Genetic Variation in Fatty Acid–Binding Protein-4 and Peroxisome Proliferator–Activated Receptor γ Interactively Influence Insulin Sensitivity and Body Composition in Males

Coleen M. Damcott, Susan P. Moffett, Eleanor Feingold, M. Michael Barmada, Julie A. Marshall, Richard F. Hamman, and Robert E. Ferrell

Obesity and type 2 diabetes are closely related, multifactorial metabolic conditions characterized by alterations in energy metabolism and glucose homeostasis, respectively. Peroxisome proliferator–activated receptor γ (PPARγ) is a ligand-dependent transcription factor that regulates genes involved in lipid and glucose homeostasis, including the adipocyte-specific fatty acid–binding protein (FABP4). In turn, FABP4 binds fatty acids and transports them to the nucleus where the FABP4/fatty acid complex activates PPARγ in a positive feedback loop. In this study, we tested the hypothesis that the polymorphisms, FABP4-376 and PPARγ Pro12Ala, interactively influence insulin sensitivity and body composition in nondiabetic, Hispanic and non-Hispanic white males (n = 314) participating in the San Luis Valley Diabetes Study (SLVDS). Although the individual sites were not statistically significantly associated with any of the outcomes, we found statistically significant interaction terms in 2-way analysis of covariance (ANCOVA) models for homeostasis model assessment of insulin resistance (HOMA-IR) (*P* = .014) and lean mass (*P* = .019). While the PPARγ Pro12Ala site was the only statistically significant predictor of fat mass in the 2-way model (*P* = .012), the FABP4 and PPARγ main effect terms individually became stronger when considered in one model compared with the analysis of each polymorphism separately. These findings provide evidence that FABP4 and PPARγ work together to influence a biologic pathway affecting insulin sensitivity and body composition, illustrating the importance of investigating the joint effect of genes in determining susceptibility for complex disease.

OBESITY AND TYPE 2 diabetes are closely related, multifactorial metabolic conditions affecting millions of people worldwide. Obesity results from imbalances in energy homeostasis due to both environmental and genetic determinants of food intake, energy expenditure, and substrate metabolism. Obesity is commonly a precursor to the development of several complex disorders, including type 2 diabetes. In the year 2000, the prevalence of obesity (body mass index [BMI] > 30) in the United States was approximately 30%.[1] Type 2 diabetes is characterized by impaired insulin sensitivity and/or abnormal insulin secretion, altering glucose homeostasis. The prevalence of type 2 diabetes in the United States is currently approximately 6% to 7%, but is increasing steadily.[2]

The fatty acid–binding proteins (FABPs) are a family of cytoplasmic proteins involved in intracellular free fatty acid transport and metabolism. They bind long-chain fatty acids with high affinities that vary with chain length and degree of saturation.[3] Nine distinct members of this gene family have been identified, including an adipose-specific isoform, FABP4 (aP2).[4] The FABPs are regulated by diet composition, hormones, and transcription factors, such as the peroxisome proliferator–activated receptors (PPARs).[3,4]

The PPARs are members of the nuclear receptor superfamily of ligand-activated transcription factors involved in lipid metabolism, glucose homeostasis, and adipocyte differentiation. There are 3 subtypes in the PPAR family, including PPARγ.[5,6] The PPARγ locus encodes 4 isoforms, which are products of alternate promoter usage.[7] When activated by fatty acids, PPARγ binds to specific response elements in the regulatory regions of genes involved in fatty acid release, transport, and synthesis, regulating transcription.[8,9] PPARγ is mainly expressed in adipose tissue and at lower levels in the muscle, liver, heart, colon, and cells of the immune system.[5,10]

Because the PPARs are located in the nucleus of the cell,[11] activation depends upon the transport of fatty acids to the nucleus. Helledie et al[12] found evidence that FABP4 and keratinocyte-FABP localize to the nucleus of adipocytes. Tan et al[13] showed that FABP4 and keratinocyte-FABP activate PPARγ and PPARβ, respectively, and function as fatty acid transporters to the nucleus where the entire FABP/fatty acid complex directly interacts with the PPARs in a ligand-dependent manner. The PPARs, in turn, regulate transcription of the FABPs through a feedback mechanism. The proximal 5′ region of mouse FABP4 contains 2 PPAR response elements with high affinity for PPARγ.[14]

