A genetic variant at the fatty acid-binding protein *aP2* locus reduces the risk for hypertriglyceridemia, type 2 diabetes, and cardiovascular disease

G. Tuncman*, E. Erbay*, X. Hom*, I. De Vivo[†‡§], H. Campos[¶], E. B. Rimm[†§¶‖], and G. S. Hotamisligil*[‖]

Departments of *Genetics and Complex Diseases, [¶]Nutrition, and [†]Epidemiology and [‡]Program in Molecular and Genetic Epidemiology, Harvard School of Public Health, Boston, MA 02115; and [§]Channing Laboratory, Department of Medicine, Harvard Medical School and Brigham and Women's Hospital, Boston, MA 02115

Communicated by Barry R. Bloom, Harvard School of Public Health, Boston, MA, March 16, 2006 (received for review December 15, 2005)

Obesity and the associated pathologies including dyslipidemia, insulin resistance, type 2 diabetes, and cardiovascular disease constitute a major threat to global human health. Yet, the genetic factors that differentially predispose individuals to this cluster of pathologies are unclear. The fatty acid-binding protein aP2 is a cytoplasmic lipid chaperon expressed in adipocytes and macrophages. Mice with aP2 deficiency are partially resistant to obesity-induced insulin resistance and type 2 diabetes, have lower circulating triglycerides, and exhibit marked protection against atherosclerosis. Here, we demonstrate a functionally significant genetic variation at the *aP2* locus in humans that results in decreased adipose tissue *aP2* expression due to alteration of the CAAT box/enhancer-binding protein binding and reduced transcriptional activity of the *aP2* promoter. In population genetic studies with 7,899 participants, individuals that carry this T-87C polymorphism had lower serum triglyceride levels and significantly reduced risk for coronary heart disease and type 2 diabetes compared with subjects homozygous for the WT allele. Taken together, our results indicate that reduction in aP2 activity in humans generate a metabolically favorable phenotype that is similar to aP2 deficiency in experimental models.

adipocyte | FABP4 | triglyceride | macrophage | metabolic syndrome

Obesity and the associated metabolic disease cluster have become one of the greatest threats to global human health. This disease cluster includes insulin resistance, dyslipidemia, type 2 diabetes, fatty liver disease, atherosclerosis, and other lesser recognized complications such as airway inflammation, gallstones, and cancer (1, 2). All of these problems have metabolic and inflammatory underpinnings and develop at the interface of genetic susceptibility and environmental factors (3). In the past decade, it has become clear that obesity triggers low-level chronic and local inflammation, predominantly emerging from adipose tissue (3). This inflammatory response is critical in the development of type 2 diabetes and potentially other metabolic diseases associated with obesity. Hence, factors critical in these pathways are likely to impact the risk for metabolic disease.

An important molecular pathway that integrates metabolic and inflammatory responses in experimental models involves the fatty acid-binding proteins (FABPs) that are present in adipocytes and macrophages (4). FABPs are a family of small cytoplasmic proteins, 14–15 kDa, conserved through evolution from *Drosophila* to humans (5). They exhibit a tissue-specific expression pattern, and often their abundance is regulated by the metabolic demands of the cells in which they are present. FABPs act as cytoplasmic lipid chaperones and play a role in the cellular trafficking of fatty acids and other lipid signals through their interaction with functional targets. Interestingly, adipocytes and macrophages express the same two FABP isoforms, aP2 (FABP4) and mal1 (FABP5) (6).

The predominant FABP present in adipocytes is aP2, with mal1 being the minor isoform (7). In contrast, both isoforms are expressed at similar levels in activated macrophages (6) and regulated by a variety of inflammatory and metabolic mediators (8, 9). The precise physiological role of these small proteins has been recognized only upon the development of genetic models to examine their function in mice (4, 10, 11). In these experimental models, deficiency of aP2 results in lowered triglycerides, increased insulin sensitivity when made obese, and protection against atherosclerosis in models of hypercholesterolemia (6, 12, 13). The impact of aP2 deficiency on atherosclerosis is predominantly the result of its action in macrophages (6). The contribution of these cell types to FABP effects on systemic glucose and lipid metabolism has not yet been fully dissected.

Taken together, FABPs, particularly aP2, serve as a locus of integration for metabolic and inflammatory pathways and play a critical role in systemic metabolic homeostasis and cardiovascular disease in experimental mice models (4, 11). Because *aP2* expression and regulation exhibits striking similarities between rodents and humans (8, 14), it is feasible to postulate that genetic variations at this locus might influence similar metabolic outcomes in humans provided that they regulate the expression of *aP2.* To test this hypothesis, we examined sequence alterations at the *aP2* locus, evaluated functional consequences and tested relevance to risk of hypertriglyceridemia, type 2 diabetes, and cardiovascular disease, the three traits that are closely linked to aP2 action, in human populations.

Results

SNP Discovery. The *aP2* gene, which has been mapped to chromosome 8q21 (Mendelain Inheritance in Man no. 600434), consists of four exons, and encodes a 132-aa protein (15, 16). The locus spans ≈7 kb of genomic DNA. To determine the potential genetic variation(s) at the *aP2* locus, we determined the genomic DNA sequence for the promoter and coding regions in randomly selected 96 men and women of primarily Caucasian descent. Through these studies, we identified and confirmed five distinct SNPs. The summary of these SNPs is shown in Table 1. Of the five, two of the sequence variants, C2600T and G4356C, were previously reported in National Center for Biotechnology Information SNP database as rs8192688 and rs1051252, respectively. All of the SNPs were outside the coding regions except for G4356C, which is a silent variant on exon 4 of *aP2*.

