American Journal of Epidemiology

Vol. 178, No. 8
DOI: 10.1093/aje/kwt108
Advance Access publication:
August 27, 2013

## Original Contribution

# Association of Polymorphisms in Natural Killer Cell–Related Genes With Preterm Birth

**Quaker E. Harmon*, Stephanie M. Engel, Andrew F. Olshan, Thomas Moran, Alison M. Stuebe, Jingchun Luo, Michael C. Wu, and Christy L. Avery**

* Correspondence to Dr. Quaker Harmon, National Institute of Environmental Health Sciences, P.O. Box 12233, MD A-305, Research Triangle Park, NC 27709 (e-mail: quaker.harmon@nih.gov).

*Initially submitted December 21, 2012; accepted for publication May 9, 2013.*

Inflammation is implicated in preterm birth, but genetic studies of inflammatory genes have yielded inconsistent results. Maternal DNA from 1,646 participants in the Pregnancy, Infection, and Nutrition Cohort, enrolled in Orange and Wake counties, North Carolina (1995–2005), were genotyped for 432 tag single-nucleotide polymorphisms (SNPs) in 30 candidate genes. Gene-level and SNP associations were modeled within strata of genetic ancestry. Six genes were associated with preterm birth among European Americans: *interleukin 12A* (*IL12A); colony-stimulating factor 2 (CSF2); interferon* $\gamma$ *receptor 2 (IFNGR2); killer cell immunoglobulin–like receptor, three domain, long cytoplasmic tail, 2 (KIR3DL2); interleukin 4 (IL4);* and *interleukin 13 (IL13)*. Of these, relatively strong single-SNP associations were seen in *IFNGR2* and *KIR3DL2*. Among the 4 genes related to natural killer cell function, 2 (*IL12A* and *CSF2)* were consistently associated with reduced risk of prematurity for both European and African Americans. SNPs tagging a locus control region for *IL4* and *IL13* were associated with an increased risk of spontaneous preterm birth for European Americans (rs3091307; risk ratio = 1.9; 95% confidence interval: 1.4, 2.5). Although gene-level associations were detected only in European Americans, single-SNP associations among European and African Americans were often similar in direction, though estimated with less precision among African Americans. In conclusion, we identified novel associations between variants in the natural killer cell immune pathway and prematurity in this biracial US population.

African Americans; European Continental Ancestry Group; genetics; genetic association studies; inflammation; natural killer cells; premature birth

Abbreviations: CSF, colony-stimulating factor; DNA, deoxyribonucleic acid; FDR, false discovery rate; IFN, interferon; IL, interleukin; KIR, killer cell immunoglobulin–like receptor; LD, linkage disequilibrium; NK, natural killer cells; RR, risk ratio; SKAT, SNP-Set Kernel Association Test; SNP, single-nucleotide polymorphism; TNF, tumor necrosis factor.

Preterm birth, which affects approximately 12% of US births (1), has significant medical and societal implications, including increased risk of neonatal death and both acute and chronic medical and neurocognitive disease (2). Although the earliest preterm births have the most severe outcomes (3), late preterm births comprise the majority (74.1%) (4) of preterm births and also are associated with increased risk of death and respiratory difficulties (5).

Having had a previous preterm birth is one of the strongest maternal risk factors for a subsequent preterm birth. In addition to environmental exposures that might persist between pregnancies, this risk could reflect innate susceptibility factors from maternal genes (6–8). Although several candidate pathways have been identified (9), inflammatory pathways have been a particular focus because pregnancy is a state of altered immune function (10–13). Pregnancy appears to involve a global shift toward defensive immunity (14–17). In particular, T cells and natural killer (NK) cells decrease in both number and function over the course of pregnancy (14), and both cell types have been linked to initiation of labor before term (18, 19). Candidate genes involved in inflammation have been examined in several diverse populations (20–38), although many of the studies have had small case groups, and few have addressed population stratification. Given differences in allelic frequencies in genes related to inflammatory cytokines (39), as well as differences in the risk of preterm birth (26–29, 40)

among whites and African Americans, genetic ancestry is of particular importance. Existing studies with larger case groups (28–32, 41) have had few single-nucleotide polymorphisms (SNPs) per gene and often have not included regulatory regions that flank genes.

Using the Pregnancy, Infection, and Nutrition Cohort, we expanded candidate genes to include lesser-studied genes in the critical T cell and NK cell pathways and deepened the per-gene SNP coverage while addressing issues of population stratification. We additionally improved on previous studies by incorporating novel statistical methodology to identify gene-based associations, and we used inverse probability of selection modeling to account for any differences between the genotyped and parent cohort. In this article, we describe our findings for polymorphisms in 30 candidate genes and the risk of preterm birth.

## MATERIALS AND METHODS

### Study population

We used a nested case-control subset of the Pregnancy, Infection, and Nutrition Cohort, a prospective pregnancy cohort designed to assess antenatal risk factors for preterm birth described by Savitz et al. (42). Women were recruited between 1995 and 2005 through Wake County Human Services Department (Raleigh, North Carolina), Wake Medical Center (Raleigh, North Carolina), and University of North Carolina (Chapel Hill, North Carolina) prenatal clinics. Exclusion criteria at enrollment included age less than 16 years, inability to speak English, delivery not planned to occur at the recruitment hospital, multiple gestations, and lack of a telephone.

Maternal blood for genetic analysis was obtained at the first study visit. Covariates were collected through self-administered questionnaires or telephone interviews in the late second and third trimesters. Pregnancy outcome information was abstracted from the medical record after delivery. All participants gave informed consent, and the institutional review boards of the University of North Carolina School of Medicine, Chapel Hill, North Carolina, and Wake Medical Center, Raleigh, North Carolina, approved the study.

Cases and controls for this study were selected among eligible women from the entire Pregnancy, Infection, and Nutrition Cohort ($n = 5,169$) as a part of a larger study investigating multiple reproductive outcomes and gene pathways. Initial eligibility criteria included consent for genetic analysis ($n = 3,539$), collection of a suitable biological specimen ($n = 3,289$), self-reported maternal race of white or African American ($n = 3,075$), and known infant birth date, resulting in 3,065 (59.3%) women who were eligible for selection into our study. Of the eligible women, all preterm cases with sufficient DNA were genotyped ($n = 347$; 92% of eligible preterm births). Term births selected for genotyping included births with other reproductive outcomes of interest (gestational hypertension, small for gestational age), as well as women with uncomplicated pregnancies ($n = 1,299$). In total, 1,646 women were genotyped.