A proline (C) to alanine (G) substitution at codon 12 of the PPARγ2 isoform was reported by Yen et al.[15] There is evidence suggesting that the product of the alanine-encoding allele at codon 12 has reduced transcriptional and adipogenic activity in vitro, which could lead to lower adipose tissue mass.[16] In addition, the alanine-encoding allele was found to be associated with lower lipolysis and greater insulin sensitivity in a group of lean nondiabetic subjects.[17] Association studies of the Pro12Ala site involving BMI have shown variable results.[18-23] However, in a large study of Scandinavian individuals, the Pro12Ala variant was the only polymorphism out of 16 common single nucleotide polymorphisms (SNPs) in candidate

From the Department of Human Genetics, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA; and the Department of Preventive Medicine and Biometrics, University of Colorado Health Sciences Center, Denver, CO.

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C.M.D. and S.P.M. contributed equally to this work.

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Address reprint requests to Coleen M. Damcott, PhD, University of Maryland School of Medicine, Department of Endocrinology, Diabetes, and Nutrition, 660 W. Redwood St, Baltimore, MD 21201.

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Table 1. Genotype Counts and Allele Frequencies

		Genotype Counts	Common Allele Frequency	*P* Value: Hardy-Weinberg Test
FABP4p-376	AA	237	0.857	.815
	AC	76		
	CC	8		
PPARγ2	CC	267	0.894	.992
	CG	63		
	GG	4		

genes that were previously reported to influence diabetes risk to show a reproducible association with reduced diabetes risk.[24]

Because FABP4 and PPARγ are involved in a feedback loop that regulates lipid and glucose metabolism pathways, variation in those genes may influence risk of developing obesity and/or type 2 diabetes. In the present study, we tested the hypothesis that genetic variation in FABP4 and PPARγ interactively influence insulin sensitivity and body composition in nondiabetic participants in the San Luis Valley Diabetes Study (SLVDS).

MATERIALS AND METHODS

Study Subjects

The SLVDS is a prospective study of the natural history, incidence, and risk factors of type 2 diabetes and its complications in Hispanic and non-Hispanic white individuals living in the geographically isolated San Luis Valley of Colorado. Participants in the SLVDS were evaluated for glucose tolerance status by a 2-hour oral glucose tolerance test at baseline (1984 to 1988) and 2 follow up visits (1988 to 1992, 1997 to 1998).[25] Subjects for the current study were nondiabetic at the baseline visit and were seen at the second follow up visit. A total of 314 males and 348 females were genotyped for polymorphisms in FABP4 and PPARγ. Only males are presented in this report because few statistically significant associations with the outcome variables of interest were observed in females, suggesting the presence of sex-specific effects. Fat mass and lean mass were estimated in grams using dual energy x-ray absorptiometry (DEXA).[26] Homeostasis model assessment of insulin resistance (HOMA-IR), a derived measure of insulin resistance, was calculated using fasting insulin and glucose levels (glucose · insulin/22.5).[27] Informed consent was obtained from all subjects, and the University of Colorado Health Sciences Center Institutional Review Board approved all of the protocols.

Mutation Detection and Genotyping

Approximately 500 bp of the region 5′ of the FABP4 initiation codon was screened for variation by direct sequencing in 12 laboratory control samples (Genbank BAC Clone #AC018616). Two single nucleotide polymorphisms were detected in this region: an A to C substitution at −376 bp and an A to C substitution 2 bp downstream at −374 bp. These polymorphisms were in high linkage disequilibrium; therefore, only the FABP4 −376 site was genotyped. Genotypes at FABP4 −376 were assigned using fluorescence polarization.[28] The primers used for amplification of genomic DNA by standard polymerase chain reaction (PCR) methods were: 5′CGAGGCAGTTCTTATGTTCC3′ and 5′TGACAGCTTAATGCTCAGTGC3′. The detection primer used for the single base extension reaction was 5′CATAACTGCAATTTAAATAACACCCC3′. The Analyst AD instrument (Molecular Devices Corp, Sunnyvale, CA) was used to read the fluorescent signals for genotyping and the Allele Caller software (Molecular Devices Corp) was used to assign genotypes.