Conflict of interest statement: No conflicts declared.

Freely available online through the PNAS open access option.

Abbreviations: C/EBP, CAAT box/enhancer-binding protein; CHD, coronary heart disease; FABP, fatty acid-binding protein.

[‖]To whom correspondence may be addressed. E-mail: erimm@hsph.harvard.edu or ghotamis@hsph.harvard.edu.

Table 1. The sequence variations found in *aP2* gene

Polymorphism	Position, bp	rs no.	Allele freq., %
T → C	−87	—	4.69
C → T	2600	8192688	16.67
T → C	2613	—	0.52
Ins G	4265	—	16.67
G → C	4356	1051252	0.52

The promoter and coding region of the *aP2* gene, including the intron/exon boundaries, were sequenced in 96 male and female subjects whom were predominantly Caucasian. The list of sequence variations observed in this group is shown. When available, the existing SNP database presence is indicated with reference number (rs no.). The nucleotide position shown is based on reverse-strand human *aP2* sequence with respect to transcriptional initiation site.

We also identified a unique variant at position −87, which was a base alteration of T to C (T-87C) in the 5′ promoter region of human aP2 gene and was in moderate linkage disequilibrium with C2600T and InsG4265 SNPs (D′ = 0.6995 and 0.6912, respectively). T-87C is predicted to be located at the CAAT box/enhancer-binding protein (C/EBP) binding site (Fig. 1*a*). Because this site is critically important for the expression and regulation of murine *aP2* gene (17), we examined the potential functional consequences of this genetic variation and its association with metabolic disease risk in humans.

C/EBPα Binding to WT and T-87C Variant of the *aP2* Promoter. Because this SNP alters the cognate C/EBP recognition site, we first examined potential alterations in the ability of C/EBPα to bind to this site on the human *aP2* promoter. A DNA fragment containing the target promoter sequences of *aP2* (nucleotides −194 to −16) was radiolabeled to examine binding of C/EBPα protein to its cognate DNA. In control experiments, a dose-dependent binding of C/EBPα was observed with an increasing amount of protein (Fig. 1*b*). The specific interaction of C/EBPα was determined by the formation of a supershift with the addition of a C/EBPα antibody to the reaction. The binding of C/EBPα also was effectively competed by the addition of unlabeled oligonucleotide competitors that contain the C/EBPα binding (Fig. 1*b*). Having established these controls, we then tested whether the T-87C variant differed in its ability to interact with C/EBPα protein. We performed EMSA with C/EBPα and its cognate DNA by including increasing amounts of unlabeled competing DNA oligonucleotides with the WT or variant *aP2* promoter sequences (Fig. 1*c*). These experiments demonstrated that the DNA sequence harboring the T-87C variant exhibited significantly reduced competitive-binding capacity to C/EBPα compared with WT *aP2* promoter sequence (Fig. 1*c*). At all doses of competition, there was ≥50% reduction in the ability of the mutant sequence to bind to C/EBPα compared with that of the WT.

Activity of the WT and T-87C Variant of the *aP2* Promoter. Next, we examined the impact of the T-87C variant on the activity of the *aP2* promoter. HEK-293 cells were cotransfected with reporter constructs where luciferase gene expression was driven by either WT or mutant *aP2* promoter alleles and control or C/EBPα expressing vector. As shown in Fig. 2, the activity of the WT promoter could be detected, albeit at relatively low levels in these

