

# Linkage of Plasma Adiponectin Levels to 3q27 Explained by Association With Variation in the *APM1* Gene

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We performed a genome-wide linkage scan of plasma adiponectin levels in 569 nondiabetic participants in the Amish Family Diabetes Study. The highest logarithm of odds (LOD) score (2.13;  $P = 0.0009$ ) occurred on chromosome 3q27 between markers D3S1602 and D3S1580, which flank *APM1/ACDC*, the adiponectin gene. The *APM1* +2019 A/- insertion/deletion polymorphism in the 3' untranslated region (single nucleotide polymorphism [SNP] +2019; deletion allele frequency 0.30 in Amish) showed strong association with adiponectin levels in a dosage-dependent manner in a direction consistent with that reported in previous studies, with deletion heterozygosity increasing adiponectin levels by  $1.3 \pm 0.5 \mu\text{g/ml}$  and deletion homozygosity increasing levels by  $3.0 \pm 0.8 \mu\text{g/ml}$  ( $P < 0.0001$ ). Two other SNPs, rs2241766 and rs1501299, showed moderate association. In a subset of 523 subjects genotyped for both SNP +2019 and rs2241766, including the *APM1* SNP +2019 genotype as a covariate reduced the linkage signal at 3q27 by 1.26 LOD units (from 2.22 to 0.96) and including both SNPs reduced the signal by 1.51 LOD units (to 0.71). These findings, combined with a two-point LOD score of 2.35 for SNP +2019, provide evidence that variation in *APM1* is responsible for linkage of adiponectin levels to 3q27 in the Old Order Amish. *Diabetes* 54:268–274, 2005

**A**diponectin is a fat-secreted hormone involved in insulin sensitivity, free fatty acid oxidation, and inhibition of inflammation (1,2). Circulating adiponectin levels are decreased in obesity (3), diabetes (4), hypertension (5), and dyslipidemia (6), which are components of the metabolic syndrome, or insulin resistance syndrome. Adiponectin is one of a number of adipose tissue–secreted proteins collectively called

adipocytokines, the discovery of which in the past decade has radically changed the view of adipose tissue from that of a simple energy storage depot to that of an active endocrine organ. Also known as ACRP30, AdipoQ, and GBP28, adiponectin was discovered in 1995 and 1996 as the most abundantly expressed sequence tag in an adipose mRNA library (7–9). The chromosomal region 3q27, which contains *APM1* (also known as *ACDC*), the gene encoding adiponectin, has been linked to several features of the metabolic syndrome (10) and diabetes (11). Adiponectin levels have been significantly linked to chromosomes 3 (12,13), 5 and 14 (14), and 9 (15) and suggestively linked to others (14,15). Several investigators have characterized single nucleotide polymorphisms (SNPs) in the adiponectin gene and found one or more of these SNPs to be associated with diabetes (16) and other metabolic syndrome features, as well as with adiponectin levels themselves (16–18).

We measured adiponectin levels in participants in the Amish Family Diabetes Study (AFDS) to 1) confirm and further define the relation between adiponectin levels and other metabolic syndrome components (19) and 2) identify loci harboring genetic variants that may influence adiponectin levels (and thus insulin resistance and type 2 diabetes). After finding that our strongest linkage signal for adiponectin levels in our genome scan occurred in the chromosomal region containing *APM1*, the adiponectin structural gene, we performed association and linkage analysis between *APM1* variation and adiponectin levels. Our findings suggest that sequence variation in *APM1* influences adiponectin levels.

## RESEARCH DESIGN AND METHODS

Recruitment for the AFDS began in early 1995 with the goal of identifying genes influencing the risk of type 2 diabetes and related traits. The study protocol was approved by the Institutional Review Board at the University of Maryland School of Medicine, and informed consent was obtained from each study participant. With the help of liaisons from the Old Order Amish community, we identified individuals with type 2 diabetes. These probands and their first- and second-degree relatives and spouses age 18 years or older were invited to participate. All subjects could be connected into a single 14-generation pedigree (20). Among the 691 individuals recruited and included in a 10-cM genomewide scan, there were 70 subjects with type 2 diabetes (25 prevalent cases and 45 incident cases) contained in 43 sibships (including 40 affected subjects in 13 multiplex sibships). Stored fasting plasma from 627 subjects (including 58 with diabetes) of the 691 scanned subjects was available for measuring adiponectin levels. Because of the potential influence of diabetes and its treatment on adiponectin levels, we excluded type 2 diabetic subjects from the analysis, leaving a total of 569 subjects for the linkage analysis. Fasting plasma adiponectin levels were measured in an

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AFDS, Amish Family Diabetes Study; LD, linkage disequilibrium; QTL, quantitative trait locus; SNP, single nucleotide polymorphism; SOLAR, Sequential Oligogenic Linkage Analysis Routines; UTR, untranslated region.

