

# Effects of Genetic Variation in the Human Retinol Binding Protein-4 Gene (*RBP4*) on Insulin Resistance and Fat Depot–Specific mRNA Expression

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**OBJECTIVE**—Serum retinol binding protein 4 (RBP4) is a new liver- and adipocyte-derived signal that may contribute to insulin resistance. Therefore, the *RBP4* gene represents a plausible candidate gene involved in susceptibility to type 2 diabetes.

**RESEARCH DESIGN AND METHODS**—In this study, the *RBP4* gene was sequenced in DNA samples from 48 nonrelated Caucasian subjects. Five novel and three known single nucleotide polymorphisms (SNPs) were identified. Furthermore, five recently reported SNPs were genotyped in 90 subjects. Six SNPs, representative of their linkage disequilibrium groups, were then genotyped in 934 diabetic and 716 nondiabetic subjects.

**RESULTS**—A haplotype of six common SNPs (A-G-G-T-G-C) was significantly increased in 934 case subjects with type 2 diabetes compared with 537 healthy control subjects with normal glucose tolerance ( $P = 0.02$ ; odds ratio 1.37 [95% CI 1.05–1.79]). Furthermore, in the cohort of 716 nondiabetic Caucasian subjects, carriers of the A-G-G-T-G-C haplotype had significantly higher mean fasting plasma insulin and 2-h plasma glucose than subjects without the haplotype. Two single SNPs (rs10882283 and rs10882273) were also associated with BMI, waist-to-hip ratio, and fasting plasma insulin, and several SNPs were associated with circulating free fatty acids (all adjusted  $P < 0.05$ ). In addition, subjects carrying a previously reported diabetes-associated haplotype had significantly higher mRNA levels in visceral adipose tissue (adjusted  $P < 0.05$ ) in a subgroup of nondiabetic subjects ( $n = 170$ ) with measurements of *RBP4* mRNA expression in visceral and subcutaneous fat depots.

**CONCLUSIONS**—Our data indicate a role of RBP4 genetic variation in susceptibility to type 2 diabetes and insulin resistance, possibly through an effect on *RBP4* expression. *Diabetes* 56:3095–3100, 2007

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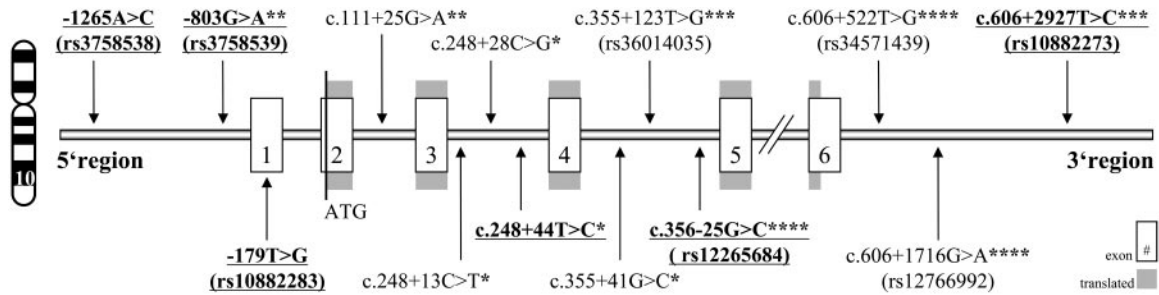
FFA, free fatty acid; LD, linkage disequilibrium; RBP4, serum retinol binding protein 4; SNP, single nucleotide polymorphism; WHR, waist-to-hip ratio.

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**S**erum retinol binding protein 4 (RBP4) is a new adipocyte-derived signal linking adipose tissue dysfunction to systemic insulin resistance and thereby likely contributing to the pathogenesis of type 2 diabetes. Serum RBP4 is elevated in insulin-resistant mice, as well as in humans, with obesity and type 2 diabetes and can be normalized by insulin-sensitizing drugs (1). Moreover, RBP4 serum levels highly correlate with the degree of insulin resistance in subjects with obesity, impaired glucose tolerance, or type 2 diabetes, as well as in nonobese subjects with family history of type 2 diabetes (2). Recently, we found increased *RBP4* mRNA expression in visceral compared with subcutaneous adipose tissue and serum RBP4 concentrations correlated with *RBP4* mRNA expression, intra-abdominal fat mass, total body fat mass, A1C, and insulin resistance (3).