### Outcome assessment

The first ultrasound performed before 22 weeks' gestation was used to calculate gestational age at delivery. For women without an early ultrasound (9.3%), self-reported last menstrual period was used. Preterm birth was defined as a live birth before 37 completed weeks of gestation. Subtype of preterm birth was assessed by physician review (42) of the hospital record to identify events (e.g., preterm labor, spontaneous rupture of membranes) immediately preceding delivery. Although consideration of each subtype of preterm birth would be optimal (43), the present prospective study lacked sufficient numbers. However, spontaneous preterm birth, which includes both preterm labor and preterm prelabor rupture of membranes, was considered as an additional outcome.

### Gene selection

Thirty candidate genes were selected from the innate and adaptive immune system, with a focus on representing T helper 1 and T helper 2 cytokines and their regulators, inflammatory mediators (including tumor necrosis factor (TNF) signaling), and NK cells. In particular, we aimed to represent inflammatory genes for which marked changes in protein concentrations across trimesters have been demonstrated, because dysregulation in these genes could result in initiation of parturition before term (14, 15).

### Genotyping

Whole blood was collected and centrifuged, and the buffy coat fraction was stored in cell preparation tubes and placed in −80°C storage. DNA was extracted with an Applied Biosystems automated DNA extractor (Foster City, California) and Qiagen Gentra Puregene chemistry (Valencia, California).

A custom 1,536 Illumina GoldenGate (San Diego, California) panel was created, which included the 30 genes (432 SNPs) from this study as well as genes from angiogenesis, apoptosis, and cell cycle pathways. TagZilla (44) was used to choose tag SNPs for 2 populations (European and Yoruban; HapMap build 27), with allowance for 20-kb upstream and 10-kb downstream margins and with tags restricted to those with minor allele frequencies of at least 10% (in one population) and linkage disequilibrium (LD) with $r^2 > 0.8$.

Genotyping was conducted at the University of North Carolina Mammalian Genotyping Core (Chapel Hill, North Carolina), and genotypes were called with Illumina GenomeStudio software. Genotyping was performed on 1,646 samples. Individuals were dropped when less than 95% of SNPs were successfully called ($n = 11$). We further excluded unintentional duplicate samples ($n = 3$), congenital anomalies ($n = 24$), and stillbirths ($n = 10$), resulting in 1,598 women included in the analysis.

Poor genotyping quality (<95% call rate) resulted in the loss of 39 SNPs. Quality control included duplicate Pregnancy, Infection, and Nutrition samples and standardized samples from Corriel (Camden, New Jersey) CEPH (Centre d'Etude du Polymorphisme Humain) trios on each plate. A single base-pair discrepancy and no Mendelian errors were found in the 393 duplicate or trio samples. Hardy-Weinberg equilibrium was assessed with SAS version 9.2 (SAS Institute, Inc., Cary, North Carolina) among noncases stratified by genetic ancestry. One SNP (*IL-2*: rs10027390) significantly violated Hardy-

Weinberg equilibrium ($P < 10^{-5}$) in African-American noncases and was dropped from analysis for this ancestry group.

Tagging for 2 genetic ancestry populations resulted in redundant (LD > 0.8) tag SNPs in the European-American population. Haploview (45) (Broad Institute, Cambridge, Massachusetts) was used to calculate LD (with $r^2$) and to generate LD heatmaps.

A panel of 157 ancestry-informative markers was chosen to estimate genetic ancestry with the use of SNPs tested in a similar North Carolina biracial population (46). STRUCTURE, version 2.3.3 (47) (University of Chicago, Chicago, Illinois) was used to quantify genetic ancestry, with the assumption of 2 underlying populations. Genetic ancestry was then used to stratify the analyses (European-American ancestry and African-American ancestry), and continuous percent African-American ancestry was included in all models.

### Statistical analysis

Covariates were selected on the basis of previous studies and an examination of a directed acyclic graph. Possible covariates included maternal smoking, age, body mass index, and socioeconomic status. In addition, several additional demographic, pregnancy health, and study characteristics were included for the calculation of selection probabilities.

We used a 2-stage analysis approach to examine the influence of inflammatory genes on preterm birth. Stage 1 was a gene-level analysis that used the SNP-Set Kernel Association Test (SKAT) (48) with a linear kernel (analogous to logistic regression with an additive genetic effect). Gene-level analysis is particularly useful in genetically diverse populations in which different tag SNPs are in LD with the causal SNPs. SKAT also allows for complex SNP interactions, permits covariate adjustment, and does not penalize SNPs with opposing associations (increased or decreased risk) within a single gene. SNPs were grouped by gene into SNP sets, and genes in close proximity (within 25 kbp) were analyzed together. Persons with at least 1 missing genotype were dropped from the relevant SNP set but were included in other SNP sets for which they had complete data. On average, 98% of participants were included in each SNP set, with a range of 92%–100% (Web Table 1, available at http://aje.oxfordjournals.org/). Hypothesis testing in SKAT was conducted with a variance component score test of the null hypothesis that the general function for the SNP set equals zero (49). Analyses were performed within strata defined by genetic ancestry and were additionally adjusted for percent African-American ancestry. A false discovery rate (FDR) of 20% was used to identify genes that progressed to Stage 2. The FDR was calculated as a $Q$ value (50) with R, version 2.13.1 (R Foundation for Statistical Computing, Vienna, Austria) (p.adjust function, FDR). Cases included all preterm births, with a subset analysis of spontaneous preterm birth. Controls included term births without gestational hypertension or small-for-gestational-age complications (Table 1, "Disease Free").

The goal of Stage 2 was to identify the individual SNPs in each gene responsible for the significant SNP set association $P$ values estimated in Stage 1. Given the prevalence of preterm birth in this population (13.5%), risk ratios were estimated from the entire genotyped cohort with a log-linear risk model. Inverse probability weighting was used so that the estimates reflected the eligible population. Cases were all preterm births, with a subset analysis of spontaneous preterm births. Controls were all genotyped term births (Table 1, "Term Births"). Briefly, the probability of being selected into the genotyped sample ($n$ = 1,646) was calculated for all eligible women ($n$ = 3,065) by the use of a logistic model that included all covariates in Table 1 as well as additional demographic and study-related characteristics. The inverse of these selection probabilities was used to weight the analysis. Robust variance estimators were used. Given the dependence between Stage 1 and Stage 2, top-ranking SNPs from Stage 2 were reported on the basis of the consistency of the observed associations.

## RESULTS

The final analysis set included 1,598 individuals. Women were predominantly white (62.6%), nonsmoking (74.4%), and well educated (52% with at least high school education), with a mean body mass index of 26.5 kg/m$^2$ and a mean age of 26.1 years at the start of pregnancy (Table 1). In the underlying cohort, preterm birth occurred in 13.5% of births, and 62.1% of these were spontaneous. The eligible and genotyped population did not differ appreciably from the entire cohort (Web Table 2).

