

## ORIGINAL ARTICLE

# Retinol-Binding Protein 4 and Insulin Resistance in Lean, Obese, and Diabetic Subjects

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## ABSTRACT

**BACKGROUND**

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Insulin resistance has a causal role in type 2 diabetes. Serum levels of retinol-binding protein 4 (RBP4), a protein secreted by adipocytes, are increased in insulin-resistant states. Experiments in mice suggest that elevated RBP4 levels cause insulin resistance. We sought to determine whether serum RBP4 levels correlate with insulin resistance and change after an intervention that improves insulin sensitivity. We also determined whether elevated serum RBP4 levels are associated with reduced expression of glucose transporter 4 (GLUT4) in adipocytes, an early pathological feature of insulin resistance.

**METHODS**

We measured serum RBP4, insulin resistance, and components of the metabolic syndrome in three groups of subjects. Measurements were repeated after exercise training in one group. GLUT4 protein was measured in isolated adipocytes.

**RESULTS**

Serum RBP4 levels correlated with the magnitude of insulin resistance in subjects with obesity, impaired glucose tolerance, or type 2 diabetes and in nonobese, nondiabetic subjects with a strong family history of type 2 diabetes. Elevated serum RBP4 was associated with components of the metabolic syndrome, including increased body-mass index, waist-to-hip ratio, serum triglyceride levels, and systolic blood pressure and decreased high-density lipoprotein cholesterol levels. Exercise training was associated with a reduction in serum RBP4 levels only in subjects in whom insulin resistance improved. Adipocyte GLUT4 protein and serum RBP4 levels were inversely correlated.

**CONCLUSIONS**

RBP4 is an adipocyte-secreted molecule that is elevated in the serum before the development of frank diabetes and appears to identify insulin resistance and associated cardiovascular risk factors in subjects with varied clinical presentations. These findings provide a rationale for antidiabetic therapies aimed at lowering serum RBP4 levels.

**T**YPE 2 DIABETES IS CAUSED BY RESISTANCE to insulin action in multiple tissues, accompanied by failure of the pancreatic beta cells to compensate sufficiently by increased insulin secretion.<sup>1</sup> Measurement of insulin resistance provides an early and strong predictor of type 2 diabetes.<sup>2</sup> Even in the absence of hyperglycemia or diabetes, insulin resistance constitutes an important risk factor for cardiovascular disease and early death.<sup>3</sup> Obesity, which has reached epidemic proportions worldwide, is a major cause of insulin resistance.<sup>4</sup> However, insulin resistance does not develop in all obese persons, and genetic background contributes strongly to insulin resistance, even in nonobese persons.<sup>5</sup>

In insulin-resistant states, the expression of the glucose-transporter 4 (GLUT4), the principal insulin-stimulated glucose transporter, is down-regulated selectively in adipocytes and not in skeletal muscle<sup>6</sup>; this results in impaired insulin-stimulated glucose transport in adipocytes.<sup>6</sup> These defects precede glucose intolerance.<sup>6,7</sup> However, the consequences of decreased GLUT4 expression in adipocytes have been unclear, since adipose tissue contributes little to whole-body glucose disposal.<sup>6</sup> Genetic knockout of GLUT4 selectively in adipocytes of mice<sup>8</sup> results in increased serum levels of retinol-binding protein 4 (RBP4).<sup>9</sup> Injection of purified RBP4 into mice or transgenic overexpression of RBP4 in mice impairs insulin signaling in muscle and induces the expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase in the liver.<sup>9</sup> RBP4 is the only specific transport protein for retinol (vitamin A) in the circulation.<sup>10,11</sup> Elevated RBP4 levels have been reported in people with type 2 diabetes.<sup>12,13</sup> In an earlier study, we found that serum RBP4 levels are increased in many insulin-resistant states induced by genetic and dietary factors.<sup>9</sup> Therefore, we assessed whether serum RBP4 levels are correlated with the magnitude of insulin resistance and cardiovascular risk factors and whether a therapeutic intervention that improves insulin sensitivity is associated with a reduction in serum RBP4 levels.

## METHODS

### STUDY GROUPS

Overweight and obesity were defined according to the World Health Organization criteria on the basis of the body-mass index (the weight in kilograms divided by the square of the height in me-

ters).<sup>14</sup> Definitions of normal glucose tolerance, impaired glucose tolerance, and type 2 diabetes were based on the 1997 American Diabetes Association criteria for glucose values obtained after an overnight fast and a two-hour oral glucose-tolerance test (OGTT), conducted with a standard loading dose of 75 g.<sup>15</sup> For clamp studies, the rate of glucose disposal was defined as the glucose infusion rate during the final 30 minutes.<sup>16</sup> Standard techniques were used to measure plasma glucose, serum or plasma insulin, and serum lipids.<sup>17,18</sup> Written informed consent was obtained from each subject. Samples were collected and clamp studies were conducted between 1996 and 2004.

### MEASUREMENT OF SERUM RBP4

Serum RBP4 was measured by an enzyme-linked immunosorbent assay (ELISA) (ALPCO Diagnostics) in groups 1 and 3 and by quantitative Western blotting<sup>9</sup> with purified human RBP4 standards in group 2. Immunodetection was performed with a polyclonal antibody to human RBP4 (DakoCytomation). ELISA samples were run in duplicate, and Western blotting samples were run once. The coefficient of variation for interassay replicate samples was less than 7 percent for ELISA and less than 10 percent for quantitative Western blotting.

### GROUP 1

The experimental protocol for group 1 was approved by the Committee on Human Investigation of the University of California, San Diego. Subjects were recruited as previously described,<sup>17</sup> and serum RBP4 levels were measured. All lean subjects, none of whom were diabetic, and all non-diabetic overweight or obese subjects had normal glucose tolerance. Until several weeks before the study, diabetic subjects received treatment with metformin, sulfonylurea, or insulin, alone or in combination, but not treatment with thiazolidinediones. All diabetes-specific medications were withdrawn at least two weeks before studies were performed. Subjects with type 2 diabetes were otherwise well and were not taking other medications known to influence glucose metabolism. The euglycemic-hyperinsulinemic clamp protocol has been described previously.<sup>17</sup>

### GROUP 2

For group 2, the study was approved by the ethics committee of the University of Leipzig. Sixty white