The previously reported Pro12Ala polymorphism in the PPARγ gene (C to G transversion) was detected using an engineered BstUI restriction enzyme site. The primers used for amplification were: 5′GCCAATTCAAGCCCAGTC3′ and 5′GATATGTTTGCAGACAGTGTATCAGTGAAGGAATCGCTTTCCG3′. Digestion products were resolved on 2% agarose gels containing ethidium bromide and visualized under ultraviolet (UV) illumination. Fragment sizes were assigned by comparison to known size markers.

Statistical Analyses

Allele frequencies for each polymorphic site were estimated by gene counting.[29] Fit to the expectations of Hardy-Weinberg equilibrium was tested using chi-square tests. Analysis of covariance (ANCOVA) was used to test for cross-sectional associations between each single locus and HOMA-IR, lean mass, and fat mass. Ethnicity, age, physical activity, and smoking status were included as covariates based on significant correlation with the outcome variables. Two-way ANCOVA was used to explore the multilocus effects and interactions between the FABP4 −376 and PPARγ Pro12Ala sites on each outcome variable. Because ethnicity was a strong predictor for each outcome variable, the 2-way ANCOVA were repeated for the ethnic groups separately. All statistical analyses were performed using the SPSS (Chicago, IL) statistical software package version 10.1 for Windows.

RESULTS

Through direct sequencing, 2 single nucleotide polymorphisms were identified in the 5′ region of FABP4: an A to C substitution at −376 bp and an A to C substitution 2 bp downstream at −374 bp. These polymorphisms were nearly in complete linkage disequilibrium (172 of 174 chromosomes had matching genotypes at the 2 sites); therefore, only the FABP4 −376 bp variant was genotyped for this study. The PPARγ Pro12Ala variant, originally reported by Yen et al,[15] was also genotyped. Table 1 shows the genotype counts and allele frequencies for the FABP4 −376 and PPARγ Pro12Ala polymorphic sites. There was no statistically significant deviation from Hardy-Weinberg equilibrium for either site. Due to the small number of individuals homozygous for the less common allele at both sites, the rare homozygotes were pooled with the heterozygotes for analysis purposes.

Table 2 shows the genotype means and *P* values from the ANCOVA of the individual polymorphisms versus HOMA-IR,

Table 2. Genotype Means From Single Loci ANCOVA

	FABP4-376			PPARγ Pro12Ala		
	AA	AC + CC	*P* Value	CC	CG + GG	*P* Value
Adjusted fat mass (g)	22,987	24,025	.279	22,894	25,008	.051
Adjusted lean mass (g)	54,186	54,093	.906	54,121	54,242	.894
Adjusted HOMA-IR	17.8	17.4	.776	17.8	18.3	.761

NOTE. Means were adjusted for ethnicity, age, physical activity, and smoking status.

Table 3. Two-Way ANCOVA for HOMA-IR

Model	β-Coefficients	*P* Value	R^2 (*P* value)
All males			
Constant	27.2	<.001	.065 (.004)
FABP4 −376 (AA)	−8.0	.158	
PPARγ Pro12Ala (CC)	−8.3	.123	
FABP4 (AA) * PPARγ (CC)	10.2	.014	
Ethnicity (Hispanic)	3.6	.011	
Age (yr)	−0.06	.299	
Physical activity (kcal/kg/h)	−0.02	.065	
Smoking status (ever smoked)	2.3	.114	
Non-Hispanic white males			
Constant	20.3	.005	.053 (.114)
FABP4 −376 (AA)	−6.4	.182	
PPARγ Pro12Ala (CC)	−4.1	.855	
FABP4 (AA) * PPARγ (CC)	7.4	.076	
Age (yr)	0.06	.432	
Physical activity (kcal/kg/h)	−0.02	.073	
Smoking status (ever smoked)	2.5	.112	
Hispanic males			
Constant	50.5	<.001	.088 (.094)
FABP4 −376 (AA)	−14.6	.401	
PPARγ Pro12Ala (CC)	−20.2	.053	
FABP4 (AA) * PPARγ (CC)	20.9	.034	
Age (yr)	−0.2	.090	
Physical activity (kcal/kg/h)	−0.02	.311	
Smoking status (ever smoked)	3.7	.227	

