR (95% CI)
Females				
G-954C	24 (18) ^b	4 (6) ^b	0.042	3.4 (1.1, 13.8)
Wild-type	111 (82)	62 (94)		
Total sample				
G-954C	24 (17)	7 (10)	0.16	1.9 (0.7, 5.1)
Wild-type	120 (83)	66 (90)		

^a Analysis of the G-954C NOS2 promoter polymorphism was performed in 144 African-American SLE and 73 African-American control subjects from North Carolina and South Carolina. The frequency of those who had at least one G-954C allele versus those who were wild-type was compared in SLE and control subjects. P values for Fisher's exact test and the odds ratio (OR) with 95% confidence interval (CI) for each comparison are reported.

^b Three case subjects and one control subject were homozygous for the G-954C promoter polymorphisms.

the African-American samples from both sexes revealed an increased incidence of G-954C among SLE cases, but the difference was not statistically significant (Table 1).

Sera 3NT levels among SLE case and control African-American female subjects with and without the G-954C NOS2 promoter polymorphism. Subjects with SLE and the G-954C polymorphism had higher sera 3NT levels (SLE with G-954C = $7.18 \pm 2.14\%$, SLE with wild-type = $6.45 \pm 2.80\%$, control with G-954C = $6.77 \pm 4.03\%$, and control with wild-type = $5.82 \pm 2.28\%$ of standard). SLE subjects with the G-954C polymorphism had significantly greater sera 3NT levels than wild-type controls ($p = 0.04$). 3NT levels in wild-type and G-954C SLE subjects were not statistically different. All subjects (both SLE and control subjects) with the G-954 polymorphism had higher sera 3NT levels than all wild-type subjects ($7.09 \pm 2.53\%$ vs $6.23 \pm 2.63\%$ of standard; $p = 0.19$). Using 2-way ANOVA, neither the G-954C polymorphism nor SLE status of subject affected 3NT levels in a statistically significant manner. However, given the limited numbers of patients in each group, the low power of the ANOVA analysis (5%) was not likely to detect statistically significant differences.

Frequency of CCTTT repeat NOS2 promoter polymorphisms in SLE cases and control subjects. Figure 2 compares the frequency of CCTTT repeat alleles among African-American female SLE cases and controls. There was significant evidence of heterogeneity among African-American females in the number of CCTTT repeats (likelihood ratio chi-square, $p = 0.01$; data not shown). The frequency of individual CCTTT repeat alleles was determined for African-American female SLE cases and controls. In this population, the distribution of alleles among African-American cases and controls was significantly different ($p = 0.013$). The frequency of CCTTT-8 was significantly different between 86 SLE cases and 40 con-

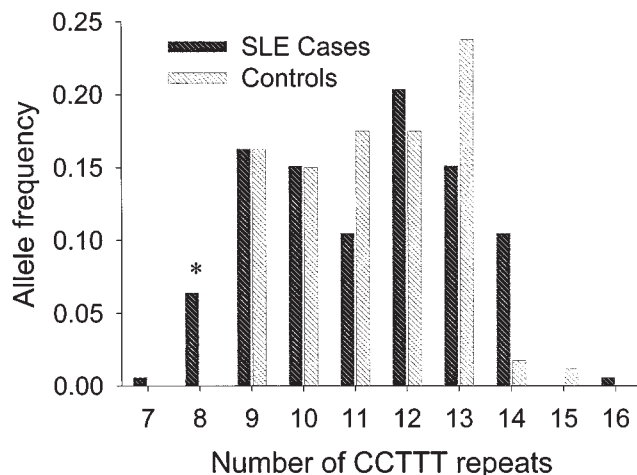


Figure 2. Frequency histogram of CCTTT repeats (CCTTT-#) among female African-American SLE cases and controls. DNA from 86 African-American female SLE patients and 40 African-American female controls in the Carolina Lupus Study was analyzed for CCTTT repeat polymorphisms of the NOS2 promoter region (2.5 kb 5' to the NOS2 start site). The x-axis depicts the number of CCTTT microsatellite repeats, and the y-axis depicts the frequency of each allele in this population. * $p = 0.03$ by Fisher's exact test between SLE cases and controls.