RBP4 is encoded by the *RBP4* gene, which maps to chromosome 10q23-q24, a region that has been linked to increased risk for type 2 diabetes in different populations (4,5). Despite known physiology as well as chromosomal location, to date, very few studies on the effects of genetic variation in the *RBP4* gene on increased metabolic risk in humans have been reported (6,7). We therefore investigated whether genetic variants within the *RBP4* gene might be responsible for observed changes in *RBP4* mRNA expression and whether it may affect obesity/type 2 diabetes and pathophysiologically relevant traits in humans. We screened the gene for prevalent and functionally relevant variants and genotyped six informative single nucleotide polymorphisms (SNPs) in 934 patients with type 2 diabetes and 716 healthy German Caucasian subjects. To identify genetic variants, all six exons (National Center for Biotechnology Information reference NM\_006744), including intron/exon splicing sites, the 5' region (~1,500 bp upstream of the first translation initiation site, which also included the 5' untranslated region [exon 1 and the part of exon 2] and intron 1), and the 3' untranslated region, were sequenced in DNA samples from 48 nonrelated Caucasian subjects (24 nondiabetic with normal glucose tolerance and 24 with type 2 diabetes). Eight SNPs (five novel and three known database SNPs) were identified (Fig. 1). Three SNPs were in the 5' region, and five SNPs were in introns. Based on a recent publication (7), we selected five additional SNPs (rs36014035, rs12265684, rs34571439, rs12766992, and rs10882273) with minor allele frequency >5% in Caucasian samples (7), which we genotyped in 90 subjects, including all 48 sequenced DNA samples.



**FIG. 1.** Scheme of the *RBP4* gene with analyzed genetic variants. Underlined SNPs were genotyped in German Caucasians and analyzed for association with type 2 diabetes, obesity, and related phenotypes. Positions of SNPs in exons are based on the cDNA sequence NM\_006744 (National Center for Biotechnology Information, GenBank). Positions of SNPs in the 5' region are relative to the first translation initiation site. Minor allele frequency: rs3758538, C = 0.19; rs3758539, A = 0.18; rs10882283, G = 0.30; c.111+25G>A, A = 0.18; c.248+13C>T, T = 0.08; c.248+28C>G, G = 0.08; c.248+44T>C, C = 0.08; c.355+41G>C, C = 0.08; rs36014035, G = 0.37; rs12265684, C = 0.20; rs34571439, G = 0.20; rs12766992, A = 0.20; and rs10882273, C = 0.37. Groups of SNPs marked by the same number of asterisks are in complete LD with each other.

We estimated linkage disequilibrium (LD) among the variants (EMLD software; available from <https://epi.mdan-derson.org/~qhuang/software/pub.htm>) (8) (supplemental Fig. 1 [available in an online appendix at <http://dx.doi.org/10.2337/db07-1647>]). Among these variants, c.248+13C>T, c.248+28C>G, c.248+44T>C, and c.355+41G>C were in complete LD; rs3758539 was in complete LD with c.111+25G>A; rs36014035 was in LD with rs10882273; and rs12265684, rs34571439, and rs12766992 were in LD among each other (Fig. 1).

For association studies, only c.248+44T>C, rs3758539, rs12265684, and rs10882273 were selected as representative variants for all four LD groups and genotyped in all subjects for association analyses. In addition, rs3758538 and rs10882283, which were unique among SNPs, were also selected for further association analyses. The genotype distributions for all SNPs were consistent with Hardy-Weinberg equilibrium.

Using the PHASE v.2.1 software (9,10), we identified five common haplotypes among the six different SNPs genotyped in all subjects (Table 1). These five haplotypes, A-G-T-T-G-T, A-G-G-T-G-C, A-A-G-T-C-C, C-G-T-T-G-T, and C-G-T-C-G-C accounted for ~90% of the all observed haplotypes, where haplotypes are defined by the composition of alleles at each SNP in following order: rs3758538, rs3758539, rs10882283, c.248+44T>C, rs12265684, rs10882273. No single variant was associated with type 2 diabetes in 934 case subjects and 537 healthy control subjects with normal glucose tolerance (supplemental Table 1 of the online appendix), but the A-G-G-T-G-C haplotype frequency was significantly increased in type 2

