

ACDC/Adiponectin Polymorphisms Are Associated With Severe Childhood and Adult Obesity

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Common single nucleotide polymorphisms (SNPs) in the *ACDC* adiponectin encoding gene have been associated with insulin resistance and type 2 diabetes in several populations. Here, we investigate the role of SNPs -11,377C>G, -11,391G>A, +45T>G, and +276G>T in 2,579 French Caucasians (1,229 morbidly obese and 1,350 control subjects). We found an association between severe forms of obesity and -11,377C (odds ratio 1.23, $P = 0.001$) and +276T (1.19, $P = 0.006$). Surprisingly, alternative alleles -11,377G and +276G have been previously reported as risk factors for type 2 diabetes. Transmission disequilibrium tests showed a trend in overtransmission (56.7%) of a risk haplotype 1_(C)-1_(G)-1_(T)-2_(T) including -11,377C and +276T in 634 obesity trios ($P = 0.097$). Family-based analysis in 400 trios from the general population indicated association between obesity haplotype and higher adiponectin levels, suggesting a role of hyperadiponectinemia in weight gain. However, experiments studying the putative roles of SNPs -11,377C>G and +276G>T on *ACDC* functionality were not conclusive. In contrast, promoter SNP -11,391G>A was associated with higher adiponectin levels in obese children ($P = 0.005$) and in children from the general population (0.00007). In vitro transcriptional assays showed that -11,391A may increase *ACDC* activity. In summary, our study suggests that variations at the *ACDC*/adiponectin gene are associated with risk of severe forms of obesity. However, the mechanisms underlying these possible associations are not fully understood. *Diabetes* 55:545-550, 2006

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EMSA, electrophoretic mobility shift assay; FLVS, Fleurbaix-Laventie Ville Santé; SNP, single nucleotide polymorphism; TDT, transmission disequilibrium test.

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Adiponectin is a potent insulin-sensitizing adipokine that acts on several peripheral tissues. In contrast to leptin, plasma adiponectin is reduced in obese children (1) and adults (2), and low adiponectin levels correlate with increased risk for type 2 diabetes (2). However, hypoadiponectinemia associates more with insulin resistance than with the degree of obesity (3). Associations between the adiponectin encoding gene (*ACDC*) variants and insulin resistance, type 2 diabetes, and/or cardiovascular diseases were reported in several but not all studies (4,5). The strongest associations are seen in two promoter single nucleotide polymorphisms (SNPs) -11,377C>G and -11,391G>A, the exon 2 synonymous SNP +45T>G, and the intronic SNP +276G>T. Alleles showing higher risk for type 2 diabetes associated with decreased adiponectin levels (6,7). Associations between *ACDC* SNPs and BMI have been previously described (8), but their contribution to risk for severe forms of obesity is not known. Here, we report genetic evidence for the role of *ACDC* SNPs in the risk for childhood and morbid adult obesity. We also provide functional data supporting the role for promoter SNP -11,391G>A in the modulation of adiponectinemia.

RESEARCH DESIGN AND METHODS

We genotyped 424 nuclear families including 634 obese children (defined as BMI >97th percentile for age and sex). Ninety-five unrelated obese children recruited in Toulouse were also genotyped (online appendix 1 [available from <http://diabetes.diabetesjournals.org>]). We used 534 unrelated obese children for case/control studies and 634 obesity trios (two parents and one obese child) for transmission disequilibrium test (TDT) analyses of obesity status and quantitative traits. Control set 1 was composed of 655 individuals (BMI <27 kg/m² and fasting glucose <5.6 mmol/l) recruited in Lille or through the Fleurbaix-Laventie Ville Santé (FLVS) study. We genotyped 695 unrelated morbidly obese adults (BMI ≥40 kg/m² and 75% were obese children or adolescents) recruited in Lille or Paris and compared them with age- and sex-matched control subjects (control set 2) (BMI <25 kg/m² and fasting glucose <5.6 mmol/l) selected among participants of the DESIR study. Data were also available for 224 nuclear families representative of the Northern France general population (FLVS study), from which we selected 400 trios and 197 unrelated lean children. The genetic study was approved by ethical committees of Hôtel Dieu Hospital in Paris and Centre Hospitalier Régional Universitaire in Lille. Insulin sensitivity-related phenotypes were analyzed in a subgroup of normal glucose-tolerant obese ($n = 599$) and FLVS ($n = 427$) children. Adiponectin levels were analyzed in 334 obese and in 367 FLVS children, where data were available. Homeostasis model assessment of insulin resistance was calculated as fasting insulin/[22.5e^{-ln(fasting glucose)}].