### Stage 1: gene set analysis

Proximity of genes resulted in 24 SNP sets, with *interleukin (IL)-13* (*IL13*) and *IL-4 (IL4)* considered jointly and *lymphotoxin α (TNF superfamily member 1)* (*LTA)* and *TNF* considered jointly in the SKAT analysis. Genes with a single SNP *(transforming growth factor, beta 1 (TGFB1)* and *beta 3 (TGFB3)*) or with SNPs genotyped solely for replication purposes (*IL-1A* and *IL-1B,* each with 2 SNPs) were not included in the Stage 1 gene-level analysis.

For preterm birth, 4 SNP sets *(interferon (IFN) γ receptor 2 (IFNGR2); IL12A; killer cell immunoglobulin–like receptor (KIR), three domain, long cytoplasmic tail, 2 (KIR3DL2);* and *colony-stimulating factor 2 (CSF2))* associated with NK cells and 1 SNP set associated with T helper 2 immunity (*IL13/IL4*) met the FDR criteria of 20% (Table 2) among European Americans. *IFNGR2* and *IL13/IL4* also met the FDR criteria for spontaneous preterm birth. No SNP set met criteria for either outcome among African-American participants.

### Stage 2: single-SNP analysis

The 5 SNP sets identified in Stage 1 (90 SNPs) and 7 SNPs not included in Stage 1 were examined in Stage 2 in both European and African Americans. Tables 3 and 4 present the risk ratios and 95% confidence intervals for the strongest single-SNP results. Results for all of the single SNPs included in Stage 2 can be found in Web Table 3. Variant allele frequencies can be found in Web Tables 4 and 5. In the assessment of covariates, nested models adjusted only for percent genetic ancestry were compared with fully and singly adjusted models for a subset of the SNPs with the strongest associations. Adjustment for all or any of the covariates failed to change the point estimates (>10%) (results not shown). Web Table 6 provides

**Table 1.** Demographic Characteristics of Mothers Genotyped in the Pregnancy, Infection, and Nutrition Cohort, North Carolina, 1995–2005

| Characteristic | Genetic Ancestry[a] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Preterm Case | | | | Disease Free | | | | Term Birth[b] | | | |
| | European American ($n$ = 194) | | African American ($n$ = 134) | | European American ($n$ = 409) | | African American ($n$ = 204) | | European American ($n$ = 813) | | African American ($n$ = 457) | |
| | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| Maternal age, years | | | | | | | | | | | | |
| <25 | 94 | 48.5 | 50 | 37.3 | 215 | 52.6 | 68 | 33.3 | 401 | 49.3 | 159 | 34.8 |
| 25–34 | 77 | 39.7 | 73 | 54.5 | 133 | 32.5 | 132 | 64.7 | 294 | 36.2 | 281 | 61.5 |
| ≥35 | 23 | 11.9 | 11 | 8.2 | 61 | 14.9 | 4 | 2.0 | 118 | 14.5 | 17 | 3.7 |
| Smoking[c] | | | | | | | | | | | | |
| No | 115 | 66.5 | 95 | 80.5 | 302 | 77.4 | 148 | 84.6 | 541 | 71.0 | 325 | 81.9 |
| Yes | 58 | 33.5 | 23 | 19.5 | 88 | 22.6 | 27 | 15.4 | 221 | 29.0 | 72 | 18.1 |
| Missing | 21 | 10.8 | 16 | 11.9 | 19 | 4.6 | 29 | 14.2 | 51 | 6.3 | 60 | 13.1 |
| Body mass index[d] | | | | | | | | | | | | |
| <18.5 | 14 | 7.5 | 8 | 6.5 | 23 | 5.7 | 16 | 8.3 | 41 | 5.2 | 30 | 7.1 |
| 18.5–24.9 | 95 | 50.8 | 43 | 34.7 | 250 | 62.2 | 79 | 40.9 | 431 | 54.9 | 158 | 37.3 |
| 25–29.9 | 44 | 23.5 | 28 | 22.6 | 71 | 17.7 | 39 | 20.2 | 146 | 18.6 | 85 | 20.1 |
| ≥30 | 34 | 18.2 | 45 | 36.3 | 58 | 14.4 | 59 | 30.6 | 167 | 21.3 | 151 | 35.6 |
| Missing | 7 | 3.6 | 10 | 7.5 | 7 | 1.7 | 11 | 5.4 | 28 | 3.4 | 33 | 7.2 |
| Poverty index[e] | 331 (254) | | 132 (94) | | 367 (238) | | 144 (123) | | 343 (234) | | 148 (126) | |
| Missing | 23 | 11.9 | 17 | 12.7 | 24 | 5.9 | 37 | 18.1 | 57 | 7.0 | 94 | 20.6 |
| Marital status | | | | | | | | | | | | |
| Married | 134 | 69.1 | 39 | 29.3 | 313 | 76.5 | 32 | 15.7 | 587 | 72.2 | 83 | 18.2 |
| Unmarried | 60 | 30.9 | 94 | 70.7 | 96 | 23.5 | 172 | 84.3 | 226 | 27.8 | 374 | 81.8 |
| Missing | 0 | | 1 | 0.7 | 0 | | 0 | | 0 | | 0 | |
| Education, years | | | | | | | | | | | | |
| ≥13 | 105 | 54.1 | 56 | 41.8 | 275 | 67.2 | 68 | 33.3 | 498 | 61.3 | 172 | 37.6 |
| ≤12 | 89 | 45.9 | 78 | 58.2 | 134 | 32.8 | 136 | 66.7 | 315 | 38.8 | 285 | 62.4 |
| Parity | | | | | | | | | | | | |
| Nulliparous | 88 | 45.4 | 49 | 36.8 | 189 | 46.4 | 88 | 43.1 | 398 | 49.1 | 219 | 47.9 |
| Multiparous | 106 | 54.6 | 84 | 63.2 | 218 | 53.6 | 116 | 56.9 | 413 | 50.9 | 238 | 52.1 |
| Missing | 0 | | 1 | 0.7 | 2 | 0.5 | 0 | | 2 | 0.2 | 0 | |
| Spontaneous preterm | | | | | | | | | | | | |
| Yes | 103 | 53.1 | 65 | 48.5 | | | | | | | | |

[a] Genetic ancestry was determined from 148 ancestry-informative markers and the STRUCTURE software package.
[b] Term births include disease-free term births plus term births with other outcomes of interest: small for gestational age, gestational hypertension, and preeclampsia.
[c] Self-reported smoking during months 1–6 of pregnancy. Values in the no and yes rows are expressed as a percent of those with nonmissing data.
[d] Prepregnancy body mass index was calculated as self-reported prepregnancy weight in kilograms divided by measured height in meters squared. Values within categories of body mass index are expressed as a percent of those with nonmissing data.
[e] Poverty index reflects household income and the number of household members expressed as the percent of the local poverty limit. Mean (standard deviation) is presented.

the single-SNP associations for all genotyped markers not included in Stage 2.

