Associations between Common Genetic Polymorphisms in Angiopoietin-Like Proteins 3 and 4 and Lipid Metabolism and Adiposity in European Adolescents and Adults

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Context: Plasma-borne angiopoietin-like proteins (ANGPTL) act as endocrine factors on their target tissues. Because ANGPTL3 and ANGPTL4 play important roles in lipid metabolism and the regulation of adiposity in mice, we hypothesized that genetic variability at the *ANGPTL3* and *ANGPTL4* genes loci might influence lipid metabolism and fat deposition in humans.

Objective: The aim of the study was to examine the association between *ANGPTL3* and *ANGPTL4* genetic polymorphisms and metabolic phenotypes in adolescent and adult samples.

Design and Participants: Two independent population-based studies, one composed of 1144 adolescents (mean age, 14.8 $\pm$ 1.4 yr) from nine European countries (the HELENA study) and the other composed of 1155 adults (age range, 35–65 yr) from Northern France (the MONICA Lille study), were genotyped for one *ANGPTL3* polymorphism and four *ANGPTL4* polymorphisms.

Results: The *ANGPTL3* rs11207997 polymorphism (minor allele frequency, 0.32) was associated with lower plasma HDL-cholesterol and apolipoprotein A-I levels in both adolescents ($P = 0.0004$, $P = 0.00006$, respectively) and adults ($P = 0.03$, $P = 0.02$, respectively). The *ANGPTL4* rs4076317 polymorphism (minor allele frequency, 0.29) was associated with a higher percentage of body fat ($P = 0.02$) in adolescents and a higher waist-to-hip ratio (in interaction with the peroxisome proliferator-activated receptor γ Pro12Ala polymorphism) in adults ($P = 0.0004$).

Conclusion: The present study underlines the role of ANGPTL3 in HDL-cholesterol metabolism as early as in adolescence. Our data also suggest possible associations between *ANGPTL4* polymorphisms and body fat, but these findings require replication. **(*J Clin Endocrinol Metab* 94: 5070–5077, 2009)**

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Abbreviations: ANGPTL, Angiopoietin-like protein; ApoA1, apolipoprotein A-I; ApoB, apolipoprotein B; BMI, body mass index; GWAS, genome-wide association studies; HDL, high-density lipoprotein; LD, linkage disequilibrium; LDL, low-density lipoprotein; LPL, lipoprotein lipase; MAF, minor allele frequency; NeuroD1, neurogenic differentiation factor 1; PPAR, peroxisome proliferator-activated receptor; SNP, single nucleotide polymorphism; TG, triglyceride.

Angiopoietin-like proteins (ANGPTL) are a family of secreted proteins that was discovered a decade ago. They are characterized by the presence of two structural domains shared with the angiopoietins (a coiled-coil domain and a fibrinogen-like domain), and unlike angiopoietins, their inability to bind the TIE2 (a tyrosine kinase with Ig and epithelial growth factor homology domains) receptor. Seven members have been identified so far and denominated as ANGPTLs 1 to 7 (1). Although it is known that ANGPTLs act as endocrine factors on target tissues, the corresponding receptors and signaling pathways have yet to be determined.

Angptl3 and Angptl4 are the most closely related members of this protein family. Both inhibit lipoprotein lipase (LPL) activity and prompt a decrease in very low density lipoprotein (LDL)-triglyceride (TG) clearance (2, 3). Furthermore, Angptl3 and Angptl4 stimulate lipolysis in adipose tissue and thus lead to the release of free fatty acids and glycerol from adipocytes (4, 5). Concordantly, mice lacking *Angptl3* and/or *Angptl4* exhibit hypotriglyceridemia, whereas overexpression or iv injection of Angptl3 or Angptl4 increases plasma TG and free fatty acid levels (5–7). Furthermore, Angptl3 inhibits endothelial lipase, which hydrolyzes high-density lipoprotein (HDL) phospholipids and decreases plasma HDL levels (8), and Angptl4 reduces hepatic cholesterol uptake by inhibiting LPL and hepatic lipase, leading to increased cholesterol synthesis in the liver (9). Double-knockout mice die prenatally or do not survive beyond 2 months of age and present nearly undetectable TG levels and low cholesterol levels— demonstrating the essential role of Angptl3/4 in the regulation of circulating TG and lipoproteins (7).