Genotyping. SNPs -11,377C>G and -11,391G>A were genotyped by Light-Cycler technology (Roche), and SNPs +45T>G and +276G>T were genotyped by Taqman technology (Applied Biosystems). Control set 2 genotypes were provided by the DESIR group. Genotyping error rates calculated from

TABLE 1
Association of SNPs in *ACDC* with childhood obesity and severe adult obesity

	Genotype			Allele frequencies		<i>P</i> value
	CC	CG	GG	C	G	
-11,377C>G (rs 266729)						
Obese children	302 (0.58)	188 (0.36)	27 (0.05)	0.77	0.23	0.025
Control set 1	327 (0.51)	261 (0.41)	43 (0.06)	0.73	0.27	1.24 (1.03–1.50)
Severely obese adults	411 (0.59)	247 (0.35)	37 (0.05)	0.77	0.23	0.022
Control set 2	377 (0.54)	263 (0.38)	55 (0.08)	0.73	0.27	1.22 (1.03–1.45)
All obese	713 (0.59)	435 (0.36)	64 (0.05)	0.77	0.23	0.001
All control subjects	704 (0.53)	524 (0.38)	98 (0.08)	0.73	0.27	1.23 (1.08–1.39)
-11,391G>A (rs17300539)						
Obese children	423 (0.80)	90 (0.17)	6 (0.01)	0.90	0.10	0.30
Control set 1	535 (0.83)	100 (0.15)	5 (0.01)	0.91	0.09	1.16 (0.87–1.54)
Severely obese adults	560 (0.80)	126 (0.18)	9 (0.01)	0.90	0.10	0.41
Control set 2	569 (0.82)	121 (0.17)	5 (0.01)	0.91	0.09	1.11 (0.87–1.42)
All obese	983 (0.81)	216 (0.18)	15 (0.01)	0.90	0.10	0.19
All control subjects	1104 (0.83)	221 (0.16)	10 (0.01)	0.91	0.09	1.13 (0.94–1.36)
+45T>G (rs2241766)						
Obese children	344 (0.74)	117 (0.25)	9 (0.02)	0.86	0.14	0.61
Control set 1	421 (0.75)	131 (0.23)	11 (0.02)	0.86	0.14	1.06 (0.83–1.37)
Severely obese adults	468 (0.74)	147 (0.23)	14 (0.02)	0.86	0.14	0.29
Control set 2	536 (0.77)	144 (0.21)	15 (0.02)	0.87	0.13	1.13 (0.90–1.41)
All obese	841 (0.74)	270 (0.24)	23 (0.02)	0.86	0.14	0.27
All control subjects	957 (0.79)	275 (0.22)	26 (0.02)	0.87	0.13	1.10 (0.93–1.30)
+276G>T (rs1501299)						
Obese children	229 (0.50)	188 (0.41)	43 (0.09)	0.70	0.30	0.08
Control set 1	308 (0.55)	209 (0.37)	43 (0.08)	0.74	0.26	1.19 (0.97–1.44)
Severely obese adults	316 (0.50)	269 (0.42)	48 (0.07)	0.71	0.29	0.038
Control set 2	380 (0.55)	279 (0.40)	36 (0.05)	0.75	0.25	1.20 (1.01–1.42)
All obese	545 (0.50)	457 (0.42)	91 (0.08)	0.71	0.29	0.006
All control subjects	688 (0.55)	488 (0.39)	79 (0.06)	0.74	0.26	1.19 (1.05–1.36)