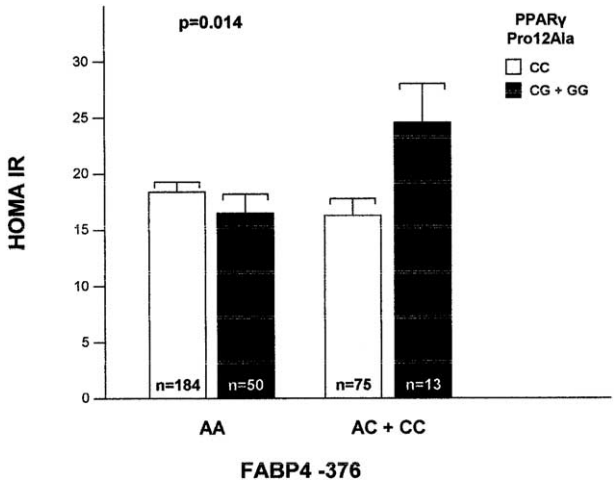

Fig 1. Bar chart of adjusted means for HOMA-IR in the FABP4 −376/PPARγ Pro12Ala genotype groups. HOMA-IR was derived from fasting glucose and insulin measured from plasma collected after a 12-hour fast. Error bars represent the SEM. The *P* value represents the interaction effect.

lean mass, and fat mass. The PPARγ site was marginally associated with fat mass ($P = .051$). Otherwise, no statistically significant associations were observed between the outcome variables and the individual sites. The biologic relationship between FABP4 and PPARγ suggests that the effects of polymorphisms in these genes may interactively modify one another. Thus, 2-way ANCOVA was used to further explore multilocus effects and interactions between the FABP4 and PPARγ variants on HOMA-IR, lean mass, and fat mass.

Table 3 shows the 2-way ANCOVA results for HOMA-IR in all males, non-Hispanic males, and Hispanic males. In the model for the entire male cohort, the interaction between the FABP4 and PPARγ sites was a statistically significant predictor of HOMA-IR ($P = .014$). This model explains approximately 6.5% of the variation in HOMA-IR. Figure 1 shows the adjusted means for HOMA-IR for each FABP4/PPARγ multilocus genotype group. Individuals with the less common allele at both sites showed an average increase of 6.2 to 8.3 HOMA-IR units when compared with all other genotype groups. In the model for Hispanic males, the interaction between the FABP4 and PPARγ sites was statistically significantly associated with HOMA-IR ($P = .034$). The interaction term was not statistically significantly associated with HOMA-IR in non-Hispanic males ($P = .076$).

Table 4 shows the results of 2-way ANCOVA for lean mass. For all males, the interaction between the FABP4 and Pro12Ala sites was a statistically significant predictor of lean mass ($P = .019$). Overall, the model explains approximately 33% of the variation in lean mass, which is largely due to the explanatory power of age and ethnicity. Figure 2 shows the adjusted means for lean mass in each FABP4/PPARγ multilocus genotype group. When compared with individuals in all other genotype groups, carriers of the less common allele at both sites showed an average 2.9 to 4.1 kg increase in lean mass. The genotypic effects on lean mass in non-Hispanic males were very similar to those observed in the overall male model. In the Hispanic

Table 4. Two-Way ANCOVA for Lean Mass (g)