However, Angptl3 and Angptl4 may not be redundant because they are not always expressed in the same tissues or at the same time. Whereas Angptl3 expression is restricted to the liver (regardless of the nutritional status) (10), Angptl4 is expressed in adipose tissue, liver, and (to a lesser extent) in intestine and placenta, and its transcription is strongly induced by fasting conditions (11). Indeed, Angptl4 expression is regulated by peroxisome proliferator-activated receptors (PPARs) (12). Angptl3 functions seem to be restricted to lipid metabolism, whereas Angptl4 has a broader sphere of activity. Angptl4 overexpression in mice caused a 50% reduction in adipose tissue weight, partly by stimulating fatty acid oxidation and uncoupling in fat (5). Furthermore, Angptl4 seems to mediate the microbial regulation of peripheral fat storage (13). Lastly, Angptl4 also appears to play a role in glucose metabolism, although this finding remains controversial (5, 7, 9, 14).

In humans, the *ANGPTL3* and *ANGPTL4* genes are located on chromosomes 1p31.3 and 19p13.2, respectively. Single nucleotide polymorphisms (SNPs) nearby *ANGPTL3* have been associated with plasma TG levels (15–17), and SNPs in *ANGPTL4* have been associated with plasma HDL-cholesterol levels (17) in recent genome-wide association studies (GWAS). The goal of our study was to explore the impact of the common sequence variability in *ANGPTL3* and *ANGPTL4* on some metabolic phenotypes (anthropometric parameters and plasma glucose, insulin, and lipid levels) in both adult and adolescent samples. After selecting all the known common SNPs of each gene, we performed association studies in two independent population-based samples: 1) the Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA)-Cross Sectional Study, composed of 1144 European adolescents; and 2) the MONICA Lille study, composed of 1155 French adults.

Subjects and Methods

The HELENA study

Participants were recruited as part of the HELENA study (http://www.helenastudy.com) performed from 2006 to 2007 in nine European countries (Greece, Germany, Belgium, France, Hungary, Italy, Sweden, Austria, and Spain) as previously described (18). The protocol was approved by the appropriate ethics committee in each center. Written, informed consent was obtained from each subject and both of his/her parents or legal representatives. Participation in the study was voluntary. The sample included a total of 3865 adolescents (mean age, 14.8 ± 1.4 yr) recruited through their schools; the latter were randomly selected according to a proportional cluster sampling methodology taking into account geographical repartition within each city, private/public school ratio, and number of classes by school (19). Participants were barefoot and in underwear, and anthropometric measurements were taken by trained researchers. Waist and hip circumferences were measured three consecutive times and with a nonelastic tape (Seca 200) to the nearest 0.1 cm. The body mass index (BMI) was calculated. The percentage of body fat was estimated from skin-fold measurements, according to Slaughter *et al.* (20). Physical activity over a 1-wk period was evaluated using accelerometers (Actigraph MTI, model GT1M; Manufacturing Technology Inc., Fort Walton Beach, FL) (21).

One third of the classes (n = 1155) were randomly selected for blood collection. Blood samples were drawn at school according to a standardized collection protocol (after a 10-h overnight fast) and were sent to the Analytical Laboratory at the University of Bonn [Institut für Ernährungs und Lebensmittelwissenschaften] (Germany) for subsequent biochemical measurements (22). Serum TG, HDL and LDL cholesterol, and glucose levels were measured on the Dimension RxL clinical chemistry system (Dade Behring, Schwalbach, Germany) with enzymatic methods. Serum apolipoprotein A-I (ApoA1) and apolipoprotein B (ApoB) concentrations were measured in an immunochemical reaction with a BN II analyzer (Dade Behring). Blood for DNA extraction was collected in EDTA K3 tubes and sent to the Genomic Analysis Laboratory at the Institut Pasteur of Lille (Lille, France). DNA was extracted from white blood cells with the Puregene kit (QIAGEN, Courtaboeuf, France). Genotyping was performed on an Illumina system, using GoldenGate technology (Illumina, Inc., San Diego, CA). The genotyping success rates were 97.2%

for rs11672433, 98.8% for rs11207997, and above 99.6% for the other SNPs. Data on BMI were available for 1144 subjects.