**Table 1. Relationship of Serum RBP4 Levels to Insulin Resistance and Associated Metabolic Values.\***

		Group 1										
Clinical Features	No. and Sex of Subjects	RBP4 — $\mu\text{g/ml}$ (range)	Age — yr (range)	BMI (range)	GDR — mg/kg/min (range)†	Insulin — $\mu\text{U/ml}$ (range)‡	Glucose — mg/dl (range)§	Hemoglobin — % (range)	Triglycerides — mg/dl (range)¶	Glycated Hemoglobin — % (range)	Waist-to-Hip Ratio (range)	
Lean (controls)	5 M	23.8±1.0 (23.0–24.3)	38±11 (20–48)	23.8±0.5 (23.0–24.3)	14.7±2.2 (12–17)	3.7±1.7 (2.5–6.7)	90±4 (84–93)	5.0±0.2 (4.8–5.3)	99±33 (71–151)	5.0±0.2 (4.8–5.3)	0.40	
Obese, no diabetes	7 M	39.4±5.0 (35.1–48.9)	48±9 (37–63)	32.4±3.2 (30.0–37.3)	8.5±2.3 (5–13)	16.5±5.8 (9.2–25.9)	96±7 (88–108)	5.5±0.6 (4.4–6.1)	212±141 (53–416)	5.5±0.6 (4.4–6.1)	0.40	
Obese, type 2 diabetes**	9 M	40.8±10.8 (24.9–50.3)	58±8 (46–68)	31.6±4.5 (27.7–40.9)	6.5±1.3 (5–9)	16.6±4.2 (4.2–50.2)	132±34 (95–209)	6.8±1.1 (5.3–8.5)	168±93 (55–354)	6.8±1.1 (5.3–8.5)	0.40	
Spearman correlation coefficient (R) with serum RBP4 (95% CI)			0.28 (–0.17 to 0.64)	0.64 (0.30 to 0.84)	–0.55 (–0.79 to –0.16)	0.72 (0.41 to 0.88)	0.40 (–0.03 to 0.70)	0.53 (0.14 to 0.78)	0.40 (–0.02 to 0.71)	0.53 (0.14 to 0.78)	0.40	
P value			0.22	0.001	0.009	<0.001	0.06	0.01	0.06	0.01	0.06	
		Group 2										
Clinical Features	No. and Sex of Subjects	RBP4 — $\mu\text{g/ml}$ (range)	Age — yr (range)	BMI (range)	Waist-to-Hip Ratio (range)	GDR — mg/kg/min (range)	Insulin — $\mu\text{U/ml}$ (range)	Glucose — mg/dl (range)	Hemoglobin — % (range)	Glycated Hemoglobin — % (range)	HDL Cholesterol — mg/dl (range)‡‡	Systolic Blood Pressure — mm Hg (range)
Normal glucose tolerance (controls)	20 (9 M/11 F)	26.3±7.1 (14.0–33.9)	33±11 (21–58)	24.3±1.5 (21.7–27.5)	0.9±0.02 (0.7–1.1)	13.5±3.0 (9–18)	4.8±2.5 (2.3–12.9)	94±9 (73–108)	5.2±0.1 (5.0–5.5)	5.2±0.1 (5.0–5.5)	53±12 (40–77)	123±10 (105–140)
Impaired glucose tolerance	20 (9 M/11 F)	59.9±32.8 (28.9–149.0)	56±12 (25–73)	29.8±3.9 (25.0–42.1)	1.2±0.04 (0.8–1.4)	3.4±1.6 (1–6)	50.2±35.7 (2.3–131.8)	103±10 (77–125)	5.7±0.2 (5.4–6.2)	5.7±0.2 (5.4–6.2)	40±7 (33–53)	137±6 (126–146)
Type 2 diabetes	20 (11 M/9 F)	63.2±24.9 (29.6–129.1)	53±7 (38–65)	30.7±3.2 (27.4–38.4)	1.3±0.03 (1.1–1.4)	3.8±1.7 (2–8)	23.1±15.4 (8.3–76.1)	113±11 (90–133)	6.4±0.4 (5.9–7.1)	6.4±0.4 (5.9–7.1)	37±8 (23–47)	139±5 (129–150)
Spearman correlation coefficient (R) with serum RBP4 (95% CI)			0.25 (–0.01 to 0.47)	0.62 (0.43 to 0.75)	0.68 (0.49 to 0.82)	–0.78 (–0.86 to –0.65)	0.57 (0.37 to 0.72)	0.46 (0.24 to 0.64)	0.65 (0.47 to 0.77)	0.65 (0.47 to 0.77)	–0.55 (–0.70 to –0.34)	0.62 (0.44 to 0.76)
P value			0.06	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	<0.001	<0.001	<0.001
		Group 3										
Clinical Features	No. and Sex of Subjects	RBP4 — $\mu\text{g/ml}$ (range)	Age — yr (range)	BMI (range)	GDR — mg/kg/min (range)	Insulin — $\mu\text{U/ml}$ (range)	Glucose — mg/dl (range)	Triglycerides — mg/dl (range)	Systolic Blood Pressure — mm Hg (range)			

Nonobese relatives of subjects with type 2 diabetes	26 M	30.8±7.4 (17.1–45.1)	44±8 (29–57)	25.5±2.0 (20.9–29.0)	8.2±3.4 (3–17)	9.8±3.2 (3.0–15.7)	92±9 (79–115)	88±48 (57–141)	123±13 (104–152)
Spearman correlation coefficient (R) with serum RBP4 (95% CI)		0.16 (–0.24 to 0.52)	0.15 (–0.25 to 0.51)	–0.59 (–0.80 to –0.23)	0.71 (0.47 to 0.85)	0.26 (–0.15 to 0.59)	0.71 (0.40 to 0.86)	0.50 (0.13 to 0.74)	0.009
P value		0.45	0.46	0.002	<0.001	0.21	<0.001	0.009	

\* Blood for all studies was drawn with the subject in the fasting state. Data are given as means ±SD. Kruskal–Wallis analysis of variance with pairwise comparisons was used to determine differences among the three clinical categories in groups 1 and 2. RBP4 denotes retinol-binding protein 4, BMI body-mass index, GDR glucose disposal rate, and HDL high-density lipoprotein.  
 † To convert values for glucose disposal to micromoles per kilogram per minute, multiply by 5.549.  
 ‡ To convert values for insulin to picomoles per liter, multiply by 7.175.  
 § To convert values for glucose to millimoles per liter, multiply by 0.05549.  
 ¶ To convert values for triglycerides to millimoles per liter, multiply by 0.01129.  
 || P<0.05 for the comparison with lean control subjects in group 1 or control subjects with normal glucose tolerance in group 2.  
 \*\* Two subjects in the obese, type 2 diabetes group had body-mass-index values of less than 30 at the time the blood was drawn for this study.  
 †† P<0.05 for the comparison between obese subjects without diabetes and obese subjects with diabetes in group 1 or between subjects with normal glucose tolerance and subjects with type 2 diabetes in group 2.  
 ††† To convert values for HDL cholesterol to millimoles per liter, multiply by 0.0259.