diabetic compared with control subjects ( $P = 0.02$ ; odds ratio [OR] 1.37 [95% CI 1.05–1.79]) (Table 1). Furthermore, in the cohort of 716 nondiabetic Caucasian subjects, the rs10882283 G-allele was associated with higher BMI and waist-to-hip ratio (WHR) (adjusted  $P < 0.05$  in a recessive mode of inheritance), and the rs10882273 C-allele was significantly associated with increased BMI, plasma insulin, and circulating free fatty acid (FFA) concentrations (adjusted  $P < 0.05$ ) (Table 2). Because these two alleles are part of the diabetes risk haplotype (A-G-G-T-G-C), it is not surprising that subjects carrying the A-G-G-T-G-C haplotype had significantly higher mean fasting plasma insulin and 2-h plasma glucose levels than subjects without the haplotype (Table 3), which further supports the observed association with type 2 diabetes. We are aware that we have not corrected our statistical analyses for the number of comparisons made (given the number of tested traits and six SNPs); the results therefore must be interpreted with caution. However, our data strongly support the findings reported by Craig et al. (7), who investigated the *RBP4* gene and its role in type 2 diabetes in two Caucasian cohorts (Utah and Arkansas) and in individuals of African-American ancestry. Similarly to our present study, they showed significant effects of *RBP4* genetic variation on insulin resistance in Caucasians. In addition, one haplotype was significantly increased in subjects with type 2 diabetes, and the authors therefore suggest that noncoding variants may increase diabetes susceptibility and may contribute to insulin resistance (7). Although the haplotype composition in our present study was extended by two additional variants (rs3758538 and rs10882283), the

**TABLE 1**  
Association of *RBP4* haplotypes with type 2 diabetes

	Subjects with type 2 diabetes	Subjects without type 2 diabetes	$\chi^2$	<i>P</i>	OR (95% CI)
<i>n</i>	934	537			
Sex (M/F)	477/457	205/332			
Age (years)	64 ± 0.4	46 ± 0.7			
BMI (kg/m <sup>2</sup> )	29.2 (28.9–29.5)	26.1 (25.7–26.4)			
	Frequency (%)				
A-G-T-T-G-T	58.3	56.9	0.554	0.46	1.06 (0.91–1.23)
A-G-G-T-G-C	10.9	8.2	5.469	0.02	1.37 (1.05–1.79)
A-A-G-T-C-C	12.7	15.0	3.093	0.08	0.83 (0.66–1.02)
C-G-T-T-G-T	4.2	3.3	1.545	0.21	1.30 (0.86–1.92)
C-G-T-C-G-C	4.8	3.8	1.448	0.23	1.27 (0.86–1.85)

Haplotype frequencies were compared using the  $\chi^2$  test. Haplotypes are defined by the composition of alleles at each SNP in following order: rs3758538, rs3758539, rs10882283, c.248+44T>C, rs12265684, and rs10882273.

TABLE 2  
Metabolic characteristics of subjects without type 2 diabetes grouped by *RBP4* variant genotypes