The MONICA Lille study

Participants were recruited as part of the World Health Organization (WHO)-MONICA population survey performed from 1995 to 1997 in the Lille Urban Community in Northern France (n = 1195) as previously described (23). The sample included individuals aged 35–65 randomly selected from electoral rolls after stratification by town size, gender, and age to obtain 200 participants for each gender and each 10-yr age group (WHO-MONICA Project protocol) (24). The study protocol was approved by the local ethics committee. After signing an informed consent form, participants filled out a standard questionnaire, and physical measurements were taken by a specially trained nurse. Physical activity was defined as at least 15 min of walking a day and/or daily lifting or carrying heavy objects at work and/or doing sport or physical exercise for more than 2 h/wk. Current cigarette smokers were defined as individuals reporting at least one cigarette per day. Total alcohol intake (in milliliters of alcohol) was calculated as the sum per week from wine, beer, cider, and spirits. A 20-ml blood sample was drawn on disodium EDTA after a 10-h overnight fast for 1155 subjects. Lipid and lipoprotein levels were all measured at the Purpan Hospital Biochemical Laboratory (Toulouse, France). The genotyping was performed using the restriction fragment length polymorphism method (see Supplementary Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The genotyping success rate was above 98.7% for all SNPs.

SNP selection

SNPs were extracted from the National Center for Biotechnology Information (NCBI) dbSNP and HapMap (data release 23a/phase II, March 2008) databases. For *ANGPTL3*, the seven validated SNPs described in the 10-kbp genomic region in the NCBI database were all genotyped in the CEU (CEPH Utah residents with ancestry from Northern and Western Europe) panel from the HapMap Project. Only one SNP (rs11207997) had a minor allele frequency (MAF) higher than 0.05. Hence, rs11207997 (MAF 0.30) was the only SNP that could be analyzed in association studies for *ANGPTL3*.

There were six validated *ANGPTL4* SNPs with a MAF higher than 0.02 reported in the NCBI dbSNP database: rs4076317, rs2278236, rs7255436, rs1044250 (Thr266Met), rs35061979 (Arg278Gln), and rs11672433 (Pro389Pro). In $HapMap_{CEU}$, rs2278236 was a perfect proxy for rs7255436 ($r^2 = 1$), and therefore rs2278236 was chosen a tagSNP of this block. rs1044250, rs4076317, rs10404615, and rs11672433 were independent SNPs ($r^2 < 0.8$). Because the linkage disequilibrium (LD) of rs35061979 with the other SNPs was not known, we typed rs35061979 in 100 subjects from the MONICA Lille study, but it could not be detected and was not pursued. Thus, the rs4076317, rs2278236, rs1044250, and rs11672433 SNPs (accounting for over 99.9% of *ANGPTL4* common genetic variability) were further analyzed in association studies.

Statistical analyses

Statistical analyses were performed with SAS statistical software, version 8 (SAS Institute Inc., Cary, NC). To obtain a normal data distribution, log-transformation was used for TG and insulin levels in both studies and also for glucose levels in the MONICA sample. In MONICA Lille, intergroup comparisons of quantitative variables were made using a general linear model (GLM) procedure, except for gene-gene interaction analyses for which the number of subjects was small in one of the four groups (n = 18); we first performed a rank transformation on the anthropometric variables as described by Conover and Iman (25) and then used a multivariate general linear model procedure. In HELENA, the effects were homogenous across centers because there was no significant genotype × center interaction ($P > 0.05$). Reported *P* values are nominal and were systematically adjusted for confounding variables. In MONICA Lille, confounding variables were age, gender, smoking status, alcohol consumption, and physical activity for anthropometric variables, with the addition of BMI for biochemical variables. In HELENA, we used age, gender, and center as covariates for anthropometric variables, with the addition of BMI for biochemical variables. Statistical significance was considered as $P \leq 0.05$.

Results

Table 1 shows the clinical characteristics of the subjects from the HELENA and MONICA Lille studies. We focused on the associations between the *ANGPTL* SNPs with anthropometric measurements and plasma lipids, glucose, and insulin levels significant in both studies.

Study of ANGPTL3's genetic variability

The MAF for the *ANGPTL3* rs11207997 SNP was 0.30 in the HELENA study and 0.34 in the MONICA Lille sample. The genotype distribution did not deviate from the Hardy-Weinberg equilibrium in either sample ($P > 0.54$). We detected significant associations between rs11207997 and plasma HDL-cholesterol and ApoA1 levels (Table 2).