men and women with normal glucose tolerance, impaired glucose tolerance, or type 2 diabetes (20 per group) were randomly selected from 469 people screened by OGTT as participants in a health survey.<sup>18</sup> Before screening, the subjects had no history of type 2 diabetes, gestational diabetes, insulin resistance, or the metabolic syndrome and had not previously been treated with medications for diabetes. Thus, those with abnormal glucose metabolism had newly received the diagnosis on the basis of fasting glucose levels and the results of OGTT. In addition, subjects with normal glucose tolerance had no family history of diabetes. Other exclusion criteria are described elsewhere.<sup>18</sup>

The subjects underwent supervised physical training (60 minutes of bicycling and running per day for at least three days per week). At baseline and after four weeks of training, but at least three days after the last exercise session, fasting blood samples were obtained for metabolic assays. Before and after training, the body-mass index and waist-to-hip ratio were determined; the percentage of body fat was measured by dual-energy x-ray absorptiometry; a euglycemic–hyperinsulinemic clamp study was performed; and a graded bicycle ergometry study to volitional exhaustion was performed. The highest oxygen uptake per minute ( $\dot{V}O_{2MAX}$ ) was measured. The clamp protocol and assays for adiponectin, leptin, interleukin-6, and C-reactive protein have been described previously.<sup>18,19</sup>

The subjects were divided into three groups on the basis of the magnitude of the change in insulin sensitivity during the clamp study (glucose disposal rate) with exercise training. Subjects in the lowest third had small or no improvements in the rate of glucose disposal (mean [±SD] increase, 0.6±0.3 mg per kilogram per minute, or less than 15 percent over baseline) and were categorized as having a marginal insulin-sensitivity response to exercise training. Similar results were obtained with a quartile-based ranking system (data not shown).

**GROUP 3**

For group 3, the study was approved by the ethics committee of Göteborg University and was conducted in a manner consistent with the principles of the Declaration of Helsinki. Healthy men with at least one first-degree relative with type 2 diabetes were recruited by advertisements in local newspapers. The inclusion criteria were an age of

25 to 55 years, a body-mass index of 22 to 30, normal glucose tolerance, fasting triglyceride level less than 150 mg per deciliter (1.7 mmol per liter), and the absence of known endocrine or metabolic disease. The subjects were asked to abstain from alcohol and excessive physical exercise for two days before each examination or specimen-collection day. The clamp protocol has been described previously.<sup>20</sup> Several days before the clamp study, subcutaneous adipose-tissue biopsies were performed and adipocytes were immediately isolated by collagenase digestion. GLUT4 was measured in lysates by Western blotting.<sup>20,21</sup>

#### STATISTICAL ANALYSIS

The results are expressed as means  $\pm$ SD. Linear correlations and nonparametric statistical tests were performed with Analyse-it (version 1.71, Analyse-it Software), and independently confirmed with StatView (version 5.0, SAS Institute). All reported P values are two-tailed and are not adjusted for multiple testing. Multivariate regression analysis was performed with Data Desk/XL (version 1.1, DataDescription).

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## RESULTS

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#### SERUM RBP4 IN OBESE SUBJECTS WITH AND THOSE WITHOUT TYPE 2 DIABETES

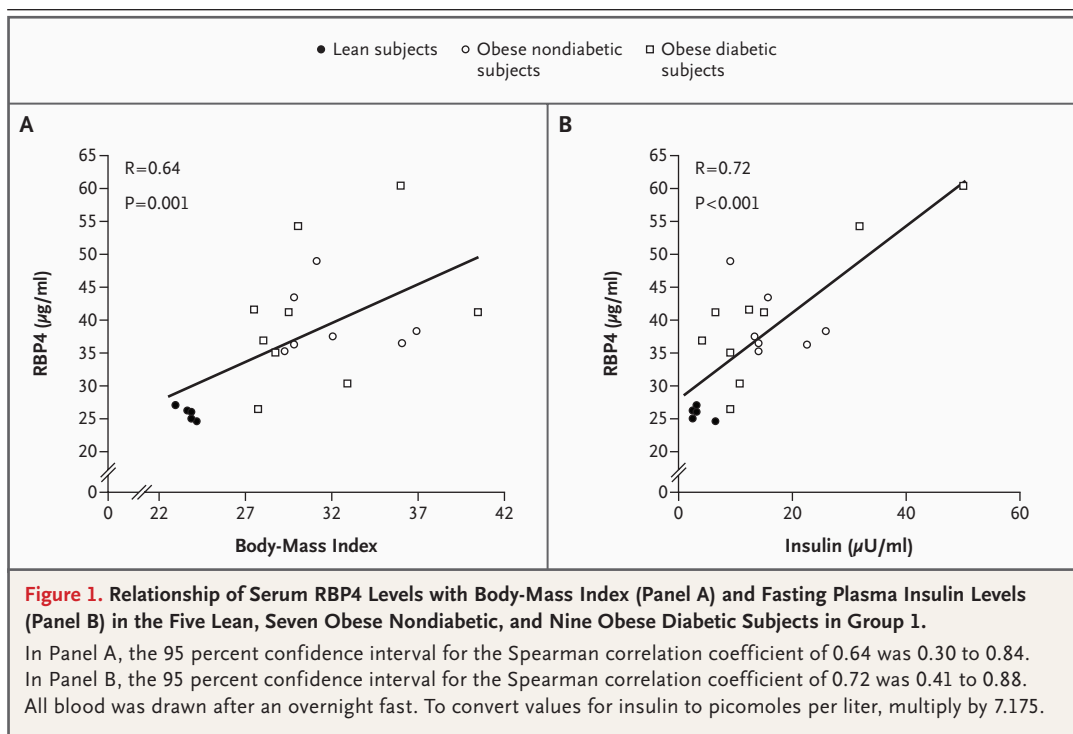
Obese, nondiabetic subjects and obese subjects with type 2 diabetes had similar body-mass index values; all but two subjects had a body-mass index of 30 or greater (Table 1). The average rate of glucose disposal during the clamp study was 42 percent lower in obese, nondiabetic subjects and 56 percent lower in subjects with type 2 diabetes than in lean control subjects. The mean serum RBP4 level in group 1 control subjects was consistent with reported normal values in healthy inhabitants of vitamin A-sufficient regions (Table 1).<sup>10</sup> The mean serum RBP4 levels were elevated in both nondiabetic and diabetic obese subjects and serum RBP4 levels correlated positively with body-mass index (Fig. 1A) ( $P=0.001$ ) and fasting insulin levels (Fig. 1B) ( $P<0.001$ ) and correlated inversely with the rate of glucose disposal (Table 1) ( $P=0.009$ ). Multivariate regression analysis showed that the RBP4 level correlated with the rate of glucose disposal independently of age ( $P=0.004$ ) but not independently of body-mass index ( $P=0.06$ ); RBP4 also correlated with fasting insulin independently of the rate of glucose dis-

posal ( $P=0.003$ ). The serum RBP4 level correlated with the glycated hemoglobin value but not with the fasting glucose level (Table 1). Thus, even in the absence of hyperglycemia or diabetes, serum levels of RBP4 were elevated and were correlated with body-mass index and insulin resistance in overweight and obese subjects.