Model	β-Coefficients	*P* Value	R^2 (*P* value)
All males (n = 297)			
Constant	76,973	<.001	.330 (<.001)
FABP4 −376 (AA)	−4,927	.140	
PPARγ Pro12Ala (CC)	−3,778	.210	
FABP4 (AA) * PPARγ (CC)	4,930	.019	
Ethnicity (Hispanic)	−6,578	<.001	
Age (yr)	−204	<.001	
Physical activity (kcal/kg/h)	6	.191	
Smoking status (ever smoked)	890	.234	
Non-Hispanic white males (n = 180)			
Constant	67,257	<.001	.120 (.001)
FABP4 −376 (AA)	−5,281	.084	
PPARγ Pro12Ala (CC)	−3,985	.464	
FABP4 (AA) * PPARγ (CC)	6,060	.020	
Age (yr)	−146	.002	
Physical activity (kcal/kg/h)	3	.615	
Smoking status (ever smoked)	2,140	.032	
Hispanic males (n = 117)			
Constant	65,189	<.001	.295 (<.001)
FABP4 −376 (AA)	64	.641	
PPARγ Pro12Ala (CC)	−848	.993	
FABP4 (AA) * PPARγ (CC)	1,664	.659	
Age (yr)	−261	<.001	
Physical activity (kcal/kg/h)	5	.492	
Smoking status (ever smoked)	−689	.568	

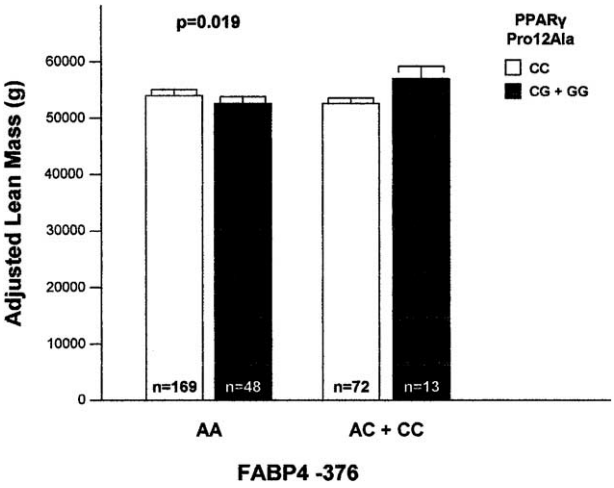

Fig 2. Bar chart of adjusted means for lean mass in the FABP4 −376/PPARγ Pro12Ala genotype groups. Lean mass was measured in grams using DEXA. Error bars represent the SEM. The *P* value represents the interaction effect.

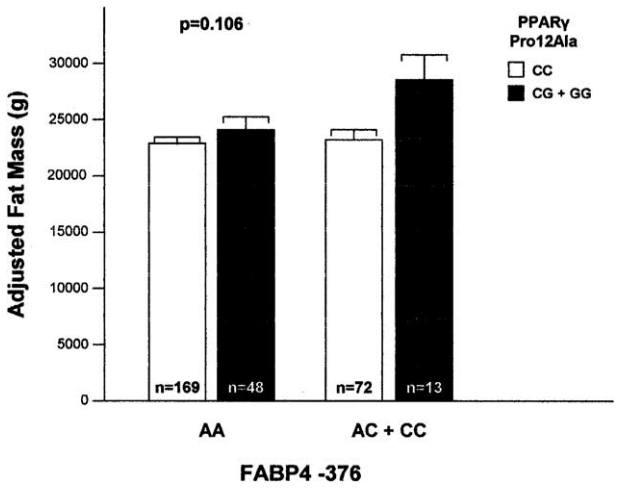

Fig 3. Bar chart of adjusted means for fat mass in the FABP4 −376/PPARγ Pro12Ala genotype groups. Fat mass was measured in grams using DEXA. Error bars represent the SEM. The *P* value represents the interaction effect.

group, on the other hand, there was no significant genotypic effect on lean mass; however, there were only 3 carriers of both the FABP4 C allele and the PPARγ G allele. Because this group is predicted to show the largest genotypic effect, splitting the cohort by ethnicity reduced the power to detect these effects.