TABLE 1. Characteristics of subjects from HELENA and MONICA Lille

	HELENA	MONICA Lille
n	1144	1155
Males/females (%)	48.0/52.0	50.7/49.3
Age (yr)	14.8 ± 1.4	51.3 ± 8.5
Weight (kg)	58.4 ± 13.0	74.3 ± 15.5
BMI (kg/m²)	21.3 ± 3.8	26.6 ± 5.0
Waist (cm)	72.3 ± 9.3	91.0 ± 13.9
Waist/hip ratio	0.794 ± 0.07	0.885 ± 0.096
Body fat (%)	23.8 ± 9.8	NA
Triglyceride (mmol/liter)	0.79 ± 0.40	1.41 ± 1.14
HDL-cholesterol (mmol/liter)	1.43 ± 0.27	1.50 ± 0.48
LDL-cholesterol (mmol/liter)	2.45 ± 0.65	3.78 ± 1.03
ApoA1 (g/liter)	1.51 ± 0.22	1.73 ± 0.31
ApoB (g/liter)	0.65 ± 0.16	1.21 ± 0.30
Glucose (mmol/liter)	5.00 ± 0.40	5.55 ± 1.54
Insulin (μIU/ml)	9.93 ± 5.88	11.93 ± 7.92
Dyslipidemia (%)	NA	49.7
Hypertension (%)	NA	45.1
Coronary heart disease (%)	NA	2.5
Diabetes mellitus (%)	NA	10.9

Data are expressed as means ± SD or percentage. NA, Not available.

TABLE 2. Association between *ANGPTL3* rs11207997 and anthropometric and biochemical phenotypes

	HELENA						MONICA Lille					
	CC	CT	TT	*P* trend	*P* (CT/TT *vs.* CC)	*P* (TT *vs.* CC/CT)	CC	CT	TT	*P* trend	*P* (CT/TT *vs.* CC)	*P* (TT *vs.* CC/CT)
n	549	478	104				499	503	140			
BMI (kg/m²)	21.4 ± 3.7	21.4 ± 3.9	20.6 ± 3.4	0.39	0.87	0.10	26.5 ± 5.2	26.6 ± 4.9	27.1 ± 4.6	0.33	0.58	0.24
Waist/hip ratio	0.796 ± 0.06	0.794 ± 0.08	0.788 ± 0.06	0.54	0.62	0.60	0.882 ± 0.10	0.889 ± 0.09	0.887 ± 0.09	0.04	0.12	0.06
Body fat (%)	23.9 ± 9.6	24.0 ± 10.0	22.6 ± 9.4	0.80	0.54	0.62		NA				
Triglyceride (mmol/liter)[a]	0.79 ± 0.39	0.79 ± 0.40	0.80 ± 0.50	0.73	0.77	0.78	1.43 ± 1.26	1.38 ± 1.14	1.19 ± 0.65	0.15	0.30	0.15
HDL-cholesterol (mmol/liter)[a]	1.46 ± 0.28	1.41 ± 0.27	1.38 ± 0.26	**0.0001**	**0.0004**	**0.02**	1.50 ± 0.50	1.53 ± 0.47	1.45 ± 0.43	**0.03**	0.48	**0.03**
LDL-cholesterol (mmol/liter)[a]	2.45 ± 0.63	2.45 ± 0.66	2.48 ± 0.67	0.63	0.86	0.42	3.79 ± 1.02	3.70 ± 1.03	3.77 ± 1.06	0.41	0.23	0.89
ApoA1 (g/liter)[a]	1.53 ± 0.22	1.48 ± 0.22	1.48 ± 0.22	**0.00007**	**0.00006**	0.06	1.74 ± 0.33	1.74 ± 0.30	1.69 ± 0.31	0.06	0.78	**0.02**
ApoB (g/liter)[a]	0.65 ± 0.16	0.65 ± 0.16	0.67 ± 0.17	0.60	0.77	0.29	1.23 ± 0.29	1.21 ± 0.30	1.20 ± 0.31	0.77	0.47	0.75
Glucose (mmol/liter)[b]	5.02 ± 0.38	4.99 ± 0.41	5.04 ± 0.44	0.50	0.85	0.23	5.39 ± 1.20	5.35 ± 0.88	5.27 ± 0.82	0.25	0.27	0.13
Insulin (μIU/ml)[b]	9.86 ± 5.75	10.07 ± 6.01	9.93 ± 6.15	0.11	0.18	0.04	12.06 ± 8.96	11.64 ± 6.98	11.76 ± 7.76	0.54	0.41	0.34

Data are expressed as means ± SD. *P* values are adjusted for confounding variables. Significant *P* values are indicated in *bold*. NA, Not available.