#### EFFECTS OF EXERCISE TRAINING IN SUBJECTS WITH IMPAIRED GLUCOSE TOLERANCE

Subjects with impaired glucose tolerance or type 2 diabetes underwent exercise training to determine the effects of an insulin-sensitizing therapy on serum RBP4. In group 2, subjects with diabetes had higher fasting blood glucose levels but lower insulin levels than subjects with impaired glucose tolerance, a finding consistent with diminished beta-cell compensation for insulin resistance (Table 1). The baseline glucose disposal rate was reduced in all subjects with impaired glucose tolerance or diabetes as compared with the rate in controls, a finding indicating insulin resistance. The subjects in group 2 (Table 1) with impaired glucose tolerance or diabetes had higher baseline levels of plasma leptin, plasma C-reactive protein, plasma interleukin-6, and serum free fatty acids than control subjects (data not shown), whereas they had lower plasma levels of high-density lipoprotein (HDL) cholesterol (Table 1) and adiponectin (data not shown), a finding consistent with those of other reports.<sup>22,23</sup> Although they had the same degree of insulin resistance, subjects with diabetes had higher baseline levels of C-reactive protein, interleukin-6, and free fatty acids than those with impaired glucose tolerance (data not shown).

Serum RBP4 levels were higher in subjects with impaired glucose tolerance or diabetes than in controls (Fig. 2A). There was no apparent effect of sex on serum RBP4 levels (Fig. 2A). RBP4 levels correlated inversely with the rate of glucose disposal (Fig. 2B), even after the combined contributions of age and body-mass index had been controlled for by multivariate regression analysis ( $P<0.001$ ). In group 2, the serum RBP4 level correlated positively with the fasting glucose level, insulin level, glycated hemoglobin value, and systolic blood pressure and correlated inversely with the HDL cholesterol level (Table 1). Also in group 2, the serum RBP4 level was more highly correlated with the waist-to-hip ratio than with the body-mass index (Table 1) or the percentage of body

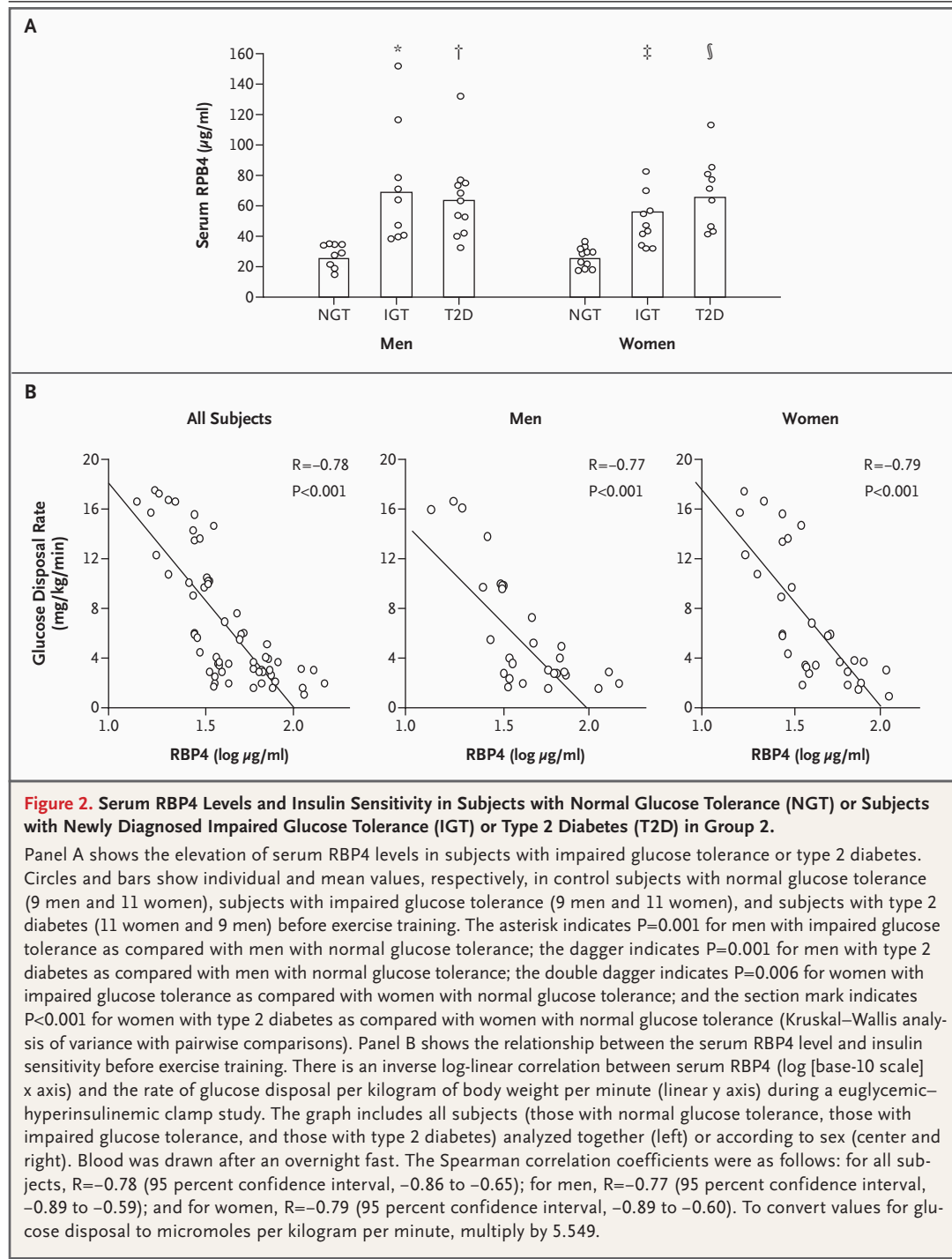


fat (data not shown); this observation suggests a specific association between the serum RBP4 level and abdominal obesity, a known risk factor for insulin resistance and cardiovascular disease.<sup>24-26</sup>

