LPIN2 Is Associated With Type 2 Diabetes, Glucose Metabolism, and Body Composition

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OBJECTIVE —To identify the type 2 diabetes gene located at chromosome 18p11.

RESEARCH DESIGN AND METHODS —We investigated the region in a young genetically isolated population by genotyping 34 single nucleotide polymorphisms (SNPs) in 78 case subjects and 101 control subjects. Two SNPs were selected and followed up in two cohorts. The first cohort came from a general Dutch population. In this cohort, association with type 2 diabetes was investigated using 616 type 2 diabetic case subjects and 2,890 control subjects; association with oral glucose tolerance test data was performed in 361 normoglycemic people. Association with fat distribution was studied in the second replication cohort, consisting of 836 people from the genetically isolated population.

RESULTS —At the initial step, we found that the common C allele of SNP rs3745012 was associated with type 2 diabetes (odds ratio 2.01, $P = 0.03$). This SNP is located at the 3′ untranslated region of the *LPIN2* gene, which is a plausible candidate for type 2 diabetes and obesity. In the cohort from the general Dutch population, we demonstrated that rs3745012 interacts with BMI in determination of type 2 diabetes: whereas in subjects with high BMI, the common C allele is associated with type 2 diabetes, the same allele exhibits a neutral or protective effect in lean subjects ($P = 0.05$ overall effect, $P = 0.02$ interaction). Most remarkably, rs3745012 strongly affected composite insulin sensitivity index ($P = 0.006$ for overall effect, $P = 0.004$ for interaction). In the second replication cohort, we found that the allele C of rs3745012 increases trunk-to-legs fat mass ratio ($P = 0.001$) and may affect other fat-related measurements.

CONCLUSIONS —rs3745012 SNP of the *LPIN2* gene is associated with type 2 diabetes and fat distribution. ***Diabetes* 56: 3020–3026, 2007**

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DEXA, dual-energy X-ray absorptiometry; ERF, Erasmus Rucphen Family; ISI, insulin sensitivity index; OGTT, oral glucose tolerance test; PPAR, peroxisome proliferator–activated receptor; SNP, single nucleotide polymorphism; UTR, untranslated region; WHR, waist-to-hip ratio.

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The majority of type 2 diabetic patients are obese, and obesity by itself may be a cause of insulin resistance (1,2). An important determinant of insulin resistance is not only total fat mass, but also fat distribution (3,4). Given the same degree of obesity, insulin sensitivity varies largely depending on visceral adipose tissue content (5,6). Abdominal obesity measures and visceral fat content may be better predictors of insulin resistance when compared with BMI (7). Also, abnormally decreased adipose tissue mass, such as that observed in lipodystrophy, leads to severe insulin resistance in humans (8,9) and animal models (10–13).

Several known genes for type 2 diabetes are also implicated in lipodistrophy. Examples include peroxisome proliferator–activated receptor γ (*PPARG*) (14), which plays a critical role in the differentiation of preadipocytes to mature fat cells (15). A common 12Pro polymorphism in this gene is associated with increased risk of type 2 diabetes in the general population (16,17), and rare deleterious variants lead to familial partial lipodystrophy (9,18). Another example is the lamin A gene, which is implicated in rare Dunnigan-type familial partial lipodystrophy (8). This gene also plays a role in type 2 diabetes and related phenotypes (19–22). The *LPIN1* gene was recently shown to be associated with serum insulin levels and BMI in a Finnish population (23). This gene appeared as a possible candidate involved in human glucose metabolism because it is one of the human homologs of mouse *Lpin1* gene. *Fdl* mice, in which *Lpin1* is deleted, have diminished adipose tissue mass and multiple pathologies, including insulin resistance, fatty liver, and progressive neuropathy of peripheral nerves (11,13). The protein encoded by *Lpin1*, Lipin, is required upstream of PPARG for normal adipocyte differentiation (24).

We reported a genome-wide scan using a combined association and linkage test in a sample of type 2 diabetic patients derived from a recently genetically isolated population from the Southwest of the Netherlands (25). Evidence for association with type 2 diabetes was found for one region at chromosome 18p11 ($P = 0.001$ at marker D18S63). The locus at 18p was originally found to be linked to type 2 diabetes in families from Finland and Southern Sweden (26) and was confirmed in a second series derived from the Southwest of the Netherlands (27). In all three studies, the effect of the 18p region was strongest in the obese subpopulation.

One of the human homologs of mouse *Lpin1* gene, *LPIN2*, is located in the 18p11 region. In this study, we

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examine the association between type 2 diabetes and related traits and *LPIN2* gene in three cohorts.