Data are *n* (frequency) or odds ratio (95% CI), unless otherwise indicated. We used genotypes from 1,229 morbidly obese (534 obese children with BMI >97th percentile and 695 obese adults BMI \geq 40 kg/m²) and 1,350 control (control set 1 = 655 and control set 2 = 695) subjects. Case/control analyses were performed using the χ^2 test. Odds ratios and *P* values indicated are for the allelic model (df = 1). For the pooled data, we used the Mantel-Haenszel test.

duplicate genotypes of 260 individuals were 0.01% for SNPs -11,377C>G, -11,391G>A, and +276G>T and 0.00% for SNP +45T>G. All SNPs were in Hardy-Weinberg equilibrium.

Adiponectin measurements. Adiponectin levels were measured in sera from 334 obese children and 367 children from the FLVS study with a commercial assay kit (LINCO Research) in P.E.S.'s laboratory.

Electrophoretic mobility shift assays. Nuclear extracts were obtained as previously described (9) from differentiated 3T3-L1 cells (10). DNA probes were labeled with T4 kinase (Roche) using [γ -³²P] ATP. Binding reactions were performed in 20 μ l of 50% glycerol, 20 mmol/l Tris/HCl, pH 7.5, 100 mmol/l KCl, 2 μ g dI-dC, 1 mmol/l dithiothreitol, and 10 μ g nuclear extract. Probes (50,000 cpm) were added to the binding mixture and incubated for 30 min at 20°C. The DNA-protein complexes were resolved on 5% polyacrylamide at 4°C. Gels were dried and exposed to X-ray films (Kodak). Quantification of the signal was performed with NIH Image software (available from <http://rsb.info.nih.gov/nih-image/>).

Transfection reporter constructs. *ACDC* promoters were generated by PCR of 1.3 kb of genomic DNA from three homozygous subjects using an expand high-fidelity PCR system (Roche). PCR products were cloned into pGL3-basic (Promega). Competent bacteria were transformed with the constructs. All plasmids used for cell transfection were controlled by bidirectional sequencing.

Cell culture and luciferase assay. COS-7 cells grown in Dulbecco's modified Eagle's media (Life Technologies) with 10% fetal bovine serum and 25 μ g/ml gentamicin at 37°C and 5% CO₂ were transfected with 2 μ g (TransFast reagent; Promega). Transfection efficiencies were normalized by 50 ng pRL-TK, the Renilla luciferase vector. A Dual-Luciferase Reporter Assay (Promega) was performed after 48 h incubation.

Statistical analysis. Case/control analyses used the χ^2 test, and *P* values were empirically computed with the CLUMP program (11). Allelic means for quantitative traits (corrected for BMI, age, and sex), haplotypes, allelic TDT, and quantitative TDT were analyzed by UNPHASED software (12), effects of

SNPs on haplotype association by THESIAS software (13), and relative luciferase activities by SPSS 10.1.

Data are given as means (*n*). Analyses were performed in allelic model for traits corrected for BMI, age, and sex using the Qtphase subprogram of UNPHASED (df = 1).

