

Association of the Estrogen Receptor- α Gene With the Metabolic Syndrome and Its Component Traits in African-American Families

The Insulin Resistance Atherosclerosis Family Study

Carla J. Gallagher,^{1,2,3} Carl D. Langefeld,⁴ Candace J. Gordon,² Joel K. Campbell,⁴ Josyf C. Mychalecky,^{2,4,5,6,7} Michael Bryer-Ash,⁸ Stephen S. Rich,^{6,7} Donald W. Bowden,^{1,2,5} and Michèle M. Sale^{2,5,6,9,10}

OBJECTIVE—We previously detected an association between a region of the estrogen receptor- α (ESR1) gene and type 2 diabetes in an African-American case-control study; thus, we investigated this region for associations with the metabolic syndrome and its component traits in African-American families from the Insulin Resistance Atherosclerosis Family Study.

RESEARCH DESIGN AND METHODS—A total of 17 single nucleotide polymorphisms (SNPs) from a contiguous 41-kb intron 1–intron 2 region of the ESR1 gene were genotyped in 548 individuals from 42 African-American pedigrees. Generalized estimating equations were computed using a sandwich estimator of the variance and exchangeable correlation to account for familial correlation.

RESULTS—Significant associations were detected between ESR1 SNPs and the metabolic syndrome ($P = 0.005$ to $P = 0.029$), type 2 diabetes ($P = 0.001$), insulin sensitivity ($P = 0.0005$ to $P = 0.023$), fasting insulin ($P = 0.022$ to $P = 0.033$), triglycerides ($P = 0.021$), LDL ($P = 0.016$ to $P = 0.034$), cholesterol ($P = 0.046$), BMI ($P = 0.016$ to $P = 0.035$), waist circumference ($P = 0.012$ to

$P = 0.023$), and subcutaneous adipose tissue area ($P = 0.016$).

CONCLUSIONS—It appears likely that ESR1 contributes to type 2 diabetes and CVD risk via pleiotropic effects, leading to insulin resistance, a poor lipid profile, and obesity. *Diabetes* 56: 2135–2141, 2007

Variants in the estrogen receptor- α (ESR1) gene have been associated with components of the metabolic syndrome, including obesity (1), HDL cholesterol (2), LDL metabolism (3), blood pressure (4,5), and type 2 diabetes (1). A genome scan for type 2 diabetes using 638 affected African-American sibling pairs from 247 families revealed the greatest evidence for linkage (logarithm of odds [LOD] 2.26) at 163.5 cM on chromosome 6q (6); the support (LOD-1) interval contains the ESR1 gene at 154 cM. A recent study by our group found evidence for association of the intron 1–intron 2 region of ESR1 with type 2 diabetes in African Americans and European Americans (7).

Animal models of the ESR1 gene support pleiotropic effects on phenotypes related to diabetes and cardiovascular disease (CVD) risk because male and female *esr1* knockout mice exhibit insulin resistance, impaired glucose tolerance, and obesity (8). A human male with an ESR1-null mutation had insulin resistance, impaired glucose tolerance, obesity, and increased height (9).

Previously reported associations of the ESR1 gene with components of the metabolic syndrome and type 2 diabetes motivated the current investigation. The Insulin Resistance and Atherosclerosis (IRAS) Family Study is designed to identify the genetic basis of insulin resistance and visceral adiposity as components of the pathway that lead to type 2 diabetes and atherosclerosis (10). We focused on African-American individuals of the IRAS Family Study because both linkage in the 6q region (6) and the association between ESR1 and type 2 diabetes in our previous study (7) were the strongest in African Americans. We evaluated 17 single nucleotide polymorphisms (SNPs) across a contiguous 41-kb region spanning ESR1 intron 1–intron 2 for associations with metabolic syndrome and its components, as well as type 2 diabetes, in 548 African-American subjects.

From the ¹Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, North Carolina; the ²Center for Human Genomics, Wake Forest University School of Medicine, Winston-Salem, North Carolina; the ³Department of Health Evaluation Sciences, Penn State College of Medicine, Hershey, Pennsylvania; the ⁴Division of Public Health Sciences, Wake Forest University School of Medicine, Winston-Salem, North Carolina; the ⁵Department of Internal Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina; the ⁶Center for Public Health Genomics, University of Virginia, Charlottesville, Virginia; the ⁷Department of Public Health Sciences, University of Virginia, Charlottesville, Virginia; the ⁸Division of Endocrinology, Diabetes and Metabolism, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California; the ⁹Department of Medicine, University of Virginia, Charlottesville, Virginia; and the ¹⁰Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia.

Address correspondence and reprint requests to Michèle M. Sale, PhD, Center for Public Health Genomics, University of Virginia, P.O. Box 800717, Charlottesville, VA 22908. E-mail: msale@virginia.edu.

Received for publication 21 July 2006 and accepted in revised form 1 May 2007.

Published ahead of print at <http://diabetes.diabetesjournals.org> on 18 May 2007. DOI: 10.2337/db06-1017.

Additional information for this article can be found in an online appendix at <http://dx.doi.org/10.2337/db06-1017>.

CVD, cardiovascular disease; ESR1, estrogen receptor- α ; FPG, fasting plasma glucose; GEE, generalized estimating equation; PDT, pedigree disequilibrium test; SAT, subcutaneous adipose tissue; SNP, single nucleotide polymorphism; QPDT quantitative PDT; VAT, visceral adipose tissue.