RESEARCH DESIGN AND METHODS

Study population. Two series of patients and control subjects were studied. The first was derived from a young genetically isolated population located in the Southwest of the Netherlands. The characteristics of the population regarding linkage disequilibrium and drift are described elsewhere (28–30). The original sample was formed by 79 small families (117 patients) that could be traced to a common ancestor within 13 generations. The diagnosis of type 2 diabetes was based on American Diabetes Association criteria (31). The details of the recruitment procedure and analysis are described elsewhere (25). The study protocol was approved by the medical ethics committee of the Erasmus Medical Centre Rotterdam, and written consent was obtained from all participants.

In the present study, we used 78 patients who were probands in our previous research and who were not or were remotely (>10 meioses) related. The unrelated spouses of the type 2 diabetic patients, together with the spouses of patients from other ongoing studies, were used as control subjects ($n = 101$). This schema justifies the use of the sample as independent case and control subjects.

No significant differences in sex ratio or age between patients and control subjects were observed ($P > 0.1$). The BMI was significantly higher in patients (30.21) than in control subjects (27.53, $P = 0.03$).

The second series was derived from the Rotterdam Study. This is a large cohort from outbred population that is aimed to study prevalence, incidence, and determinants of chronic disease in the elderly (32). The medical ethics committee of the Erasmus Medical Centre Rotterdam approved the study protocol. Baseline examinations, including a detailed interview, physical examination, and blood sampling, were conducted between 1990 and 1993.

In the Rotterdam Study, 3,506 people were genotyped. Diagnosis of type 2 diabetes was made based on the use of antidiabetic medication or random glucose level >11.1 mmol/l, according to the previous work of Rietveld et al. (33). Of the investigated group, 616 were affected. The effect of sex was not significant. Type 2 diabetic people were on average older (73.46 vs. 68.09 years in control subjects, $P < 0.001$) and had higher BMI (26.76 vs. 26.14 kg/m^2 in control subjects, $P < 0.01$) and waist-to-hip ratio (WHR) (0.93 vs. 0.90 in control subjects, $P < 0.001$).

Within the Rotterdam Study, oral glucose tolerance test (OGTT) was performed for 361 normoglycemic people, thus making fasting glucose and insulin and postload glucose and insulin at 30 and 120 min available.

To study body fat distribution, we used data on 836 diabetes-free individuals from the family-based Erasmus Rucphen Family (ERF) study. The study protocol was approved by the medical ethics board of Erasmus Medical Centre Rotterdam. The ERF study consists of ~3,000 people who are descendants of 22 couples who lived in the second half of the 19th century.

Measurements. In the Rotterdam study, at baseline examination, information concerning health status and drug use was obtained using a computerized questionnaire. Height and weight were measured, and BMI (kg/m^2) was calculated. Blood sampling and storage have been described elsewhere (34). Serum was separated by centrifugation and quickly frozen in liquid nitrogen. Baseline measurements were performed on nonfasting blood samples. Glucose levels were measured by the glucose hexokinase method in fasting serum and postload serum samples (35).

Glucose level was measured in millimoles per liter and insulin in milliunits per liter. As indicator of insulin resistance we used HOMA-IR, as suggested by Matthews et al. (36):

$$\text{HOMA-IR} = \text{FGL} \times \text{FIL}/22.5$$

where FGL is fasting glucose level and FIL is fasting insulin level. As indicator of insulin sensitivity, which uses the data from the OGTT, we used the index suggested by Matsuda and DeFronzo (37):

$$\text{ISI} = 10{,}000/\sqrt{(\text{FGL} \times \text{FIL} \times \text{MEAN_GL_IN_OGTT} \times \text{MEAN_IL_IN_OGTT})}$$

where MEAN_GL_IN_OGTT and MEAN_IL_IN_OGTT are mean glucose and insulin levels in OGTT, respectively. In the ERF study, anthropometric measurements and total-body imaging (using dual-energy X-ray absorptiometry [DEXA]) were done with participants in their underwear and without shoes. Using soft tape, with the subject in standing position, waist circumference was measured midway between the lower costal margin and the iliac crest, while hip circumference was taken at the hip's widest diameter. WHR was then computed. Height and weight were also measured. BMI was calculated as weight in kilograms divided by the square of height in meters. All DEXA scans were performed in a calibrated Prodigy total body fan-beam densitometer and analyzed with the enCORE 2002 software v.6.70.021 (GE Lunar, Madison, WI). Other parameters estimated by DEXA include fat and lean mass measurements for total body, trunk, and legs.

Genotyping. Genomic DNA for each individual was extracted from 10 ml of whole blood using the Puregene kit (Gentra Systems, Minneapolis, MN) and protocol recommended by the manufacturer.

In the initial scan, all single nucleotide polymorphisms (SNPs) were genotyped using the Pyrosequencing technology according to the manufacturer's instructions (Biotage, Uppsala, Sweden). For each assay, a standard PCR was performed in a total volume of 10 μl using 20 ng of genomic DNA, 10 pmol of each primer, one of which was biotinylated at the 5′ end, and AmpiTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). A full list of primer sequences can be found in Supplementary Table 1 (available in an online appendix at http://dx.doi.org/10.2337/db07-0338).