[a] Subjects treated with lipid-lowering therapy were excluded in MONICA Lille.

[b] Subjects treated with antidiabetic drugs were excluded in MONICA Lille.

Indeed, in the HELENA study, *T* allele bearers had significantly lower HDL-cholesterol and ApoA1 levels, compared with *CC* subjects (HDL, 1.40 ± 0.27 *vs.* 1.46 ± 0.28 mmol/liter; $P = 0.0004$; ApoA1, 1.48 ± 0.22 *vs.* 1.53 ± 0.22 g/liter; $P = 0.00006$). These associations were not modified by further adjustment for physical activity or for pubertal status (data not shown). In the MONICA Lille study, *TT* subjects displayed lower HDL-cholesterol and ApoA1 levels, compared with *C* allele bearers (HDL-cholesterol, 1.44 ± 0.43 *vs.* 1.51 ± 0.48 mmol/liter; $P = 0.03$; ApoA1, 1.68 ± 0.30 *vs.* 1.74 ± 0.31 g/liter; $P = 0.02$). Given that 1) *ANGPTL3* is a direct target gene of the LXR nuclear receptor (26), and 2) we have previously shown that the *LXRα* rs11039155 (−6 G/A) SNP was associated with plasma HDL-cholesterol levels in the MONICA Lille study (27), we checked whether the association between *ANGPTL3* rs11207997 and HDL-cholesterol could be modulated by the *LXRα* rs11039155 SNP. However, no significant interaction between these two SNPs on HDL-cholesterol levels could be detected ($P = 0.29$).

Study of ANGPTL4's genetic variability

The MAFs for the rs4076317, rs2278236, rs1044250, and rs11672433 SNPs were 0.29, 0.48, 0.29, and 0.14, respectively, in HELENA and 0.28, 0.46, 0.30, and 0.16 in MONICA Lille. Hardy-Weinberg equilibrium was observed for all four SNPs in the MONICA Lille study and in all but one center for the HELENA sample. The exceptions were rs4076317 and rs2278236 in the HELENA Vienna sample ($P = 0.011$ and $P = 0.047$, respectively), which may be due to the small number of subjects recruited (n = ~100).

We used Thesias (http://ecgene.net/genecanvas) (28) and Haploview (http://www.broadinstitute.org/haploview/haploview) software to evaluate the LD between the four *ANGPTL4* SNPs in the MONICA Lille study (Table 3). All SNPs were in weak LD with the others ($r^2 < 0.50$). Similar LD pattern was observed in HELENA (data not shown).

We did not find any association between rs2278236, rs1044250, or rs11672433 and any of the studied phenotypes in either the HELENA or the MONICA Lille sample

TABLE 3. LD (D′ and r^2) between the four *ANGPTL4* SNPs in the MONICA Lille study

SNP (frequency)	rs4076317 (0.28)	rs2278236 (0.46)	rs1044250 (0.30)	rs11672433 (0.16)
rs4076317		+1.0	−1.0	−1.0
rs2278236	0.44		−1.0	−1.0
rs1044250	0.17	0.37		−1.0
rs11672433	0.07	0.15	0.08	

D′ values are displayed in the *upper right corner* and r^2 values in the *lower left corner*.

(data not shown). In contrast, we found a significant association between rs4076317 and the percentage of body fat in the HELENA study; *GG* subjects had a significantly higher percentage of body fat than *C* allele bearers (26.2 ± 11.3 *vs.* 23.6 ± 9.6%; $P = 0.02$) (Table 4). This association was still present after further adjustment for physical activity or pubertal status and removal of the Vienna center, in which the Hardy-Weinberg equilibrium was not respected (data not shown).

