

ASSOCIATION OF A POLYMORPHISM IN THE β_3 -ADRENERGIC-RECEPTOR GENE WITH FEATURES OF THE INSULIN RESISTANCE SYNDROME IN FINNS

ELISABETH WIDÉN, M.D., MARKKU LEHTO, M.Sc., TIMO KANNINEN, B.Sc., JEREMY WALSTON, M.D.,
ALAN R. SHULDINER, M.D., AND LEIF C. GROOP, M.D.

Abstract Background. Because visceral obesity predicts insulin resistance, we studied whether alterations in the gene encoding for the β_3 -adrenergic receptor in visceral fat are associated with insulin resistance.

Methods. We studied the frequency of a cytosine-to-thymidine mutation that results in the replacement of tryptophan by arginine at position 64 (Trp64Arg) of the β_3 -adrenergic receptor by restriction-enzyme digestion with BstOI in 335 subjects from western Finland, 207 of whom were nondiabetic and 128 of whom had non-insulin-dependent diabetes mellitus (NIDDM). We also determined the frequency of the mutation in 156 subjects from southern Finland. Sensitivity to insulin was measured by the hyperinsulinemic-euglycemic clamp technique in 66 randomly selected nondiabetic subjects.

Results. In the subjects from western Finland, the frequency of the mutated allele was similar in the nondiabetic subjects and the subjects with NIDDM (12 vs. 11 percent). The mean age of the subjects at the onset of di-

abetes was lower among those with the mutation than those without it (56 vs. 61 years, $P=0.04$). Among the nondiabetic subjects, those with the mutation had a higher ratio of waist to hip circumference ($P=0.02$), a greater increase in the serum insulin response after the oral administration of glucose ($P=0.05$), a higher diastolic blood pressure (82 vs. 78 mm Hg, $P=0.01$), and a lower rate of glucose disposal during the clamp study (5.3 vs. 6.5 mg [29 vs. 36 μmol] per kilogram of body weight per minute; $P=0.04$) than the subjects without the mutated allele. In an analysis of sibling pairs, the siblings with the mutation generally had higher waist:hip ratios ($P=0.05$) and higher responses of blood glucose and serum insulin after the oral administration of glucose than their siblings without the mutation ($P=0.02$ and $P=0.005$, respectively).

Conclusions. The Trp64Arg allele of the β_3 -adrenergic receptor is associated with abdominal obesity and resistance to insulin and may contribute to the early onset of NIDDM. (*N Engl J Med* 1995;333:348-51.)

RESISTANCE to insulin in skeletal muscle has often been attributed to concomitant obesity, particularly abdominal obesity,¹⁻⁵ and associated with glucose intolerance, hypertension, and dyslipidemia.⁶ Whether abdominal obesity leads to insulin resistance or the reverse, or whether both conditions are a consequence of a third factor, possibly genetic, is not known. The theory of a genetic basis is supported by findings of insulin resistance and increased abdominal obesity in first-degree family members of patients with non-insulin-dependent diabetes mellitus (NIDDM)⁷ (and unpublished data). There are several putative candidates for this genetic factor, including the recently cloned *ob* gene,⁸ tumor necrosis factor- α ,⁹ and the β_3 -adrenergic-receptor gene.¹⁰

The β_3 -adrenergic receptor is expressed in visceral fat in humans¹¹ and is considered responsible for increases in lipolysis and the delivery of free fatty acid into the portal vein.¹² An increase in visceral fat mass, in turn, correlates with resistance to insulin in skeletal muscle.¹³ An abnormality in the β_3 -adrenergic receptor could therefore explain the link between abdominal obesity and insulin resistance. To test this hypothesis, we studied nondiabetic subjects and subjects with NIDDM to detect polymorphism in the gene for the

β_3 -adrenergic receptor, which is described by Walston et al. in this issue of the *Journal*,¹⁴ and related the receptor genotypes to estimates of insulin sensitivity and abdominal obesity.