The biotinylated PCR product was bound to magnetic streptavidin-coated Dynabeads (Dynal, Oslo, Norway) by shaking at 65°C for 15 min and then denatured in 0.5 mol/l NaOH. DNA bound to the Dynabeads was transferred between solutions using the magnetic PSQ Sample Tool (Biotage). After washing to remove excess NaOH, the single-stranded template was hybridized to 15 nmol sequencing primer by incubating at 80°C for 2 min and cooling for 15 min. The pyrosequencing reaction was then carried out in a PSQ96 instrument (Biotage) using the PSQ SNP Reagent kit (Biotage). Genotypes were analyzed using the PSQ96 Analysis Software (Biotage).

In the ERF and Rotterdam Study, genotyping was performed using Taqman allelic discrimination Assay-By-Design (Applied Biosystems) (38). Forward and reverse primer sequences and the minor groove binding probes are presented in Supplementary Table 2. We used the reverse-strand design for SNP9 and SNP10. The assays used 5 ng genomic DNA and 5-μl reaction volumes. The amplification and extension protocol was as follows: an initial activation step of 10 min at 95°C preceded 40 cycles of denaturation at 95°C for 15 s and annealing and extension at 50°C for 60 s. Allele-specific fluorescence was then analyzed on an ABI Prism 7900HT Sequence Detection System with SDS v. 2.1 (Applied Biosystems). Based on the analysis of 5% of blind duplicates, there was a 100% concordance in genotyping this polymorphism.

Expression analysis. To access functionality of the potential miR-371 target site at the *LPIN2* 3′ untranslated region (UTR), we fused 780 bp of the *LPIN2* 3′ UTR to a firefly luciferase reporter gene and injected mRNA derived from this construct, together with a Renilla luciferase control reporter, in the zebrafish embryo. In this experiment, reporter signal is expected to decrease if micro RNA (miRNA)-target interaction happens (39,40).

To study expression of *LPIN2*, whole-blood samples were collected from seven participants of the Rotterdam Study. Ten milliliters of peripheral venous blood was drawn from each donor into heparin-containing Vacutainer tubes (BD Biosciences, Breda, the Netherlands). Selective lysis of erythrocytes was achieved by the addition of three volumes of hypotonic buffer (150 mmol/l ammonium chloride, 10 mmol/l potassium bicarbonate, and 1 mmol/l EDTA disodium salt) to whole blood and incubation on ice for 20 min. Nucleated blood cells were then collected with centrifugation at 500*g* for 10 min at 4°C and washed twice with ice-cold PBS before RNA extraction. Total RNA was extracted from leukocytes with RNABee reagent (Tel-Test, Friendswood, TX) according to the manufacturer's protocol. RNA quality was determined with an A_{260}-to-A_{280} ratio and capillary electrophoresis on an Agilent 2100 Bioanalyzer automated analysis system (Agilent Technologies, Palo Alto, CA). Hybridization of the RNA to Affymetrix GeneChip Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA) was performed by the microarray core facility of the Erasmus MC Center for Biomics according to Affymetrix specifications. Background subtraction, probe signal summarization, and normalization were calculated according to the RMA algorithm using the R Bioconductor (41). *LPIN2* gene is represented by three probe sets on Affymetrix U133 plus2 microarray: probe sets 202459_s_at and 202460_s_at target different regions of the 3′ UTR; the probe set 244799_s_at targets the exon IV of *LPIN2* transcript. The position of Affymetrix target sequences for *LPIN2* gene on genomic alignment is shown in Supplementary Fig. 1.

Statistical analysis. ANOVA and Wilcoxon tests were used for crude comparison of means between groups. For single SNPs, the crude comparison of frequencies between case and control subjects was performed using the Fisher's exact test. Adjusted analysis of relation between type 2 diabetes status and genotypes was performed using logistic regression. For quantitative traits, linear regression analysis was performed. In population-based cohorts, all analyses were performed using freely available R 1.9.0 software (available from http://www.r-project.org/).

In the family-based ERF study, we assessed association using a variance-components model, as implemented in the SOLAR software (42). The null hypothesis of no association was formulated using the polygenic model with covariates. The hypothesis of association was obtained by adding SNP (coded as −1 for AA, 0 for AB, and 1 for BB) as a covariate. To test significance of association, the likelihood ratio test was performed: under the null, twice the

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TABLE 1
Association between SNP located in chromosome 18p11 region and type 2 diabetes in the genetically isolated population