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additional 23 nondiabetic subjects not included in the original genome-wide scan/linkage analysis; these subjects were included in the association analysis (for a total of 592 subjects).

**Phenotypes.** Blood samples were obtained from an antecubital vein after an overnight fast at the Amish Research Clinic in Strasburg, Pennsylvania, or in subjects' homes. Adiponectin levels were measured in fasting plasma samples using a commercial radioimmunoassay kit (Linco) according to the manufacturer's instructions. The manufacturer reports coefficients of variation of 6.90–9.25 and 1.78–6.21% for inter- and intra-assay precision, respectively. Samples were diluted at 1:250 (10  $\mu$ l plasma in 2,500  $\mu$ l milliQ water).  $\gamma$  Counts were measured for 1 min on an automated  $\gamma$  counter (Packard Cobra-II Auto Gamma). All samples were assayed in duplicate, and results of samples with  $\gamma$  counts differing by >10% were discarded and the samples retested. Assays in which adiponectin levels for quality control samples did not fall into the expected range were discarded. Other phenotyping for the AFDS has been previously described in detail (20).

**Short tandem repeat genotyping.** Genotyping was performed on DNA extracted from leukocytes using a screening set of 373 highly polymorphic microsatellite short tandem repeat markers on the 22 autosomes and the X chromosome from the ABI Prism Linkage Mapping Set (Applied Biosystems Division/Perkin-Elmer, Foster City, CA). The mean marker heterozygosity was 0.75 (range 0.33–0.91). The average intermarker distance was 9.7 cM. The largest gap between markers was 25.4 cM, occurring on chromosome 7. The genotyping error rate based on blind replicates was 0.16% on average.

**Candidate gene SNP genotyping.** The adiponectin structural gene, *APMI* or *ACDC*, is ~17 kb in length and contains three exons, one noncoding and two coding. From public databases and previous publications (16,17), we selected five SNPs (rs266729 [promoter]), rs182052 [promoter], rs822396 [intron 1], rs2241766 [exon 2], and rs1501299 [intron 2]) and one single base pair insertion/deletion polymorphism 2,019 bp downstream of the ATG start codon in the 3' untranslated region (hereafter denoted as SNP +2019). These polymorphisms were chosen because they were distributed throughout the gene, had been previously reported to be common, and in some studies had been found to be associated with diabetes and related phenotypes in Caucasian populations.

The five SNPs were genotyped using the SNPstream Ultra High Throughput Genotyping System (Beckman Coulter, Fullerton, CA) according to the manufacturer's instructions. Briefly, the target genomic sequences containing the SNPs were amplified as part of 12-plex PCRs, which were subjected to an extension reaction using 5'-tagged extension primers and fluorescent dye-labeled terminators. In a thermal cycled extension step, the primers hybridized to the specific amplicons one base adjacent to the SNP site and were extended by one base at the 3' end with a fluorescently labeled nucleotide. These reaction products were transferred to an array plate, where each of the 12 extension products in the multiplex reaction were sorted by hybridization of the unique 5'-tag sequence to its complementary probe immobilized in a mini-array within each well. The plates were imaged by the SNPscope (Beckman Coulter). SNPs were called by comparing the two fluorescent signals. The error rates for these SNPs based on 5% blind replicates were 0%, except for SNPs rs2241766 and rs1501299, which had error rates of 1.2 and 4.8%, respectively.

SNP +2019 was genotyped using the PSQ HS 96A Pyrosequencer according to the manufacturer's methods (Pyrosequencing, Uppsala, Sweden; www.pyrosequencing.com). Briefly, a PCR product was generated from a primer pair that included one primer covalently coupled to biotin, the biotinylated template was bound to streptavidin-coated Sepharose HP beads, and this mixture was then annealed to a sequencing primer. Stepwise elongation of a sequencing primer strand upon sequential addition of a specified sequence of deoxynucleotide triphosphates and the degradation of nucleotides by apyrase were carried out simultaneously. As the sequencing reaction progressed, the DNA strand was extended and the sequence was determined from the measured signal output of light upon nucleotide incorporation. The resulting peaks in the pyrogram were analyzed using Pyrosequencing software. The error rate for SNP +2019 based on 5% blind replicates was 0%.

**Sequencing.** To determine if SNPs found to be associated with variation in adiponectin levels were marking other functional SNPs, particularly those altering an amino acid sequence, we sequenced the entire coding region of *APMI*, which comprises most of exon 2 and a portion of exon 3, in a subset of 24 individuals enriched for the presence of associated polymorphisms. Sequencing was performed on an ABI 3700 DNA sequencer.