© 2007 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

RESEARCH DESIGN AND METHODS

IRAS Family Study subjects. The study design, recruitment, and phenotyping of the IRAS Family Study have previously been described in detail (10). Studies were conducted under institutional review board approval at each participating institution and adhered to the Declaration of Helsinki. All participants provided informed consent. Briefly, multigenerational African-American and Hispanic families were initially recruited from probands of the original IRAS cohort (11). Ascertainment of the proband was based on the sample size of available family members (with a target of four living full siblings and five living offspring of these siblings). Ascertainment was supplemented with additional non-IRAS families recruited from the general population. Families were not selected based on any phenotypic criteria. Participants were from Los Angeles, California (African American), San Luis Valley, Colorado (rural Hispanic), and San Antonio, Texas (urban Hispanic). The analyses reported here were conducted using only the 42 multigenerational self-reported African-American families from Los Angeles containing 548 individuals.

Metabolic syndrome. Individuals were classified as having the metabolic syndrome using the definition of the 2001 National Cholesterol Education Program Adult Treatment Panel III (12); i.e., the presence of any three of the following: fasting plasma glucose (FPG) ≥ 100 mg/dl or known diabetes, serum triglycerides ≥ 150 mg/dl, HDL cholesterol < 40 mg/dl in men or < 50 mg/dl in women, waist circumference > 102 cm in men or 88 cm in women, or blood pressure $\geq 130/85$ mmHg or treated hypertension. We expanded our investigations to encompass the available traits related to each of these phenotypic clusters, as detailed below.

Diabetes and glucose homeostasis traits. Diabetes was diagnosed using the American Diabetes Association criteria of FPG values ≥ 126 mg/dl and/or current use of diabetes medications. In the 42 African-American families used in this study, 12.6% of family members had type 2 diabetes. Individuals with type 2 diabetes were excluded for analyses of glucose homeostasis traits.

The following traits were tested for association: FPG, fasting plasma insulin, acute insulin response to glucose (AI_{R_g}), and insulin resistance, expressed as the insulin sensitivity index (S_i). Glucose values were obtained after a minimum 8-h fast. Plasma glucose and insulin levels were measured at the University of Southern California (Los Angeles, CA) using the glucose oxidase technique on an autoanalyzer and the insulin dextran-charcoal immunoassay (13). Insulin sensitivity was assessed by the frequently sampled intravenous glucose tolerance test, using a reduced sampling protocol (14). Glucose in the form of a 50% solution (0.3 g/kg) and regular human insulin (0.03 units/kg) were injected through an intravenous line at 0 and 20 min, respectively. Blood was collected at -5, 2, 4, 8, 19, 22, 30, 40, 50, 70, 100, and 180 min. AI_{R_g} was defined as the mean insulin increment in the plasma insulin concentration above the basal in the first 8 min after the administration of glucose. S_i was calculated by mathematical modeling methods (MINMOD) (15).

Lipid traits. Lipid traits tested for association included triglycerides, HDL, LDL, and total cholesterol levels. Plasma was separated from blood collected after a 12-h fast and stored at -70°C before analysis. Total cholesterol and triglycerides were measured using enzymatic methods. LDL cholesterol was calculated using the Friedewald equation (16) if triglyceride was < 400 mg/dl or otherwise by ultracentrifugation. HDL cholesterol was measured using the direct method (17).

Obesity and adiposity traits. Obesity traits used for association analyses included waist circumference, BMI, visceral adipose tissue (VAT), and subcutaneous adipose tissue (SAT). Height and waist circumference were measured to the nearest 0.5 cm and weight to the nearest 0.1 kg. BMI was calculated as the weight in kilograms divided by the square of the height in meters. Abdominal fat mass was measured at the L2/L3 and L4/L5 vertebral region by computed tomography. Scans were read for VAT and SAT at the Department of Radiology, University of Colorado Health Sciences Center (Denver, CO). Bowel fat was subtracted from the VAT and L4/L5 measures used in these analyses. A small number of participants were missing L4/L5 data but had L2/L3 data; for these participants, L4/L5 data were imputed from the L2/L3 data using a simple linear model.

Blood pressure. Blood pressure traits tested for associations with ESR1 SNPs were systolic and diastolic blood pressure and pulse pressure (systolic blood pressure minus diastolic blood pressure). Seated blood pressure was measured in triplicate following a 5-min rest period using a standard sphygmomanometer. The average of the second and third blood pressure measurements was used. Analyses were not adjusted for antihypertensive medications because data were available for less than one-third of participants.

SNP genotyping. Total genomic DNA was purified from whole blood using PUREGENE DNA isolation kits (Gentra, Minneapolis, MN). The 17 SNPs across intron 1-intron 2 region of the ESR1 gene were genotyped using a MassARRAY system (Sequenom, San Diego, CA) (18), and primer sequences

are shown in supplementary Table 1 (available in an online appendix at <http://dx.doi.org/10.2337/db06-1017>). Seventeen replicate pairs were 100% concordant for all SNPs. Nine of the 17 SNPs had been genotyped in the HapMap Yoruba sample (HapMap data release no. 19). Using the locations of rs6902771 and rs11155819 to define the boundaries of the region of interest and Tagger (19), these 9 SNPs tagged the 27 HapMap SNPs in this region with minor allele frequency $> 5\%$ at the following levels: 85.2% with $r^2 > 0.2$, 70.4% with $r^2 > 0.5$, and 55.6% with $r^2 > 0.8$.

Association analyses. Each pedigree had been previously examined for consistency of stated family structure (20). SNPs were evaluated for Mendelian consistency using PedCheck (21), with two genotypes converted to missing. Maximum likelihood estimates of allele frequencies were computed using the largest set of unrelated individuals and tested for departures from Hardy-Weinberg equilibrium.