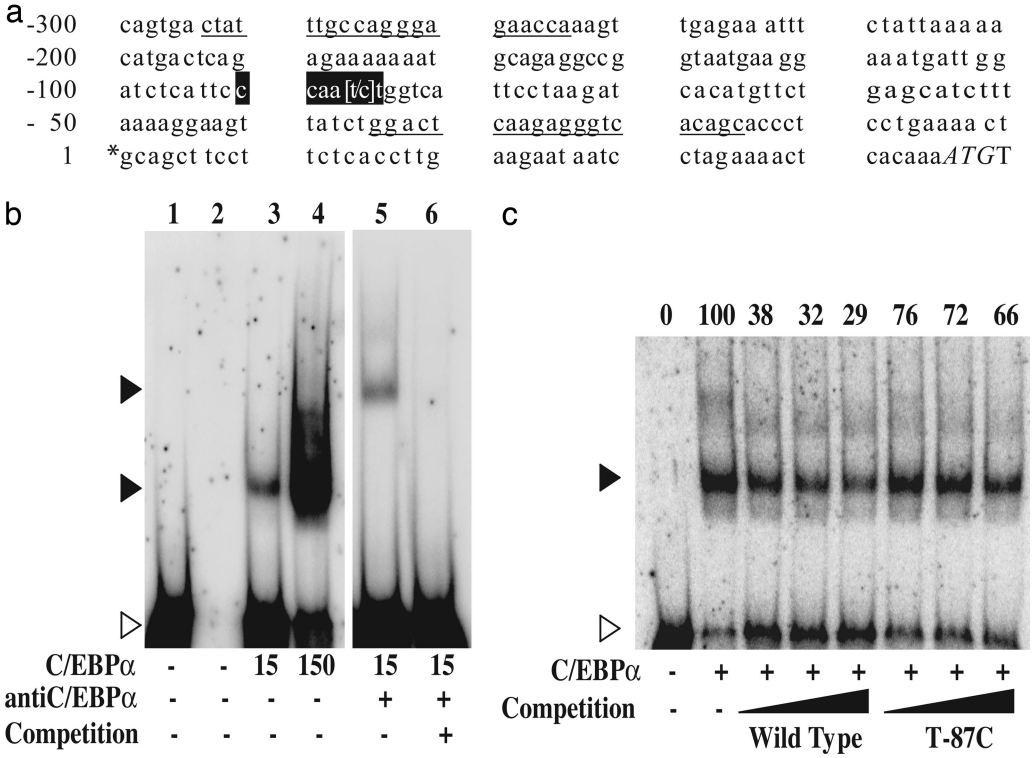

Fig. 1. Partial sequence of human *aP2* promoter sequence and EMSA. (*a*) Sequence of the reverse complementary strand of human *aP2* promoter region. [t/c] is the polymorphic site, sequences underlined are the primer sequences used for generating probes and inserts, * denotes the transcription start site, exon 1 is with capitalized letters, and the bold sequence in box denotes the predicted C/EBPα binding site. (*b*) Interaction between C/EBPα and WT *aP2* promoter sequence examined by EMSA. Dose-dependent binding of C/EBPα protein to target sequence (lanes 1–4), super shifting by an antibody raised against C/EBPα protein (lane 5), and binding competition with unlabeled WT DNA sequence (lane 6) are shown. (*c*) Comparison of C/EBPα binding to WT and T-87C sequences determined by competing with increasing amounts of WT and T-87C unlabeled oligonucleotides. Filled arrowheads show C/EBPα–DNA complexes, and open arrowheads show the free probe. The percentage decrease in binding is shown at the top of *C*.

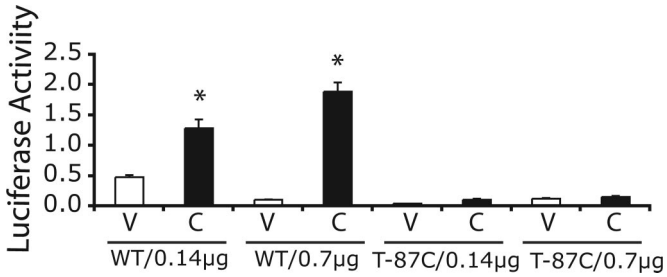

Fig. 2. The effect of T-87C variant on *aP2* promoter activity. A luciferase reporter gene under the control of WT and T-87C *aP2* promoter was used to examine transcriptional activity in the presence or absence of C/EBPα. Luciferase activity is plotted after normalizing to transfection efficiency with the indicated amounts of DNA. *, Statistically significant difference when compared to T-87C promoter activity; $P = 0.006$ for WT C/EBPα (0.14 μg) and $P = 0.003$ for WT C/EBPα (0.7 μg). V, vector control; C, presence of C/EBPα.

cells in the absence of exogenously added C/EBPα. This low baseline promoter activity level was similar between the two genotypes. When these experiments were performed in the presence of different levels of exogenously expressed C/EBPα, a 3- to 10-fold increase was observed in the WT promoter activity. However, there was only a minimal (1.5-fold) increase in the mutant *aP2* promoter upon addition of C/EBPα. These experiments clearly demonstrate that the T-87C variation reduced the binding ability of C/EBPα to its recognition site at this location and significantly impaired transcriptional activation of *aP2* promoter by C/EBPα in cultured cells.

Genotype–Phenotype Studies in Population Cohorts. Based on the results of our functional characterization, we expanded our studies on this SNP to two large ongoing prospective studies designed to identify genetic and lifestyle predictors of chronic disease. The genotype at nucleotide −87 of the *aP2* promoter was determined in a total of 7,899 subjects (3,050 female and 4,849 male) from the Nurses' Health Study and Health Professionals Follow-up Study cohorts described below. The heterozygosity prevalence for the T-87C variant was 4.3% with an allele frequency of 2.2%, and the population was found to be in Hardy–Weinberg equilibrium $[(0.98 + 0.02)^2 = 1]$ for this polymorphism (Table 2).

We assessed the *aP2* genotype in men and women with available plasma biomarkers of cardiovascular disease. After controlling for diet and other lifestyle characteristics, fasting triglyceride levels were significantly lower by 21.5 mg/dl (12%) in subjects who were carriers of this polymorphism ($P = 0.009$; Table 3). Other lipid, inflammatory, and metabolic markers that were measured were not associated with the *aP2* genotype.

We next examined the impact of this variant on coronary heart disease (CHD) in the subpopulations from these cohorts that were previously selected for nested case-control studies of CHD. The outcome for this analysis was incident CHD, defined as nonfatal myocardial infarction or fatal CHD. In this group, we found an allele prevalence of 2.2%. Compared with WT, participants heterozygous for the *aP2* polymorphism had an odds ratio for CHD of 0.66 (95% confidence interval: 0.46, 0.97; $P = 0.03$) (Fig. 3). Hence, the presence of this *aP2* variant was associated with significantly reduced risk for coronary heart disease.