The response of whole-body insulin sensitivity to one month of exercise training was variable; some subjects had little or no improvement in insulin sensitivity. Changes in serum RBP4 levels correlated inversely with changes in the rate of glucose disposal (Fig. 3A). In contrast, changes in the rate of glucose disposal did not correlate with changes in levels of fasting plasma insulin ( $R=0.11$ ,  $P=0.48$ ) or fasting plasma glucose ( $R=0.07$ ,  $P=0.65$ ) or plasma glucose obtained after an OGTT ( $R=-0.02$ ,  $P=0.92$ ). In a post hoc analysis of the response to exercise training, subjects were grouped into thirds according to the magnitude of the change in the rate of glucose disposal. Subjects in the lowest third had the least improvement in the rate of glucose disposal (mean change,  $0.6 \pm 0.3$  mg per kilogram of body weight per minute, or less than 15 percent above baseline) and were classified as having a marginal response of insulin sensitivity to exercise training (Table 2 and Fig. 3B). The remaining subjects (upper two thirds) had a greater increase in the mean rate of glucose disposal, a result suggesting improved insulin sensitivity after exercise training.

RBP4 levels decreased from baseline levels after exercise training in all subjects in the highest third except for one subject who had a normal baseline RBP4 level (Fig. 3B). In contrast, serum RBP4 levels did not change or increased in all but one subject in the lowest third. In the middle third, RBP4 levels decreased in 11 subjects, albeit only slightly in some, and increased in 2 subjects. The upper two thirds were grouped for metabolic analyses (Table 2). Aerobic conditioning, assessed by  $\dot{V}O_{2\text{MAX}}$  during exercise, increased to a similar extent in both those with marginal insulin sensitivity (lowest third) and those with improved insulin sensitivity (upper two thirds; data not shown). In subjects with either marginal or improved insulin sensitivity, exercise training was associated with reductions in body-mass index, body-fat percentage, waist-to-hip ratio, and fasting insulin levels and an increase in HDL cholesterol levels (Table 2). However, only subjects with improved insulin sensitivity had a significant improvement in fasting glucose levels and glucose levels during an OGTT (Table 2).

Exercise training increased plasma adiponectin levels and lowered C-reactive protein levels to the same extent regardless of whether insulin sensitivity improved (Table 2). Exercise training was not associated with changes in leptin or inter-



**Figure 2.** Serum RBP4 Levels and Insulin Sensitivity in Subjects with Normal Glucose Tolerance (NGT) or Subjects with Newly Diagnosed Impaired Glucose Tolerance (IGT) or Type 2 Diabetes (T2D) in Group 2.

Panel A shows the elevation of serum RBP4 levels in subjects with impaired glucose tolerance or type 2 diabetes. Circles and bars show individual and mean values, respectively, in control subjects with normal glucose tolerance (9 men and 11 women), subjects with impaired glucose tolerance (9 men and 11 women), and subjects with type 2 diabetes (11 women and 9 men) before exercise training. The asterisk indicates  $P=0.001$  for men with impaired glucose tolerance as compared with men with normal glucose tolerance; the dagger indicates  $P=0.001$  for men with type 2 diabetes as compared with men with normal glucose tolerance; the double dagger indicates  $P=0.006$  for women with impaired glucose tolerance as compared with women with normal glucose tolerance; and the section mark indicates  $P<0.001$  for women with type 2 diabetes as compared with women with normal glucose tolerance (Kruskal–Wallis analysis of variance with pairwise comparisons). Panel B shows the relationship between the serum RBP4 level and insulin sensitivity before exercise training. There is an inverse log-linear correlation between serum RBP4 (log [base-10 scale] x axis) and the rate of glucose disposal per kilogram of body weight per minute (linear y axis) during a euglycemic–hyperinsulinemic clamp study. The graph includes all subjects (those with normal glucose tolerance, those with impaired glucose tolerance, and those with type 2 diabetes) analyzed together (left) or according to sex (center and right). Blood was drawn after an overnight fast. The Spearman correlation coefficients were as follows: for all subjects,  $R=-0.78$  (95 percent confidence interval,  $-0.86$  to  $-0.65$ ); for men,  $R=-0.77$  (95 percent confidence interval,  $-0.89$  to  $-0.59$ ); and for women,  $R=-0.79$  (95 percent confidence interval,  $-0.89$  to  $-0.60$ ). To convert values for glucose disposal to micromoles per kilogram per minute, multiply by 5.549.

leukin-6 levels. Therefore, a change in the RBP4 level in response to exercise training in a given subject predicted the degree of improvement in insulin sensitivity with greater specificity than did the responses of other adipokines or markers of inflammation that are altered in obesity, type 2 diabetes, or both.