Table 5 shows the 2-way ANCOVA models for fat mass in all males, non-Hispanic males, and Hispanic males. The model for the entire male cohort included the Pro12Ala site ($P = .012$) as a statistically significant predictor of fat mass. The FABP4 site ($P = .070$) and the interaction between the 2 sites ($P = .106$) were not statistically significantly associated with fat mass. Although the interaction term is not statistically significant, the FABP4 and PPARγ main effect terms individually become stronger when considered in one model compared with the analysis of each polymorphism separately. Approximately 6.5% of the variation in fat mass is explained by this model. Figure 3 shows the adjusted means for fat mass for each multilocus genotype group in the SLVDS males. Carriers of the less common allele at both sites showed an average 4.4 to 5.6 kg greater fat mass when compared with individuals in all other genotype groups. For the non-Hispanic males, the FABP4 site was a statistically significant predictor of fat mass ($P = .014$), while the PPARγ site ($P = .086$) and the interaction between the 2 sites ($P = .085$) were not statistically significant. The model for the Hispanic males showed no statistically significant contribution of these 2 sites to variation in fat mass.

Table 5. Two-Way ANCOVA for Fat Mass (g)

Model	β-Coefficients	*P* Value	R^2 (*P* value)
All males (n = 297)			
Constant	35,071	<.001	.065 (.006)
FABP4 −376 (AA)	−4,485	.070	
PPARγ Pro12Ala (CC)	−5,390	.012	
FABP4 (AA) * PPARγ (CC)	4,205	.106	
Ethnicity (Hispanic)	−2,650	.004	
Age (yr)	−42	.283	
Physical activity (kcal/kg/h)	−3	.625	
Smoking status (ever smoked)	1,146	.217	
Non-Hispanic white males (n = 180)			
Constant	28,871	<.001	.070 (.047)
FABP4 −376 (AA)	−6,458	.014	
PPARγ Pro12Ala (CC)	−5,329	.086	
FABP4 (AA) * PPARγ (CC)	5,324	.085	
Age (yr)	31	.561	
Physical activity (kcal/kg/h)	−7	.360	
Smoking status (ever smoked)	2,349	.047	
Hispanic males (n = 117)			
Constant	31,334	<.001	.068 (.250)
FABP4 −376 (AA)	1,436	.462	
PPARγ Pro12Ala (CC)	−2,694	.384	
FABP4 (AA) * PPARγ (CC)	902	.858	
Age (yr)	−125	.038	
Physical activity (kcal/kg/h)	−4	.695	
Smoking status (ever smoked)	−131	.935	

DISCUSSION

The purpose of this study was to examine potential interactive effects of genetic variation in FABP4 and PPARγ on measures of insulin sensitivity and body composition in non-Hispanic and Hispanic males from the SLVDS. In the entire male cohort, we found that individually the FABP4 −376 and the PPARγ Pro12Ala variants were not statistically significantly associated with measures of insulin sensitivity and body composition; however, statistically significant effects were observed when both sites were considered in one model. In particular, carriers of the less common alleles at FABP4 −376 and Pro12Ala showed greater HOMA-IR indices, lean mass, and fat mass. In the 2-way ANCOVA models, the interaction term between the FABP4 and PPARγ sites was a statistically significant predictor of both HOMA-IR and lean mass. Al-

though the model for fat mass did not show similar evidence of an interactive effect, the Pro12Ala variant was a statistically significant predictor of fat mass when considered in 2-way ANCOVA with FABP4. When the ethnic groups were analyzed separately, the genotypic effects were similar for most of the outcomes; however, the Hispanic males showed no statistically significant association with measures of body composition, which probably resulted from the small number of carriers of the less common alleles at both sites.

The outcome variables in this study were chosen with the idea that genes sharing common biologic pathways work together to influence physiologically related phenotypes. When the 2-way ANCOVA models are compared, it is apparent that the β-coefficients for the genotypic effects are similar in direction and magnitude for HOMA-IR, lean mass, and fat mass. In addition, similar trends in the mean values for the multilocus genotype groups were observed for each outcome (see Figs 1, 2, and 3). Although the interaction term in the fat mass model was not statistically significant, we may be lacking power to detect interactive effects between the FABP4 and PPARγ loci on fat mass. We used simulation methods to estimate the power of our sample to detect this interaction assuming that the model we fit is the true model and found it to be 33%. While we cannot draw definitive conclusions about fat mass, our study provides evidence that variation in FABP4 and PPARγ may influence a common biologic pathway affecting HOMA-IR, lean mass, and possibly fat mass.