To test for association, a series of generalized estimating equations (GEEs) (22) were computed. Familial correlation was accounted for using a sandwich estimator of the variance and exchangeable correlation. For type 2 diabetes, glucose homeostasis, lipids, and blood pressure traits, tests were computed adjusting for age, sex, and BMI. For metabolic syndrome and obesity traits, adjustments were made only for age and sex. For each quantitative trait, a family of power transformations conditional on the above covariates (23) was explored. To minimize the heterogeneity of variance, the phenotypes were transformed to best approximate the normality assumptions. We computed four tests of association for each SNP: the overall test of genotypic association with 2 d.f. and the statistical contrasts defined by three genetic models—dominant, additive, and recessive. Associations were considered significant if $P < 0.05$ for the 2 d.f. Where this was the case, the three genetic models were then considered to provide information on possible mode of inheritance. Potential influential points and outliers have been checked. The analyses were performed using SAS software (SAS Institute, Cary, NC).

To avoid the potential increase of type I error rate, we also estimated the empirical P values for the single SNP GEE association analyses. We used the gene dropping approach implemented in Mendel version 5.7 (24) to simulate 10,000 datasets based on the IRAS Family Study pedigree structure under the null hypothesis of no association between phenotype and genotype data. The empirical P value was determined as the proportion of simulated data sets with statistics more extreme than the observed value. Only empirical P values are presented because this approach is more conservative and adjusts for deficiencies in the large sample approximation of the GEE method.

Haplotype analyses. To test for haplotypic associations, pedigree disequilibrium test (PDT) or quantitative PDT (QPDT) analyses (25) were performed. Metabolic syndrome and type 2 diabetes PDT analyses were unadjusted for covariates, while quantitative trait QPDT analyses were adjusted for age, sex, and BMI (glucose homeostasis, lipid, and blood pressure traits) or age and sex (obesity traits). Analyses of two-, three-, and four-SNP haplotype moving windows were conducted. Because there was a small number ($n = 53$) of unrelated African-American founders in the IRAS Family Study, linkage disequilibrium (LD) structure of the region of the ESR1 gene under investigation was determined using 635 unrelated African-American control subjects who were not participants in the IRAS Family Study (supplementary Fig. 1), as described in Gallagher et al. (7). Four haplotype blocks were identified: rs6902771–rs7774230 (five SNPs in intron 1 plus rs7774230 in intron 2; block 1), rs1709181–rs11155818 (block 2), rs827417–rs1709183 (intron 2; block 3), and rs1033182–rs11155819 (intron 2; block 4). Haplotype association analyses were conducted for these four haplotype blocks. Additional ad hoc haplotype analyses were conducted where multiple SNPs were associated with the same trait.

RESULTS

Demographic information and mean trait values for all traits investigated for association are summarized in Table 1. The mean age of the family members was 42.9 ± 14.1 years, and mean BMI was 30.0 ± 6.8 kg/m². Of the African-American participants, 60% were female.

Metabolic syndrome and type 2 diabetes. Five SNPs showed significant associations with the metabolic syndrome ($P = 0.006$ to $P = -0.029$) (Table 2). Only the most significant result ($P < 0.05$) across the four tests is presented for each SNP in the tables. These SNPs are located in haplotype block 1 (rs6902771 and rs8340799), block 3 (rs2431260), and block 4 (rs1033182 and rs2175898). SNP rs1033182 (located in haplotype block 4) was also associated with type 2 diabetes (Table 2). Single

TABLE 1
Demographic summary of African-American subjects in the IRAS Family Study

Phenotype	Subjects with data	Means \pm SD	Median
Age (years)	525	42.9 \pm 14.1	41.4
Female (%)	548	59.5	—
Metabolic syndrome (%)	532	20.7	—
Diabetes (%)	532	12.6	—
Fasting glucose (mg/dl)	464	94.8 \pm 9.8	93.0
Fasting insulin (μ U/ml)	465	14.3 \pm 11.8	11.0
AIR _g (μ U/dl)	451	987.8 \pm 827.8	734.8
S _i	452	1.64 \pm 1.18	1.41
Triglycerides (mg/dl)	526	79.6 \pm 62.9	64.0
LDL cholesterol (mg/dl)	525	113.1 \pm 32.3	110
HDL cholesterol (mg/dl)	526	46.9 \pm 12.4	45.0
Total cholesterol (mg/dl)	526	176.0 \pm 35.7	174.0
Waist circumference (cm)	523	91.2 \pm 14.9	91.1
BMI (kg/m ²)	523	30.0 \pm 6.8	29.0
Visceral area L4/L5 (cm ²)	484	91.8 \pm 57.5	81.7
Subcutaneous area L4/L5 (cm ²)	484	355.1 \pm 190.4	321.9
Systolic blood pressure (mmHg)	526	118.6 + 18.6	115.0
Diastolic blood pressure (mmHg)	526	74.9 + 10.3	74.0

Data are *n* unless otherwise indicated.

SNP QPDT analyses of rs1033182 showed evidence for transmission distortion with S_i ($P = 0.038$) and disposition index ($P = 0.048$) (data not shown).

PDT moving windows two-, three-, and four-SNP haplotype analyses of metabolic syndrome did not produce any significant association results. Two three-SNP haplotypes were associated with type 2 diabetes (Table 3). These haplotypes are both located in intron 2 and overlap at rs712221; however, none contained the associated single SNP rs1033182.