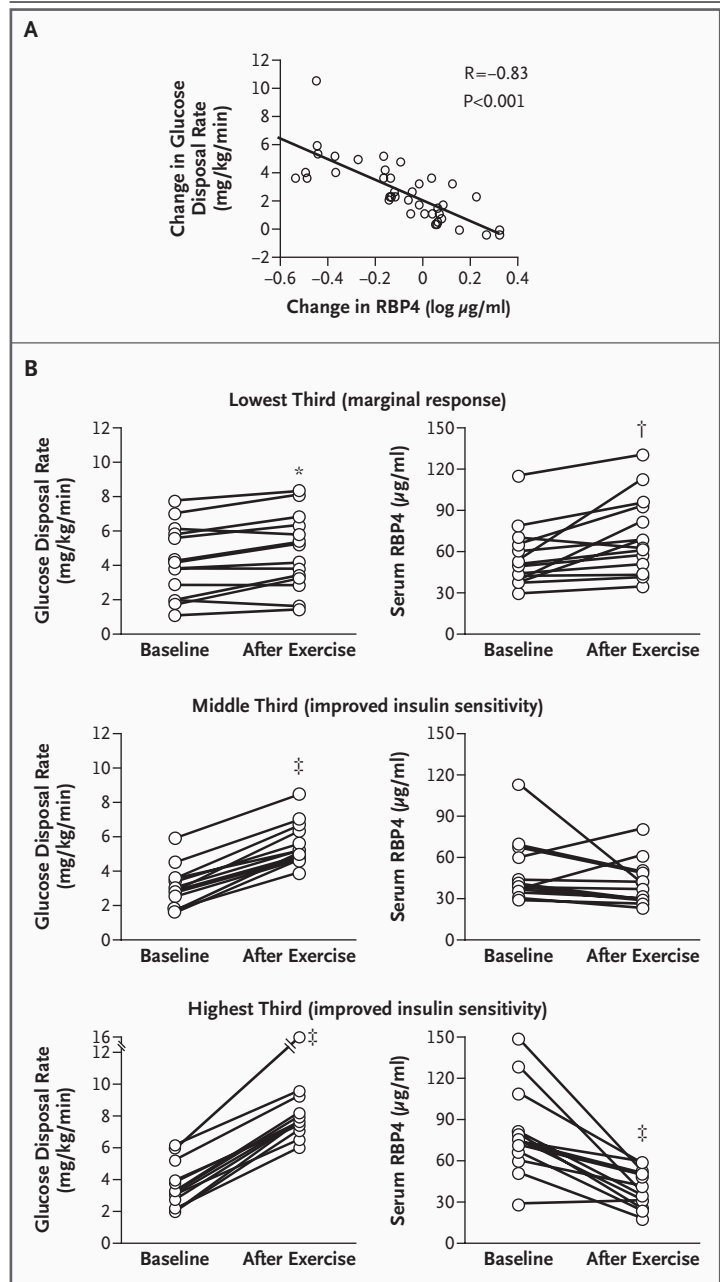
**SERUM RBP4 AND GENETIC RISK OF TYPE 2 DIABETES**

To determine whether RBP4 is elevated when insulin resistance is present in persons without obesity or clinically apparent disease, we studied nonobese, normoglycemic men with at least one first-degree relative with type 2 diabetes.<sup>20</sup> The rate

**Figure 3.** Changes in Serum RBP4 Levels and Insulin Sensitivity after Exercise Training in Subjects with Newly Diagnosed Impaired Glucose Tolerance or Type 2 Diabetes in Group 2.

Panel A shows coordinate and inverse responses of the serum RBP4 level and insulin sensitivity of individual subjects to exercise training. The correlation between changes in serum RBP4 levels (log [base-10 scale] x axis) and changes in the glucose disposal rate (linear y axis) caused by one month of exercise training are shown; Spearman correlation coefficient  $R = -0.83$  (95 percent confidence interval,  $-0.91$  to  $-0.70$ ). Panel B shows insulin sensitivity (glucose disposal rate, left) and serum RBP4 levels (right) in individual subjects separated into thirds on the basis of the response of glucose disposal rate to exercise training. Baseline and after exercise refer to clamp studies or sampling of blood before exercise training and after one month of exercise training. Studies were performed at least three days after the last exercise session. The asterisk indicates  $P = 0.005$ , the dagger  $P = 0.002$ , and the double dagger  $P < 0.001$  for the change in glucose disposal rate or RBP4 after exercise training as compared with baseline (Wilcoxon signed-rank test). To convert values for glucose disposal to micromoles per kilogram per minute, multiply by 5.549.

of glucose disposal during a euglycemic-hyperinsulinemic clamp study is a strong predictor of diabetes in such persons, who are at high risk for type 2 diabetes.<sup>21,27-29</sup> These subjects had a wide range of insulin sensitivities, hyperinsulinemia, dyslipidemia, and hypertension (Table 1), a finding consistent with the variable contributions of their lifestyles and genetics to their risk of type 2 diabetes and the metabolic syndrome. Serum RBP4 levels correlated inversely with the rate of glucose disposal (Fig. 4A) and correlated positively with fasting insulin levels, the insulin level in response to the OGTT, fasting triglyceride level, and systolic blood pressure (Fig. 4B). Similar correlations were observed between RBP4 level and these metabolic values in female offspring of persons with type 2 diabetes (data not shown). Multivariate regression analysis showed that the serum RBP4 level correlated with the rate of glucose disposal independently of both age and body-mass index ( $P = 0.009$ ) and with the triglyceride level independently of the rate of glucose disposal ( $P < 0.001$ ). Subjects in group 3 had a restricted range of body-mass-index values and had normal fasting glucose levels (Table 1) and glucose levels after an OGTT (data not shown). The RBP4 level did not correlate with these values or with age (Table 1) or waist-to-hip ratio (data not shown).



GLUT4 messenger RNA and protein are reduced in adipocytes in many insulin-resistant states,<sup>6</sup> often long before type 2 diabetes develops.<sup>20</sup> We previously reported that genetic knockout of GLUT4 selectively in the adipocytes of mice is sufficient to induce the expression of RBP4 in adipose tissue, increase serum levels of RBP4, and induce systemic insulin resistance.<sup>8,9</sup> However, we cannot eliminate the possibility that the secretion of RBP4 from liver or other tissues may also be increased. To determine whether reduced

**Table 2. Effects of Exercise Training on Metabolic Values and Serum Adipokine Levels in Subjects with Newly Diagnosed Impaired Glucose Tolerance or Type 2 Diabetes.\***

Measurement	Marginal Response (N=14)†			Improved Insulin Sensitivity (N=26)‡		
	Baseline	Post-Exercise Training	Wilcoxon P Value	Baseline	Post-Exercise Training	Wilcoxon P Value
GDR (mg/kg/min)§	4.1±2.0	4.7±2.1	0.005	3.4±1.0	7.1±1.7¶	<0.001
BMI	31.2±3.7	30.5±3.4	0.04	30.3±2.3	29.4±0.7	<0.001
Waist-to-hip ratio	1.29±0.14	1.25±0.10	0.005	1.22±0.10	1.18±0.11	<0.001
Fasting glucose (mg/dl)	108±13	106±10	0.18	106±7	99±6	0.007
2-hr OGTT glucose (mg/dl)	218±9	205±17	0.08	194±27	176.4±31	0.02
Fasting insulin (μU/ml)**	64.1±30.6	39.6±18.4	0.002	74.3±46.2	44.4±29.2	<0.001
RBP4 (μg/ml)	55.5±19.7	65.9±22.8	0.002	69.8±26.5	40.1±10.5¶	<0.001
Leptin (pmol/liter)	19.9±12.4	17.8±17.3	0.30	18.8±11.9	17.9±10.5	0.41
Adiponectin (μg/ml)	3.6±1.7	5.8±2.0	<0.001	3.4±1.0	6.1±1.7	<0.001
Interleukin-6 (pg/ml)	6.5±3.4	5.9±3.1	0.07	4.8±2.4	5.1±2.4	0.08
C-reactive protein (μg/dl)	0.7±0.4	0.4±0.3	0.005	0.5±0.2	0.2±0.1¶	<0.001
Free fatty acids (mmol/liter)	0.49±0.24	0.44±0.17	0.17	0.58±0.17	0.52±0.25	0.07
HDL cholesterol (mg/dl)††	34±5	39±6	0.004	36±4	42±4	<0.001