Two SNPs in *IL12A* (rs6441282 and rs692890) showed consistent associations (risk ratio (RR) = 0.7–0.8) for both ancestry groups and both outcomes, with the variant allele conferring reduced risk of both preterm and spontaneous preterm birth. The variant alleles were the minor alleles for European-American women but were more common in African-American women (Web Table 4). These 2 SNPs were in strong LD among African-American women but not among European-American women (Figure 1). Several SNPs had consistent results for both outcomes among European-American women only: rs503582, rs7653097, and rs755004 (RR = 1.3–1.5), and rs13064168, rs609907, and rs17826053 (RR = 0.6–0.8). SNP

**Table 2.** $Q$[a] Values From SNP-Set Kernel Association Test Analysis for Each Gene Set Stratified by Genetic Ancestry in the Pregnancy, Infection, and Nutrition Cohort, North Carolina, 1995–2005

| Gene Symbol | European American[b] | | African American[b] | |
|---|---|---|---|---|
| | Preterm | Spontaneous | Preterm | Spontaneous |
| *IFNGR2* | 0.06[c,d] | 0.18[c] | 0.93 | 1.00 |
| *IL13* and *IL4*[e] | 0.10[c] | 0.01[c,d] | 1.00 | 1.00 |
| *KIR3DL2* | 0.10[c] | 0.43 | 0.89 | 1.00 |
| *IL12A* | 0.10[c] | 0.44 | 0.89 | 0.64 |
| *CSF2* | 0.14[c] | 0.67 | 0.89 | 0.82 |
| *IL18* | 0.40 | 0.44 | 0.93 | 1.00 |
| *GATA3* | 0.40 | 0.48 | 0.89 | 1.00 |
| *IL10* | 0.40 | 0.48 | 0.89 | 0.82 |
| *IL12B* | 0.54 | 0.81 | 0.93 | 1.00 |
| *IL6* | 0.54 | 0.79 | 0.93 | 0.92 |
| *KIR2DL4* | 0.54 | 0.43 | 0.89 | 1.00 |
| *IL15* | 0.66 | 0.67 | 0.93 | 0.89 |
| *LTA* and *TNF*[e] | 0.70 | 0.70 | 0.48 | 1.00 |
| *IFNG* | 0.75 | 1.00 | 0.89 | 0.64 |
| *TBX21* | 0.75 | 1.00 | 0.89 | 0.92 |
| *NFKB1* | 0.75 | 1.00 | 0.93 | 1.00 |
| *TNFRSF1B* | 0.75 | 0.67 | 0.89 | 0.64 |
| *IL8* | 0.75 | 0.67 | 0.93 | 0.82 |
| *KIR3DL3* | 0.75 | 0.67 | 0.89 | 0.64 |
| *KLDR1* | 0.85 | 1.00 | 0.89 | 1.00 |
| *IL6R* | 0.88 | 0.67 | 0.93 | 1.00 |
| *IL2* | 0.89 | 0.81 | 0.89 | 0.82 |
| *IL8RB* | 1.00 | 0.81 | 0.93 | 1.00 |
| *CXCL10* | 1.00 | 0.81 | 0.89 | 1.00 |

[a] $Q$ values represent the proportion of false positives (number of false rejections divided by total number of rejections).

[b] Genetic ancestry was determined from 148 ancestry-informative markers and the STRUCTURE software package. All models were additionally adjusted for percentage Yoruban ancestry.

[c] Meets false discovery rate criterion of <0.2.

[d] Bonferroni $P$ value for $P = 0.05$ and 24 gene sets = 0.002. The $P$ value for *IL13* and *IL4* met this criterion for spontaneous preterm birth among European-American mothers. The $P$ value for *IFNGR2* met this criterion for preterm birth among European-American mothers.

[e] Genes were considered in the same model because of proximity.

rs4680536 showed an increased risk of spontaneous preterm birth only (RR = 1.3; 95% confidence interval: 1.0, 1.7).

Two LD blocks that cover *CSF2* and *IL3* in European-American women (Figure 2) show an association between *CSF2* and preterm birth. A group of 3 SNPs (rs25881, rs25882, and rs27438; $r^2 > 0.8$), including a missense variant, conferred increased risk (RR = 1.3–1.4) for preterm birth only. A second LD block (rs721121, rs4705916, rs743564, and rs6898270; $r^2 > 0.85$ in European-American women) was associated with generally reduced risk of preterm birth for both European- and African-American women. For African-American women, there was a strengthening of the risk reduction for spontaneous preterm birth for rs721121 and rs4705916 (RR = 0.57; 95% confidence interval: 0.4, 0.9).

Upstream tags for *CSF2* also captured SNPs that are more closely associated with *IL-3* (*IL3*). One SNP in *IL3* (rs31481) was associated with an increased risk of preterm birth among both ancestry groups (RR = 1.3–1.4). Among European-American women only, a downstream SNP (rs11575022) was associated with an increased risk of both preterm and spontaneous preterm birth (RR = 1.6 and 1.4, respectively).

*IL13* and *IL4* are in close proximity on chromosome 5 and were jointly tagged. In both genes, associations were generally found for European-American women only, and these associations were strongest for spontaneous preterm birth. For *IL13*, a cluster of 3 SNPs in strong LD (rs7737470, rs3091307, and rs1881457; $r^2 > 0.8$) (Figure 3) had a risk ratio of 1.8–1.9 for spontaneous preterm birth. A second cluster in LD (rs2243204, rs2243210, rs2243218, and rs2243219; $r^2 > 0.7$) had a risk ratio of 1.3–1.5. The results for *IL4* were more varied with both protective and risk alleles. One cluster of SNPs in strong LD (rs2243267, rs2243270, and rs11242123; $r^2 > 0.8$) showed both risk (RR = 1.6–1.8) and protective variants (rs2243250) (RR = 0.6). A single SNP (rs11242122) downstream of *IL4* showed a particularly strong protective association for spontaneous preterm birth, with a risk ratio of 0.54 (95% confidence interval: 0.4, 0.7).

SNP associations for *KIR3DL2* were found only for European-American women. Two downstream SNPs (rs11672983 and rs3816051) showed consistently increased risk of both preterm and spontaneous preterm births (RR = 1.3–1.4). A single SNP (rs4806457) showed a risk association with preterm birth only (RR = 1.7; 95% confidence interval: 1.1, 2.7).