Table 2. Genotype frequencies

Gender	WT/WT	WT/T-87C	T-87C/T-87C	Total
Female	2,910 (95.41)	139 (4.56)	1 (0.03)	3,050
Male	4,645 (95.79)	202 (4.17)	2 (0.04)	4,849
Total	7,555 (95.65)	341 (4.32)	3 (0.04)	7,899

Frequency of the T-87C genotypes observed in the total population studied. Upon identification by sequencing, the T-87C SNP was studied utilizing a TaqMan-based screen. Allele distribution is shown in absolute numbers and percentages (in parentheses).

Table 3. Least-square means for lipid, inflammatory, and glycemic parameters by *aP2* genotype

	WT/WT	WT/T-87C	*P*
Triglycerides, mg/dl	176.7	153.6	0.009
CRP, mg/liter	2.81	2.89	0.83
IL-6, pg/ml	2.84	4.97	0.50
TNFR1, pg/ml	1236	1187	0.25
TNFR2, pg/ml	2492	2534	0.66
LDL-C, mg/dl	137.0	133.9	0.27
HDL-C, mg/dl	50.9	51.1	0.90
ApoB, mg/dl	114.4	109.6	0.06
Hemoglobin A1c, %	6.29	6.17	0.32

The multivariate model is controlled for age, BMI, activity, smoking, calories, saturated fat, transfat, fasting hours, and study groups. *, For triglycerides, we only used the participants with fasting samples. For each blood parameter the number of samples with available data ranged from 1,167 (for IL-6 and TNFR1) to 3,716 for CRP.

Because FABPs regulate systemic glucose metabolism and insulin action in obese mice, we further explored the association between the *aP2* variant and risk of diabetes. We found a marginally significant reduced risk of diabetes for carriers of *aP2* variant (odd ratio = 0.75; 95% confidence interval: 0.55, 1.03; $P = 0.07$). In a stressed metabolic state, the effect of the variant may be greater. In fact, aP2 deficiency in mice exhibits its impact on insulin action and type 2 diabetes only in the presence of diet-induced or genetic obesity (18). Therefore, we asked whether this paradigm might be similar in humans and examined diabetes risk only in the obese participants. Among the 1,156 obese [body mass index (BMI) $\geq$30 kg/m^2] female and male subjects the odds of diabetes (odds ratio = 0.52; 95% confidence interval: 0.29–0.93; $P = 0.03$) was significantly reduced among carriers of the *aP2* promoter polymorphism (Fig. 4).

Impact of −87 Variant on *aP2* Gene Expression in Adipose Tissue of Carriers. Our experiments by using reporter assays and EMSA indicated that the SNP at −87 of the *aP2* promoter has a negative impact on the promoter activity and expression of the *aP2* gene (Figs. 1 and 2). Moreover, the three predominant phenotypic features of the aP2-deficient mice, reduced triglycerides, reduced

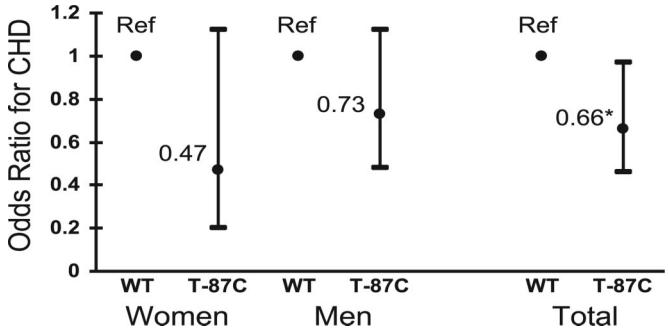

Fig. 3. Odds ratio for CHD among subjects carrying the T-87C allele compared with WT. The multivariate models were adjusted for age, BMI, activity, smoking, and gender. *, $P = 0.03$; $n = 1,462$ for women (235 cases and 1,227 controls with 2.5% and 5.2% heterozygous frequency, respectively); $n = 3,301$ for men (811 cases and 2,490 controls with 3.5% and 4.5% heterozygous frequency, respectively); Ref, reference category, T-87C represents carriers of this SNP in heterozygous state.

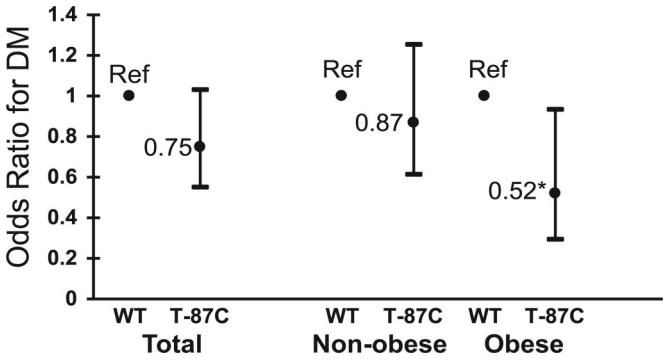

Fig. 4. Odds ratio for diabetes among subjects carrying the T-87C allele compared with the WT. The multivariate models were adjusted for age, BMI, activity, smoking, and gender. Nonobese category is for subjects whose BMI $<$ 30 kg/m^2 (n = 2,929, 1,508 cases and 1,421 controls with 4.4% and 4.5% heterozygous frequency, respectively), and the obese category is BMI $\geq$ 30 kg/m^2 (n = 1,156, 836 cases and 320 controls with 3.6% and 6.2% heterozygous frequency, respectively), *, P = 0.03; Ref, reference category, T-87C represents carriers of this SNP in heterozygous state.