\* Blood for all studies was drawn with the subject in the fasting state. Data are given as means ±SD. Subjects were ranked into thirds on the basis of improvement in their insulin sensitivity (glucose disposal rate) associated with exercise training. "Marginal response" refers to subjects in the lowest third of this ranking, who exhibited the least improvement in insulin sensitivity. "Improved insulin sensitivity" refers to the remaining subjects in the upper two thirds. Differences after one month of exercise training were analyzed by the Wilcoxon signed-rank test. GDR denotes glucose disposal rate, BMI body-mass index, and HDL high-density lipoprotein.

† Among the 14 subjects with a marginal response, 8 were men and 6 were women; 5 had impaired glucose tolerance and 9 had type 2 diabetes; and the average age was 55±7 years.

‡ Among the 26 subjects with improved insulin sensitivity, 11 were men and 15 were women; 15 had impaired glucose tolerance and 11 had type 2 diabetes; and the average age was 54±7 years.

§ To convert values to micromoles per kilogram per minute, multiply by 5.549.

¶ P<0.05 for the comparison of either baseline values or those after exercise training between the two groups (marginal response or improved insulin sensitivity) by the Wilcoxon signed-rank test.

|| To convert values to millimoles per liter, multiply by 0.05549.

\*\* To convert values to picomoles per liter, multiply by 7.175.

†† To convert values to millimoles per liter, multiply by 0.0259.

adipocyte levels of GLUT4 might contribute to elevated serum RBP4 and insulin resistance in humans, we measured GLUT4 protein in isolated subcutaneous adipocytes of subjects in group 3. The level of adipocyte GLUT4 protein correlated positively with the rate of glucose disposal and correlated inversely with the serum RBP4 level (Fig. 4C and 4D). These data provide support for the existence of a mechanistic link between reduced GLUT4 protein in adipocytes, elevated serum RBP4, and insulin resistance.

#### DISCUSSION

RBP4, a molecule secreted by adipocytes and liver, may contribute to systemic insulin resistance.<sup>9</sup>

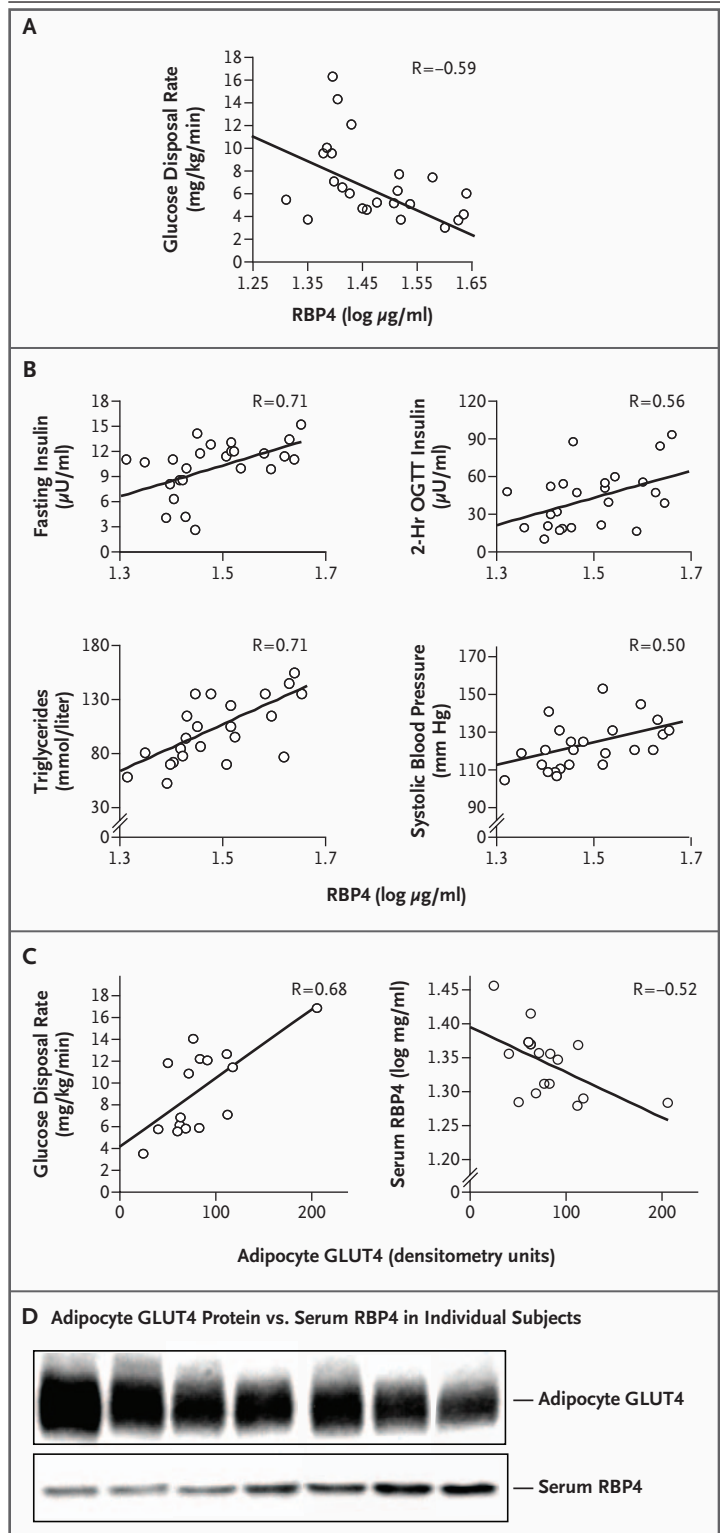
We found that the magnitude of increase in serum RBP4 correlates with insulin resistance among humans with obesity, impaired glucose tolerance, or type 2 diabetes and among nonobese, nondiabetic subjects with strong family histories of type 2 diabetes. The serum RBP4 level is correlated with a cluster of cardiovascular risk factors accompanying insulin resistance as part of the metabolic syndrome.<sup>23</sup> Even though the serum RBP4 level correlated with body-mass index, the relationship between the serum RBP4 level and insulin resistance was independent of obesity, and nonobese, insulin-resistant subjects also exhibited increased serum RBP4 levels. In these nonobese subjects, decreased expression of GLUT4 in adipocytes predicts increased serum RBP4 levels