Among European-American women, 2 LD clusters in *IFNGR2* were seen (Figure 4). rs9978223, rs2268241, and rs9808753 ($r^2 > 0.9$) all showed a reduced risk, with a strengthening of the association for spontaneous preterm birth (RR = 0.6–0.7). An additional cluster of 2 SNPs (rs9808685 and rs2834210; $r^2 = 0.97$) showed a consistent risk association with both preterm and spontaneous preterm births (RR = 1.3).

## DISCUSSION

We undertook an investigation of T-cell and NK cell–related gene variants in a biracial pregnancy cohort. In addition to novel findings in *KIR3DL2*, our increased tagging of T helper 1 and T helper 2 genes revealed important associations with preterm birth.

Several of the genes highlighted in our study are important for NK cells and their function (*IL12A, IFNGR2, CSF2,* and *KIR3DL2*). NK cells and the cytokines associated with them (IL-12, IL-15, IL-6, IFN-γ, TNFα, and CSF2) have been documented to change dramatically over the course of pregnancy (14, 15) and might be involved closely with immune tolerance to the developing placenta and adequate placental implantation early in pregnancy. KIR genes, which regulate the killing function of NK cells, are particularly important in allowing trophoblast cells to evade destruction by NK cells during placental development. Indeed, KIR

**Table 3.** Relative Risk of Preterm and Spontaneous Preterm Birth for Natural Killer Cell–Related Single SNPs Among Mothers Stratified by Genetic Ancestry in the Pregnancy, Infection, and Nutrition Cohort, North Carolina, 1995–2005[a]

| Gene/SNP[c] | European American[b] | | | | African American[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | Preterm | | Spontaneous Preterm | | Preterm | | Spontaneous Preterm | |
| | RR | 95% CI | RR | 95% CI | RR | 95% CI | RR | 95% CI |
| *IL12A* | | | | | | | | |
| rs503582 | 1.3 | 1.1, 1.7 | 1.3 | 1.0, 1.7 | 1.1 | 0.8, 1.4 | 1.0 | 0.7, 1.4 |
| rs7653097 | 1.5 | 1.0, 2.1 | 1.4 | 0.9, 2.3 | 1.0 | 0.7, 1.4 | 0.9 | 0.6, 1.5 |
| rs13064168 | 0.6 | 0.4, 0.8 | 0.7 | 0.4, 1.0 | 1.1 | 0.7, 1.7 | 1.3 | 0.7, 2.4 |
| rs609907[d] | 0.6 | 0.5, 0.8 | 0.7 | 0.5, 1.0 | 1.1 | 0.8, 1.6 | 1.2 | 0.7, 2.0 |
| rs2647929 | 1.1 | 0.9, 1.4 | 1.1 | 0.8, 1.5 | 1.1 | 0.8, 1.6 | 1.6 | 1.0, 2.4 |
| rs9811792 | 1.1 | 0.9, 1.4 | 1.1 | 0.8, 1.5 | 1.1 | 0.8, 1.4 | 1.5 | 1.0, 2.2 |
| rs7372767 | 1.1 | 0.9, 1.4 | 1.1 | 0.9, 1.5 | 1.3 | 0.9, 1.7 | 1.7 | 1.1, 2.6 |
| rs6441282 | 0.8 | 0.6, 1.0 | 0.8 | 0.6, 1.0 | 0.8 | 0.6, 1.0 | 0.7 | 0.5, 1.0 |
| rs692890 | 0.7 | 0.6, 0.9 | 0.7 | 0.5, 0.9 | 0.8 | 0.6, 1.1 | 0.7 | 0.5, 1.0 |
| rs755004 | 1.3 | 1.0, 1.7 | 1.5 | 1.1, 2.1 | 0.6 | 0.3, 1.2 | 0.6 | 0.2, 1.7 |
| rs17826053 | 0.7 | 0.5, 1.0 | 0.7 | 0.5, 1.1 | 1.0 | 0.7, 1.4 | 0.7 | 0.4, 1.2 |
| rs4680536 | 1.1 | 0.9, 1.4 | 1.3 | 1.0, 1.7 | 1.1 | 0.8, 1.4 | 1.0 | 0.7, 1.4 |
| *IFNGR2* | | | | | | | | |
| rs6517167 | 1.3 | 1.0, 1.7 | 1.1 | 0.7, 1.6 | 1.1 | 0.8, 1.4 | 0.9 | 0.6, 1.3 |
| rs9978223 | 0.7 | 0.5, 0.9 | 0.6 | 0.4, 1.0 | 1.1 | 0.8, 1.4 | 1.1 | 0.7, 1.5 |
| rs2268241 | 0.6 | 0.5, 0.9 | 0.6 | 0.3, 0.9 | 1.2 | 0.9, 1.6 | 1.3 | 0.9, 1.8 |
| rs9808685 | 1.3 | 1.1, 1.7 | 1.3 | 1.0, 1.8 | 1.0 | 0.8, 1.3 | 0.9 | 0.6, 1.3 |
| rs2834210 | 1.3 | 1.0, 1.6 | 1.3 | 0.9, 1.7 | 1.0 | 0.8, 1.4 | 0.9 | 0.6, 1.3 |
| rs9808753 | 0.7 | 0.5, 1.0 | 0.6 | 0.4, 1.0 | 1.2 | 0.9, 1.6 | 1.1 | 0.7, 1.8 |
| rs2834213 | 1.1 | 0.9, 1.4 | 1.1 | 0.8, 1.5 | 1.2 | 0.7, 2.1 | 1.8 | 0.9, 3.5 |
| *KIR3DL2* | | | | | | | | |
| rs4806457 | 1.7 | 1.1, 2.7 | 1.5 | 0.7, 3.1 | 1.0 | 0.5, 1.8 | 0.7 | 0.3, 1.7 |
| rs11672983 | 1.3 | 1.0, 1.5 | 1.3 | 1.0, 1.7 | 0.8 | 0.6, 1.0 | 0.9 | 0.6, 1.4 |
| rs3816051 | 1.4 | 1.2, 1.7 | 1.4 | 1.1, 1.9 | 0.9 | 0.6, 1.1 | 0.9 | 0.6, 1.3 |
| *IL3* | | | | | | | | |
| rs31481 | 1.4 | 1.1, 1.8 | 1.0 | 0.7, 1.5 | 1.3 | 0.9, 1.8 | 1.2 | 0.7, 2.0 |
| rs11575022 | 1.6 | 1.1, 2.3 | 1.5 | 0.9, 2.6 | 1.1 | 0.7, 1.5 | 1.0 | 0.6, 1.7 |
| *CSF2* | | | | | | | | |
| rs721121 | 0.8 | 0.7, 1.0 | 0.9 | 0.7, 1.2 | 0.7 | 0.5, 1.0 | 0.6 | 0.4, 0.9 |
| rs4705916 | 0.8 | 0.6, 1.0 | 0.8 | 0.6, 1.1 | 0.7 | 0.5, 1.0 | 0.6 | 0.4, 0.9 |
| rs743564 | 0.8 | 0.6, 1.0 | 0.9 | 0.6, 1.2 | 0.7 | 0.5, 1.0 | 0.8 | 0.5, 1.2 |
| rs25881 | 1.4 | 1.0, 1.8 | 1.2 | 0.8, 1.7 | 1.0 | 0.8, 1.4 | 0.9 | 0.6, 1.4 |
| rs25882 | 1.3 | 1.0, 1.7 | 1.1 | 0.7, 1.6 | 1.1 | 0.8, 1.5 | 1.0 | 0.7, 1.6 |
| rs27438 | 1.3 | 1.0, 1.7 | 1.1 | 0.8, 1.6 | 0.9 | 0.7, 1.1 | 0.8 | 0.6, 1.2 |
| rs6898270 | 0.8 | 0.6, 1.0 | 0.9 | 0.6, 1.2 | 0.8 | 0.6, 1.2 | 0.8 | 0.5, 1.3 |