Study name	dbSNP name	Position	Change A>B	Control subjects AA	Control subjects AB	Control subjects BB	Case subjects AA	Case subjects AB	Case subjects BB	OR (95% CI)	*P* value
SNP1	rs493422	192,494	C>T	50	35	3	35	27	3	1.12 (0.64–1.96)	0.69
SNP2	rs1275329	846,696	A>T	63	22	5	47	16	1	0.76 (0.38–1.47)	0.43
SNP3	rs2856966	897,710	A>G	50	36	7	36	31	4	1.03 (0.61–1.73)	1.00
SNP4	rs160048	1,111,731	G>T	44	40	5	44	24	3	0.69 (0.39–1.19)	0.19
SNP5	rs313020	1,873,404	A>T	25	46	17	21	31	11	0.87 (0.53–1.42)	0.64
SNP6	rs770226	2,487,437	T>C	33	45	12	21	28	15	1.33 (0.82–2.17)	0.24
SNP7	rs680666	2,681,426	A>G	32	35	23	21	32	10	0.86 (0.53–1.40)	0.56
LPN213	rs7231822	2,896,086	G>A	30	54	11	24	31	10	1.03 (0.64–1.67)	0.91
SNP8	rs606491	2,899,608	C>T	88	3	0	63	5	1	3.18 (0.71–19.39)	0.11
LPN214	rs649624	2,900,578	C>T	69	20	3	46	15	2	1.08 (0.54–2.14)	0.87
LPN215	rs1985	2,907,223	A>T	42	45	7	32	24	8	0.99 (0.59–1.66)	1
SNP9	rs3745012	2,910,288	C>T	48	40	5	44	18	1	**0.51 (0.27–0.94)**	**0.03**
SNP10	rs3737514	2,910,725	T>A	76	16	1	49	12	0	0.98 (0.43–2.33)	1.00
LPN216	rs2282635	2,911,444	A>G	48	39	8	35	28	2	0.80 (0.46–1.37)	0.44
LPN219	rs647195	2,926,036	G>A	28	49	17	26	32	7	1.44 (0.89–2.35)	0.13
LPN223	rs641287	2,936,833	T>A	44	42	8	32	29	4	1.12 (0.67–1.89)	0.71
LPN229	rs8094920	2,973,306	G>A	63	28	2	50	12	1	1.66 (0.82–3.53)	0.15
LPN220	—	2,985,195	C>T	38	44	12	31	27	7	0.81 (0.49–1.34)	0.4
SNP11	rs591549	3,532,247	G>A	43	36	11	21	33	11	1.54 (0.94–2.53)	0.07
SNP12	rs562121	4,225,179	G>C	23	41	26	23	33	8	**0.58 (0.36–0.94)**	**0.02**
SNP13	rs280995	4,429,291	A>G	48	37	7	34	31	7	1.18 (0.71–1.96)	0.54
SNP14	rs1619055	4,580,320	T>C	26	41	25	16	35	17	1.05 (0.66–1.68)	0.91
SNP15	rs1719945	5,506,106	G>A	61	28	3	44	20	0	0.82 (0.42–1.55)	0.55
SNP16	rs558673	5,898,869	G>A	60	25	5	40	28	3	1.30 (0.74–2.30)	0.34
SNP17	rs341179	6,428,187	A>G	25	48	18	22	33	13	0.89 (0.56–1.43)	0.65
SNP18	rs478879	6,998,269	C>T	48	39	3	39	18	7	1.00 (0.57–1.74)	1.00
SNP19	rs517800	7,424,689	G>C	67	19	1	51	15	1	1.06 (0.50–2.21)	0.86
SNP20	rs331418	7,806,276	C>T	29	46	15	21	28	15	1.13 (0.70–1.84)	0.64
SNP21	rs546102	8,717,770	T>C	48	31	11	30	30	10	1.33 (0.81–2.19)	0.28
SNP22	rs1785560	9,106,080	T>C	63	26	0	49	18	2	1.11 (0.57–2.15)	0.75
SNP23	rs329029	9,452,875	C>T	32	47	8	28	31	5	0.83 (0.50–1.38)	0.46
SNP24	rs621707	9,666,409	G>A	70	19	1	48	16	0	1.08 (0.50–2.28)	0.86
SNP25	rs264229	10,967,539	A>G	25	42	23	11	42	12	1.08 (0.67–1.74)	0.82
SNP26	rs457329	11,297,693	C>T	73	12	2	45	18	2	2.01 (0.96–4.29)	0.051

Significant *P* values (≤0.05) and ORs are indicated in bold. *P* values comes from the Fisher's exact test. SNP, SNPs typed in the first batch; LPN, SNPs typed in the second batch. Position in base pairs, as given in Ensembl database. OR from allelic 2×2 table, the allele listed first in "Change" column is used as a reference.

difference between log-likelihood of these nesting models is distributed as χ^2 with 1 degree of freedom (df). Total, trunk, and leg lean tissue mass, lean mass index, and the ratio between trunk and leg fat tissue masses were log transformed. Total, trunk, and leg fat tissue mass, fat mass index, and ratio between trunk and leg fat tissue masses underwent square root transformation. During the analysis, we considered nominal *P* values ≤0.05 as significant.