In the MONICA Lille study, the percentage of body fat was not available, but *GG* subjects had a higher waist-to-hip ratio than *C* allele bearers (0.892 ± 0.09 *vs.* 0.885 ± 0.10; $P = 0.02$). Given that 1) *ANGPTL4* is a direct target gene of PPARs (12), and 2) we have previously reported that the *PPARG* Pro12Ala (rs1801282 C > G) polymorphism was associated with adiposity parameters in the MONICA Lille study (29), we looked at whether this *ANGPTL4* SNP interacted with the *PPARG* Pro12Ala polymorphism regarding anthropometric measurements. In the MONICA Lille sample, we detected a significant gene-gene interaction ($P = 0.007$) between *ANGPTL4* rs4076317 (used in a recessive model) and *PPARG* Pro12Ala (used in a dominant model) for the waist-to-hip ratio. Indeed, the group of subjects carrying both the *PPARG Ala12* allele and the *ANGPTL4* rs4076317 *GG* genotype (n = 18) had a higher waist-to-hip ratio ($P = 0.0004$) than the three other subject groups (Fig. 1). In the HELENA study, no such gene-gene interaction could be detected, possibly because of the youth of the subjects.

Discussion

In the present work, we examined the associations between *ANGPTL3* and *ANGPTL4* common genetic variability and metabolic traits in adolescent and adult population-based studies. rs11207997 (the only frequent SNP described in the *ANGPTL3* gene) was consistently associated with lower plasma HDL-cholesterol and ApoA1 levels in both adolescents and adults. Several GWAS have described associations between three intergenic SNPs nearby the *ANGPTL3* locus (rs1748195, rs12130333, and rs10889353) and plasma TG levels (15–17). These SNPs are located at 13.6 kbp upstream and 47 kbp and 121 kbp downstream from the *ANGPTL3* gene, respectively but, together with rs11207997, belong to the same haplotype block (see Supplementary Fig. 1). Contrary to these GWAS, we did not detect significant association between rs11207997 and plasma TG levels. However, the effect size on TG levels described in these GWAS is too small to be detected in our study. Indeed, Willer *et al.* (15) reported a 7 mg/dl difference in TG levels between alleles,

TABLE 4. Association between *ANGPTL4* rs4076317 and anthropometric and biochemical phenotypes

	HELENA						MONICA Lille					
	CC	CG	GG	*P* trend	*P* (CG/GG *vs.* CC)	*P* (GG *vs.* CC/CG)	CC	CG	GG	*P* trend	*P* (CG/GG *vs.* CC)	*P* (GG *vs.* CC/CG)
n	593	439	112				584	474	90			
BMI (kg/m²)	21.5 ± 3.7	21.1 ± 3.7	21.7 ± 4.1	0.25	0.07	0.64	26.6 ± 5.1	26.6 ± 4.8	27.3 ± 5.5	0.23	0.50	0.11
Waist/hip ratio	0.793 ± 0.07	0.793 ± 0.07	0.806 ± 0.08	0.38	0.59	0.15	0.885 ± 0.1	0.885 ± 0.09	0.892 ± 0.09	0.32	0.98	**0.02**
Body fat (%)	23.6 ± 9.6	23.5 ± 9.6	26.2 ± 11.3	0.29	0.83	**0.02**		NA				
Triglyceride (mmol/liter)[a]	0.80 ± 0.43	0.76 ± 0.35	0.83 ± 0.43	0.97	0.87	0.52	1.44 ± 1.22	1.32 ± 1.11	1.33 ± 0.84	0.28	0.11	0.71
HDL-cholesterol (mmol/liter)[a]	1.41 ± 0.27	1.45 ± 0.27	1.42 ± 0.29	0.20	0.09	0.98	1.52 ± 0.50	1.52 ± 0.46	1.39 ± 0.44	0.19	0.96	0.08
LDL-cholesterol (mmol/liter)[a]	2.46 ± 0.65	2.43 ± 0.62	2.47 ± 0.73	0.83	0.68	0.85	3.74 ± 1.01	3.76 ± 1.01	3.78 ± 1.25	0.93	0.72	0.81
ApoA1 (g/liter)[a]	1.49 ± 0.22	1.52 ± 0.23	1.50 ± 0.20	0.20	0.09	0.97	1.74 ± 0.31	1.74 ± 0.31	1.66 ± 0.29	0.28	0.71	0.11
ApoB (g/liter)[a]	0.66 ± 0.16	0.65 ± 0.15	0.65 ± 0.17	0.44	0.35	0.88	1.20 ± 0.30	1.19 ± 0.28	1.22 ± 0.35	0.68	0.86	0.38
Glucose (mmol/liter)[b]	5.02 ± 0.40	5.00 ± 0.40	4.98 ± 0.37	0.19	0.23	0.34	5.37 ± 1.14	5.34 ± 0.89	5.36 ± 0.91	0.91	0.87	0.74
Insulin (μIU/ml)[b]	9.89 ± 5.91	9.90 ± 5.98	10.22 ± 5.37	0.88	0.84	0.99	11.91 ± 7.81	11.77 ± 8.34	11.64 ± 7.25	0.53	0.36	0.38

Data are expressed as means ± SD. *P* values are adjusted for confounding variables. Significant *P* values are indicated in *bold*. NA, Not available.