RESULTS

Case/control analysis in 534 obese children and 655 control adults (control set 1) showed significant association between childhood obesity and the -11,377C allele (odds ratio 1.24 [1.03–1.50], *P* = 0.025) (Table 1). Similar results were obtained for -11,377C in 695 morbidly obese adults and in 695 age- and sex-matched control subjects (control set 2) (1.22 [1.03–1.45], *P* = 0.022). Pooled analysis of 2,579 French Caucasian subjects (1,229 morbidly obese and 1,350 control subjects) confirmed that the -11,377C allele is a risk factor for severe forms of obesity in our populations (1.23 [1.08–1.39], *P* = 0.001). Allele +276T was also associated with severe obesity (1.19 [1.05–1.36], *P* = 0.006), while -11,391G>A and +45T>G did not show evidence of association in our study. We genotyped SNPs -11,377C>G and +276G>T in a limited subset of 197 unrelated lean children from the north of France and obtained similar frequencies for the -11,377C and +276T alleles as seen in our control adults (0.74 vs. 0.73 and 0.28 vs. 0.26, respectively), which rules out a significant bias due to age.

TABLE 2
Association of haplotypes for ACDC SNPs in 1,229 obese vs. 1,350 control subjects

Haplotype					Frequencies (n haplotypes)		Case/control	
	-11,377C>G	-11,391G>A	+45T>G	+276G>T	Obese	Control	Odds ratio	P
1 _(C)	1 _(G)	1 _(T)	1 _(G)	0.36 (748)	0.38 (927)	1*	0.17	
1 _(C)	1 _(G)	1 _(T)	2 _(T)	0.20 (424)	0.17 (424)	1.24	0.009	
2 _(G)	1 _(G)	1 _(T)	1 _(G)	0.20 (422)	0.23 (568)	0.92	0.007	
1 _(C)	1 _(G)	2 _(G)	1 _(G)	0.10 (217)	0.08 (199)	1.35	0.03	
1 _(C)	2 _(A)	1 _(T)	2 _(T)	0.07 (144)	0.06 (155)	1.15	0.45	

Haplotype frequencies were estimated and compared between case and control subjects using the Cocophase subprogram of UNPHASED. Haplotypes with frequency >0.05 are presented. Overall P value = 0.03 (df = 11). *Odds ratio was not available for the “all-wild” haplotype because it was the reference haplotype.

We analyzed haplotypes and identified an “at-risk” haplotype, including the -11,377C and +276T alleles (1_(C)-1_(G)-1_(T)-2_(T)), more frequent in obese than in control subjects (0.20 vs. 0.17, respectively; odds ratio 1.24, P = 0.009, Table 2). In contrast, the 2_(G)-1_(G)-1_(T)-1_(G) haplotype was more frequent in control than in obese subjects (0.23 vs. 0.20, respectively; 0.92, P = 0.007). The effect of each SNP on haplotype associations was assessed using THE-SIAS software and showed that both associated SNPs contribute to the at-risk haplotype association. Using regression analyses, we obtained similar results (data not shown).

We also tested in our childhood obesity population familial association using TDT in 634 trios. We did not observe distortion of transmission for individual SNPs. However, haplotype TDT showed a trend in overtransmission of the obesity risk haplotype 1_(C)-1_(G)-1_(T)-2_(T) in obese trios (56.7%, P = 0.097, Table 3).

We assessed ACDC SNP effect on BMI, insulin sensitivity, and adiponectinemia in obese children and in children of similar age and representative of the general population (FLVS children). Single SNP analysis showed that allele -11,377C was associated with lower fasting insulin (P = 0.018) and lower homeostasis model assessment of insulin resistance (P = 0.013) in obese children and in trend of association with higher adiponectinemia in FLVS children (P = 0.087) (appendix 2). Allele +276T was associated with higher adiponectin levels in obese children (P = 0.03), but this finding was not observed in FLVS children (P = 0.303). Allele -11,391A was associated with higher adiponectinemia in obese children (P = 0.005), and this

finding was strongly replicated in FLVS children (P = 0.00007).

Using quantitative family-based analysis, haplotype 1_(C)-2_(A)-1_(T)-2_(T), including -11,391A and obesity risk alleles -11,377C and +276T, was associated with higher adiponectin levels in obese (P = 0.008, Table 3) but not in FLVS (P = 0.49) children. Obesity risk haplotype (1_(C)-1_(G)-1_(T)-2_(T)) did not show evidence of association with adiponectin levels in obese children (P = 0.38). However, this haplotype was associated with higher adiponectinemia in FLVS children (P = 0.03).