Abbreviations: CI, confidence interval; RR, risk ratio; SNP, single-nucleotide polymorphism.

[a] Variant allele and frequency are available in Web Table 4.

[b] Based on genetic ancestry. All models were additionally adjusted for percentage of Yoruban ancestry.

[c] SNPs are arranged by base pair position within each gene.

[d] Estimate for preterm birth among European Americans has $P < 0.0005$ (Bonferroni correction for 100 SNPs in Stage 2).

**Table 4.** Relative Risk of Preterm and Spontaneous Preterm Birth for T Helper 2 Single SNPs Among Mothers Stratified by Genetic Ancestry in the Pregnancy, Infection, and Nutrition Cohort, North Carolina, 1995–2005[a]

| Gene/SNP[c] | European American[b] | | | | African American[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | Preterm | | Spontaneous Preterm | | Preterm | | Spontaneous Preterm | |
| | RR | 95% CI | RR | 95% CI | RR | 95% CI | RR | 95% CI |
| *IL13* | | | | | | | | |
| rs7737470[d] | 1.4 | 1.1, 1.8 | 1.9 | 1.4, 2.5 | 1.1 | 0.8, 1.4 | 1.0 | 0.6, 1.5 |
| rs3091307[d] | 1.4 | 1.1, 1.8 | 1.9 | 1.4, 2.6 | 1.0 | 0.7, 1.3 | 0.9 | 0.7, 1.3 |
| rs1881457[d] | 1.5 | 1.1, 1.9 | 1.8 | 1.3, 2.5 | 1.0 | 0.7, 1.3 | 1.1 | 0.7, 1.8 |
| rs1295686 | 0.8 | 0.6, 1.1 | 0.7 | 0.5, 1.0 | 1.1 | 0.9, 1.5 | 1.2 | 0.8, 1.7 |
| rs20541 | 1.2 | 0.9, 1.6 | 1.5 | 1.1, 2.1 | 0.9 | 0.6, 1.2 | 0.8 | 0.5, 1.3 |
| rs848 | 1.2 | 0.9, 1.6 | 1.5 | 1.0, 2.0 | 1.1 | 0.8, 1.4 | 1.1 | 0.8, 1.4 |
| rs1295683 | 1.3 | 0.9, 1.7 | 1.5 | 1.0, 2.2 | 0.8 | 0.5, 1.3 | 0.7 | 0.4, 1.4 |
| rs2243204 | 1.2 | 0.9, 1.8 | 1.5 | 0.9, 2.3 | 0.9 | 0.7, 1.2 | 0.9 | 0.6, 1.3 |
| rs2243210 | 1.0 | 0.7, 1.6 | 1.3 | 0.8, 2.2 | 0.8 | 0.6, 1.1 | 0.8 | 0.5, 1.3 |
| rs2243218 | 1.2 | 0.8, 1.7 | 1.5 | 1.0, 2.3 | 1.0 | 0.7, 1.3 | 1.0 | 0.7, 1.5 |
| rs2243219 | 1.2 | 0.9, 1.8 | 1.5 | 1.0, 2.3 | 1.0 | 0.8, 1.3 | 0.9 | 0.7, 1.3 |
| *IL4* | | | | | | | | |
| rs2243250 | 0.7 | 0.5, 0.9 | 0.6 | 0.4, 0.9 | 1.0 | 0.8, 1.4 | 1.0 | 0.7, 1.5 |
| rs2243263 | 1.2 | 0.9, 1.7 | 1.6 | 1.1, 2.3 | 1.1 | 0.8, 1.5 | 1.2 | 0.8, 1.9 |
| rs2243267 | 1.5 | 1.1, 2.0 | 1.8 | 1.2, 2.6 | 1.1 | 0.8, 1.5 | 1.2 | 0.8, 1.9 |
| rs2243270 | 1.4 | 1.1, 1.9 | 1.7 | 1.1, 2.5 | 0.9 | 0.7, 1.2 | 0.9 | 0.6, 1.3 |
| rs11242122[d] | 0.7 | 0.6, 0.9 | 0.5 | 0.4, 0.7 | 1.0 | 0.8, 1.3 | 0.8 | 0.5, 1.2 |
| rs11242123 | 1.4 | 1.0, 1.9 | 1.7 | 1.2, 2.6 | 1.0 | 0.8, 1.4 | 1.1 | 0.7, 1.6 |

Abbreviations: CI, confidence interval; RR, risk ratio; SNP, single-nucleotide polymorphism.

[a] Variant allele and frequency are available in Web Table 5.

[b] Based on genetic ancestry. All models were additionally adjusted for percentage of Yoruban ancestry.

[c] SNPs are arranged by base pair position within each gene.

[d] Estimates for spontaneous preterm birth among European Americans have $P < 0.0005$ (Bonferroni correction for 100 SNPs in Stage 2).

genes have been implicated in both recurrent miscarriage and preeclampsia (51–53). Although the present study is the first to examine their association with preterm birth, previous studies have genotyped other NK-related genes (e.g., *IL12A, IFNGR2, CSF2*, *IL13,* and *IL4*), albeit with fewer SNPs per gene. The positive findings in the present study relative to the previous null findings could be due to more extensive coverage of the genes and the enhanced power of our Stage 1 gene-level analysis.