[a] Subjects treated with lipid-lowering therapy were excluded in MONICA Lille.

[b] Subjects treated with antidiabetic drugs were excluded in MONICA Lille.

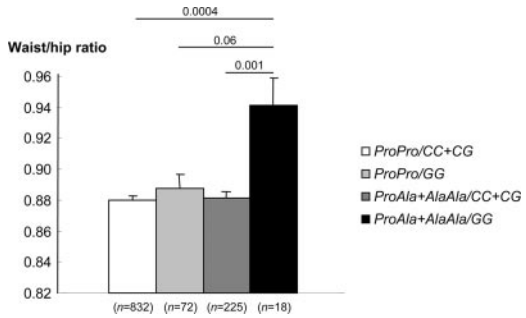

FIG. 1. Analysis of the gene-gene interaction between the *PPARG* Pro12Ala and *ANGPTL4* rs4076317 *C/G* polymorphisms. Data are expressed as means adjusted for age, gender, smoking status, alcohol consumption, and physical activity, ± SEM. Nonparametric tests were used. Subjects carrying the *PPARG ProPro* genotype and the *ANGPTL4* rs4076317 *C* allele (n = 832) are represented in *white*. Subjects carrying the *PPARG ProPro* and the *ANGPTL4* rs4076317 *GG* genotypes (n = 72) are represented in *light gray*. Subjects carrying the *PPARG Ala12* and the *ANGPTL4* rs4076317 *C* alleles (n = 225) are represented in *dark gray*. Subjects carrying both the *PPARG Ala12* allele and the *ANGPTL4* rs4076317 *GG* genotype (n = 18) are represented in *black*. The *P* value for the *PPARG* x *ANGPTL4* interaction was 0.007. $P = 0.0004$ for the comparison between *ProAla+AlaAla/GG* and the three other groups.

but the statistical power of the MONICA Lille study was only 29% to detect such a difference.

Unlike the present study, these GWAS did not report any significant associations between *ANGPTL3* SNPs and HDL-cholesterol levels. Nevertheless, in the GWAS of Kathiresan *et al.* (16), the rs10889353 SNP in very high LD with rs11207997 ($r^2 = 0.88$) was associated with lower plasma HDL-cholesterol ($P = 0.04$), LDL-cholesterol ($P = 0.016$), total cholesterol ($P = 0.00024$), ApoB ($P = 0.014$), and ApoC3 ($P = 0.004$) levels in Framingham Heart Study second-generation participants. Our results may be overestimated but are coherent with these findings and with the role of ANGPTL3 in HDL metabolism: 1) ANGPTL3 inhibits endothelial lipase (8), which is a key regulator of HDL metabolism, that hydrolyzes HDL phospholipids and decreases plasma HDL levels (30, 31); and 2) human plasma ANGPTL3 levels are correlated with plasma HDL-cholesterol levels but not plasma TG levels (8), suggesting that the role of ANGPTL3 in regulation of HDL metabolism may be as important as its role in regulation of TG metabolism.

The rs11207997 polymorphism is located within the promoter region of the *ANGPTL3* gene, 1286 bp upstream from the transcription start site. Using Genomatix MatInspector and ElDorado software (www.genomatix.de) (32), we observed that a binding site for neurogenic differentiation factor 1 (NeuroD1) was identified in the presence of the rs11207997 C allele (matrix similarity = 1.00; core similarity = 0.98) and abolished in the presence of the minor T allele. By RT-PCR, we checked whether ANGPTL3 and NeuroD1 were coexpressed in brain (NeuroD1's main site of expression) or liver (ANGPTL3's main site of expression). NeuroD1 mRNA could be detected in brain but not in liver and vice versa for ANGPTL3 mRNA (Supplementary Fig. 2), ruling out the hypothesis whereby NeuroD1 could regulate ANGPTL3 expression. Therefore, further studies are needed to unravel the molecular mechanisms underlying the genetic associations.