RESULTS

Fine-mapping of 18p11 in a young genetically isolated population. With 26 SNPs, we covered the telomeric region 18p11.22–18p11.32 spanning from 0.2 to 12.3 Mb. Table 1 shows SNPs typed, their position, counts of subjects with different genotypes, and ORs with *P* values. In total, 78 type 2 diabetic patients and 101 control subjects were genotyped. The average heterozygosity of the SNP markers was 38% with a minimum of 6% and maximum of 50%. The average coverage was one SNP per 465 kb, with a maximal gap of 1.3 Mb (Table 1 and Supplementary Fig. 2*A* and *B*).

In our previous analysis, the strongest association was found for D18S63 (position 3,428,540 in the Ensembl database) and D18S1105 (position 1,823,900), flanking the *LPIN2* gene region. In the current SNP analysis, association was found for two SNPs, SNP9 and SNP12 (Table 1). For SNP9, after adjustment for sex and age, the common allele C was associated with odds ratio (OR) 2.01 (95% CI 1.09–3.71, $P = 0.03$); the frequency of C was 0.84 in case subjects and 0.73 in control subjects. For SNP12, the G allele was associated with type 2 diabetes (OR = 1.66, $P = 0.03$, after adjustment for age and sex).

The region showing association with type 2 diabetes (SNP9–SNP12) contains six hypothetical and seven known genes: *EMILIN2*, *LPIN2*, *MYOM1*, *MRCL3*, *MRLC2*, *TGIF*, and *DLGAP1*. From these seven, *LPIN2* was studied further because of the association of its 3′ UTR SNP9 with type 2 diabetes and the analogy to mouse *Lpin1*, the gene involved in insulin resistance in the *fdl* mouse (13,43). According to the HapMap data, *LPIN2* is included into a large block of linkage disequilibrium; the boundaries of the block coincide well with the gene boundaries (Supplementary Fig. 3). We typed eight additional SNPs in the *LPIN2* gene (Table 1; SNPs with study name starting with "LPN;" Supplementary Fig. 2*B*). However, no strong association with additional SNPs was found.

Role of *LPIN2* in the general population. We next conducted a study in a large outbred population, the

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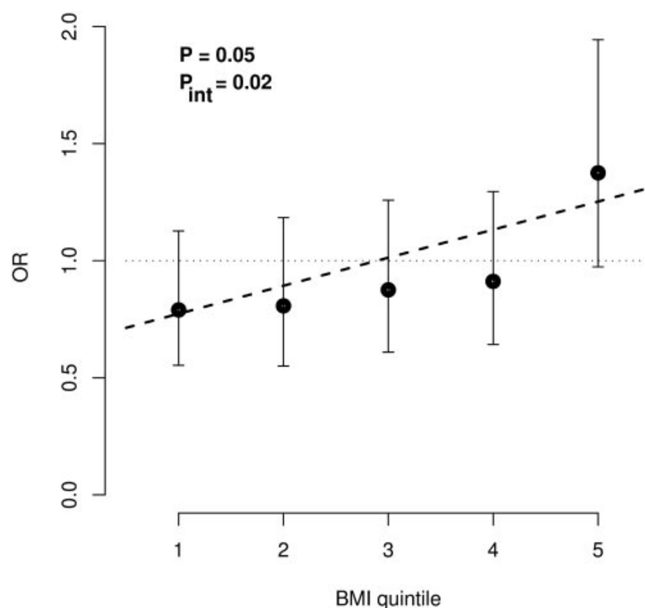

FIG. 1. Effect of SNP9C allele (adjusted for age, age^2, sex, and WHR) on the risk of type 2 diabetes by BMI quintiles. OR and 95% CI are shown by BMI quintile. Line indicates the ORs expected from the model of additive effect of SNP9C in linear interaction with BMI quintile. *P* corresponds to the *P* value of the test of significance of SNP9 effect and its interaction with BMI (2 d.f.). P_{int} corresponds to the *P* value of the test of interaction (1 d.f.).

Rotterdam Study. We genotyped 3,506 participants (616 with type 2 diabetes) of the Rotterdam Study for SNP9 and SNP10. Both SNP9 and SNP10 were in Hardy-Weinberg equilibrium ($P > 0.1$). Given the findings that the type 2 diabetes locus at the 18p11 region interacts with BMI in other populations (25–27), we allowed for interaction in the analysis. We defined BMI groups as five quintiles (cutoff points at BMI 23.32, 25.04, 26.79, and 28.93 kg/m^2), coded as 0, 1, 2, 3, or 4. The model consisted of this covariate, SNP9, their interaction, and additional covariates. The interaction of SNP9 with BMI was significant ($P = 0.02$; Fig. 1). Also, the overall test of the effect of SNP9 and BMI was significant ($P = 0.05$). Interestingly, although the common C allele is the risk allele at high BMI (OR 1.38, 95% CI 0.97–1.94 in upper quintile; frequency of C in case subjects 0.79, in control subjects 0.74), the situation is reverse at low BMI (Fig. 1). The ORs and case/control frequencies observed in the highest BMI quintile are similar to those seen in the genetically isolated population among predominantly obese patients (mean BMI of 30.21 vs. 26.76 kg/m^2 in participants of the Rotterdam Study).