Genotype	rs3758538					rs3758539					rs10882283					c248 + 44T>C		
	M/A	A/C	C/C	G/G	G/A	A/A	T/T	T/G	G/G	T/T	T/C	C/C						
<i>n</i>	530	173	13	465	226	25	332	288	96	611	102	3						
Sex (M/F)	224/306	74/99	6/7	195/270	106/120	5/20	151/181	127/161	37/59	276/335	43/59	1/2						
Age (years)	46 ± 0.6	48 ± 1.1	47 ± 2.8	47 ± 0.7	47 ± 1.0	45 ± 3.1	46 ± 0.8	46 ± 0.9	47 ± 1.5	46 ± 0.6	49 ± 1.4	43 ± 2.4						
BMI (kg/m <sup>2</sup> )	26.8 (26.4-27.2)	27.3 (26.6-28.2)	28.1 (26.1-30.3)	26.9 (26.5-27.3)	27.1 (26.4-27.8)	26.5 (24.7-28.4)	26.5 (26.0-26.9)	27.3 (26.7-27.9)	27.2 (26.2-28.2)*	26.9 (26.5-27.3)	27.3 (26.4-28.2)	26.1 (23.3-29.3)						
WHR	0.94 (0.93-0.96)	0.93 (0.9-0.97)	0.91 (0.81-1.01)#	0.94 (0.92-0.95)	0.95 (0.92-0.97)	0.92 (0.85-0.99)	0.93 (0.91-0.95)	0.95 (0.92-0.97)	0.94 (0.9-0.98)*	0.94 (0.92-0.96)	0.93 (0.90-0.97)	0.81 (0.51-1.29)						
Fasting plasma glucose (mmol/l)	5.35 ± 0.02	5.35 ± 0.04	5.43 ± 0.12	5.36 ± 0.02	5.34 ± 0.03	5.26 ± 0.08	5.36 ± 0.03	5.33 ± 0.03	5.36 ± 0.05	5.36 ± 0.02	5.32 ± 0.04	5.16 ± 0.18						
Fasting plasma insulin (pmol/l)	44 (39-50)	47 (37-59)	33 (19-55)	42 (37-49)	51 (41-62)	31 (16-59)	39 (33-46)	48 (40-58)	49 (36-68)	45 (40-51)	51 (38-68)	12 (2.9-47)						
2-h plasma glucose (mmol/l)	6.71 (6.57-6.85)	6.70 (6.45-6.96)	6.44 (5.53-7.50)	6.73 (6.58-6.89)	6.64 (6.43-6.85)	6.38 (5.85-6.96)	6.68 (6.49-6.87)	6.60 (6.41-6.79)	7.00 (6.63-7.39)	6.69 (6.56-6.83)	6.72 (6.39-7.07)	5.80 (3.63-9.26)						
A1C (%)	5.45 (5.41-5.48)	5.51 (5.44-5.58)	5.46 (5.28-5.65)	5.45 (5.42-5.49)	5.48 (5.43-5.53)	5.41 (5.25-5.67)	5.47 (5.43-5.52)	5.43 (5.39-5.48)	5.52 (5.44-5.61)	5.45 (5.42-5.48)	5.54 (5.45-5.64)	5.33 (4.65-6.11)						
FFAs (mmol/l)	0.28 (0.26-0.30)	0.28 (0.24-0.32)	0.24 (0.10-0.53)	0.26 (0.24-0.29)	0.30 (0.27-0.34)	0.30 (0.18-0.51)#*	0.26 (0.23-0.29)	0.29 (0.26-0.33)	0.31 (0.25-0.38)	0.28 (0.26-0.30)	0.28 (0.24-0.34)	0.16 (0.05-0.56)						
<i>n</i>	298	105		262	127	14	187	162	54	344	59							
Glucose infusion rate (μmol · kg <sup>-1</sup> · min <sup>-1</sup> )	50.8 (46.3-55.6)	48.1 (40.3-57.3)		51.4 (46.5-56.9)	47.8 (41.3-55.4)	58.9 (37.5-92.7)	50.8 (45.0-57.2)	50.9 (44.5-58.3)	43.1 (33.4-55.7)	51.1 (47.0-55.7)	45.4 (35.2-58.6)							
Body fat (%)	27.5 (26.4-28.7)	28.2 (26.1-30.4)		27.4 (26.2-28.7)	28.2 (26.2-30.2)	28.6 (23.4-35.0)	27.1 (25.7-28.7)	27.9 (26.1-29.9)	27.8 (24.8-31.0)	27.8 (26.7-29.0)	27.4 (25.2-29.9)							
Genotype		rs12265684			rs10882273													
<i>n</i>	462	231	23	270	351	95												
Sex (M/F)	190/272	106/125	6/17	121/149	148/203	35/60												
Age (years)	47 ± 0.7	47 ± 1.0	45 ± 3.0	46 ± 0.9	47 ± 0.8	49 ± 1.6												
BMI (kg/m <sup>2</sup> )	26.8 (26.4-27.3)	27.3 (26.6-28.0)	26.5 (24.6-28.6)	26.4 (25.8-26.9)	27.3 (26.8-27.9)	27.2 (26.2-28.3)*												
WHR	0.94 (0.92-0.95)	0.95 (0.92-0.97)	0.91 (0.84-0.98)	0.93 (0.90-0.95)	0.95 (0.93-0.97)	0.94 (0.91-0.99)												
Fasting plasma glucose (mmol/l)	5.35 ± 0.02	5.35 ± 0.03	5.30 ± 0.09	5.37 ± 0.03	5.32 ± 0.03	5.37 ± 0.05												
Fasting plasma insulin (pmol/l)	42 (37-49)	54 (44-65)	30 (16-57)	36 (30-43)	52 (44-61)	55 (40-75)#												
2-h plasma glucose (mmol/l)	6.74 (6.58-6.90)	6.66 (6.44-6.87)	6.35 (5.86-6.88)	6.70 (6.49-6.90)	6.65 (6.48-6.83)	7.00 (6.63-7.39)												
A1C (%)	5.46 (5.42-5.49)	5.50 (5.45-5.55)	5.35 (5.22-5.49)	5.45 (5.40-5.50)	5.46 (5.42-5.51)	5.53 (5.45-5.62)												
FFAs (mmol/l)	0.26 (0.24-0.29)	0.31 (0.27-0.35)	0.33 (0.19-0.58)###	0.25 (0.22-0.28)	0.30 (0.27-0.33)	0.30 (0.24-0.37)*												
<i>n</i>	260	130	13	152	197	54												
Glucose infusion rate (μmol · kg <sup>-1</sup> · min <sup>-1</sup> )	50.7 (45.8-56.2)	48.6 (42.1-56.0)	61.4 (40.1-94.1)	53.3 (47.1-60.3)	50.1 (44.5-56.4)	43.3 (34.0-55.3)												
Body fat (%)	27.3 (26.1-28.6)	28.3 (26.4-30.4)	29.1 (22.8-37.1)	27.1 (25.6-28.8)	28.1 (26.6-29.7)	27.6 (25.0-30.4)												