trols (12% cases and 0% of controls, $p = 0.03$; Table 2, Figure 2). Despite an increased frequency in SLE cases, the 14 repeat allele was not significantly different among female African-American SLE cases and controls ($p < 0.09$; data not shown). When we included African-American males in the analysis, differences in the frequency of CCTTT-8 were not significant ($p = 0.22$ by Fisher's exact test; Table 2), but differences in the frequency of CCTTT-14 were more evident (10% vs 3%, $p = 0.058$ by Fisher's exact and $p = 0.048$ by chi-square test; data not shown).

Table 2. Frequency of the NOS2 promoter CCTTT microsatellite repeat alleles in African-American SLE cases ($n = 93$) and controls ($n = 45$)^a.

	Cases, n (%)	Controls, n (%)	p	OR (95% CI)
Females				
CCTTT-8	10 (12) ^b	0 (0)	0.03	Undefined
Other alleles	76 (88)	40 (100)		
Total sample				
CCTTT-8	11 (12)	2 (4)	0.22	2.8 (0.6, 13.6)
Other alleles	82 (88)	43 (96)		

^a Analysis of the CCTTT microsatellite repeat NOS2 promoter polymorphism was performed in 93 African-American SLE and 45 African-American control subjects from North Carolina and South Carolina. The frequency of CCTTT-# was compared between SLE and control subjects. Those with at least one CCTTT-8 allele were compared to those with all other numbers of CCTTT repeats. P values for Fisher's exact test and the odds ratio (OR) with 95% confidence interval (CI) are reported for each comparison.

^b One subject (an African-American female SLE case) was homozygous for CCTTT-8.

There was no evidence of heterogeneity between Caucasian SLE cases and controls (data not shown). However, among all Caucasians CCTTT-14 was observed more frequently among SLE patients (12% vs 7%, $p = 0.062$ by Fisher's exact test and $p = 0.044$ by chi-square test; data not shown). Among non-Caucasian, non-African-American subjects, CCTTT-8 was observed in 2/7 controls and 1/9 cases.

Analysis of CCTTT microsatellite repeat NOS2 promoter polymorphism to compare Caucasian admixture in African-American case and control subjects. The frequency of individual CCTTT repeat alleles was determined for African-American ($n = 40$) and Caucasian ($n = 114$) female control subjects (Figure 3). The frequencies of the CCTTT repeat polymorphisms were significantly different between African-American female controls and Caucasian female controls ($p < 0.0001$). These data are consistent with other studies of the NOS2 promoter CCTTT repeat polymorphism in African and non-African populations^{20,29}. When analyzing for individual alleles, allele frequencies were significantly different between these groups for CCTTT-9 (16% vs 2%, $p < 0.001$; Figure 3) and CCTTT-12 (18% vs 35%, $p < 0.001$; Figure 3). The difference in frequency of these alleles can thus be used for detecting Caucasian admixture among African-Americans³⁰. The CCTTT-9 frequency is virtually identical in the African-American female cases and controls ($p = 0.36$; Figure 2). There is a small but not significantly higher frequency of the CCTTT-12 allele in African-American female SLE cases compared to African-American female controls ($p = 0.11$; Figure 2), suggesting no increased Caucasian admixture among African-American controls.