We studied the association of SNP9 with early type 2 diabetes pathology in 361 normoglycemic subjects who underwent OGTT (descriptives shown in Supplementary Table 3). For fasting glucose, insulin, and insulin resistance (HOMA-IR), the results, although insignificant, point in the same direction as those of the analysis of type 2 diabetes: the common SNP9C allele is a risk allele at high BMI, whereas in those with low BMI, it appears to decrease fasting glucose, insulin, and insulin resistance (Fig. 2).

We found a significant effect of SNP9 on glucose ($P = 0.04$) and insulin ($P = 0.02$), when studying the area under the curve, and on the insulin sensitivity index (ISI) ($P = 0.006$) (Fig. 2). For all three measures, interaction with BMI was significant. Consistent with our previous findings on type 2 diabetes, the common SNP9C allele was associated with increased risk of early pathology in participants with high BMI, whereas the effect was reversed in those with low BMI (Fig. 2).

SNP9 and body composition. In the outbred population drawn randomly from the Rotterdam Study, we found that SNP9 genotype was associated with BMI, when adjusting for sex and age. The carriers of the common C allele, which was associated with type 2 diabetes in the genetic isolate, had a significantly increased BMI when compared with TT homozygotes (difference of 0.54 kg/m^2, $P = 0.03$). At the same time, the carriers of C had lower WHR when compared with TT homozygotes ($P = 0.02$), suggesting an atypical fat distribution in TT genotype because BMI and WHR are usually positively correlated.

We further studied association between *LPIN2* and body composition in 836 diabetes-free participants of the family-based cohort derived from the EFR study. Table 2 shows studied traits and results of analysis. The family structure was adjusted for within variance components framework. Similar to the findings in the Rotterdam Study, when adjusting for age and sex, the SNP9C was associated with an increased BMI, albeit only marginally significantly (effect of 0.51 kg/m^2, $P = 0.08$). In the ERF study, SNP9C increased both waist (by 1.67 cm, $P = 0.03$) and hip circumference (by 1.03 cm, $P = 0.06$).

In the analysis of DEXA data, we found that SNP9C increased total fat mass by 1.1 kg ($P = 0.05$). This happened almost exclusively through increase in trunk fat mass (by 0.9 kg, $P = 0.01$). A strong positive effect of SNP9C was observed on various adiposity indexes: fat mass index ($P = 0.02$), ratio of total fat to total tissue ($P = 0.01$), ratio of trunk fat to total tissue ($P = 0.001$), and ratio of trunk to legs fat mass ($P = 0.001$) (Table 2).

After adjustment for trunk-to-legs fat mass ratio, the association of SNP9C with waist circumstance, fat mass, trunk fat mass, fat mass index, ratio of total fat to total tissue, and ratio of trunk fat to total tissue became insignificant, suggesting that association to these variables can be explained by the trunk-to-legs fat ratio. The same effect was observed when adjusting for the trunk fat–to–total tissue ratio. These analyses indicate that trunk-to-leg fat (or trunk fat–to–total tissue) mass ratio may be the most important endophenotype associated with SNP9 and type 2 diabetes.

Potential functionality of SNP9. The region containing SNP9 is a potential target site for miRNA miR-371 (Supplementary Fig. 4). Perfect matching of the region to the first eight bases of the miRNA, "seed" sequence, is a strong predictor for regulation of *LPIN2* by miR-371 (44). Although the target site is not conserved beyond primates, the nature of the SNP suggests conservation at the RNA level: change of the common variant (C) sequence to the rare variant T only partially affects potential miRNA-to-mRNA interaction due to possible G:U pairing in the RNA duplex. Whether G:U pairing is tolerated in the seed sequence is not clear and may vary between miRNAs (39,45,46). However, the reporter signal did not decrease after coinjection of a synthetic miR-371 duplex, as would be expected for a true miRNA-target interaction (39,40).