Data are arithmetic means ± SEM for normal variables (age and fasting plasma glucose) and geometric means (95% CI) for non-normally distributed variables. *P* values were calculated after adjusting for age and sex for the variables BMI, WHR, and body fat percentage and for age, sex, and BMI for the variables fasting plasma glucose, fasting plasma insulin, 2-h plasma glucose, glucose infusion rate, A1C, and FFAs. In the additive model, homozygotes for the major allele (MM), heterozygotes (Mm), and homozygotes for the minor allele (mm) were coded to a continuous numeric variable for genotype (as 0, 1, 2). A dominant model was defined as contrasting genotypic groups MM + Mm vs. mm, and the recessive model was defined as contrasting genotypic groups MM vs. Mm + mm. # (+\*) indicates *P* < 0.05 in additive (dominant, recessive) mode of inheritance and ## (+ +\*) indicates *P* < 0.01 in additive (dominant, recessive) mode of inheritance. Due to the low frequency of the rare allele in rs3758538 and c248+44T>C, for statistical analysis the homozygotes for minor alleles were combined with the heterozygotes; therefore, only a dominant effect on risk has been tested for the rare allele for glucose infusion rate and body fat percentage.



A-G-G-T-G-C haplotype corresponds to the diabetes risk haplotype described by Craig et al. (7). Thus, our findings provide further independent evidence for the involvement of *RBP4* gene variants in susceptibility to type 2 diabetes.

Interestingly, circulating serum FFA concentrations were significantly associated with three SNPs (Table 2). These effects were reflected in the haplotype analysis where the A-A-G-T-C-C haplotype was associated with elevated FFA levels (Table 3). Our findings are consistent with recently reported data from *RBP4* knockout mice (1). It has been shown that genetic deletion of *RBP4* in *Rbp4*<sup>-/-</sup> knockout mice results in lower levels of serum FFAs, which was suggested to be linked to their improved insulin sensitivity. However, FFAs were not changed in insulin-resistant transgenic mice with overexpressed *RBP4*, in *RBP4*-injected mice, or in adipose-*Glut4*<sup>-/-</sup> mice, suggesting that regulation of circulating FFA levels does not seem to be the principal mechanism by which *RBP4* regulates insulin sensitivity (1). Nevertheless, we believe that the association of FFAs with *RBP4* SNPs, together with the data from *RBP4*<sup>-/-</sup> knockout mice manifesting lower levels of serum FFAs provokes further studies aimed to pinpoint the mechanisms by which *RBP4* might alter circulating levels of FFA.