We investigated functional proprieties of the ACDC promoter sequences where SNPs -11,377C>G and -11,391G>A are located. We performed electrophoretic mobility shift assays (EMSA) using nuclear extracts from in vitro-differentiated adipocytes. Promoter wild-type probe (-11,377C and -11,391G) was shifted by a nuclear factor (Fig. 1B). Increasing amounts of wild-type unlabeled probe gradually unhooked the labeled probe showing specific interaction between DNA and nuclear proteins. To examine the effect of SNPs -11,377C>G and -11,391G>A on binding affinity, we performed EMSAs using probes that differed only on SNP position followed by autoradiogram quantification. The band shift with the -11,377G allele probe was 2.5-fold less intense than with the wild-type probe, which suggests decreased interaction with ACDC promoter in the presence of this allele. No band shift was observed for the -11,391A probe, indicating that this allele totally impedes the DNA/adipocyte nuclear protein interaction observed for the wild-type probe. We assessed the transcriptional activity of the

TABLE 3
Familial association of obesity and adiponectin levels in 634 obesity trios and 400 trios from the general population

Haplotypes					Adiponectinemia TDT							
					Obesity TDT				Obese children		General population children	
	-11,377	-11,391	+45	+276	T	NT	%T	P	Mean (µg/ml)	P	Mean (µg/ml)	P
1 _(C)	1 _(G)	1 _(T)	1 _(G)	125	152	45.1	0.12	6.72	0.09	11.67	0.89	
1 _(C)	1 _(G)	1 _(T)	2 _(T)	97	74	56.7	0.097	6.88	0.38	12.45	0.03	
2 _(G)	1 _(G)	1 _(T)	1 _(G)	87	81	51.8	0.66	7.13	0.31	11.46	0.12	
1 _(C)	1 _(G)	2 _(G)	1 _(G)	51	54	48.6	0.77	7.72	0.05	12.29	0.66	
1 _(C)	2 _(A)	1 _(T)	2 _(T)	28	30	48.3	0.80	8.98	0.008	15.21	0.49	

Transmissions of haplotypes were analyzed using the Tdtphase subprogram of UNPHASED, which assesses haplotype transmission rates in trios and tests for deviation from the expected 50% transmission. Familial associations of haplotypes with adiponectinemia were tested using the Qpdtphase subprogram of UNPHASED. Using a χ² test, the transmission rate of haplotype (1_(C)-1_(G)-1_(T)-2_(T)) in obesity trios (56.7%) was compared with the transmission rate observed in general population trios (39% instead of the theoretical rate of 50%) (P value = 0.005). %T, percentage of transmitted haplotypes; NT, number of untransmitted haplotypes; T, number of transmitted haplotypes.