**Statistical analysis.** Although virtually all subjects could be connected into a single 14-generation pedigree, we divided the sample into 40 discrete families for linkage analysis to reduce computational burden. These 40 families contained 2–59 genotyped and phenotyped individuals and provided a large number of relative pairs, including 281 parent-offspring pairs, 971 sibling pairs, 888 avuncular (aunt/uncle–niece/nephew) pairs, and 983 first-cousin pairs.

Heritability, linkage, and association analyses were carried out using a variance components methodology. For heritability and linkage analyses, we partitioned variation in the adiponectin trait into components attributable to environmental covariates, the additive effects of genes (i.e., residual heritability), and a specific quantitative trait locus (QTL, the linkage component). These analyses were conducted using maximum likelihood procedures as implemented in the Sequential Oligogenic Linkage Analysis Routines (SOLAR) program (21). The residual heritability was modeled as a function of the expected genetic covariances between relatives, and the QTL effect was modeled as a function of the identity-by-descent probabilities at the marker locus. Allele frequencies were estimated from the data using maximum likelihood methods (21). The hypothesis of linkage was evaluated by the likelihood ratio test, which determines whether the locus-specific effect is significantly >0 ( $H_0$ , [null hypothesis]:  $\sigma^2_{QTL} = 0$  vs.  $H_A$  [alternative hypothesis]:  $\sigma^2_{QTL} > 0$ ). In each model, we simultaneously adjusted for the effects of sex and sex-specific age and age squared. Because of the sensitivity of variance components to violations of normality, the three adiponectin data points whose values were >3 SD from the mean were truncated and their values set to the value corresponding to 3 SD from the mean (Winsorization; 22). To estimate the probability of obtaining a false-positive result and to obtain empirical logarithm of odds (LOD) scores, we used the LODADJ routine in SOLAR to generate 100,000 fully informative unlinked markers. We evaluated evidence for linkage of adiponectin levels to these simulated markers and defined the probability of obtaining a false-positive result as the proportion of the 100,000 replicates for which a LOD score greater than or equal to that observed for the original linked locus was obtained. All LOD scores in this study were obtained by converting the empirical *P* value obtained by simulation to its corresponding LOD score [ $LOD = \chi^2 \div [2 * \ln(10)]$ ].

Before association analysis, genotypes were checked for Mendelian consistency using the Pedcheck software package (23) and inconsistencies (<0.5% of genotypes) were resolved or removed. Using variance components, we estimated the effects of the SNP genotype on adiponectin levels, adjusting for sex and sex-specific age and age squared. Genotype was parameterized according to the number of copies of the minor allele the subject carried (genotype = 0 if major allele homozygote, 1 if heterozygote, and 2 if minor allele homozygote). Parameter estimates were obtained conditional on the pedigree structure. For each SNP, the likelihood ratio test was used to compare a model that included the *APMI* SNP as a covariate with a model that did not include the SNP.

Allele frequencies were estimated by the maximum likelihood estimates conditioned on the pedigree structure as implemented in SOLAR. Linkage disequilibrium (LD) was estimated using an algorithm implemented in the software package Zaplo, which uses both pedigree and LD information to find all possible haplotype configurations of the data under a zero-recombinant assumption (24).

To test whether the *APMI* association explained the linkage to 3q27, variance components linkage analysis was repeated with the associated *APMI* polymorphism genotype as a covariate. The gene-dropping option of Mendel 5.0 (25) was used to simulate the segregation 10,000 times of an unlinked SNP with the same frequency as the *APMI* polymorphism. These simulated SNP genotypes were then used as covariates in 10,000 repetitions of the linkage analysis to attempt to assess the significance of the LOD change that occurred with the inclusion of the *APMI* variant as a covariate.

**Power estimation.** We evaluated the power of our sample to detect linkage to QTLs accounting for 20, 25, and 30% of the total variation in a model trait using simulation, as previously described (26). For each of the three effect sizes, 200 QTLs were simulated and the power to detect linkage was defined as the proportion of replicates for which we obtained LOD scores greater than selected values (3.0 and 2.0).

## RESULTS

**Heritability and genome scan.** The heritability of the plasma adiponectin trait, adjusted for sex and sex-specific age and age squared, was estimated at  $0.55 \pm 0.08$ .