SNPs in *IL12A* and *CSF2* were associated with prematurity in both ancestry groups. IL12A is an important stimulator of NK cells and results in production of both TNF-α and IFN-γ (54). As an upstream regulator of NK cells, IL12 can stimulate the release of TNF-α, which increases in amniotic fluid with the onset of labor. TNF-α is associated with membrane degradation, cervical ripening, and uterine contractions (16). Upstream changes in the regulation of NK cells could have a significant impact on TNF-α levels in women with an otherwise normally functioning *TNF* gene. CSF2 is also released from NK cells, and levels are suppressed in the second and third trimesters in uncomplicated pregnancies (14). Of interest, 2 of the *CSF2* SNPs (rs4705916 and rs721121) with consistent associations with spontaneous preterm birth in both European and African Americans flank a possible regulatory region on chromosome 5 that regulates both *CSF2* and *IL3* (55).

The 2 remaining NK-associated genes (*KIR3DL2* and *IFNGR2*) were associated with preterm birth only among European Americans. IFNGR2 is the receptor for IFN-γ, which is an important NK cell cytokine. *IFNGR2* expression changes over the course of normal pregnancy (17), and dysregulation of *IFNGR2* through polymorphisms could influence the timing of parturition through its role in multiple immune-related cell lines. Although *KIR3DL2* might be implicated directly in placental implantation (51), 2 SNPs that were used to tag *KIR3DL2* are closer to a gene related to immunoglobulin A response (*Fc fragment of IgA, receptor for* (*FCAR*)). *FCAR* has shown altered expression over the course of pregnancy (17), though the significance of this change is unknown. More extensive examination of *FCAR* is warranted to distinguish which gene these tags are capturing.

In addition to the genes related to NK cells, T helper 2 cytokines *IL13* and *IL4* had strong gene-level and single-SNP associations. These genes, as well as *IL-5*, are geographically

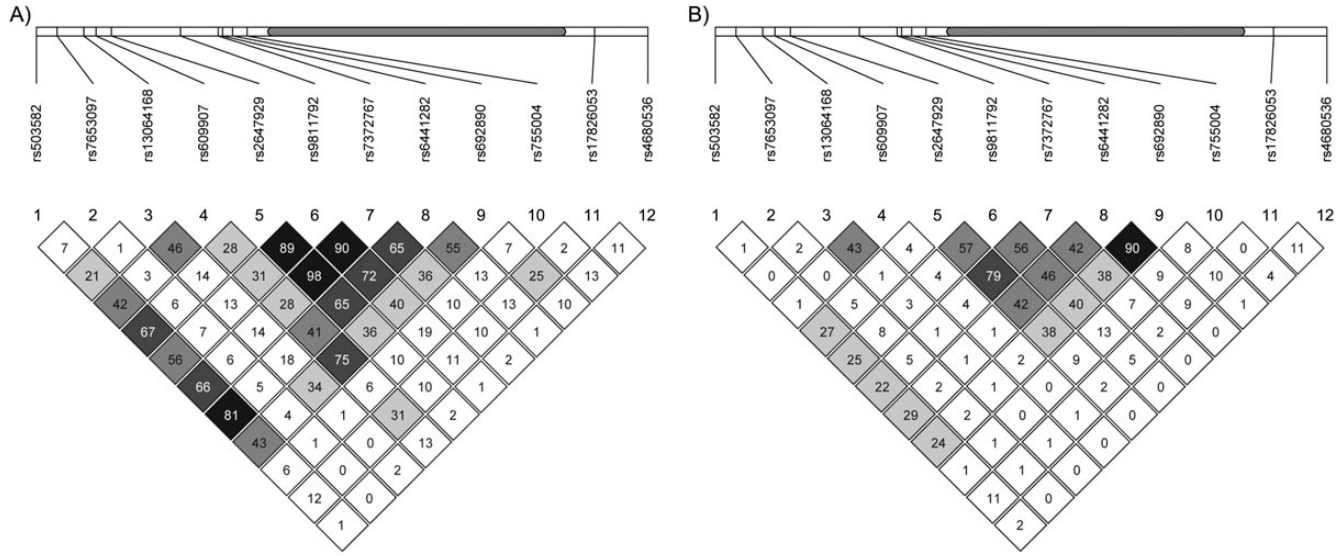


**Figure 1.** Linkage disequilibrium in *interleukin-12a* (*IL12A*) within European-American (A) and African-American (B) mothers in the Pregnancy, Infection, and Nutrition Cohort, North Carolina, 1995–2005. Values represent $r^2$ for pairs of single-nucleotide polymorphisms, with darker boxes having stronger correlations. Gray bars represent approximate location of *IL12A*.

close on chromosome 5 and share regulatory elements located between *RAD50* (RAD50 homolog (*S. cerevisiae*)) and *IL13* (56). The strongest associations for *IL13* appear for 3 SNPs that are in strong LD ($r^2 = 0.9$) with each other and share strong LD with several untyped SNPs within both *RAD50* and an intergenic locus control region. Because this regulatory region has the potential to influence expression of *IL13, IL4,* and *IL5*, further investigation of this region would be worthwhile.

Both gene- and SNP-level results differed for groups stratified by genetic ancestry. It is possible that fewer African-American participants, weaker patterns of LD, and population substructure that reduces effective sample size could explain the lack of an association with any of the candidate genes we explored. Nevertheless, despite our inability to detect a significant gene-level association for African Americans, the similar single-SNP results for *CSF2* and *IL12A* suggest that similar genes are important for both groups. Given the differences in the frequency of many of the variant alleles (Web Tables 4 and 5) and the variation in the social and environmental exposure distributions between African and European Americans, attention to both population stratification and gene-by-environment interactions will be important in future studies.

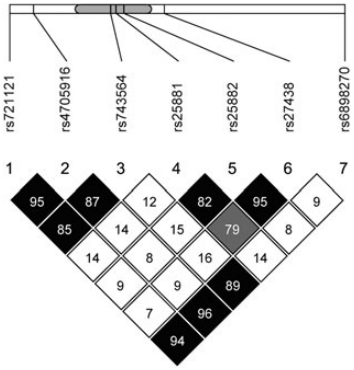


**Figure 2.** Linkage disequilibrium in *colony-stimulating factor 2* (*CSF2*) within European-American mothers in the Pregnancy, Infection, and Nutrition Cohort, North Carolina, 1995–2005. Values represent $r^2$ for pairs of single-nucleotide polymorphisms, with darker boxes having stronger correlations. Gray bar represents approximate location of *CSF2*.