We studied expression of *LPIN2* in seven healthy individuals from the Rotterdam Study. Four of these were homozygous for the SNP9C, and three were C/T heterozygotes. Using exact Wilcoxon's nonparametric test, we showed that the probe sets targeting the 3′ UTR did not differ in expression between T carriers and noncarriers

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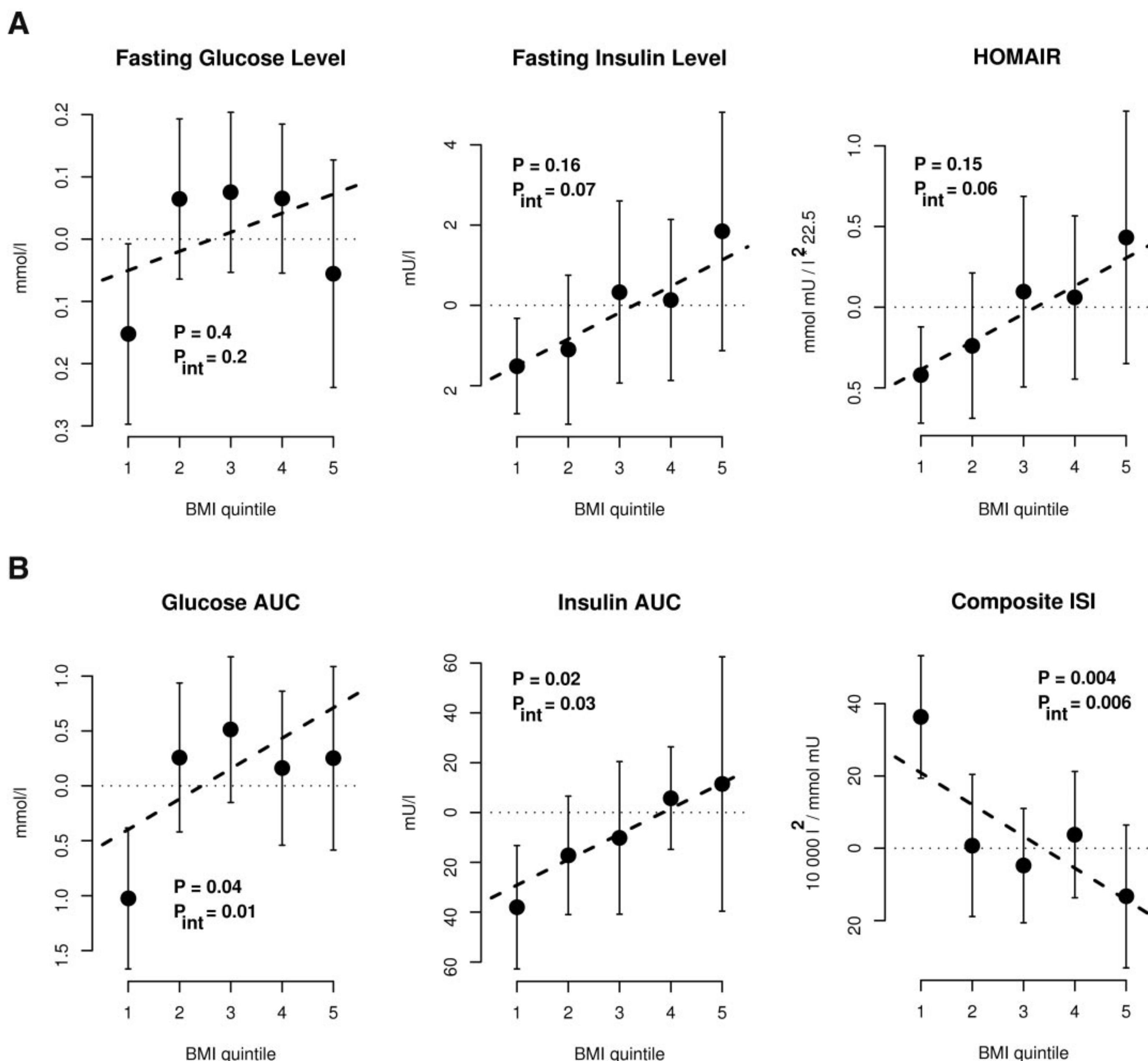

FIG. 2. Effect of SNP9C allele (adjusted for age, age^2, sex, and WHR) on glucose and insulin measurements by BMI quintiles. Mean effect and 95% CI are shown by BMI quintile. Line shows the mean expected from the model.

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($P > 0.2$). However, the probe set 244799_s_at, located in exon IV, did show marginally significant ($P = 0.06$) down-regulation in the carriers of the T allele.

DISCUSSION

In our previous study in a young genetically isolated population, we found evidence for a type 2 diabetes locus at chromosome 18p11. This region was also previously reported by Parker et al. (26) in a Scandinavian study and by van Tilburg et al. (27) in the general Dutch population. Both studies suggested that the effect of the locus is modified by BMI.