The maximum LOD score in the entire genome scan was 2.13 ( $P = 0.0009$ ), occurring on chromosome 3q between markers D3S1602 and D3S1580 (Fig. 1 and Table 1). We observed four other regions of suggestive linkage (Table 1): 7q11–21, near marker D7S669 (LOD = 1.87,  $P = 0.0017$ ); 9q34, between markers D9S290 and D9S164 (LOD = 1.54,  $P = 0.0039$ ); 10p12–14, near marker D10S548 (LOD = 1.73,  $P = 0.0024$ ); and 16p13, near marker D16S407 (LOD = 1.69,  $P = 0.0026$ ).

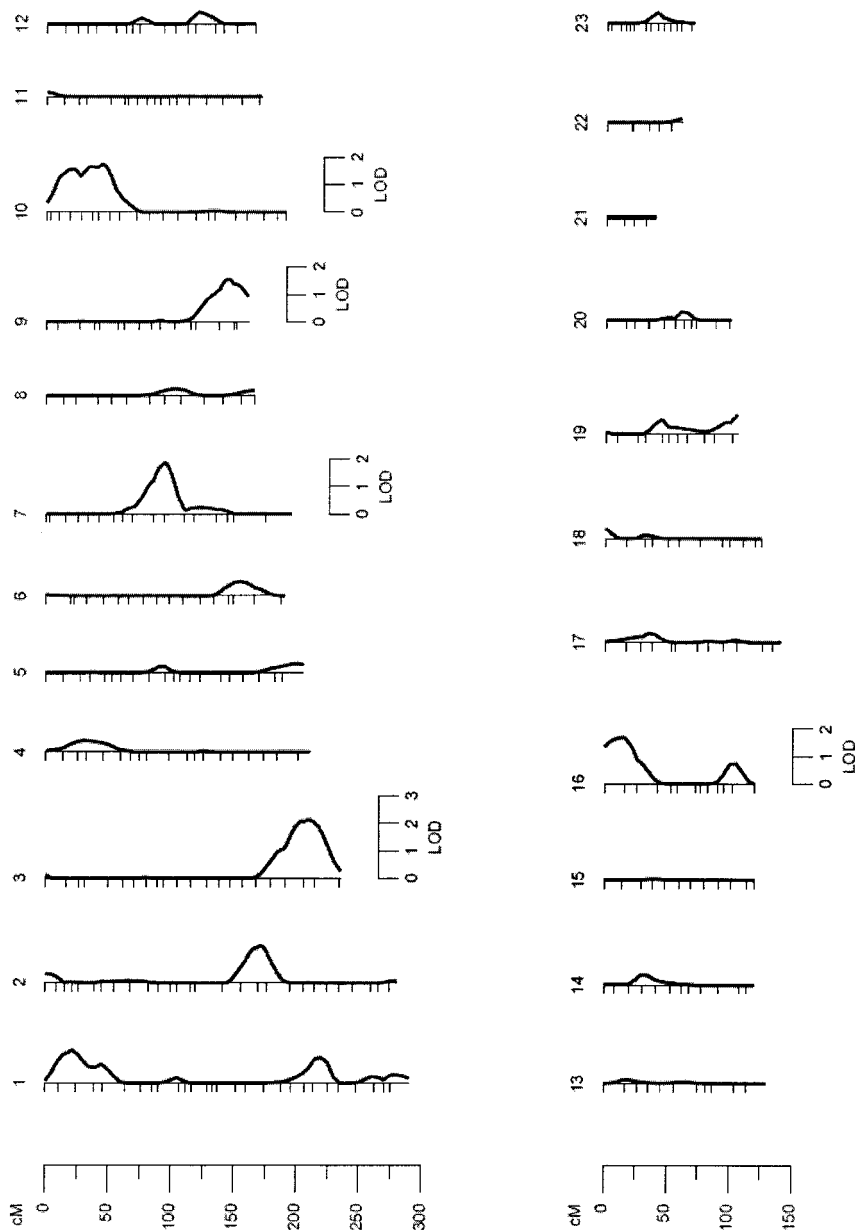


FIG. 1. Genome-wide scan results by chromosome.

**Power estimation.** The results of the power simulation indicated that the sample provided reasonable power (78%) to detect linkage (at LOD scores  $>3.0$ ) for a QTL accounting for  $\geq 30\%$  of the total trait variance. For a QTL accounting for 25% of the variance, power would be 49% to detect a LOD score  $>3.0$  and 80% for a LOD score  $>2.0$ . The power was much lower for a QTL accounting for only 20% of the variance (27% for a LOD score  $>3.0$  and 57% for a LOD score  $>2.0$ ).