The present study takes a novel approach to the analysis of high-dimensional data. The use of SKAT to identify genes of interest reduces multiple comparisons, allows for complex SNP interactions, and incorporates information from rare markers. Using SKAT allowed us to address our primary goal, which was to identify high-priority *genes* for future study, irrespective of the fact that few SNPs within those genes might have met a strict multiple-testing threshold. With our increased inclusion of genes related to natural killer cells and expanded margins around all genes, we were able to identify novel genes and regulatory regions that might not be identified through single-SNP analysis of markers that are chosen solely from known coding regions.

This study has several limitations that should be considered in future studies examining genetic associations with preterm and spontaneous preterm birth. Preterm birth is a heterogeneous phenotype (43, 57), and genetic studies have taken various approaches to defining both overall and spontaneous preterm birth, which makes comparison across studies difficult. Theoretically, associations will be estimated most precisely in homogenous phenotypes; however, there is a balance between the gain

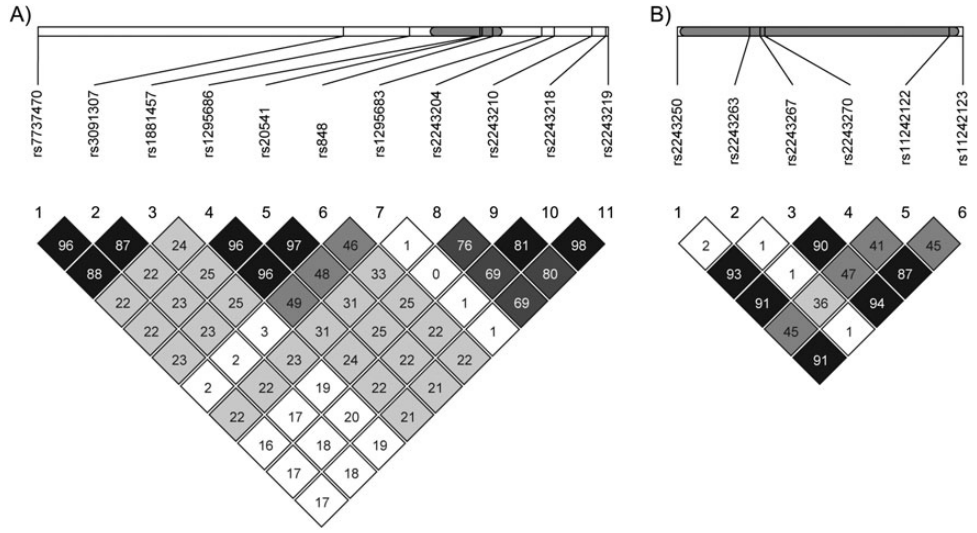


**Figure 3.** Linkage disequilibrium in *interleukin-13* (*IL13*) (A) and *interleukin-4* (*IL4*) (B) within European-American mothers in the Pregnancy, Infection, and Nutrition Cohort, North Carolina, 1995–2005. Values represent $r^2$ for pairs of single-nucleotide polymorphisms, with darker boxes having stronger correlations. Gray bars represent approximate location of *IL13* and *IL4*.

in case homogeneity and the loss in power due to small case populations (43). Although we often observed similar effect sizes in spontaneous and total preterm births, in some circumstances, subgroup associations were magnified or attenuated. The lack of a clear pattern in effect estimates indicates the possibility of subtype-specific association in some but not all genes. As the number of genetic studies of preterm birth grows, careful attention to outcome classification will be needed to interpret the assembled research.

Although inclusion of fetal DNA is important to understand all possible pathways to preterm birth, we did not have access to fetal DNA in the present study and were unable to explore main effects or interactions with fetal DNA. However, the findings of this study support population-based studies that suggest that maternal genetics could play an independent role in the risk of preterm birth. Additionally, tag SNPs themselves are not expected to be causal, and further fine mapping of genomic regions identified by this study will be necessary to identify the causal SNPs.

In summary, this study broadened coverage of polymorphisms in genes related to inflammation and explored novel genes related to NK cells. Genes associated with NK cells (*IL12A, IFNGR2,* and *KIR3DL2*) were novel findings, and the results suggested that further examination of the regulatory regions associated with cytokines on 5q31 (*IL4, IL13, IL3,* and *CSF2*) could be fruitful.

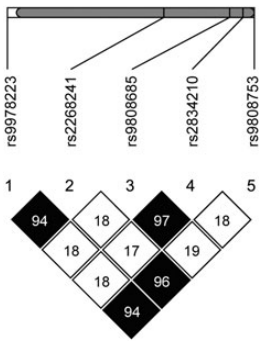


**Figure 4.** Linkage disequilibrium in *interferon γ receptor 2* (*IFNGR2*) within European-American mothers in the Pregnancy, Infection, and Nutrition Cohort, North Carolina, 1995–2005. Values represent $r^2$ for pairs of single-nucleotide polymorphisms, with darker boxes having stronger correlations. Gray bar represents approximate location of *IFNGR2*.

## ACKNOWLEDGMENTS

Author affiliations: Department of Epidemiology, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, North Carolina (Quaker E. Harmon, Stephanie M. Engel, Andrew F. Olshan, Christy L. Avery); Department of Microbiology, Mt. Sinai School of Medicine, New York, New York (Thomas Moran); Department of Obstetrics and Gynecology, University of North Carolina School of Medicine, University of North Carolina, Chapel Hill, North Carolina (Alison M. Stuebe); Genomics and Bioinformatics Core, Carolina Center for Genome Sciences, Department of Genetics, University of North Carolina School of Medicine, University of North Carolina, Chapel Hill, North Carolina (Jingchun Luo); and Department of Biostatistics, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, North Carolina (Michael C. Wu).

This work was supported by the National Institute of Child Health and Development (R21 HD060207 to Stephanie

M. Engel); National Institute of Environmental Health Sciences (2-T32-ES07018 and P30ES010126 to Andrew F. Olshan); and the Mount Sinai Center for Children's Environmental Health. The Pregnancy, Infection, and Nutrition Cohort was supported by the National Institutes of Health (HD28684, HD28684A, HD37584, HD39373, and RR00046).

We gratefully acknowledge the significant contributions of the following personnel: Dr. Patricia Basta, Laboratory Director, Biospecimen Processing Facility, University of North Carolina, Chapel Hill, North Carolina, for DNA extraction; Kathryn Carrier, Project Manager, University of North Carolina, Chapel Hill, North Carolina, for specimen management; Kevin Jacobs, National Cancer Institute/National Institutes of Health, Rockville, Maryland, for help with plate design; and Michael Andre and Amanda Floyd Beaty, Genomics Core, University of North Carolina, Chapel Hill, North Carolina, for genotyping.

Conflict of interest: none declared.

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