Metabolic syndrome components and related traits. Four of 17 SNPs showed evidence of significant association with S_i ($P = 0.0005$ to $P = 0.023$) as summarized in Table 4. These four SNPs are in high LD in the founders from the African-American IRAS Family Study families (pairwise D' values 0.86–1.00) and all are located within haplotype block 1 (supplementary Table 1), which contains a total of six genotyped SNPs (rs6902771–rs7774230). Two of the four SNPs (rs6902771 and rs7774230) were also associated with fasting insulin ($P = 0.033$ and $P = 0.022$, respectively). In addition, associations between rs4870056 and rs2234693 with fasting insulin approached significance ($P = 0.052$ [under an additive model for rs4870056] and $P = 0.051$ [dominant model for rs2234693]; data not

TABLE 2
Significant single SNP associations of ESR1 intron 1–intron 2 SNPs with the metabolic syndrome and type 2 diabetes

Phenotype	SNP	Major/minor alleles	P	Model	Odds ratio (95% CI)
Metabolic syndrome	rs6902771	C/T	0.012	2 d.f.	
Metabolic syndrome	rs9340799	A/G	0.029	Additive	1.53 (1.05–2.27)
Metabolic syndrome	rs2431260	G/C	0.005	Dominant	2.51 (1.31–4.80)
Metabolic syndrome	rs1033182	G/A	0.020	2 d.f.	
Type 2 diabetes	rs1033182	G/A	0.001*	2 d.f.	
Metabolic syndrome	rs2175898	A/G	0.006	Dominant	2.51 (1.30–4.84)

Data are adjusted for age and sex. *Adjusted for age, sex, and BMI. Empirical P values and the model for the most significant association are shown.

TABLE 3
Significant unadjusted PDT haplotype associations with type 2 diabetes and SNPs in intron 2 of the ESR1 gene

Haplotype	Z	P	Frequency
rs1709181, rs12664989, and rs712221*			
CGT	−2.07	0.039	0.50
CGA	0	0	0.02
CCT	1.36	0.174	0.42
CCA	1.94	0.052	0.002
TGA	0	0	<0.001
TCT	1.94	0.052	<0.001
TCA	1.94	0.052	0.053
rs712221, rs1514348, and rs11155818†			
TGG	−0.22	0.827	0.07
TGA	−1.66	0.098	0.42
TTG	1.82	0.069	0.41
TTA	−1.70	0.089	0.03
AGG	−1.00	0.317	0.001
AGA	−1.00	0.317	0.009
ATG	1.94	0.052	0.07

*Global test, $P = 0.021$. †Global test, $P = 0.049$.

shown). The mean trait value is shown for each of the three genotypes (Table 4). For the two SNPs associated across both traits, alleles associated with a higher fasting insulin (rs6902771 allele 2 and rs7774230 allele 1) are also associated with reduced S_i, although genetic models differ. Haplotypes consisting of all SNPs associated with one of the glucose homeostasis traits (i.e., rs6902772 and rs7774230 for fasting insulin and rs6902771, rs4870056, rs2234693, and rs7774230 for S_i) were not significantly associated with these traits (data not shown).

Three of the 17 SNPs showed evidence of significant association with at least one lipid measure ($P = 0.016$ to $P = 0.046$) (Table 4). SNPs rs9322331 and rs12664989 are in high LD in the IRAS Family Study founders as are rs12664989 and rs712221 (both $D' = 1$), whereas the D' value between rs9322331 and rs712221 is only 0.31. In the 635 African-American control subjects, the D' values for the pairwise comparisons described were 0.82, 0.69, and 0.50, respectively (7). All three SNPs show an association with LDL levels ($P = 0.016$ to $P = 0.034$). For rs9322331, allele 2 is associated with higher triglycerides and higher LDL cholesterol, whereas allele 2 of rs12664989 is associated with both lower LDL and total cholesterol. Although rs12664989 and rs712221 are both located in haplotype block 2, the two-SNP haplotype containing these SNPs was not significantly associated with LDL cholesterol levels (data not shown).

Three of the 17 SNPs showed evidence of significant

TABLE 4
Significant single SNP associations in intron 1–intron 2 of ESR1 gene with glucose homeostasis, lipid, and adiposity traits

SNP	Major/ minor alleles	Glucose homeostasis traits						Lipid traits						Adiposity traits																						
		Fasting insulin			S ₁			LDL			Triglycerides			Waist			SAT																			
		P	1/1	2/2	1/2	2/2	1/1	1/2	2/2	1/1	1/2	2/2	P	1/1	2/2	1/2	2/2	P	1/1	2/2																
rs6902771	C/T	0.033*	12.8 ± 8.6 (111)	14.3 ± 13.0 (242)	15.9 ± 12.4 (98)	0.0005†	1.92 ± 1.35 (109)	1.56 ± 1.12 (235)	1.54 ± 1.15 (95)	0.034*	110.7 ± 32.2 (358)	117.5 ± 32.6 (137)	123.1 ± 29.5 (16)	0.016†	91.6 ± 15.0 (410)	88.4 ± 14.7 (93)	—	0.012†	30.8 ± 6.5 (111)	30.8 ± 6.7 (266)	29.3 ± 7.4 (129)	28.3 ± 6.1 (93)	28.7 ± 6.0 (82)	0.023†	109.0 ± 30.8 (163)	112.3 ± 32.9 (249)	121.4 ± 32.6 (68)	0.046†	177.5 ± 34.9 (423)	167.6 ± 38.1 (88)	—	—	—			
rs4870056	A/G	—	—	—	—	0.0007‡	1.60 ± 1.11 (104)	1.58 ± 1.16 (245)	1.83 ± 1.29 (91)	0.016†	114.8 ± 31.5 (422)	104.3 ± 33.2 (88)	—	0.023†	91.5 ± 15.0 (433)	89.0 ± 14.1 (82)	—	0.023†	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
rs2234693	C/T	—	—	—	—	0.023‡	—	—	—	0.029*	109.0 ± 30.8 (163)	112.3 ± 32.9 (249)	121.4 ± 32.6 (68)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
rs7774230	T/C	0.022*	16.9 ± 14.6 (117)	13.0 ± 7.5 (253)	14.5 ± 16.8 (83)	0.003‡	1.56 ± 1.10 (113)	1.58 ± 1.15 (246)	1.94 ± 1.35 (82)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Data are means ± SD (n). *Additive model; †dominant model; ‡recessive model; §2 d.f. test; empirical P value and model for the most significant association is shown; ||genotypes 1/2 and 2/2 were combined where there were <10 counts in a 2/2 cell.