METHODS

Study Subjects

All the subjects were participants in the ongoing Bothnia Study in western Finland, which began in 1990 with the goal of identifying early metabolic defects and genetic alterations in families with NIDDM. In this study, 128 subjects with NIDDM (67 women and 61 men) among whom there were no first-degree relationships and 207 similarly unrelated nondiabetic subjects (108 women and 99 men) were randomly selected from the population of western Finland. We studied the polymorphism of the β_3 -adrenergic receptor that results in the replacement of tryptophan with arginine at position 64 (Trp64Arg) in 17 normoglycemic pairs of siblings of the same sex, one of each pair being homozygous for the Trp64 allele and the other being heterozygous for the mutation, and compared the effect of the polymorphism on obesity and glucose metabolism within each pair. The allelic frequencies associated with the β_3 -adrenergic receptor were also determined in a group of 79 subjects with NIDDM and 77 nondiabetic subjects, all from southern Finland. The study was approved by the local ethics committees, and all subjects gave their informed consent.

Metabolic Characterization of the Subjects

Glucose tolerance was assessed by administering 75 g of glucose orally after an overnight fast. Venous-blood samples were drawn 10 minutes before the glucose ingestion, at the time of the ingestion, and 30, 60, and 120 minutes thereafter for the determination of blood glucose and serum insulin concentrations. Fat-free mass was measured by infrared spectroscopy of the outer layer of the biceps on the dominant arm with a Futrex 5000 device (Futrex, Gaithersburg, Md.). The subject's waist was measured with a soft tape midway between the lowest rib and the iliac crest. The hip circumference was measured at the widest part of the gluteal region. Blood pressure was measured in the right arm after 30 minutes of rest, with the subject seated. A subgroup of subjects randomly selected from the group

From the Department of Endocrinology, University of Lund, Lund, Sweden (E.W., M.L., T.K., L.C.G.); the Fourth Department of Medicine, Helsinki University Hospital, Helsinki, Finland (E.W.); and the Division of Geriatric Medicine and Gerontology, Johns Hopkins University School of Medicine, Baltimore (J.W., A.R.S.). Address reprint requests to Dr. Groop at the Department of Endocrinology, Malmö University Hospital, 205 02 Malmö, Sweden.

Supported by a grant (10858) from the Swedish Medical Research Council and by the Sigrid Juselius Foundation, the Novo Nordisk Foundation, the Crafoord Foundation, the Medical Society of Finland, the Pahlsson Foundation, and the Perklén Foundation.

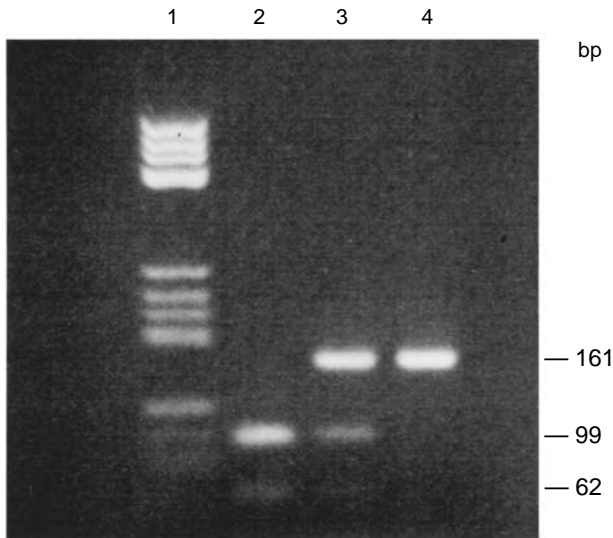


Figure 1. Detection of Trp64Arg Polymorphism of the β_3 -Adrenergic Receptor by PCR and Analysis of Restriction-Fragment-Length Polymorphism.

The PCR products (210 bp) were digested with the restriction enzyme *Bst*OI and visualized by staining with ethidium bromide. Lane 1 shows a molecular-weight marker (*pBr322/Hae* III); lane 2, a Trp64 homozygote; lane 3, a Trp64/Arg64 heterozygote; and lane 4, an Arg64 homozygote.

from western Finland were studied by the hyperinsulinemic–euglycemic clamp technique (in which the insulin concentration was raised by 80 μ U per milliliter), as described elsewhere.⁷

Assays

Blood glucose concentrations after oral glucose administration were measured by a hexokinase method (Boehringer–Mannheim, Mannheim, Germany). Plasma glucose concentrations during the clamp study were measured by a glucose oxidase method (Beckman Glucose Analyzer II, Beckman Instruments, Fullerton, Calif.). Serum insulin concentrations were measured by a radioimmunoassay (Pharmacia, Uppsala, Sweden) with an interassay coefficient of variation of 7.5 percent. The increased area under the curve for the concentrations of glucose and insulin was calculated by the trapezoidal rule. Serum concentrations of total cholesterol, high-density lipoprotein (HDL) cholesterol (after precipitation), and triglycerides were measured with a Cobas Mira analyzer (Hoffmann–LaRoche, Basel, Switzerland).