type 2 diabetes, and coronary heart disease, also were captured among the human carriers of the *aP2*–87 SNP. Therefore, a critical and definitive link between these observations lies with the demonstration of altered *in vivo aP2* expression because of *aP2*–87 in humans.

To address this critical question, we genotyped an additional 1,247 subjects from which adipose tissue needle-biopsy samples has been collected along with blood samples (19) for a nonfatal MI case-control study in Costa Rica. Among these subjects, we identified 31 individuals heterozygous for *aP2*–87 and matched WT carriers according to age, sex, dietary habits, and region of residence. After RNA isolation and cDNA synthesis from these samples, we established a real-time quantitative PCR assay for human *aP2* gene. The assay was further validated with a mixing experiment by pooling RNA from aP2 expressing (adipose) and nonexpressing tissues at precalculated ratios followed by RT-PCR analyses. In this experiment, the quantitative experimental measurement was in perfect agreement with the aP2 ratio in premixed samples (r = 0.98). After this validation, we examined the levels of *aP2* expression in tissue samples obtained from subjects with the T-87C compared with WT controls. As shown in Fig. 5, subjects carrying the T-87C polymorphism had a 63% reduction in adipose tissue *aP2* mRNA expression levels compared with those with the WT allele (0.078 versus 0.21 respectively; P = 0.017). Hence, the T-87C is a loss-of-function allele

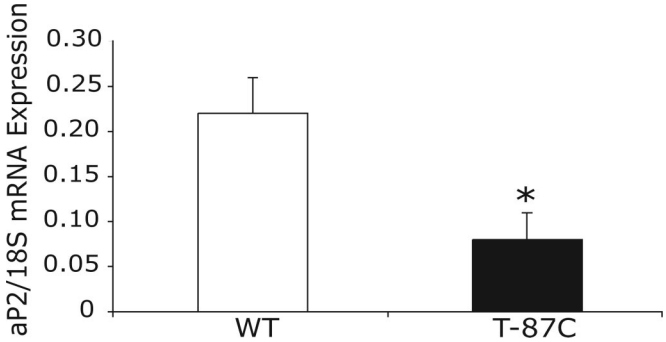

Fig. 5. The impact of T-87C SNP on *aP2* expression in human adipose tissue. The level of *aP2* mRNA expression is determined by quantitative real-time PCR in adipose tissue samples obtained from individuals with WT (n = 22) and T-87C (n = 22) *aP2* promoter alleles. *, P = 0.017.

in vivo and results in reduction of adipose tissue *aP2* mRNA expression in humans.

Discussion

The global prevalence of obesity and the associated metabolic disease cluster is increasing rapidly. In the United States, 65.7% of adults and 16% of children are overweight and 23.7% of adults have metabolic syndrome, an aggregation of comorbidities, including obesity, insulin resistance, dyslipidemias, and hypertension. The presence of metabolic syndrome introduces great risk for coronary events, stroke, type 2 diabetes, and other chronic health problems. These pathological states have strong inflammatory underpinnings (3). Integrated metabolic abnormalities emerge from the action of common pathways to metabolic and inflammatory cells, the prototype example is between adipocytes and macrophages in obesity. Hence, molecular pathways controlling the responses of these cells are of critical importance in understanding the underlying mechanism(s) of metabolic syndrome.

In recent years, results obtained from experimental models demonstrated that FABPs integrate metabolic and immune responses and link the inflammatory and lipid-mediated pathways that are critical in metabolic syndrome (9). In particular, the adipocyte/macrophage FABP isoform, *aP2*, when deleted in mice demonstrates three major phenotypes: moderately reduced triglyceride levels, protection against type 2 diabetes when the animals were made obese, and reduced atherosclerosis despite hypercholesterolemia (6, 18). Similar to mice, aP2 is expressed in human adipocytes and regulated by differentiation. It is also detected in activated macrophages, and aP2 level is elevated in human atherosclerotic lesions. Interestingly, *aP2* expression in macrophages is negatively regulated by statins (20). As directly shown in mice models, reduction in aP2 abundance is metabolically beneficial; if similar in humans then alleles that reduce *aP2* expression or activity might confer resistance to some or all of these parameters in humans. In this study, we have searched for polymorphisms associated with regulation of *aP2* expression in humans and tested this hypothesis in a large prospective population study. Our studies revealed a functionally significant SNP, T-87C, in the 5′ promoter region of *aP2*, which disturbs C/EBPα binding and subsequent transcriptional activation of this promoter by C/EBPα. Hence, the T-87C allele represents a "loss-of-function" allele of *aP2* in humans. Consistent with this inference, subjects that carry this *aP2* allele exhibit reduced serum triglycerides and significantly reduced risk for type 2 diabetes and cardiovascular diseases. In other words, the phenotypic outcome of the T-87C allele in humans is highly reminiscent of the phenotype of aP2 deficiency in the mice. In general, we were able to replicate our findings from our cohort of women and in our cohort of men. However, these subjects were healthy, free-living, mostly Caucasian populations. Confirmation from other populations, especially those with a higher variant allele prevalence is warranted.