TABLE 5
Significant QPDT haplotype associations, adjusted for age, sex, and BMI, in intron 1–intron 2 region of ESR1 gene

Haplotype	Z	P	Frequency	Mean (mg/dl)
rs827417 and rs2431260 (n = 482)*				
AG	2.19	0.029	0.79	94.85
AC	-2.25	0.024	0.10	94.24
GG	-0.73	0.464	0.10	92.80
rs712221, rs1514348, and rs11155818 (n = 545)†				
TGG	2.14	0.032	0.57	47.82
TGA	0.17	0.862	0.12	49.72
ATG	-2.17	0.030	0.31	45.60

*Global test, P = 0.03; †Global test, P = 0.045.

association with one or more adiposity traits (P = 0.012 to P = 0.035) (Table 4). In the founders from the families, rs6902771 and rs2175895 and rs2431260 and rs2175898 are in high LD (D' = 1), whereas the LD between rs6902771 and rs2431260 is 0.56. The D' values between these SNP pairs are lower in the African-American control subjects (7), with D' = 0.67, 0.84, and 0.30, respectively, each located in distinct haplotype blocks (blocks 1, 3, and 4) (supplementary Table 1). All three SNPs were significantly associated with BMI (P = 0.016 to P = 0.035). Allele 2 of rs2431260 and rs2175898 was associated with lower BMI, smaller waist circumference, and, in the case of rs2431260 only, reduced SAT. Haplotypes containing rs6902771, rs2431260, and rs2175898 were not significantly associated with BMI (data not shown). No significant single SNP or haplotypic associations were detected with traits relating to blood pressure.

In two-, three-, and four-SNP haplotype moving window QPDT analyses (adjusted for age, sex, and BMI), two haplotypes showed evidence of association (Table 5). One two-SNP haplotype containing rs827417 and rs2431260 was associated with fasting glucose (P = 0.031), and a three-SNP haplotype (rs712221–rs11155818) was associated with HDL cholesterol levels (P = 0.045). Apart from rs712221, contained within the three-SNP haplotype associated with HDL levels (P = 0.045) and also independently associated with LDL levels, none of the SNPs in these two associated haplotypes showed evidence of single SNP association. None of the QPDT moving windows analyses adjusted for age and sex showed associations with obesity or adiposity traits. Similarly, none of the four haplotype blocks (shown in supplementary Table 1) (7) showed association with traits of interest in adjusted QPDT analyses. The pleiotropic effects of the SNPs in this region of ESR1 on components of the metabolic syndrome and related phenotypes are summarized in Table 6.

DISCUSSION

On the basis of prior evidence for association with type 2 diabetes (7) and published associations with component traits of the metabolic syndrome (1–5), we evaluated a region spanning intron 1–intron 2 of the ESR1 gene for association with type 2 diabetes, the metabolic syndrome, and its components in African-American families from the IRAS Family Study. Single SNP and haplotype association analyses provided evidence that SNPs in this region are associated with type 2 diabetes and metabolic syndrome

TABLE 6
Summary of pleiotropic effects of SNPs across ESR1 intron 1–intron 2 region

SNP	Metabolic syndrome	Type 2 diabetes	Fasting insulin	S_i	Triglycerides	LDL	Cholesterol	BMI	Waist circumference	SAT
rs6902771	X		X	X				X		
rs4870056				X						
rs9322331					X	X				
rs2234693				X						
rs9340799	X									
rs7774230			X	X						
rs1709181										
rs12664989						X	X			
rs712221						X				
rs1514348										
rs11155818										
rs827417										
rs2431260	X							X	X	X
rs1709183										
rs1033182	X	X								
rs2175898	X							X	X	
rs11155819										

X represents a positive association (reported in Tables 2 and 4).

and also with quantitative measures of insulin resistance, lipid profile, and adiposity.

Three of the five SNPs associated with metabolic syndrome (Table 2) were also associated with multiple components of the metabolic syndrome: rs6902771 with fasting insulin, S_i , and BMI; rs22431260 with BMI, waist circumference, and SAT; and rs2175898 with BMI and waist circumference (Table 4). SNP rs9340799, also known as the *XbaI* polymorphism, was associated with metabolic syndrome in African-American families from the IRAS Family Study (Table 2), although associations with individual quantitative metabolic traits were not detected. Association between rs1033182 and type 2 diabetes, first detected in an African-American case-control population (7), was observed in this independent African-American family-based sample ($P = 0.001$ for genotypic association, adjusted for age, sex, and BMI) and showed a transmission distortion in relation to S_i . Additionally, two three-SNP haplotypes in intron 2 also showed association with type 2 diabetes (Table 3), suggesting that multiple ESR1 variants may contribute to diabetes risk. In nondiabetic family members, an association between FPG and a two-SNP haplotype contained within haplotype block 3 and 2.7 kb upstream of rs1033182 (possibly tagging an ungenotyped SNP in this region) was also noted (Table 5).

Four of the six SNPs in haplotype block 1 (supplementary Table 1) (7) demonstrated association with S_i , and two of these SNPs also displayed an association with fasting insulin (Table 4). The six-SNP haplotype showed association with type 2 diabetes in our previous study of African-American case and control subjects (7). The current results may shed some light on how ER- α is involved in glucose homeostasis because variants in this region of the ESR1 gene appear to affect an insulin resistance phenotype but are not involved in insulin secretion since associations were not observed with AIR_g. An influence of ESR1 on S_i is consistent with the finding that mice with a nonfunctional *esr1* gene (8,9) and the only human male identified without a functional copy of ESR1 (9) exhibit insulin resistance. Minor allele homozygotes of rs4870056, rs2234693, and rs7774230 have increased S_i (Table 4). Because ER- α has pleiotropic influences, including effects

on reproductive fitness (26), common “risk” alleles may have been retained in the population due to positive selective pressure unrelated to their effect on the metabolic phenotypes investigated. Alternatively, common major alleles of rs2431260 and rs2175898, associated with increased BMI and waist circumference (Table 4), may reflect past selection pressures favoring high-energy storage.