Detection of the Trp64Arg Polymorphism

The polymerase chain reaction (PCR) was carried out in a volume of 15 μ l containing 25 ng of genomic DNA from leukocytes; 10 pmol each of the primers BSTNUP (5'CGCCCAATACCGCAACAC) and BSTNDOWN (5'CCACCAGGAGTCCCATCACC); 200 μ M each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate, in 1.5 mM magnesium chloride; 10 mM TRIS–hydrochloric acid (pH 9.0); 50 mM potassium chloride; 0.1 percent Triton X-100; 4 percent formamide (pH 8.0); and 0.5 unit of *Taq* polymerase (Promega, Madison, Wis.). The PCR reactions (Perkin-Elmer 9600, Norwalk, Conn.) began with denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 61°C for 30 seconds, extension at 72°C for 30 seconds, with a final extension at 72°C for 10 minutes.

The amplified PCR products were digested with the addition of 5 μ l of a mixture containing 30 mM TRIS–hydrochloric acid (pH 7.9),

30 mM magnesium chloride, 150 mM sodium chloride, and 5 units of *Bst*OI, a restriction enzyme specific for the sequence CC(A/T)GG (Promega); this mixture was incubated at 60°C for two hours. The digested samples were separated by electrophoresis through a 3 percent agarose gel (Multipurpose agarose, Appligene, Illkirch, France) and visualized by staining with ethidium bromide.

Statistical Analysis

The results are presented as means \pm SD. Differences between group means were tested by Student's t-test, and for variables that were not normally distributed, by the Mann–Whitney U test. The chi-square test was used to compare frequencies. Differences between sibling pairs were tested by the nonparametric Wilcoxon's test. Correlations were calculated with Spearman's rank-correlation test.

RESULTS

Frequency of the Trp64Arg Polymorphism among β_3 -Adrenergic-Receptor Alleles

Digestion of the 210-base-pair (bp) PCR product with *Bst*OI produced fragments of the following sizes: 99, 62, 30, 12, and 7 bp in Trp64 homozygotes; 161, 99, 62, 30, 12, and 7 bp in Trp64/Arg64 heterozygotes; and 161, 30, 12, and 7 bp in Arg64 homozygotes (Fig. 1). The smallest of these fragments (30, 12, and 7 bp) were too small to be resolved on the gel. There was no difference between the diabetic and nondiabetic subjects in the frequency of the Trp64 and Arg64 alleles (Table 1). Two nondiabetic subjects from western Finland were homozygous for Arg64, as were two diabetic subjects from southern Finland.

Association between the Trp64Arg Polymorphism, Sensitivity to Insulin, and Obesity

Nondiabetic subjects with the Arg64 allele generally had higher ratios of waist to hip circumference than nondiabetic Trp64 homozygotes ($P=0.02$), despite similar values for body-mass index (the weight in kilograms divided by the square of the height in meters) and fat mass (Table 2). This difference was primarily due to differences among women. Nondiabetic subjects with the Arg64 mutation also had higher concentrations of blood glucose and serum insulin two hours after the oral ingestion of glucose than did the nondiabetic Trp64 homozygotes and had higher diastolic blood pressures than the Trp64 homozygotes ($P=0.01$). Sen-

Table 1. Frequency of the Trp64 and Arg64 Alleles among Nondiabetic Subjects and Subjects with NIDDM in Two Areas of Finland.*

ALLELE	WESTERN FINLAND		SOUTHERN FINLAND	
	NONDIABETIC SUBJECTS (N = 207)	SUBJECTS WITH NIDDM (N = 128)	NONDIABETIC SUBJECTS (N = 79)	SUBJECTS WITH NIDDM (N = 77)
	no. (%) of alleles			
Arg64	50 (12)	27 (11)	22 (14)	13 (8)
Trp64	364 (88)	229 (89)	136 (86)	141 (92)

*The risk ratio associated with the presence of the Arg64 allele in subjects with NIDDM was 0.9 (95 percent confidence interval, 0.5 to 1.4) for subjects from western Finland and 0.6 (95 percent confidence interval, 0.3 to 1.2) for subjects from southern Finland.