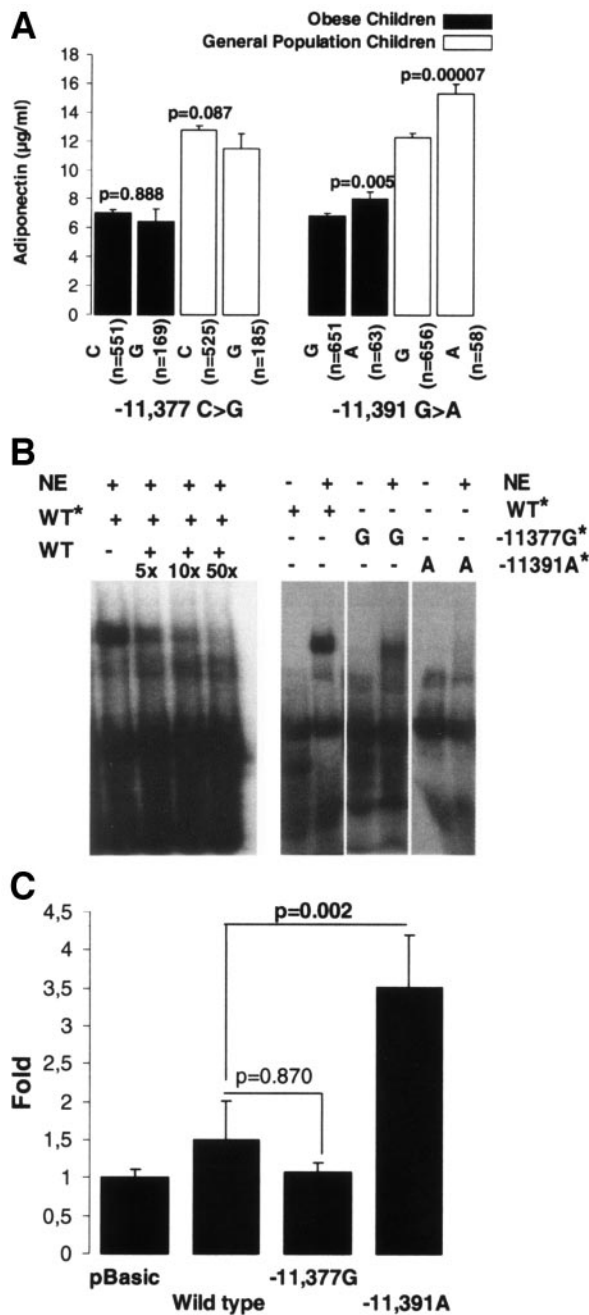


FIG. 1. *ACDC* promoter SNPs effect on circulating adiponectin. **A:** adiponectin levels in obese and general population children. Adiponectin means for promoter SNPs $-11,377C>G$ and $-11,391G>A$ are presented [allele (*n*)]. Means corrected for BMI, age, and sex were compared in an allelic model using Qtpase subprogram of UNPHASED (DF = 1). **B:** EMSAs of the *ACDC* promoter region harboring SNPs $-11,377C>G$ and $-11,391G>A$. Nuclear proteins were extracted from 3T3-L1 differentiated adipocytes and incubated with radio-labeled double strand DNA fragments. Wild type probe (WT) was the frequent alleles ($-11,377C$ and $-11,391G$) probe. Experiments were performed in five replicates. NE: nuclear extracts; *radiolabeled probe. **C:** Gene reporter experiments in order to assess the role of *ACDC* promoter SNPs on transcriptional activity. COS7 cells were transfected by plasmid coding the luciferase protein under human *ACDC* promoter. Transfection efficiency was normalized by cotransfecting the different constructions with plasmid encoding *Renilla* luciferase. Relative luciferase activities of nine replicates were compared using Mann-Whitney test. Results are expressed as fold activity of the pBasic plasmid.

ACDC promoter according to SNPs $-11,377C>G$ and $-11,391G>A$ through luciferase tests (Fig. 1C). The $-11,377G$ allele had lower transcriptional activity than the wild-type probe, which includes the $-11,377C$ obesity-associated allele, but this effect was not significant. In contrast, the $-11,391A$ promoter had a 2.5-fold increase in transcriptional activity ($P = 0.002$), which was in accordance with the association between this allele and higher adiponectin levels observed in children. Genomatix analyses (available from <http://www.genomatix.de/>) failed to predict any potential transcription factor binding sites, suggesting that $-11,377C>G$ and $-11,391G>A$ may locate in a novel *cis* element recognized by putative nuclear repressors.

We performed an EMSA on the intronic sequence harboring SNP $+276G>T$ and obtained a specific interaction with adipocyte nuclear factors, but the $+276T$ allele had no effect on this interaction (data not shown).