Significant associations were detected between ESR1 SNPs and triglycerides and LDL and total cholesterol levels (Table 4), with nominal association between a three-SNP haplotype and HDL cholesterol (Table 5). All SNPs associated with lipid traits, except for rs9322331 (block 1, intron 1), were located in intron 2 within haplotype block 2. Associations have been reported between ESR1 polymorphisms in intron 1 and exon 4 and LDL particle size and small LDL cholesterol (3,27–29) and total cholesterol in adolescent females (29). Herrington et al. (2) found that rs2234693 modified the effects of hormone replacement therapy on levels of HDL cholesterol in postmenopausal women. After reaching sexual maturity, *esr1* knockout mice display elevated total cholesterol, with an increase in smaller LDL particles (30).

SNPs in the ESR1 gene were also associated with measures of adiposity, specifically BMI, waist circumference, and SAT (Table 4). Estrogens are known to play an important role in body fat distribution. Male and female *esr1* knockout mice have large increases in white adipose tissue compared with wild-type mice (8,31). Linkage of waist circumference to this region of chromosome 6 has been reported in the Framingham Heart Study (32). Although we did not see an association between rs2234693 (the ESR1 *PvuII* polymorphism) or rs9340799 (*XbaI*) and adiposity traits in this population, associations between these ESR1 SNPs and BMI and/or waist circumference have been reported (33–35). Associated SNPs in our study are located in regions flanking these markers. We observed an association between rs2431260 and SAT. Low estrogen levels are associated with increased abdominal and subcutaneous fat (36), and estrogen has been found to upregulate expression of ER- α in subcutaneous adipocytes (37). Pedersen et al. (38) found that estrogens act through

ER- α to upregulate the number of antilipolytic α 2A-adrenergic receptors in subcutaneous fat depots, which could explain how estrogens maintain fat distribution.

Although rs2234693 (*PvuII*) allele C has been associated with lower BMI (30), waist circumference (32), and lower small LDL concentration (27,28), this allele was associated with reduced S_i in this study. The rs9340799 (*XbaI*) minor allele G has been associated with reduced waist circumference (32), LDL cholesterol, and apolipoprotein B (3) but showed an additive risk for metabolic syndrome in the IRAS Family Study families. The modest associations observed ($0.02 > P > 0.03$) could be the result of type I error. Alternatively, these alleles may have differential effects on these traits or be in LD with a functional SNP present on different haplotypic backgrounds.

Unless there is a systematic bias, associations with quantitative traits are unlikely to be confounded by admixture. Haplotypic analyses were performed using family-based methods that overcome potential biases due to stratification. Empirical P values are presented to adjust for deficiencies in the large sample approximation of the GEE method; however, corrections for multiple comparisons were not applied due to correlations between SNPs and traits. One potential adjustment is a Bonferroni correction for the four haplotype blocks examined. If we apply this correction, the following associations remain significant: metabolic syndrome (rs6902771, $P = 0.048$; rs2431260, $P = 0.020$; rs2175898, $P = 0.024$), type 2 diabetes (rs1033182, $P = 0.004$), and S_i (rs6902771, $P = 0.002$; rs4870056, $P = 0.028$; rs7774230, $P = 0.012$), while an association with waist circumference (rs2431260, $P = 0.048$) is of borderline significance. This approach may be overly conservative considering correlations between blocks; however, the significance of the results should be considered with these issues in mind.

Because the majority of published studies only genotyped rs9340799 (*XbaI*) and rs2234693 (*PvuII*) in ESR1 intron 1, it is possible that our higher SNP density in this region, together with the decreased LD observed in the African-American population (7), have more precisely localized association signals with certain traits investigated in this study. Given the highly polymorphic nature of the ESR1 gene and broad impact of ER- α and estradiol on human physiology, it is plausible that different variants (or haplotypes) within introns 1 and 2 (tagged by SNPs or haplotypes genotyped in this study) may produce a range of splice variants and/or alter transcription factor binding sites. Genotyped SNPs are not located within putative consensus splice donor/acceptor or branch sites and are some distance from exon 2 (rs9340799 is 350 bp proximal to exon 2 and rs7774230 317 bp distal). In independent sequencing studies of 48 African-American type 2 diabetic case and 48 African-American control subjects, we did not detect any polymorphisms in ESR1 exon 2 (7). Wang et al. (39) described a novel splice variant, ER- α 36, containing a small, noncoding alternate exon 1 located in ESR1 intron 1 that is spliced to exons 2–6, then to a novel downstream “exon 9.” Like the ER- α 46 isoform, which lacks exon 1 (40), translation is initiated from a sequence located in exon 2. ER- α 36 is postulated to act as a dominant-negative inhibitor of transcriptional activation mediated by the common 66 kDa isoform, ER- α 66 (39). Additionally, splice variants lacking exon 2 have been described in normal and tumor tissues (41), and in vitro studies indicate that these isoforms are transcriptionally inactive (42). Although rs6902771 is distal to alternate exon 1, if polymorphisms

within the ESR1 intron 1–intron 2 region (possibly in LD with associated SNPs) alter ER- α 36 alternate exon 1, ER- α 36, or ER- α 46 intron 1 promoters, or exon 2 skipping, this in turn could impact transcription of downstream genes affecting metabolic phenotypes. Herrington et al. (2) noted that the rs2234693 C-allele produces a potential binding site for the myb family of transcription factors, although it is unknown whether this allele influences transcriptional activation of ER- α .