**Candidate gene analysis.** The two markers flanking our strongest linkage signal, D3S1602 and D3S1580, flank a 2.5-Mb region that contains the structural gene (*APM1*) encoding adiponectin. This observation motivated us to evaluate whether sequence variation in *APM1* might influence adiponectin levels in this population. We genotyped five SNPs and an insertion/deletion polymorphism (SNP +2019) distributed throughout the *APM1* gene and known to be polymorphic in Caucasian populations (16,17) and then performed single SNP association analyses. As shown in Table 2, the strongest association occurred with SNP

+2019, the single nucleotide insertion/deletion polymorphism in the 3' untranslated region. Heterozygosity for the insertion/deletion polymorphism was associated with an increase in the adiponectin level of  $1.0 \pm 0.5 \mu\text{g/ml}$ , and deletion homozygosity was associated with an increase of  $3.0 \pm 0.8 \mu\text{g/ml}$  ( $P < 0.0001$  for additive model). The rs2241766 and rs1501299 SNPs showed moderate association in a dosage-dependent manner with adiponectin levels ( $P = 0.001$  and  $0.014$ , respectively). When individuals with the rs2241766 GT or GG genotypes were excluded from the analysis (remaining  $n = 460$ ), the association of SNP +2019 with adiponectin levels persisted, with  $\beta_{12} = 1.1 \pm 0.5 \mu\text{g/ml}$ ,  $\beta_{22} = 3.5 \pm 1.0 \mu\text{g/ml}$ , and  $P = 0.0004$ , suggesting that the SNP +2019 association with adiponectin levels is largely independent of rs2241766. However, when rs2241766 was added to a model in the full set already containing SNP +2019, the log likelihood increased from  $-1,109.28$  to  $-1,107.65$  for a nearly significant  $P$  value of 0.07, suggesting an independent effect of rs2241766. The further addition of rs1501299 to the two-

TABLE 1  
Multipoint linkage analysis peaks with LOD >1.5 (corresponding to  $P < 0.005$ )

Chromosome location	Distance (cM)	Closest marker(s)	LOD ( $P$ )	Nearby linkages in related traits in Old Order Amish	Nearby linkages to related traits in other populations
3q27	209	D3S1602/D3S1580	2.13 (0.0009)	—	Multiple metabolic syndrome QTLs (BMI, waist, hip, weight, insulin, insulin:glucose): LOD = 2.4–3.5 @ 189–203 cM (10) Diabetes: MLS = 4.67 and MLB-LOD = 3.43 @ D3S1580 (11)
7q11–21	94	D7S669	1.87 (0.0017)	Disinhibition (eating behavior): LOD = 1.65 @ 109 cM (31) Glucose 60: LOD = 1.45 @ 100 cM (32)	—
9q34	144	D9S290	1.54 (0.0039)	Total serum cholesterol: LOD = 1.61 @ 131 cM (33)	—
10p12-p14	44	D10S548	1.73 (0.0024)	BMI-adjusted leptin: LOD = 2.73 @ 71 cM (26)	Adiponectin levels: LOD = 1.9 @ 67 cM (27) Obesity: MLS = 4.85 @ 55 cM (28) BMI: LOD <sub>FA</sub> = 1.7 @ 20 cM (29)
16p13	15	D16S407	1.69 (0.0026)	BMI: LOD = 1.66 @ 1 cM (26) Leptin: LOD = 1.72 @ 16 cM (26) Fasting glucose to fasting insulin: LOD = 1.54 @ 26 cM (34)	—

LOD<sub>FA</sub>, paternal LOD; MLB, maximum likelihood binomial; MLS, maximum likelihood score.

SNP model did not alter the association. The SNPs rs266729, rs182052, and rs822396 showed no association with adiponectin levels.

Including the additive effect of SNP +2019 in the variance components model decreased the variance in adiponectin levels by 2.2%, and including both SNP +2019 and rs2241766 decreased the variance by 3.2% from baseline. The combination of all six SNPs resulted in a decrease of 4.4% from baseline.

As shown in Table 3, the three associated SNPs are in strong LD with one another, as estimated by pairwise  $D'$  coefficients (0.81–0.96), but weakly predictive of one another (because of differing allele frequencies), as indicated by low to moderate pairwise  $R^2$  estimates (0.02–0.61). The rs2241766 G allele, which is much less common than the SNP +2019-delA allele (0.08 vs. 0.30), is found almost exclusively in one of the haplotypes containing the SNP +2019-delA allele (Table 4). The rs1501299-T allele is found on the majority of rs2241766-SNP2019 T-delA haplotypes and is absent from the G-delA haplotype. Taken with the association findings discussed in the preceding paragraph, the association of rs2241766 with adiponectin levels may be an independent effect or may be an enhancement of the SNP +2019 effect. Unfortunately, these two

possibilities cannot be distinguished by the association data. In contrast, the modest rs1501299 association is most likely driven by its LD with its flanking SNPs.