In the fine-typing of the 18p11 region with 34 SNPs, the association was found to be confined to the *LPIN2* gene. In a small series of case and control subjects, nominally significant association was found between type 2 diabetes and SNP9 (rs3745012), located in the 3′ UTR of the *LPIN2* gene. We extended the analysis of association between *LPIN2* and type 2 diabetes in an independent series of patients from a large population-based outbred cohort, the Rotterdam Study. In this outbred population, we observed interaction between SNP9 and BMI. Consistent with our findings in the genetically isolated population, in which patients had a high BMI, SNP9C allele was associated with type 2 diabetes in obese participants. In the Rotterdam Study, SNP9 was also associated with BMI and early type 2 diabetes pathology measured using OGTT. Lastly, in a family-based study conducted in the genetically isolated population, we demonstrated that SNP9C is associated with increased adiposity indexes, most notably trunk fat. Together, these studies provide evidence that the SNP9C allele may be a thrifty allele (47,48), i.e., it is common and

TABLE 2
Association between SNP9C and DEXA body measurements in the genetically isolated population

Trait	Mean ± SD	Heritability	Effect of SNP9C	Variance explained (%)	P value
Weights (kg)					
Lean	46.64 ± 10.26	0.52	−0.27 kg	0.12	>0.1
Fat	24.28 ± 9.26	0.27	1.1 kg	0.46	0.050
Trunk fat	13.98 ± 5.54	0.21	0.92 kg	0.78	**0.012**
Trunk lean	23.30 ± 4.80	0.42	−0.09 kg	0.05	>0.1
Leg fat	7.53 ± 3.46	0.45	0.13 kg	0.03	>0.1
Leg lean	14.80 ± 3.66	0.57	−0.12 kg	0.18	>0.1
Ratios					
FMI	9.01 ± 3.59	0.27	0.45 kg/m^2	0.72	**0.021**
LMI	16.95 ± 2.53	0.42	0.05 kg/m^2	0.04	>0.1
Fat/tissue	0.34 ± 0.09	0.30	1.22%	0.80	**0.013**
Trunk fat/tissue	0.19 ± 0.05	0.23	1.03%	1.35	**0.001**
Trunk fat/leg fat	2.02 ± 0.75	0.54	0.14	1.35	**0.001**

Analysis is adjusted for sex, age, age^2, and family structure. Heritability as reported by SOLAR. P values ≤0.05 are indicated in bold. LMI, lean mass index (kg/m^2); FMI, fat mass index (kg/m^2).

increases obesity and risk of type 2 diabetes in obese subjects.

The issue of multiple testing is worth consideration when judging the results of our study. For example, in the first stage, we tested 34 SNPs and found two to be associated to type 2 diabetes with nominal $P < 0.05$. When corrected for multiple testing, these results would have become insignificant. However, in our study, we did not rely on experiment-wise significance achieved at any stage. As suggested by others (49), we attempted to replicate our finding: in stage one we selected SNP9 and followed it up in two other cohorts, achieving positive results. Such replication procedure strongly reduces the chance of a false-positive finding (49).

The *LPIN2* gene is one of the three human homologs of the mouse *Lpin1* gene, which is responsible for the lipodystrophy in the *fld* line (13), which, among other traits, is characterized by severe insulin resistance. The product of *Lpin1* is required upstream of *PPARG* for normal adipocyte differentiation (24).

The effects of increased *Lpin1* expression on insulin resistance are opposite, depending on the tissue. Whereas *Lpin1* overexpression in muscle produces obesity-associated insulin resistance, enhanced *Lpin1* expression in mature adipocytes improves insulin sensitivity (43). A recent study of human homolog of mouse *Lpin1* gene, *LPIN1*, has shown its potential involvement in glucose metabolism (23).

LPIN2, being a member of the same lipin family as the *LPIN1* and bearing notable sequence similarity to both mouse *Lpin1* and human *LPIN1*, is therefore a plausible candidate gene for type 2 diabetes and body composition. Human *LPIN2* contains 20 exons and spans ~115 kb. The deduced *LPIN2* cDNA contains 6,245 nucleotides. A nuclear localization signal is located close to NH_2 terminus and is highly conserved among Lipin proteins. *LPIN2* is ubiquitously expressed in different tissues, including skeletal muscle (50).

The most important question is how SNP9 may be related to the risk of type 2 diabetes. SNP9 is a noncoding polymorphism located in the 3′ UTR of the gene at the third nucleotide downstream of the stop codon (Supplementary Fig. 4). Strong computational evidences suggested that the region containing SNP9 is a potential target site for miRNA miR-371. However, our experiments on coexpression of luciferase reporter and miRNA do not reveal detectable interaction between miR-371 and the *LPIN2* 3′ UTR. We therefore have to conclude that, in line with recent observations (46), perfect seed matching is not sufficient to confer regulation in case of miR-371. Alternatively, our heterologous expression system may be not sensitive enough to detect possible subtle effects of miR-371 on expression of *LPIN2*.

In our limited expression experiments, we observed increased levels of *LPIN2* expression in people homozygous for the "risk" C alleles. Although these results reach only marginal significance ($P = 0.06$), we may hypothesize that SNP9 polymorphism (or other *LPIN2* polymorphism in linkage disequilibrium with SNP9) leads to altered expression of the *LPIN2* gene. This, by analogy with the mouse model, may lead to (possibly obesity-induced) type 2 diabetes. Further investigation, including larger expression and protein studies are required to establish the link between *LPIN2*, obesity, and type 2 diabetes.

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