These results suggest that multiple variants across the intron 1–intron 2 region of the ESR1 gene may influence diabetes and CVD risk through pleiotropic effects on components of the metabolic syndrome (Table 6), specifically insulin resistance, a poor lipid profile, and obesity.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health Grants DK66358, HL060944, HL060894, HL061210, HL060931, HL061019, and HL061210 and a Career Development Award from the American Diabetes Association (to M.M.S.).

We thank the participants in the IRAS Family Study and Pam Hicks for technical assistance.

REFERENCES

1. Speer G, Cseh K, Winkler G, Vargha P, Braun E, Takacs I, Lakatos P: Vitamin D and estrogen receptor gene polymorphisms in type 2 diabetes mellitus and in android type obesity. *Eur J Endocrinol* 144:385–389, 2001
2. Herrington DM, Howard TD, Hawkins GA, Reboussin DM, Xu J, Zheng SL, Brosnihan KB, Meyers DA, Bleecker ER: Estrogen-receptor polymorphisms and effects of estrogen replacement on high-density lipoprotein cholesterol in women with coronary disease. *N Engl J Med* 346:967–974, 2002
3. Kikuchi T, Hashimoto N, Kawasaki T, Uchiyama M: Association of serum low-density lipoprotein metabolism with oestrogen receptor gene polymorphisms in healthy children. *Acta Paediatr* 89:42–45, 2000
4. Peter I, Shearman AM, Zucker DR, Schmid CH, Demissie S, Cupples LA, Larson MG, Vasan RS, D’Agostino RB, Karas RH, Mendelsohn ME, Housman DE, Levy D: Variation in estrogen-related genes and cross-sectional and longitudinal blood pressure in the Framingham Heart Study. *J Hypertens* 23:2193–2200, 2005
5. Iwai N, Tago N, Yasui N, Kokubo Y, Inamoto N, Tomoike H, Shioji K: Genetic analysis of 22 candidate genes for hypertension in the Japanese population. *J Hypertens* 22:1119–1126, 2004
6. Sale MM, Freedman BI, Langefeld CD, Williams AH, Hicks PJ, Colicigno CJ, Beck SR, Brown WM, Rich SS, Bowden DW: A genome-wide scan for type 2 diabetes in african-american families reveals evidence for a locus on chromosome 6q. *Diabetes* 53:830–837, 2004
7. Gallagher CJ, Keene KL, Mychaleckyj JC, Langefeld CD, Hirschhorn JN, Henderson BE, Gordon CJ, Freedman BI, Rich SS, Bowden DW, Sale MM: Investigation of the estrogen receptor α gene with type 2 diabetes and/or nephropathy in African American and European American populations. *Diabetes* 56:675–684, 2007
8. Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS: Increased adipose tissue in male and female estrogen receptor- α knockout mice. *Proc Natl Acad Sci U S A* 97:12729–12734, 2000
9. Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB, Korach KS: Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N Engl J Med* 331:1056–1061, 1994
10. Henkin L, Bergman RN, Bowden DW, Ellsworth DL, Haffner SM, Langefeld CD, Mitchell BD, Norris JM, Rewers M, Saad MF, Stamm E, Wagenknecht LE, Rich SS: Genetic epidemiology of insulin resistance and visceral adiposity: the IRAS Family Study design and methods. *Ann Epidemiol* 13:211–217, 2003
11. Wagenknecht LE, Mayer EJ, Rewers M, Haffner S, Selby J, Borok GM, Henkin L, Howard G, Savage PJ, Saad MF, et al: The insulin resistance atherosclerosis study (IRAS) objectives, design, and recruitment results. *Ann Epidemiol* 5:464–472, 1995
12. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults: Executive summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detec-