Increased *RBP4* gene expression in visceral adipose tissue is a likely source for elevated *RBP4* serum concentrations in patients with increased visceral fat mass and type 2 diabetes and could therefore contribute to mechanisms linking visceral fat accumulation to the development of insulin resistance (3). Therefore, we examined whether genetic variants could affect *RBP4* mRNA expression in visceral and subcutaneous fat as well as serum *RBP4* concentrations. Although we found no significant impact of the single variants on either visceral and subcutaneous mRNA expression or on serum *RBP4* (online appendix supplemental Table 2), the A-G-G-T-G-C diabetes risk haplotype carriers had a higher mean visceral and subcutaneous expression as well as serum *RBP4* concentrations compared with noncarriers. Most likely due to the small sample size, this did not reach statistical significance (adjusted  $P > 0.05$ ; online appendix supplemental Table 3). However, when we restricted the analysis to the haplotypes comprising only the variants covering the *RBP4* haplotypes previously reported by Craig et al. (7) (rs3758539, c.248+44T>C, rs12265684, and rs10882273), the type 2 diabetes-associated haplotype from the Utah study was significantly associated with increased *RBP4* mRNA expression in visceral adipose tissue (geometric mean 3,458 AU [95% CI 1,907–6,273] vs. 1,566 [953–2,574];  $P < 0.05$  after adjusting for age, sex, BMI, and percentage body fat). Although Craig et al. reported haplotypes of eight common SNPs, these eight SNPs fall into four LD groups in our study and could therefore be presented by four common SNPs (rs3758539, c.248+44T>C, rs12265684, and rs10882273). Considering the association of this haplotype with type 2 diabetes and related traits, it is noteworthy that visceral *RBP4* mRNA level was the strongest factor significantly affecting glucose infusion rate in a multivariate generalized linear model analysis also including age, sex, BMI, WHR, and percentage body fat (data not shown). This suggests a role of *RBP4* genetic variation in susceptibility to insulin resistance, possibly through an effect on *RBP4* expression. Regarding the lack of statistically significant genetic association with serum *RBP4* concentrations, we need to point out that this subgroup of subjects had a high mean BMI ( $30.0 \pm 6.9$

kg/m<sup>2</sup>), which may have masked the effect of genetic variants on serum *RBP4* levels.

Two identified SNPs (rs3758538 and rs3758539) are located 5' upstream of the translation start site in a putative promoter region. We therefore used the Transcription Element Search System (TESS; available from <http://www.cbil.upenn.edu/tess>) to examine transcriptional regulatory sequences surrounding these genetic variants, which might modify *RBP4* expression. The highly conserved region surrounding rs3758539 matches human transcriptional binding sites for MAZ (11) and R1/R2/Sp1 for the major allele G (12,13) and c-Ets-2 for the minor allele A (14). Furthermore, this SNP seems to influence the transcription efficiency in a hepatocarcinoma cell line as well as the binding efficiency of hepatocyte nuclear factor 1 $\alpha$  to the motif (6). Regarding the association of the *RBP4* haplotype with mRNA levels, this also indicates a potential functional relevance of the noncoding *RBP4* variants in the putative promoter region. However, only additional functional experiments on these SNPs might assign causality for the associated phenotypes. Alternatively, the *RBP4* haplotypes might harbor a variant, which controls *RBP4* mRNA levels but was not tested in our present study because only exons and potentially regulatory regions were sequenced.

In conclusion, consistent with previously reported findings in mice and in human studies (1,6,7), several *RBP4* SNPs and their haplotypes are likely to affect measures of insulin resistance (fasting plasma insulin and 2-h plasma glucose) and related traits (BMI, WHR, and circulating FFAs), as well as *RBP4* mRNA levels in visceral adipose tissue in humans. These effects may ultimately result in type 2 diabetes, which is in line with the observed association of the A-G-G-T-G-C haplotype with increased risk of type 2 diabetes in the present study. Thus, our data indicate a role of *RBP4* genetic variation in susceptibility to type 2 diabetes and insulin resistance, possibly through an effect on *RBP4* expression.

## RESEARCH DESIGN AND METHODS

A total of 934 patients with type 2 diabetes and 716 healthy subjects were recruited at the University Hospital in Leipzig, Germany. The healthy subjects included 269 men and 447 women (mean age  $\pm$  SD 47.2  $\pm$  14.6 years, mean BMI 27.4  $\pm$  5.3 kg/m<sup>2</sup>, mean WHR 0.96  $\pm$  0.19), and patients with type 2 diabetes included 477 men and 457 women (mean age 64.5  $\pm$  10.8 years, mean BMI 29.6  $\pm$  5.0 kg/m<sup>2</sup>, mean WHR 1.13  $\pm$  0.13). In addition, oral glucose tolerance test and fasting plasma insulin measurements were performed in all nondiabetic subjects as described elsewhere (15). Of 716 subjects, 179 had impaired glucose tolerance. Because impaired glucose tolerance is a type 2 diabetes predicting factor, only the remaining 537 subjects with normal glucose tolerance were included as healthy control subjects in the type 2 diabetes case-control study.