Table 2. Clinical Characteristics of Nondiabetic Subjects and Subjects with NIDDM, According to the Presence of the Arg64 Mutation.*

CHARACTERISTIC	NONDIABETIC SUBJECTS		SUBJECTS WITH NIDDM	
	MUTATION (N = 48)	NO MUTATION (N = 159)	MUTATION (N = 27)	NO MUTATION (N = 101)
Sex (F/M)	22/26	86/73	16/11	51/50
Family history of diabetes (% of subjects)	46	35	80	90
Age (yr)	50±15	49±14	64±10	68±11
Body-mass index†	26.4±3.7	26.0±4.0	29.1±4.6	27.7±4.0
Fat mass (% of body weight)	26.9±5.9	26.8±6.7	31.6±6.6	30.1±6.1
Waist:hip ratio				
Overall	0.92±0.08	0.89±0.09‡	0.93±0.07	0.93±0.08
Men	0.96±0.06	0.95±0.06	0.99±0.04	0.98±0.06
Women	0.86±0.05	0.83±0.07§	0.89±0.06	0.89±0.07
Age at onset of NIDDM (yr)	—	—	56±11	61±11¶
Blood pressure (mm Hg)				
Systolic	132±15	128±17	155±26	152±18
Diastolic	82±9	78±10	86±13	83±11
Blood glucose (mg/dl)				
Fasting	92±11.5	90±10.2	148±58	146±42
At 2 hr	104±23.5	95±23.0**	ND	ND
Serum insulin (mU/liter)				
Fasting	8.1±4.3	8.1±5.3	16.0±9.6	16.3±14.0
At 2 hr	59.1±52.3	41.0±34.8††	ND	ND
Increased area under curve over 2-hr period				
Glucose (mg/dl)	3294±2627	2556±2259	ND	ND
Insulin (mU/liter)	6223±4249	4832±3080‡‡	ND	ND
Serum lipids (mg/dl)				
Total cholesterol	208±33	224±47	232±42	239±63
HDL cholesterol	55±13	55±13	47±17	49±13
Triglycerides	119±59	124±62	205±127	217±193

*Plus-minus values are means ±SD. ND denotes not done, and HDL high-density lipoprotein. To convert values for glucose to millimoles per liter, multiply by 0.056; to convert values for insulin to picomoles per liter, multiply by 6; to convert values for cholesterol and HDL cholesterol to millimoles per liter, multiply by 0.026; and to convert values for triglycerides to millimoles per liter, multiply by 0.011.

†Calculated as the weight in kilograms divided by the square of the height in meters.

‡P=0.02. Except as otherwise noted, this and the following P values are for comparisons with the nondiabetic subjects with the Arg64 mutation.

§P=0.008.

¶P=0.04 for the comparison with subjects with NIDDM and the Arg64 mutation.

||P=0.01.

**P=0.006.

††P=0.005.

‡‡P=0.05.

sensitivity to insulin was assessed in 66 randomly selected nondiabetic subjects, 16 of whom were heterozygotes and 50 of whom were Trp64 homozygotes. The rate of insulin-stimulated glucose disposal was 18 percent less in the subjects with the Arg64 allele than in the Trp64 homozygotes (mean [±SD], 5.3±2.3 vs. 6.5±2.5 mg [29±13 vs. 36±14 μmol] per kilogram of body weight per minute; P=0.04).

The onset of diabetes occurred at an earlier age in the subjects with NIDDM who had the Arg64 allele than in those who did not have that allele (P=0.04). The two groups were similar with respect to body-mass index, fat mass, and waist:hip ratio (Table 2).

In the analysis of sibling pairs, the heterozygotes tended to be more obese than the Trp64 homozygotes (mean difference in weight, 6 kg; in body-mass index, 2.3; P=0.18 and P=0.20, respectively). The ratio of waist to hip circumference was generally higher in the member of each pair who had the Arg64 allele than in the one who did not (mean difference, 0.04; P=0.05) (Fig. 2). In addition, the areas under the curves for both the blood glucose and the serum insulin concen-

trations 120 minutes after the ingestion of glucose were larger for the siblings who had the Arg64 allele than for those who did not (mean difference for glucose, 2124 mg per deciliter [118 mmol per liter]; for insulin, 4071 mU per liter [24,430 pmol per liter]; P=0.02 and P=0.005, respectively) (Fig. 2).