To assess whether the association with *APMI* polymorphisms explained the linkage at 3q27, we reran the genome-wide scan using the SNP +2019 and/or rs2241766 genotypes as covariates. When SNP +2019 was included in the model, the LOD score decreased 1.26 units from 2.22 to 0.96, corresponding to an 18.2-fold (i.e.,  $10^{1.26}$ -fold) decrease in the evidence for linkage (LOD scores computed on the sample containing 523 subjects genotyped for SNP +2019 and rs2241766) (Fig. 2). When SNP rs2241766 was added to the base linkage model, there was a 1.03-unit drop in the LOD score, and when both SNP +2019 and rs2241766 were added simultaneously to the model, there was a 1.51-unit drop in the LOD score, for a total 32.4-fold decrease in the evidence for linkage. The two-SNP model provided a significantly improved fit over the model including SNP +2019 only ( $P = 0.024$ ).

Assessing whether SNP +2019 accounted for a “significant” proportion of the linkage was not straightforward. To gain some insights into this issue, we performed a simulation in which we dropped an unlinked SNP of the same frequency (0.30) 10,000 times through a 14-generation

TABLE 2  
Association of six *APMI* SNPs with adiponectin levels

SNP name	Gene region	Position (from ATG start codon)	Base change	Minor allele frequency	Mean adiponectin level ( $\mu\text{g/ml}$ )			$P$ (additive model)
					11	12	22	
rs266729	promoter	–11,374	C→G	0.47	11.8 ± 1.0 (170)	12.1 ± 0.5 (279)	11.7 ± 0.7 (104)	0.6471
rs182052	promoter	–10,066	A→G	0.45	12.0 ± 1.0 (165)	12.1 ± 0.5 (270)	11.8 ± 0.7 (116)	0.9507
rs822396	intron 1	–3,971	A→G	0.24	11.9 ± 1.0 (348)	11.4 ± 0.5 (190)	11.3 ± 0.9 (34)	0.1917
rs2241766	exon 2	+45	T→G (Gly15Gly)	0.08	11.7 ± 1.0 (463)	13.2 ± 0.6 (81)	15.0 ± 2.6 (4)	0.0010
rs1501299	intron 2	+276	G→T	0.20	11.3 ± 1.0 (345)	12.1 ± 0.5 (180)	14.3 ± 1.2 (19)	0.0140
SNP +2019	3' UTR	+2,019	insA→delA	0.30	10.8 ± 1.0 (283)	12.1 ± 0.5 (252)	13.8 ± 0.8 (50)	<0.0001

Data are means ± SE ( $n$  of individuals). Mean adiponectin levels are adjusted for age and age squared; all analyses are adjusted for sex and sex-specific age and age squared. ins, insertion; del, deletion; 11, homozygous for major allele; 12, heterozygous; 22, homozygous for minor allele.

TABLE 3  
Estimated pairwise LD for *APM1* SNPs

	rs266729	rs182052	rs822396	rs2241766	rs1501299	SNP +2019
rs266729	—	0.74	0.04	0.04	0.00	0.02
rs182052	0.99	—	0.02	0.06	0.00	0.03
rs822396	0.34	0.25	—	0.02	0.05	0.02
rs2241766	0.78	0.82	0.93	—	0.02	0.15
rs1501299	0.06	0.08	0.23	0.81	—	0.61
SNP +2019	0.24	0.26	0.15	0.90	0.96	—

Upper triangle shows  $R^2$  and lower triangle shows  $D'$ .

pedigree connecting virtually all subjects. We then repeated the linkage analysis using the simulated SNP as a covariate. None of the replicates resulted in a reduction as great as 1.26 LOD units, suggesting that association with *APM1* SNP +2019, and possibly rs2241766 and/or nearby *APM1* SNPs, is quite likely the cause of the linkage to 3q27. Furthermore, the two-point LOD score for *APM1* SNP +2019 was 2.35, whereas those for the other polymorphisms ranged from 0.01 (rs1501299) to 1.73 (rs2241766, which was also the next most significantly associated polymorphism).

Because we did not initially sequence *APM1* in an Amish sample, but rather selected polymorphisms for genotyping based on published information, we followed up the positive association results with direct sequencing analysis of the coding region (the 3' end of exon 2 and the 5' end of exon 3) to determine whether our positive associations might be tracking a potentially functional coding variant. The sample for sequencing consisted of a set of 12 relatively unrelated married couples (24 individuals, 48 alleles) in upper generations in which at least one member was heterozygous for the G allele at rs2241766. We did not detect any variants in the coding region that would explain the association.