A critical piece of evidence often challenging to gather in studies of this nature is to link the presence of a functionally significant genetic variant to a change of function *in vivo* in the individuals that carry the corresponding allele. In our study, we provide compelling evidence that the presence of T-87C allele results in significantly reduced *aP2* gene expression in the adipose tissue of carrier subjects. In this study, we did not have the opportunity to evaluate the potential impact of this variant on macrophage *aP2* expression and function. It is possible that this loss-of-function allele might have important effects on these cells that are not captured in this setting. The magnitude of changes in adipose tissue *aP2* expression is similar to haploinsufficiency in experimental mice with one targeted *aP2* allele (12). Studies on the functional consequences of *aP2* haploinsufficiency in mice are rather limited. Interestingly, mice with one

dysfunctional allele of *aP2* gene exhibit an intermediary phenotype, at least in lipid and glucose metabolism, upon exposure to a high-fat diet or in genetic obesity (12). The impact of haploinsufficiency has not yet been examined in the context of atherosclerosis and macrophage function (6, 21).

Taken together our study illustrates that an allele with reduced aP2 activity in free-living humans results in a metabolically favorable phenotype and provides an important link between the FABP function and chronic disease in humans. Although the prevalence of this allele is rather low in the general population, our findings provide insights into the pathogenesis of metabolic syndrome. They also point to the possibility that aP2 plays a similar role in human disease as in experimental mice and, therefore, chemical modification of aP2 activity in humans might represent a desirable preventive or therapeutic strategy against the devastating cluster of diseases such as type 2 diabetes and atherosclerosis.

Materials and Methods

Population. The Nurses' Health Study cohort is a prospective study of 121,700 women, 32,826 of which (43–69 years of age) provided whole-blood samples between 1989–1990. The Health Professional's Follow-up Study cohort began in 1986 and includes 51,529 male health professionals. Participants in both cohorts are followed biennially to document incident chronic disease. We had available to us for genotyping several previously conducted nested case-control studies from within these two populations (22–26). Not all participants within each substudy had plasma markers available. The institutional review board of the Brigham and Women's Hospital and the Harvard School of Public Health Human Subjects Committee approved the study protocol.

Sequencing, SNP Discovery, and Genotyping. The sequencing of the entire coding and promoter regions of *aP2* was carried out with AmpliTaq DNA Polymerase FS dye terminator method by using ABI PRISM 377 DNA Sequencer (Applied Biosystems). The primers used for sequencing were as follows: exon1F: 5′-TgCAgCTTCCTTCTCACCTTgA-3′, exon1R: 5′-CTTgCTATgTgTgCAgCCTCTC-3′, exon2F: 5′-ACACACACATTCTCTgACTTgg-3′, exon2R: 5′-gAggTTCAgggATCTTTggCTT-3′, exon3F: 5′-CCTCCCTCTgCACATTgTCATT-3′, exon3R: 5′-AgggCAAATgCCAgAgTggAAg-3′, exon4F: 5′-TATCCCCAACATTCAgAgAgAC-3′, exon4R: 5′-TATCCCACAgAATgTTgTAgAg-3′ (Integrated DNA Technologies, Coralville, IA). For each sample, sequencing was performed both with forward and reverse primers. For the T-87C promoter polymorphism, all samples were genotyped by using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in a 384-well format. The 5′ nuclease assay (TaqMan) was used to distinguish the two alleles of the *aP2* gene, involving a T→C transition at position −87 from the transcription start site (reverse complement strand). The PCR amplification was carried out on 5–20 ng of DNA by using 1× TaqMan universal PCR master mix (No Amp-erase UNG), 900 nM forward (5′-TCCTTTTAAAgATgCTCAgAACATgTgA-3′) and reverse (5′-gCCggTAATgAAggAAATgATTggA-3′) primers, 200 nM of the FAM-labeled probe (5′-CATTCCCAACTggTCAT-3′) and 200 nM of the VIC-labeled probe (5′-CTCATTCCCAATTggTCAT-3′) in a 5-μl reaction, and the polymorphic base is shown underlined. Amplification conditions on a AB 9700 dual plate thermal cycle (Applied Biosystems) were as follows: 1 cycle of 95°C for 10 min, followed by 50 cycles of 92°C for 15 s and 58°C for 1 min. TaqMan primers and probes were designed, on the reverse strand, by using the PRIMER EXPRESS 2.0 oligo design software (Applied Biosystems).