In a subgroup of 403 nondiabetic subjects, body fat content was measured by dual-energy X-ray absorptiometry. Insulin sensitivity was assessed with the euglycemic-hyperinsulinemic clamp method, as previously described (16,17).

In addition, paired samples of visceral and subcutaneous adipose tissue were obtained from a subgroup of 218 Caucasian men ( $n = 108$ ) and women ( $n = 110$ ) who underwent open abdominal surgery for gastric banding, cholecystectomy, weight reduction surgery, abdominal injuries, or explorative laparotomy (described in detail elsewhere) (18). The age ranged from 23 to 99 years and BMI from 20.8 to 54.1 kg/m<sup>2</sup>. Serum *RBP4* concentrations were also measured in these subjects. Only nondiabetic subjects ( $n = 170$ ) were included in association analyses.

All studies were approved by the ethics committee of the University of Leipzig, and all subjects gave written informed consent before taking part in the study.

**Measurement of serum *RBP4*.** *RBP4* was measured in serum by enzyme-linked immunosorbent assay (ALPCO) or by quantitative Western blotting with purified human *RBP4* standards, as described in detail elsewhere (2).

**Analysis of human RBP4 expression.** Human RBP4 mRNA expression was measured by quantitative real-time PCR in a fluorescent temperature cycler using the TaqMan assay, and fluorescence was detected on an ABI Prism 7000 sequence detector (Applied Biosystems, Darmstadt, Germany), as described in detail elsewhere (3,19).

**Sequencing of RBP4.** Sequencing of the *RBP4* gene was performed using the Big Dye Terminator (Applied Biosystems) on an automated DNA capillary sequencer (ABI Prism 3100 Avant; Applied Biosystems). Sequence information for all oligonucleotide primers used for variant screening is available upon request.

**Genotyping of RBP4 SNPs.** Genotyping of selected SNPs in all study subjects was done using the TaqMan assay (Applied Biosystems) for the variants rs3758538, rs3758539, c.248+44T>C, rs12265684, and rs10882273 and by restriction fragment-length polymorphism technique for the SNP rs10882283. Oligonucleotide sequences are available upon request. The TaqMan genotyping reaction was performed according to the manufacturer's protocol on an ABI Prism 7000 or ABI Prism 7700 sequence detector (Applied Biosystems). The restriction fragment-length polymorphism genotypes were determined by PCR amplification of the respective fragments from exon 1 of the *RBP4* gene on the GeneAmp PCR system 9700 (95°C for 5 min, 95°C for 30 s, 60°C for 1 min, and 72°C for 30 s for 35 cycles and 72°C for 10 min), subsequent digestion with the *SchI* (Fermentas Life Sciences) restriction enzyme, and size fractionation and visualization by electrophoresis. To assess genotyping reproducibility, a random ~10% selection of the sample was re-genotyped in all four SNPs; all genotypes matched initial designated genotypes.

**Statistical analyses.** Before statistical analysis, non-normally distributed parameters were logarithmically transformed to approximate a normal distribution. Differences in genotype frequencies between the diabetic and healthy control subjects were compared using logistic regression and differences in haplotype frequencies compared using the  $\chi^2$  test. Multivariate linear relationships were assessed by generalized linear regression models. *P* values <0.05 were considered statistically significant and are presented without correction for multiple hypothesis testing. Based on minor allele frequencies in the present study, we had >85% power ( $\alpha = 0.05$ ) to detect a difference in allele frequency of 6–9%, corresponding to an OR of 1.5–1.9; hence, smaller effects were likely to be missed. The analysis of associations with RBP4 serum concentrations and mRNA in adipose tissue was restricted to nondiabetic subjects to avoid diabetes status masking potential effects of the variants on these phenotypic traits. Although we performed separate analyses also in subjects with type 2 diabetes only, no associations with the above-mentioned parameters were found. This is most likely due to lacking statistical power in this very small sample size and given the very low haplotype frequencies; these data are therefore not shown. Statistical analyses were performed using the SPSS software package (SPSS, Chicago, IL) and the statistical analysis system of the SAS Institute (Cary, NC).

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