## DISCUSSION

Although we were unable to detect chromosomal loci linked to adiponectin levels significant at the  $\text{LOD} \geq 3.0$  level, five regions (3q27, 7q11–21, 9q34, 10p12–p14, and 16p13) provided evidence of suggestive linkage. The highest LOD score, 2.13, was observed in the region containing *APM1*, the adiponectin structural gene. Although *APM1* might seem an obvious candidate gene for control of adiponectin levels, a genome-wide scan for adiponectin levels in Pima Indians showed no evidence for linkage to this region (although suggestive linkage was observed between markers D3S4018 and D3S1769, which map to 3q13) (15). In contrast, strong linkage of adiponectin levels to 3q27 has been recently reported in the IRAS (Insulin Resistance Atherosclerosis Study) Family Study ( $\text{LOD} = 3.84$ ) (13). One interesting finding of that study was that evidence for linkage was provided entirely by the Hispanic-American sample ( $\text{LOD} = 5.25$ ); no evidence for linkage was seen in the African-American families ( $\text{LOD} = 0.0$ ) (13). Weak linkage to 3q27 ( $\text{LOD} = 1.3$ ) was found in another genome-wide scan of adiponectin levels in Caucasians, where much higher signals on chromosomes 5 and 14 were seen (14). Linkage in the 3q22–24 region ( $\text{LOD} = 3.2$ ), about 40 cM centromeric to 3q27, has been observed in Mexican Americans (12). Based on these findings, it is possible that

variation in *APM1* is an important determinant of circulating adiponectin levels in some populations, but not others.

Our linkages for adiponectin on chromosomes 7q11–21, 9q34, 10p12–14, and 16p13 are close to linkages we observed in the Amish for other obesity-related traits, such as total serum cholesterol, BMI-adjusted leptin, and BMI (Table 1). Although some of these traits are somewhat correlated with adiponectin levels (19), they provide additional support for the idea that these regions are true linkages rather than false-positives. The 10p region has also been reported by others to be close to linkages for adiponectin (27), obesity (28), and BMI (29), thus providing additional evidence that a gene influencing obesity and related traits resides in this region.

To confirm our suspicion that the linkage signal on 3q27 resulted from variation in the adiponectin gene itself, we performed association analysis with six previously reported SNPs (Table 2). SNP +2019 showed strong association with adiponectin levels and appeared to explain most of the linkage signal at 3q27, as accounting for this SNP led to a 57% drop in the LOD score, corresponding to an 18.2-fold drop in the evidence for linkage. This insertion/deletion polymorphism has been previously reported to be associated with adiponectin levels (16). Another group has found the corresponding insertion to be in LD with the 45T/276G haplotype previously associated with type 2 diabetes (17) and has suggested that SNP +2019 may be a causal variant via mechanisms involving regulatory elements in the 3' region of the *APM1* gene. Based on searches in UTRSite and UTRScan, the polymorphism does not reside within a known functional sequence; the closest known functional element identified is a K-box binding region 371 bp downstream. However, functional elements of the 3' untranslated region (UTR) continue to be elucidated, and the possibility that the variant alters a protein or micro-RNA binding site and/or RNA secondary structure cannot be not ruled out (30). Studies of the effect of the polymorphism on RNA production and stability along with additional characterization of the 3' UTR sequence will be needed to confirm the causative nature of this polymorphism.

TABLE 4  
Haplotype frequencies ( $\geq 1\%$ ) of associated SNPs

rs2241766	rs1501299	SNP +2019	Frequency	Cumulative frequency
T	G	insA	0.66	0.66
T	T	delA	0.22	0.88
G	G	delA	0.08	0.96
T	G	delA	0.03	0.99

ins, insertion; del, deletion.