- tion, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA* 285:2486–2497, 2001
13. Herbert V, Lau KS, Gottlieb CW, Bleicher SJ: Coated charcoal immunoassay of insulin. *J Clin Endocrinol Metab* 25:1375–1384, 1965
 14. Steil GM, Volund A, Kahn SE, Bergman RN: Reduced sample number for calculation of insulin sensitivity and glucose effectiveness from the minimal model: suitability for use in population studies. *Diabetes* 42:250–256, 1993
 15. Pacini G, Bergman RN: MINMOD: a computer program to calculate insulin sensitivity and pancreatic responsiveness from the frequently sampled intravenous glucose tolerance test. *Comput Methods Programs Biomed* 23:113–122, 1986
 16. Friedewald WT, Levy RI, Fredrickson DS: Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18:499–502, 1972
 17. Sugiuchi H, Uji Y, Okabe H, Irie T, Uekama K, Kayahara N, Miyauchi K: Direct measurement of high-density lipoprotein cholesterol in serum with polyethylene glycol-modified enzymes and sulfated alpha-cyclodextrin. *Clin Chem* 41:717–723, 1995
 18. Buetow KH, Edmonson M, MacDonald R, Clifford R, Yip P, Kelley J, Little DP, Strausberg R, Koester H, Cantor CR, Braun A: High-throughput development and characterization of a genomewide collection of gene-based single nucleotide polymorphism markers by chip-based matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Proc Natl Acad Sci U S A* 98:581–584, 2001
 19. de Bakker PI, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D: Efficiency and power in genetic association studies. *Nat Genet* 37:1217–1223, 2005
 20. Rich SS, Bowden DW, Haffner SM, Norris JM, Saad MF, Mitchell BD, Rotter JI, Langefeld CD, Wagenknecht LE, Bergman RN: Identification of quantitative trait loci for glucose homeostasis: the Insulin Resistance Atherosclerosis Study (IRAS) Family Study. *Diabetes* 53:1866–1875, 2004
 21. O'Connell JR, Weeks DE: PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am J Hum Genet* 63:259–266, 1998
 22. Zeger SL, Liang KY: Longitudinal data analysis for discrete and continuous outcomes. *Biometrics* 42:121–130, 1986
 23. Neter J, Wasserman W, Kutner M: *Applied Linear Statistical Models, Regression, Analysis of Variance, and Experimental Design*. Homewood, IL, Richard D. Irwin, 1990
 24. Lange K, Cantor R, Horvath S, Perola M, Sabatti C, Sinsheimer J, Sobel E: Mendel version 4.0: a complete package for the exact genetic analysis of discrete traits in pedigree and population data sets. *Am J Hum Genet* 69:504, 2001
 25. Dudbridge F: Pedigree disequilibrium tests for multilocus haplotypes. *Genet Epidemiol* 25:115–121, 2003
 26. Galan JJ, Buch B, Cruz N, Segura A, Moron FJ, Bassas L, Martinez-Pineiro L, Real LM, Ruiz A: Multilocus analyses of estrogen-related genes reveal involvement of the ESR1 gene in male infertility and the polygenic nature of the pathology. *Fertil Steril* 84:910–918, 2005
 27. Shearman AM, Demissie S, Cupples LA, Peter I, Schmid CH, Ordovas JM, Mendelsohn ME, Housman DE: Tobacco smoking, estrogen receptor alpha gene variation and small low density lipoprotein level. *Hum Mol Genet* 14:2405–2413, 2005
 28. Demissie S, Cupples LA, Shearman AM, Gruenthal KM, Peter I, Schmid CH, Karas RH, Housman DE, Mendelsohn ME, Ordovas JM: Estrogen receptor-alpha variants are associated with lipoprotein size distribution and particle levels in women: The Framingham Heart Study. *Atherosclerosis* 185:210–218, 2006
 29. Nordstrom P, Glader CA, Dahlen G, Birgander LS, Lorentzon R, Waldenstrom A, Lorentzon M: Oestrogen receptor alpha gene polymorphism is related to aortic valve sclerosis in postmenopausal women. *J Intern Med* 254:140–146, 2003
 30. Ohlsson C, Hellberg N, Parini P, Vidal O, Bohlooly M, Rudling M, Lindberg MK, Warner M, Angelin B, Gustafsson JA: Obesity and disturbed lipoprotein profile in estrogen receptor-alpha-deficient male mice. *Biochem Biophys Res Commun* 278:640–645, 2000
 31. Cooke PS, Heine PA, Taylor JA, Lubahn DB: The role of estrogen and estrogen receptor-alpha in male adipose tissue. *Mol Cell Endocrinol* 178:147–154, 2001
 32. Fox CS, Heard-Costa NL, Wilson PW, Levy D, D'Agostino RB Sr, Atwood LD: Genome-wide linkage to chromosome 6 for waist circumference in the Framingham Heart Study. *Diabetes* 53:1399–1402, 2004
 33. Deng HW, Li J, Li JL, Dowd R, Davies KM, Johnson M, Gong G, Deng H, Recker RR: Association of estrogen receptor-alpha genotypes with body mass index in normal healthy postmenopausal Caucasian women. *J Clin Endocrinol Metab* 85:2748–2751, 2000
 34. Okura T, Koda M, Ando F, Niino N, Ohta S, Shimokata H: Association of polymorphisms in the estrogen receptor alpha gene with body fat distribution. *Int J Obes Relat Metab Disord* 27:1020–1027, 2003
 35. Fox CS, Yang Q, Cupples LA, Guo CY, Atwood LD, Murabito JM, Levy D, Mendelsohn ME, Housman DE, Shearman AM: Sex-specific association between estrogen receptor [alpha] gene variation and measures of adiposity: the Framingham Heart Study. *J Clin Endocrinol Metab* 90:6257–6262, 2005
 36. Toth MJ, Tchernof A, Sites CK, Poehlman ET: Effect of menopausal status on body composition and abdominal fat distribution. *Int J Obes Relat Metab Disord* 24:226–231, 2000
 37. Dieudonne MN, Leneveu MC, Giudicelli Y, Pecquery R: Evidence for functional estrogen receptors alpha and beta in human adipose cells: regional specificities and regulation by estrogens. *Am J Physiol Cell Physiol* 286:C655–C661, 2004
 38. Pedersen SB, Kristensen K, Hermann PA, Katzenellenbogen JA, Richelsen B: Estrogen controls lipolysis by up-regulating alpha2A-adrenergic receptors directly in human adipose tissue through the estrogen receptor alpha. Implications for the female fat distribution. *J Clin Endocrinol Metab* 89:1869–1878, 2004
 39. Wang Z, Zhang X, Shen P, Loggie BW, Chang Y, Deuel TF: Identification, cloning, and expression of human estrogen receptor-alpha36, a novel variant of human estrogen receptor-alpha66. *Biochem Biophys Res Commun* 336:1023–1027, 2005
 40. Flouriot G, Brand H, Denger S, Metivier R, Kos M, Reid G, Sonntag-Buck V, Gannon F: Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and that is able to repress hER-alpha activation function 1. *Embo J* 19:4688–4700, 2000
 41. Poola I, Speirs V: Expression of alternatively spliced estrogen receptor alpha mRNAs is increased in breast cancer tissues. *J Steroid Biochem Mol Biol* 78:459–469, 2001
 42. Bollig A, Miksicek RJ: An estrogen receptor-alpha splicing variant mediates both positive and negative effects on gene transcription. *Mol Endocrinol* 14:634–649, 2000