Four *ANGPTL4* SNPs were analyzed in the present study. Several studies on *ANGPTL4* genetic variability have revealed associations with lipid-related phenotypes. Romeo *et al.* (33) showed that the rare E40K variant (frequency, 0.03) was associated with lower TG levels and higher HDL-cholesterol levels. This variant was also associated with lower plasma TG and LDL-cholesterol levels, higher HDL-cholesterol levels, lower BMI, and lower risk of coronary heart disease in the Atherosclerosis Risk in Communities study (34). Staiger *et al.* (35) did not find any associations between the four *ANGPTL4* SNPs investigated in the present study and metabolic traits in a low-powered study (n = 629) in which 76% of the subjects had a family history of diabetes (35). Talmud *et al.* (36) detected associations between the rs1044250 (T266M) polymorphism and lower plasma TG and higher HDL-cholesterol levels in a sample of 2772 men, although this effect was entirely due to the E40K mutation. We analyzed the rs1044250 (T266M) SNP in our population samples but did not find any association. We did not assess the effect of the E40K variant because the low MAF (0.03) of this mutation meant that only five subjects would have carried the *K40* allele in our sample. More recently, a GWAS has shown an association between rs2967605 near *ANGPTL4* and plasma HDL-cholesterol levels in approximately 35,000 individuals (17). This SNP is located 30 kbp downstream from *ANGPTL4* and is in weak LD ($r^2 < 0.33$ in HapMap) with the ones we studied and that are located within *ANGPTL4*. The association between rs2967605 and HDL-cholesterol level reported in this GWAS may therefore not be due to ANGPTL4.

The association we detected between rs4076317 in *ANGPTL4* and body fat in adolescents or waist-to-hip ratio in adults has not been shown before. However, *ANGPTL4* genetic variability has been already associated with adiposity because Folsom *et al.* (34) described an association between the *ANGPTL4* E40K rare variant and BMI. To our knowledge, no *ANGPTL4* SNPs have been significantly associated with adiposity parameters in GWAS. However, due to the required very low *P* value threshold in GWAS ($<10^{-8}$), SNPs in *ANGPTL4* might have been nominally associated with these parameters and not actually reported. In MONICA Lille, we detected a gene-gene interaction between *ANGPTL4* and *PPARG* when considering the waist-to-hip ratio. *ANGPTL4* is a direct target gene of PPARγ, a major orchestrator of adipocyte differentiation (37), and the

PPARG Pro12Ala polymorphism is known to be associated with fat mass (38). The fact that the waist-to-hip ratio was the only anthropometric parameter that came out of this gene-gene interaction suggests that the role of ANGPTL4 on fat mass might vary from one fat depot to another. However, because the number of subjects carrying minor alleles of *PPARG* and *ANGPTL4* SNPs is small, these data need to be taken with caution and replicated in other population samples. If this gene-gene interaction was confirmed, it could also explain the fact that ANGPTL4 has not been associated with obesity in GWAS. Further studies are needed to validate this hypothesis.

The association between *ANGPTL4* genetic variation and adiposity is coherent with the role of the protein in fat mass regulation. First, LPL (which is inhibited by ANGPTL4) genetic variability is associated with obesity (39). Second, Angptl4 plays an important role in the intestine as a mediator of the microbial regulation of peripheral fat storage (13). Last but not least, Angptl4 induces adipose TG lipase expression, which stimulates adipose tissue lipolysis and therefore promotes adipose tissue weight loss (5). The rs4076317 polymorphism is located within the promoter region of the *ANGPTL4* gene, 12 bp upstream from the transcription start site. According to Genomatix Eldorado software, there was no difference between the rs4076317 *C* and *G* alleles in terms of different predicted binding sites.

In conclusion, the observed associations between *ANGPTL3* polymorphism and plasma HDL-cholesterol and ApoA1 levels (as early as in adolescence) underline the role of ANGPTL3 in HDL-cholesterol metabolism in humans. Our data also suggest associations between *ANGPTL4* rs4076317 and indicators of fat mass. Although our findings warrant replication, they imply that (as in the mouse) ANGPTL4 is involved in the signaling pathways in fat and other tissues that prevent fat storage and stimulate fat mobilization.

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