## Chromosome 3

Plasma Adiponectin Levels in Genotyped Subset of 523

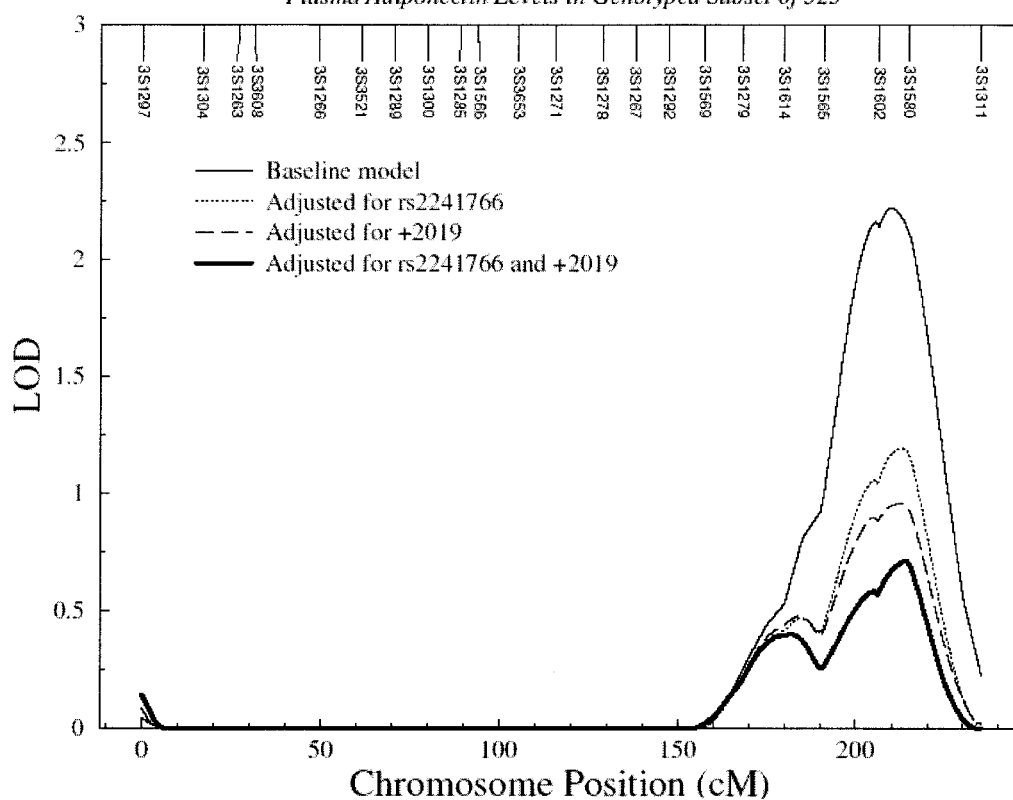


FIG. 2. Effect of adjusting for rs2241766 and SNP +2019 on LOD score at 3q27.

In addition to association with SNP +2019, there appeared to be a further source of association in rs2241766 independent from the SNP +2019 association, as evidenced by 1) an apparent additive effect between the two polymorphisms that reached statistical significance in the linkage analysis and 2) the persistence of the SNP +2019 association when the sample was restricted to those with the rs2241766 TT genotype. No common variants were detected in a set of 24 relatively unrelated individuals enriched for the presence of the rs2241766 and SNP +2019 minor alleles, increasing the likelihood that these variants directly influence adiponectin levels, although the possibility remains that variants outside the coding region are influencing the phenotype.

As in the Amish, the rs1501299-T allele has been associated with higher adiponectin levels in a Japanese population (18). The Amish data also replicate associations of the rs2241766-G, rs1501299-T, and SNP +2019-del alleles with increased adiponectin levels in the French population (16). Unlike the French (16), the Amish did not show association of the rs266729-G allele with decreased adiponectin levels; however, a follow-up analysis of the Amish in which subgroups in the lower and upper 50<sup>th</sup> BMI percentile were analyzed separately did reveal a recessive association between the rs266729-G allele and lower adiponectin levels in the high BMI (>26.4) group only ( $P = 0.014$ ).

The two-point linkage analysis of the relation of the SNPs themselves to adiponectin levels provides additional evidence that the sequence variation within *APM1* explains the 3q27 linkage, as the highest LOD score among the SNPs, 2.35, which was almost identical to the multi-

point peak score, was observed at the polymorphism (SNP +2019) with the strongest association result.

In summary, we have provided evidence for linkage of adiponectin levels to several distinct regions in the genome in the Amish. Among them, 3q27 contains the structural gene for adiponectin itself, *APM1*, in which sequence variation appears to be an important determinant of adiponectin levels. Because adiponectin is a key regulator of insulin sensitivity and other metabolic syndrome-related traits, we would expect this gene, and likely specific SNPs therein (e.g., SNP +2019, rs2241766) to be important contributors to these traits. Replication of our findings along with further investigation of sequence variation within *APM1* and functional analysis of these variants will be required to further discern the role of *APM1* as a susceptibility gene for obesity, type 2 diabetes, and the metabolic syndrome.

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## APPENDIX

Electronic database information for Internet-based resources used in this study: 1) NCBI UniSTS: <http://www.ncbi.nih.gov/entrez/query.fcgi?db=unists>; 2) UCSC Human Genome Browser Gateway: <http://genome.ucsc.edu/cgi-bin/hgGateway>; and 3) UTRScan/UTRSite: <http://bighost.area.ba.cnr.it/BIG/UTRHome/>.

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