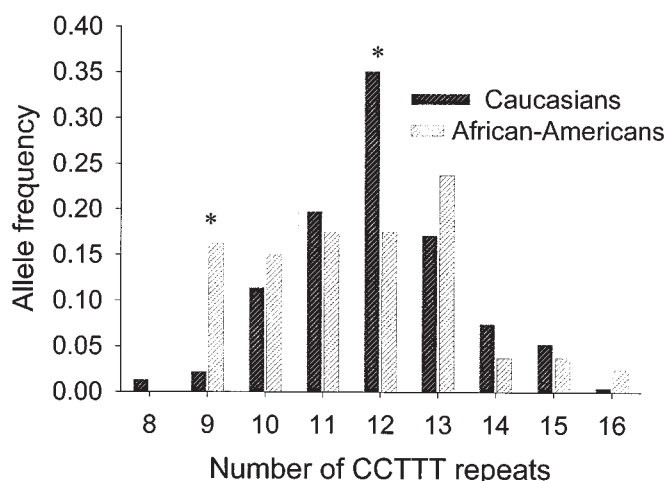


Figure 3. Frequency histogram of CCTTT repeats (CCTTT-#) among African-American and Caucasian female controls. DNA from 114 Caucasian and 40 African-American controls in the Carolina Lupus Study was analyzed for the CCTTT repeat polymorphism of the NOS2 promoter region (2.5 kb 5' to the NOS2 start site). The x-axis depicts the number of CCTTT microsatellite repeats, while the y-axis depicts the frequency of each allele in this population. * $p \leq 0.001$ by Fisher's exact test between African-American and Caucasian controls.

Association between the G-954C allele and the CCTTT-8 and CCTTT-14 alleles. We assessed linkage disequilibrium between the G-954C polymorphism and the CCTTT-8 and CCTTT-14 pentanucleotide repeat alleles by comparing the frequency of these alleles among African-American SLE cases. We found extensive linkage disequilibrium between the G-954C allele and the CCTTT-8 allele in African-American SLE cases when we analyzed females alone or both males and females. Eleven of the 13 African-American SLE cases (9 of 10 females) with CCTTT-8 alleles also had G-954C alleles ($D' = 0.89$ in females and $D' = 0.724$ for both sexes, $p < 0.0001$ in females and $p < 0.0005$ for both sexes). No linkage disequilibrium between the G-954C and CCTTT-8 alleles was detected in controls ($D' = 0.0$, as the one control that had the CCTTT-8 allele did not have a G-954C allele). There was no evidence of linkage disequilibrium between the G-954C and CCTTT-14 polymorphisms in African-American SLE cases (data not shown).

Association between SLE clinical features and NOS2 promoter polymorphisms. To determine whether the G-954C or any of the CCTTT repeat NOS2 promoter polymorphisms were associated with any specific SLE clinical or serological characteristics, we analyzed each SLE clinical characteristic against each polymorphism among African-American female SLE patients. The presence of SSB/La antibodies was associated with the G-954C NOS2 promoter polymorphism (20.8% of G-954C subjects were SSB/La positive vs 6.4% of wild-type subjects; chi-square 5.06, $p = 0.02$). Heterogeneity analysis of all CCTTT repeat polymorphisms indicated non-random association between repeat sizes and the SSB/La antibody (likelihood ratio = 26.7, $DF = 9$, $p = 0.0001$). Individually, 50% of CCTTT-8 subjects were SSB/La positive versus 5.3% of subjects with other repeat lengths (chi-square 18.9, $p \leq 0.0001$). Twenty-two percent of the CCTTT-11 individuals were SSB/La positive, while 8% of the individuals without the CCTTT-11 repeat were SSB/La positive (chi-square 4.2, $p = 0.04$). No other clinical or serological characteristics were associated with the G-954C or CCTTT repeat NOS2 promoter polymorphisms. There is no association with either allele among Caucasian or African-American male subjects.

DISCUSSION

Our data demonstrate that among African-American females in North Carolina and South Carolina, there is an association of SLE with the G-954C and CCTTT-8 NOS2 promoter polymorphisms. Our results reveal a trend towards increased NO production (measured as serum 3NT) among cases and controls with the G-954C polymorphism, but the differences were not significant. We also demonstrated significant linkage disequilibrium between the G-954C and the CCTTT-8 polymorphisms in African-American SLE cases. The extensive linkage disequilibrium in the SLE cases between the G-954C and CCTTT-8 repeat polymorphisms in the Carolinas is evidence that this haplotype background predisposes to the develop-

ment of SLE. This haplotype is rare among African-American controls as evidenced by the lack of this haplotype in the samples we studied.