DISCUSSION

ACDC SNPs have been reported as risk factors for type 2 diabetes in several populations. This study is the first to report associations of the adiponectin gene SNPs with both childhood and morbid adult obesity. The $-11,377C$ and $+276T$ allelic frequencies were very similar in both obese children and morbidly obese adults, suggesting, as already seen for SNPs in the *GAD2* (14) and *ENPP1* (15) genes, that morbid adult and childhood obesity may share part of their genetic background. Although familial association results were not strong and need further confirmation, they pointed out the absence of hidden stratification.

Previously, the alternative alleles $-11,377G$ and $+276G$ were associated with higher risk for type 2 diabetes (6,7), which suggests that severe obesity risk alleles may decrease the risk of the etiology of type 2 diabetes. The physiological mechanisms behind this genetic finding could be through the effect of the *ACDC* SNPs on adiponectin levels. Our results, and data from other studies, are in favor of the association of obesity risk alleles $-11,377C$ and $+276GT$ with higher adiponectinemia (7,16–18). This finding is surprising, as decreased adiponectin levels were described in obese subjects. However, adiponectin levels in obese insulin-sensitive subjects can be similar to lean insulin-sensitive subjects and significantly higher than in insulin-resistant patients independent of their obesity status (3). The physiological contribution of adiponectin to weight gain may be mediated by its insulin-sensitizing action in adipose tissue. Insulin signaling in the adipocyte is important for lipid storage, and adipose tissue selective insulin receptor knockout mice are protected from obesity (19). In 3T3-L1 preadipocytes, adiponectin overexpression accelerates cell proliferation and differentiation, while in mature adipocytes autocrine adiponectin increases glucose uptake and favors lipid accumulation (20). Transgenic overexpression of adiponectin in the physiological range induced morbid obesity without insulin resistance in *ob/ob* mice (J.-Y. Kim, P.E.S., unpublished data). As long-term exposure to insulin resistance would limit energy storage and weight gain (21,22), higher adiponectin-induced insulin sensitivity may increase lipid storage in adipose tissue instead of organs like liver and muscle. Consequently, hyperadiponectinemia not only protects from insulin resistance, and eventually from type 2 diabetes, but also may favor additional weight gain and may predispose to obe-

sity. Treatments in type 2 diabetic patients with peroxisome proliferator-activated receptor γ agonists improve insulin sensitivity but stimulate body fat gain (23). Several studies have reported that peroxisome proliferator-activated receptor γ or its agonists increase adiponectin expression and secretion (24,25), suggesting a putative role of adiponectin in peroxisome proliferator-activated receptor γ -induced fat gain.

Our functional experiments did not show evidence of $-11,377C$ having a role in the modulation of *ACDC* transcriptional activity. As $-11,377C$ is the wild-type allele, its presence corresponds to the basal transcriptional activity of the promoter. Our experimental design may not have enough power to detect a decrease in activity. We note that we cannot exclude linkage disequilibrium between this SNP and others at the *ACDC* locus. In contrast, we showed that the $-11,391A$ allele is associated with higher adiponectinemia, probably through enhanced *ACDC* transcriptional activity. Our results are in agreement with recent data showing that a deletion of the human promoter region where $-11,391G>A$ is located increases *ACDC* transcriptional activity (26). The lack of association for this rather infrequent SNP with severe forms of obesity is probably due to insufficient power and will require investigation in larger populations. Our EMSA experiments did not show evidence of the effect of the $+276G$ allele. The 3' untranslated region SNP $+2019delA$ is in linkage disequilibrium with $+276G>T$ in our population ($D' = 0.97$, data not shown). This SNP was previously associated with adiponectin levels and explained a 3q27 quantitative trait locus linked to adiponectinemia, suggesting that it may be functionally relevant (18).

In summary, our results suggest that the adiponectin variants predispose to severe childhood and adult obesity, but further investigations are required to determine the physiological mechanisms behind the process.

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