Plasmid Preparation and Gel Shift Assays. A DNA segment of *aP2* spanning the region between nucleotides −194 and −16 in the 5′ region of human *aP2* promoter was amplified by using primers PromFwd: 5′-CATgCgAgCTCCTATTTgCCAgggAgAACCA-3′ and PromRev: 5′-ggTAgCTAgCgCTgTgACCCTCTTgAgTCC-3′, thereby creating SacI (5′ end) and NheI (3′ end) restriction sites at the ends. After restriction enzyme digestion of the PCR product along with the pGL3 basic vector, ligation was performed by using T4 DNA Ligase (NEB, Beverly, MA). Site-directed mutagenesis was performed with aP2mutFwd: 5′-TgATTggATCTCATTCCCAACTggTCATTCCTA-3′ and aPmutRev: 5′-TtgggAATgAgATCCAATCATTT-CCTTCAT-3′ by using GeneTailor kit (Invitrogen) and confirmed by sequencing.

The 179-bp human *aP2* proximal promoter segment carrying either a WT or mutant allele was used as a DNA probe in EMSA. The EMSA reactions were performed in 30-μl volume containing 400 ng of DNA labeled with 10 μCi [γ-^{32}P]ATP (1 Ci = 37 GBq) and T4 PNK (NEB, Beverly, MA). The protein component, 15 ng of GST-C/EBPα (12 kDa carboxyl-terminal rat C/EBPα fusion protein; ref. 27), was incubated with 0.5 μg poly[d(I-C)] and ^{32}P-labeled DNA probe (2×10^4 cpm) in the presence or absence of varying quantities of specific (with or without the T/C alteration) or nonspecific unlabeled oligonucleotide competitors at room temperature for 20 min. At the end of the incubation period, the samples were run on a 4% nondenaturing polyacrylamide gel for 2 h, dried, and exposed to Imaging Screen K (Bio-Rad) for visualization by Molecular Imager FX (Bio-Rad). For super shifting, anti-C/EBPα antibody was used (Santa Cruz Biotechnology)

Promoter Activity Reporter Assays. Promoter activity assays were performed in HEK-293 cells, maintained in DMEM (GIBCO), and supplemented with 10% FBS (HyClone). Cells were cotransfected with the 179-bp WT or mutant *aP2* proximal promoter fragment (nucleotides −194 to −16) driving the expression of luciferase gene in the pGL3 luciferase reporter vector (Promega) with or without full-length C/EBPα expression vector. Transfections were performed with varying DNA concentrations at 60–70% cellular confluency by using FuGene6 reagent (Roche Applied Science, Indianapolis). Thirty-six hours after the transfections cells were washed with PBS solution, lysed and dual luciferase assay (Promega) was performed to determine activity. *Renilla* luciferase reporter was used as an internal control for transfection efficiency and to normalize the results.

Real-Time RT-PCR. Total RNA was isolated from 50 mg of adipose tissue sample by using TRIzol reagent (Invitrogen). Reverse transcription reaction was carried out with ThermoScript RT-PCR system (Invitrogen). Real-time PCR analysis was performed in a 25-μl final reaction volume with an iCycler iQ Detection System by using iQ SYBR Green Supermix (Bio-Rad). The PCR thermal cycling program was as follows: 2 min 30 s at 95°C for enzyme activation (allowing hot start), 40 cycles of 15 s at 95°C, 30 s at 58°C, and 1 min at 72°C for extension. Melting curve analysis was performed to confirm the real-time PCR products. All quantitations were normalized to the 18S rRNA level in reaction. Primer sequences used were as follows: aP2Fwd:5′-AgCACCATAACCTTAgATgggg-3′, aP2Rev: 5′-CgTggAAgTgACgCCTTTCA-3′, 18SFwd: 5′-gTAACCCgTTgAACCCCATT-3′, and 18SRev: 5′-CCATCCAATCggTAgTAgCg-3′.

Statistical Analysis. Multivariate linear regression analyses with robust variance were performed to evaluate the association between triglyceride levels and the *aP2* polymorphism. We adjusted for age, sex, BMI, activity, smoking, fasting status, and dietary intake of trans and saturated fatty acids and total calorie intake. We used unconditional logistic regression to determine

the odds ratio (95% confidence interval) for CHD and diabetes mellitus for participants with and without the variant allele. The multivariate analyses were controlled for age, sex, smoking, physical activity, and BMI. Further control for other lifestyle characteristics did not appreciably alter the odds ratio estimates. SAS 8.0 (SAS Institute, Cary, NC) was used for all analyses.

We thank the Hotamisligil laboratory for their contributions, Frank Hu and JoAnn Manson (Harvard School of Public Health) for use of their samples, and David Hunter and Hardeep Ranu of the Dana–Farber/Harvard Cancer Center High-Throughput Polymorphism Detection Core for genotyping. This work was supported in part by a grant from the Iacocca Foundation, National Institutes of Health (NIH) Grants DK52539 (to G.S.H.), HL065405 (to G.S.H. and M. Linton), and HL60692 (to H.C.), and the American Diabetes Association. Samples used from the Nurses' Health Study and Health Professionals Follow-up Study were supported by NIH Grants HL34595 (to J. Manson), CA87969 (to G. Colditz), HL35464 and AA11181 (to E.B.R.), DK58845 and HL 65582 (to F. Hu), and CA55075 (to W. Willett). G.T. is a recipient of a Postdoctoral Fellowship from the Iacocca Foundation.