The FABP4 −376 promoter variant is located in a sterol regulatory element-binding protein-1 (SREBP-1) response element as predicted by Transfac.[30] SREBP-1 is a transcription factor involved in adipocyte differentiation, fatty acid metabolism, and cholesterol homeostasis and is a known transcriptional activator of PPARγ and a suggested activator of FABP4.[31-34] The presence of the less common C allele of the FABP4 −376 polymorphism is predicted to disrupt the consensus SREBP-1 response element sequence,[30] suggesting altered binding affinity and transcriptional regulation of FABP4. Functional studies of the FABP4 promoter region would be required to confirm this inference. Assuming that the C allele at the FABP4 −376 locus disrupts SREBP-1 binding to the FABP4 promotor, presence of this allele would result in lower transcription of FABP4.

In previous studies examining the functional effects of the PPARγ Pro12Ala site in vitro, the alanine allele has been shown to reduce transcriptional activity, resulting in lower levels of lipolysis and adipogenesis.[16,17] The reduced activity of the PPARγ alanine allele would negatively affect transcription of FABP4. Thus, carriers of both the C allele at FABP4 −376 and the PPARγ alanine allele would be predicted to have lower levels of FABP4 in adipocytes. Because FABP4 delivers fatty acid ligands to PPARγ, lowered levels of FABP4 would further reduce PPARγ activity.

Several in vitro and in vivo studies have identified the FABPs as important players in cellular fatty acid uptake[35-39]; therefore, lowered FABP4 levels would presumably decrease fatty acid influx into adipocytes causing an increase in circulating fatty acid levels. Circulating fatty acids serve as an alternate fuel source to glucose and competitively inhibit glucose uptake by peripheral organs (eg, muscle and liver), leading to impaired insulin sensitivity.[40,41] In this study, individuals with the less common alleles at both the FABP4 −376 and PPARγ Pro12Ala show higher HOMA-IR indices (see Fig 1), which would be consistent with impaired insulin sensitivity in peripheral tissues through this mechanism. In addition, Shaughnessy et al[42] observed increased glucose conversion to fatty acids in adipocytes from the FABP4 knockout mouse. If adipocytes retain the ability to absorb glucose and convert it to fat for storage, this mechanism could account for the increase in fat mass and subsequent increase in lean mass observed in carriers of both the C allele at FABP4 −376 and the PPARγ alanine allele in the SLVDS males (see Figs 2 and 3).

In previous genotype/phenotype studies, several groups have found the PPARγ alanine allele to protect against type 2 diabetes.[23,24,44,45] However, a study involving Canadian Oji-Cree showed the alanine allele to be more common in diabetic women than in controls,[46] which is consistent with our finding of lower insulin sensitivity in SLVDS males with the PPARγ alanine allele in the presence of the FABP4 −376 C allele. A number of studies also found association between the PPARγ alanine allele and measures of body composition, such as increased BMI, percent body fat, and waist circumference.[19,20,43] In particular, Cole et al[19] found significantly increased BMI and waist circumference in carriers of the alanine allele in a large cohort of Mexican Americans. On the other hand, Deeb et al[21] found that the alanine allele was associated with lower BMI. In the current study, we found an increase in lean mass and fat mass in carriers of the PPARγ alanine allele on the background of the FABP4 −376 C allele. These findings suggest that it is important to consider other genes in the lipid metabolism pathways when evaluating the effects of the PPARγ Pro12Ala polymorphism.

In conclusion, we found evidence that variation in FABP4 and PPARγ interactively influence HOMA-IR and lean mass in males from the SLVDS. While we cannot make a definitive conclusion about the interactive effects on fat mass, PPARγ Pro12Ala was found to be a significant predictor of fat mass only when considered in a model with the FABP4 variant. Taken together, these findings provide evidence that FABP4 and PPARγ work together to influence a biologic pathway affecting insulin sensitivity and body composition. The synergistic action of these genes may help explain some of the inconsistencies in the PPARγ association studies and emphasizes the need to consider the effects of multiple genes on complex disorders.

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