**Figure 4. Serum Levels of RBP4, Risk Factors for Type 2 Diabetes and Cardiovascular Disease, and Adipocyte GLUT4 Protein in Nonobese, Normoglycemic Subjects with a Family History of Type 2 Diabetes in Group 3.**

Panel A shows the inverse correlation of serum RBP4 levels (log [base-10 scale] x axis) with insulin sensitivity during euglycemic-hyperinsulinemic clamp studies (glucose disposal rate, linear y axis). Spearman correlation coefficient  $R = -0.59$  (95 percent confidence interval,  $-0.80$  to  $-0.23$ ),  $P = 0.002$ . Panel B shows the association of RBP4 levels with cardiovascular risk factors, including fasting plasma insulin level, two-hour OGTT-stimulated insulin level, fasting triglyceride level, and systolic blood pressure (all linear). The Spearman correlation coefficients are as follows: for fasting insulin,  $R = 0.71$  (95 percent confidence interval,  $0.47$  to  $0.85$ ),  $P < 0.001$ ; for the insulin level after the OGTT,  $R = 0.51$  (95 percent confidence interval,  $0.28$  to  $0.74$ ),  $P < 0.001$ ; for fasting triglyceride level,  $R = 0.71$  (95 percent confidence interval,  $0.40$  to  $0.86$ ),  $P < 0.001$ ; for systolic blood pressure,  $R = 0.50$  (95 percent confidence interval,  $0.13$  to  $0.74$ ),  $P = 0.009$ . Panel C shows the positive correlation between adipocyte GLUT4 protein levels and insulin sensitivity (glucose disposal rate, left) and the inverse correlation between adipocyte GLUT4 protein levels and serum RBP4 levels (right). The Spearman correlation coefficients are as follows:  $R = 0.68$  (95 percent confidence interval,  $0.28$  to  $0.88$ ),  $P = 0.003$  for glucose disposal rate and GLUT4;  $R = -0.52$  (95 percent confidence interval,  $-0.82$  to  $-0.07$ ),  $P = 0.04$  for serum RBP4 and GLUT4. Panel D shows immunodetection of GLUT4 in isolated subcutaneous adipocytes from individual subjects (top) and serum RBP4 (bottom) in the same subject. Each lane in the blot represents a different subject in group 3. To convert values for glucose disposal to micromoles per kilogram per minute, multiply by 5.549. To convert values for insulin to picomoles per liter, multiply by 7.175. To convert values for triglycerides to milligrams per deciliter, multiply by 0.0112.

and insulin resistance. The mechanism by which a decrease in adipocyte GLUT4 results in an increase in RBP4 expression is unknown, but it might involve sensing of glucose by adipocytes.<sup>30</sup>

The correlation of serum RBP4 levels with plasma insulin levels suggests that the expression of RBP4 in adipose tissue might be a direct consequence of hyperinsulinemia. However, subjects with type 2 diabetes had lower fasting plasma insulin levels than subjects with impaired glucose tolerance with similar degrees of insulin resistance, but the RBP4 levels were similar in the two groups. Moreover, RBP4 and plasma insulin levels were dissociated in subjects who did not have an improvement in insulin sensitivity after exercise. Therefore, a primary reduction in the plasma insulin level alone does not determine



serum RBP4 levels. Nevertheless, there may be a threshold at which plasma insulin is permissive for increased RBP4 expression in adipocytes, since

the serum RBP4 level is reduced in subjects with new-onset type 1 diabetes and returns to normal after insulin treatment.<sup>31</sup>

The ability to assess a person's risk of impaired glucose tolerance and type 2 diabetes before the onset of the disease would provide a rational means for implementing preventive lifestyle interventions or pharmacologic treatment. Because the serum RBP4 level correlates with insulin resistance and the clinical signs and biochemical components of the metabolic syndrome, measurement of serum RBP4 could become a noninvasive and accessible method for assessing the risks of impaired glucose tolerance, type 2 diabetes, and cardiovascular disease. Altered levels of several adipocyte-secreted proteins (e.g., leptin and adiponectin), inflammatory cytokines (e.g., interleukin-6, monocyte chemoattractant protein 1, and tumor necrosis factor  $\alpha$ ), or inflammatory markers (e.g., C-reactive protein) have been observed in patients with obesity or insulin resistance.<sup>22</sup> Our studies suggest that the serum RBP4 level is correlated more specifically with insulin resistance and changes in insulin sensitivity than are the levels of several of these proteins (i.e., leptin, adiponectin, interleukin-6, and C-reactive protein). We observed that the RBP4 level correlated with insulin resistance, even in lean subjects, whose genetic risk for diabetes may be overlooked in some clinical settings. We studied adults, and nearly all were white; further studies are needed in more diverse groups.

Since elevated serum RBP4 levels lead to insulin resistance in mice,<sup>9</sup> our observations raise the possibility that the serum RBP4 level might contribute to systemic insulin resistance in humans. In mice, increased serum RBP4 levels impair postreceptor insulin signaling at the level of phosphoinositide-3 kinase in muscle and enhance the expression of phosphoenolpyruvate carboxykinase in liver.<sup>9</sup> Therefore, increased serum RBP4 levels in humans might contribute to impaired insulin-stimulated glucose uptake in muscle and elevated hepatic glucose production, both of which are characteristic of type 2 diabetes.<sup>1</sup> Regions near the *RBP4* locus on human chromosome 10q have been linked to hyperinsulinemia or early onset of type 2 diabetes in two populations, a finding con-

sistent with a pathogenic role for RBP4 in insulin resistance and type 2 diabetes.<sup>32,33</sup>

Since RBP4 is the principal transport protein for retinol (vitamin A) in the circulation,<sup>10,11</sup> our findings further raise the possibility that alterations of retinol metabolism might influence the action of insulin and the risk of type 2 diabetes. At present, there are no compelling data to suggest that dietary vitamin A contributes to the elevation in serum RBP4 levels observed in insulin-resistant states or to insulin resistance. However, administration of the synthetic retinoid fenretinide, an antineoplastic agent that reduces serum RBP4 and total-body retinol levels in humans,<sup>34</sup> improves insulin sensitivity and glucose tolerance in obese mice.<sup>9</sup> Therefore, it will be important to determine whether the dietary intake of retinol influences insulin sensitivity and whether lowering body retinol or RBP4 through the administration of fenretinide or related compounds improves insulin sensitivity in humans.

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