1. Saltiel, A. R. (2000) *J. Clin. Invest.* **106,** 163–164.
2. Mokdad, A. H., Ford, E. S., Bowman, B. A., Dietz, W. H., Vinicor, F., Bales, V. S. & Marks, J. S. (2003) *J. Am. Med. Assoc.* **289,** 76–79.
3. Wellen, K. E. & Hotamisligil, G. S. (2005) *J. Clin. Invest.* **115,** 1111–1119.
4. Makowski, L. & Hotamisligil, G. S. (2004) *J. Nutr.* **134,** 2464S–2468S.
5. Haunerland, N. H. & Spener, F. (2004) *Prog. Lipid. Res.* **43,** 328–349.
6. Makowski, L., Boord, J. B., Maeda, K., Babaev, V. R., Uysal, K. T., Morgan, M. A., Parker, R. A., Suttles, J., Fazio, S., Hotamisligil, G. S., *et al.* (2001) *Nat. Med.* **7,** 699–705.
7. Scheja, L., Makowski, L., Uysal, K. T., Wiesbrock, S. M., Shimshek, D. R., Meyers, D. S., Morgan, M., Parker, R. A. & Hotamisligil, G. S. (1999) *Diabetes* **48,** 1987–1994.
8. Fu, Y., Luo, N., Lopes-Virella, M. F. & Garvey, W. T. (2002) *Atherosclerosis (Shannon, Irel.)* **165,** 259–269.
9. Makowski, L., Brittingham, K. C., Reynolds, J. M., Suttles, J. & Hotamisligil, G. S. (2005) *J. Biol. Chem.* **280,** 12888–12895.
10. Maeda, K., Uysal, K. T., Makowski, L., Gorgun, C. Z., Atsumi, G., Parker, R. A., Bruning, J., Hertzel, A. V., Bernlohr, D. A. & Hotamisligil, G. S. (2003) *Diabetes* **52,** 300–307.
11. Maeda, K., Cao, H., Kono, K., Gorgun, C. Z., Furuhashi, M., Uysal, K. T., Cao, Q., Atsumi, G., Malone, H., Krishnan, B., *et al.* (2005) *Cell Metab.* **1,** 107–119.
12. Hotamisligil, G. S., Johnson, R. S., Distel, R. J., Ellis, R., Papaioannou, V. E. & Spiegelman, B. M. (1996) *Science* **274,** 1377–1379.
13. Boord, J. B., Maeda, K., Makowski, L., Babaev, V. R., Fazio, S., Linton, M. F. & Hotamisligil, G. S. (2002) *Arterioscler Thromb. Vasc. Biol.* **22,** 1686–1691.
14. Fisher, R. M., Eriksson, P., Hoffstedt, J., Hotamisligil, G. S., Thorne, A., Ryden, M., Hamsten, A. & Arner, P. (2001) *Diabetologia* **44,** 1268–1273.
15. Hunt, C. R., Ro, J. H.-S., Dobson, D. E., Min, H. Y. & Spiegelman, B. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3786–3790.
16. Baxa, C. A., Sha, R. S., Buelt, M. K., Smith, A. J., Matarese, V., Chinander, L. L., Boundy, K. L. & Bernlohr, D. A. (1989) *Biochemistry* **28,** 8683–8690.
17. Cheneval, D., Christy, R. J., Geiman, D., Cornelius, P. & Lane, M. D. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8465–8469.
18. Uysal, K. T., Scheja, L., Wiesbrock, S. M., Bonner-Weir, S. & Hotamisligil, G. S. (2000) *Endocrinology* **141,** 3388–3396.
19. Baylin, A. & Campos, H. (2004) *J. Nutr.* **134,** 3095–3099.
20. Llaverias, G., Noe, V., Penuelas, S., Vazquez-Carrera, M., Sanchez, R. M., Laguna, J. C., Ciudad, C. J. & Alegret, M. (2004) *Biochem. Biophys. Res. Commun.* **318,** 265–274.
21. Boord, J. B., Maeda, K., Makowski, L., Babaev, V. R., Fazio, S., Linton, M. F. & Hotamisligil, G. S. (2004) *Circulation* **110,** 1492–1498.
22. Shai, I., Schulze, M. B., Manson, J. E., Stampfer, M. J., Rifai, N. & Hu, F. B. (2005) *Diabetologia* **48,** 1469–1476.
23. Jiang, R., Manson, J. E., Meigs, J. B., Ma, J., Rifai, N. & Hu, F. B. (2004) *J. Am. Med. Assoc.* **291,** 711–717.
24. Jiang, R., Schulze, M. B., Li, T., Rifai, N., Stampfer, M. J., Rimm, E. B. & Hu, F. B. (2004) *Diabetes Care* **27,** 1991–1997.
25. Pai, J. K., Pischon, T., Ma, J., Manson, J. E., Hankinson, S. E., Joshipura, K., Curhan, G. C., Rifai, N., Cannuscio, C. C., Stampfer, M. J., *et al.* (2004) *N. Engl. J. Med.* **351,** 2599–2610.
26. Meyer, K. A., Conigrave, K. M., Chu, N. F., Rifai, N., Spiegelman, D., Stampfer, M. J. & Rimm, E. B. (2003) *J. Am. Coll. Nutr.* **22,** 185–194.
27. Tong, Q., Tsai, J., Tan, G., Dalgin, G. & Hotamisligil, G. S. (2005) *Mol. Cell. Biol.* **25,** 706–715.