There are 3 possible explanations for the association of these polymorphisms with SLE and for significant linkage disequilibrium between these polymorphisms. The first possibility is that the observed association between these alleles and SLE may be due to increased Caucasian admixture in the African-American control population, effectively decreasing the number of controls with African polymorphisms. African-Americans are an admixed population, with between 3% and 20% Caucasian admixture and 1–3% Native American admixture^{30,31}. We do not believe that Caucasian admixture in the control population accounts for our results because we stratified by race, and a population based sampling strategy was used to select the controls. Further, using the pentanucleotide repeat polymorphism as a marker of Caucasian admixture, we did not detect a higher degree of Caucasian admixture in African-American controls.

The second possibility is that one of these polymorphisms results in increased NOS2 expression. The higher frequency of these polymorphisms in African populations (14% for G-954C²⁰ and roughly 7% for CCTTT-8^{20,32}) compared with Caucasian populations^{20,32} supports this possibility. Our results demonstrated a trend towards an effect of the G-954C polymorphism (or a co-inherited polymorphism) on sera 3NT measures, as levels were higher among cases and controls with the G-954C polymorphism. The low number of individuals with this polymorphism reduces the power to detect statistically significant differences. In addition, the CLU study was not designed to detect levels of disease activity in SLE subjects. Our laboratory has demonstrated that among African-Americans, (1) 3NT sera levels are directly correlated with disease activity measures, and (2) SLE subjects with no disease activity have sera 3NT levels that are similar to controls¹³. Therefore, we expect sera from SLE subjects with no disease activity to reduce the differences in sera 3NT levels between SLE and control subjects. Future studies evaluating relationships between NOS2 polymorphisms and NO production *in vivo* should include disease activity as a variable.

Studies of African malaria incorporate the notion of comparing disease activity or outcome with NO production and the presence of NOS2 promoter polymorphisms. African malaria subjects who are able to substantially increase NO production during infection may have a more favorable disease outcome^{14,15}. Both the G-954C and certain CCTTT repeat polymorphisms were associated with improved malaria outcome in Gabon and Gambia, respectively^{16,17}, and blood mononuclear cells from G-954C individuals have increased basal and cytokine induced NOS activity³³. However, no reports have addressed whether the G-954C polymorphism alters NOS2 transcription, and as discussed below the G-954C polymorphism is associated with increased NO production in only some African populations²⁰. One study supports the pos-

sibility that longer forms of the NOS2 promoter CCTTT repeat are associated with increased NOS2 transcription³⁴. However, studies by Taylor, *et al*³⁵ determined that deletions of the region containing the CCTTT repeat do not alter transcription in NOS2 promoter reporter assays.

The third possibility is that these 2 alleles are linked in this population to an unidentified polymorphism that predisposes individuals to the SLE phenotype. In contrast to the studies of the G-954C and CCTTT polymorphisms in Gambia and Gabon^{16,36}, Levesque, *et al* did not identify an association of these polymorphisms with improved outcome or enhanced NO production in malaria infections in Tanzanian patients²⁰, despite an earlier study showing an association between improved outcome in malaria and increased NO production in this population¹⁴. Although this difference may be due to differences in the clinical presentation of malaria in Tanzania, Gabon, and Gambia^{16,17,20}, it is more likely that the discordance between these studies may be due to differences between these populations in an unrecognized, co-inherited polymorphism associated with the G-954C and CCTTT repeat polymorphisms.