DISCUSSION

The nondiabetic subjects with the Arg64 mutation of the gene for the β₃-adrenergic receptor had several characteristics of the insulin resistance syndrome⁶ — increased ratio of waist to hip circumference, glucose intolerance, hyperinsulinemia, and elevated blood pressure. The presence of insulin resistance was further established by measuring insulin sensitivity directly in a subgroup of the subjects. The findings of an increased ratio of waist to hip circumference and a decreased sensitivity to insulin were confirmed by an analysis of sibling pairs.

How could the β₃-adrenergic receptor be pathogenically involved in the development of insulin resistance in muscle? Increased deposits of abdominal fat could provide more free fatty acids for the synthesis of very-low-density lipoproteins in the liver, which could result in changes in the fatty-acid composition of skeletal-muscle membranes¹⁵ or higher concentrations of triglycerides in muscle. In rats, the accumulation of triglycerides in muscle is related to the impaired action of insulin.¹⁶ In humans, visceral obesity is associated with the enhanced sensitivity of visceral fat to catecholamine-induced lipolysis,¹² primarily mediated through effects on the β₃-adrenergic receptor. Visceral obesity is also associated with decreased uptake of free fatty acid by muscle and with insulin resistance in skeletal muscle — in particular, impaired synthesis of insulin-stimulated glycogen.¹³ The putative role of the β₃-adrenergic receptor in this scenario remains to be elucidated.

Insulin resistance is a major predictor of NIDDM.¹⁷ In the subjects with NIDDM who had the Arg64 allele, the onset of diabetes was significantly earlier than in the subjects who did not have this allele. These findings are consistent with those in Pima Indians, although diabetes had its onset about 20 years earlier in the Pima Indians than in our subjects.¹⁴ In contrast to the nondiabetic subjects, the diabetic subjects were not found to differ in the ratio of waist to hip circumference according to the genotype of the β₃-adrenergic receptor.

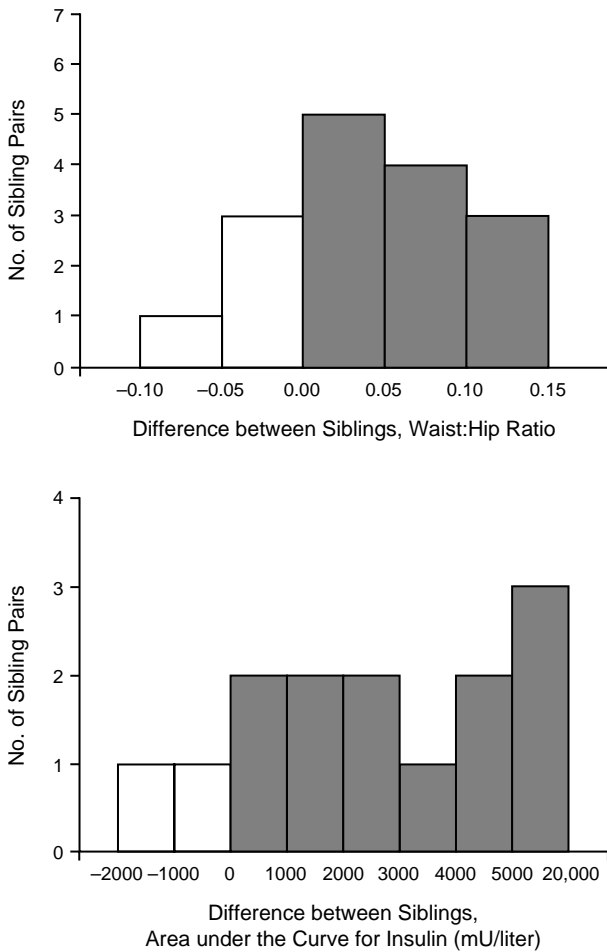


Figure 2. Differences between Siblings in the Ratio of the Waist to the Hip Circumference and in the Area under the Curve for the Serum Insulin Concentration 120 Minutes after the Oral Administration of Glucose.

In each sibling pair, one member of the pair was a Trp64/Arg64 heterozygote, and the other was a Trp64 homozygote. The value in the latter was subtracted from the value in the former to yield the difference. Positive values for the difference (shaded bars) indicate that the sibling with the Arg64 mutation was the more obese (upper panel) or the more resistant to insulin (lower panel). Only pairs for which complete data were available are shown. To convert values for insulin to picomoles per liter, multiply by 6.