The NOS2 promoter contains multiple known transcription factor binding sites³⁷. Thus, polymorphisms in several regions of the NOS2 promoter might result in changes in NOS2 transcription. NOS2 promoter region binding sites for both nuclear factor kappa B, Stat 1, and activator protein 1 are located between -5.1 kb and -8.2 kb^{37,38}. The polymorphisms we investigated in this study are located in a region of the NOS2 promoter that has not been completely mapped for transcription factor binding sites. Deletion of the -2.1 to -4.7 kb portion (containing the CCTTT repeat NOS2 promoter polymorphism) of the NOS2 promoter does not diminish cytokine stimulated promoter activity of an otherwise functional 7.2 kb NOS2 luciferase promoter construct³⁹. A 1.1 kb promoter construct (containing the G-954C NOS2 promoter polymorphism) was not inducible with lipopolysaccharide or interferon- γ (IFN- γ)³⁹. However, a recent report by Kun, *et al* indicates that unstimulated peripheral blood mononuclear cells (PBMC) from individuals with the G-954C mutation have greater NOS2 activity *in vitro* than PBMC from wild-type controls³³. These data suggest that the G-954C mutation or a co-inherited mutation affects baseline NOS2 expression.

Another manner in which NOS2 promoter polymorphisms may affect NOS2 protein expression is by altering NOS2 mRNA stability. Rodriquez-Pascual, *et al*⁴⁰ reported an influence of the 3'UTR of the NOS2 gene on mRNA stability, perhaps through an interaction between the NOS2 3'UTR and the NOS2 promoter region⁴¹. Whether this interaction with the NOS2 3'UTR occurs in the region of the G-954C and CCTTT repeat NOS2 promoter polymorphisms is unknown. Such an interaction does have precedent, as inhibitory actions of sequences in the 3'UTR on 5'UTR-driven constitutive expression of tumor necrosis factor-alpha (TNF- α) have been described⁴².

Our study is the first to illustrate an association between NOS2 promoter polymorphisms and SLE among African-American females. An affected sib-pair analysis from Minnesota⁴³ and a pedigree analysis from Oklahoma⁴⁴ did not identify loci linked with SLE on chromosome 17 near position 17 cen-q11.2, the location of the NOS2 gene⁴⁵. However, only 5% of the subjects from the Minnesota study and about one-third of the subjects in the Oklahoma study were African-American. The apparent contradiction with our results may be a reflection of different African origins of subjects in the 2 studies or may be due to the low admixture of Caucasian genes in the African-Americans from the coastal Carolinas³¹. Given the multiple genetic associations with SLE and the low penetrance of the G-954C and CCTTT-8 NOS2 promoter polymorphisms, it is unlikely that an increased risk for SLE would be detected in the region of the NOS2 gene even in the Oklahoma study. Association studies of known polymorphisms, such as the one described here, are more likely than linkage analysis to identify genetic traits associated with diseases of complex genetic origin⁴⁶. Therefore, the absence of linkage with chromosome 17 in other studies of SLE does not eliminate the NOS2 promoter as a potential site where polymorphisms may either predispose to or worsen the severity of SLE among African-Americans.

We did not determine whether the G-954C or CCTTT repeat NOS2 promoter polymorphisms were associated with other polymorphisms reported to be associated with SLE in African-Americans. Specifically, among African-Americans in the Johns Hopkins Lupus Cohort, SLE was associated with the TNF- α -308A polymorphism (OR 2.72, $p = 0.007$) in a manner that is (unlike among Caucasians) independent of the DR3 major histocompatibility complex allele⁴⁷. Because a combination of TNF- α and either or both of IFN- γ and interleukin 1 β can induce greater human NOS2 transcription⁴⁸, these 2 polymorphisms could, when present together, act in a synergistic manner to increase NOS2 expression. However, in the CLU population, there is no association between the TNF- α -308A polymorphism and SLE among African-Americans⁴⁹.

In summary, our results reveal an association between SLE and both the G-954C and CCTTT-8 NOS2 promoter polymorphisms among African-American women. These data suggest that altered genetic control of NOS2 is a risk factor for SLE in African-American females. These data do not definitively demonstrate the functional consequence of these promoter polymorphisms in this population, however. Future studies will clarify the functional significance of these polymorphisms and their relationships to inflammatory diseases such as SLE.

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