Possibly, potential differences among these patients were masked by their concomitant hyperglycemia and its treatment.

The Arg64 allele could have been expected to be more frequent in subjects with NIDDM than in nondiabetic subjects. This was not the case, however, either in this study or in the study of the Pima Indians.¹⁴ If anything, the Arg64 allele was underrepresented among

the subjects with diabetes, especially the men (risk ratio, 0.6; P=0.23). In the Pima Indians, there was an age-dependent decrease in the frequency of the Arg64 allele among men.¹⁴ One explanation for this finding is that insulin resistance is associated with increased mortality from cardiovascular causes in men with NIDDM.

In conclusion, the presence of the Arg64 allele in the first intracellular loop of the β_3 -adrenergic-receptor gene may predispose patients to abdominal obesity, which may in turn predispose them to insulin resistance and the earlier onset of NIDDM. Determining the molecular mechanisms by which this change in amino acids in the β_3 -adrenergic receptor exerts this action should provide important insights into the genetic basis of abdominal obesity and insulin resistance.

We are indebted to the research team of the Bothnia Study for their skillful assistance.

REFERENCES

- DeFronzo RA. The triumvirate: β -cell, muscle, liver: a collusion responsible for NIDDM. *Diabetes* 1988;37:667-87.
- Ferrannini E, Haffner SM, Mitchell BD, Stern MP. Hyperinsulinaemia: the key feature of a cardiovascular and metabolic syndrome. *Diabetologia* 1991; 34:416-22.
- Caro JF. Insulin resistance in obese and nonobese man. *J Clin Endocrinol Metab* 1991;73:691-5.
- Mårin P, Andersson B, Ottosson M, et al. The morphology and metabolism of intraabdominal adipose tissue in men. *Metabolism* 1992;41:1242-8.
- Pouliot M-C, Després J-P, Nadeau A, et al. Visceral obesity in men: associations with glucose tolerance, plasma insulin, and lipoprotein levels. *Diabetes* 1992;41:826-34.
- Reaven GM. Role of insulin resistance in human disease. *Diabetes* 1988;37: 1595-607.
- Eriksson J, Franssila-Kallunki A, Ekstrand A, et al. Early metabolic defects in persons at increased risk for non-insulin-dependent diabetes mellitus. *N Engl J Med* 1989;321:337-43.
- Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse *obese* gene and its human homologue. *Nature* 1994;372:425-32.
- Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor- α : direct role in obesity-linked insulin resistance. *Science* 1993;259:87-91.
- Emorine LJ, Marullo S, Briand-Sutren M-M, et al. Molecular characterization of the human β_3 -adrenergic receptor. *Science* 1989;245:1118-21.
- Krief S, Lönnqvist F, Raimbault S, et al. Tissue distribution of β_3 -adrenergic receptor mRNA in man. *J Clin Invest* 1993;91:344-9.
- Lönnqvist F, Thörne A, Nilsell K, Hoffstedt J, Arner P. A pathogenetic role of visceral fat β_3 -adrenoreceptors in obesity. *J Clin Invest* 1995;95:1109-16.
- Colberg SR, Simoneau J-A, Thaeta FL, Kelley DE. Skeletal muscle utilization of free fatty acids in women with visceral obesity. *J Clin Invest* 1995; 95:1846-53.
- Walston J, Silver K, Bogardus C, et al. Time of onset of non-insulin-dependent diabetes mellitus and genetic variation in the β_3 -adrenergic-receptor gene. *N Engl J Med* 1995;333:343-7.
- Borkman M, Storlien LH, Pan DA, Jenkins AB, Chisholm DJ, Campbell LV. The relationship between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids. *N Engl J Med* 1993;328:238-44.
- Storlien LH, Jenkins AB, Chisholm DJ, Pascoe WS, Khouri S, Kraegen EW. Influence of dietary fat composition on development of insulin resistance in rats: relationship to muscle triglyceride and ω -3 fatty acids in muscle phospholipid. *Diabetes* 1991;40:280-9.
- Martin BC, Warram JH, Krolewski AS, Bergman RN, Soeldner JS, Kahn CR. Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study. *Lancet* 1992;340:925-9.