 Human Molecular Genetics, 1998, Vol. 7, No. 9 **1431–1435**

Association between uncoupling protein polymorphisms (*UCP2–UCP3*) and energy metabolism/obesity in Pima Indians

Ken Walder*,+, Rod A. Norman+, Robert L. Hanson, Patrick Schrauwen[1], Maria Neverova[2], Chris P. Jenkinson, Juliet Easlick[2], Craig H. Warden[2], Claire Pecqueur[3], Serge Raimbault[3], Daniel Ricquier[3], Michael Harper[4], Kristi Silver[4], Alan R. Shuldiner[4], Gemma Solanes[5], Bradford B. Lowell[5], Wendy K. Chung[6], Rudolph L. Leibel[6], Richard Pratley and Eric Ravussin

Clinical Diabetes and Nutrition Section, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health, 4212 North 16th Street, Room 541, Phoenix, AZ 85016, USA, [1]Department of Human Biology, Maastricht University, Maastricht, The Netherlands, [2]Rowe Program in Genetics and Department of Pediatrics, Biological Chemistry and Medicine, University of California—Davis, Davis, CA 95616, USA, [3]CNRS/CEREMOD, 9 rue Jules Hetzel, 92190 Meudon, France, [4]Division of Diabetes, Obesity and Nutrition, University of Maryland, Baltimore, MD 21201, USA, [5]Division of Endocrinology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215, USA and [6]Division of Molecular Genetics, Columbia University, College of Physicians and Surgeons, New York, NY 10032, USA

Received May 4, 1998; Revised and Accepted June 8, 1998

The *UCP2–UCP3* gene cluster maps to chromosome 11q13 in humans, and polymorphisms in these genes may contribute to obesity through effects on energy metabolism. DNA sequencing of *UCP2* and *UCP3* revealed three polymorphisms informative for association studies: an Ala→Val substitution in exon 4 of *UCP2*, a 45 bp insertion/deletion in the 3′-untranslated region of exon 8 of *UCP2* and a C→T silent polymorphism in exon 3 of *UCP3*. Initially, 82 young (mean age = 30 ± 7 years), unrelated, full-blooded, non-diabetic Pima Indians were typed for these polymorphisms by direct sequencing. The three sites were in linkage disequilibrium ($P < 0.00001$). The *UCP2* variants were associated with metabolic rate during sleep (exon 4, $P = 0.007$; exon 8, $P = 0.016$) and over 24 h (exon 8, $P = 0.038$). Heterozygotes for *UCP2* variants had higher metabolic rates than homozygotes. The *UCP3* variant was not significantly associated with metabolic rate or obesity. In a further 790 full-blooded Pima Indians, there was no significant association between the insertion/deletion polymorphism and body mass index (BMI). However, when only individuals >45 years of age were considered, heterozygotes (subjects with the highest sleeping metabolic rate) had the lowest BMI ($P = 0.04$). The location of the insertion/deletion polymorphism suggested a role in mRNA stability; however, it appeared to have no effect on skeletal muscle *UCP2* mRNA levels in a subset of 23 randomly chosen Pima Indians. In conclusion, these results suggest a contribution from *UCP2* (or *UCP3*) to variation in metabolic rate in young Pima Indians which may contribute to overall body fat content later in life.

INTRODUCTION

Recently, two new mitochondrial uncoupling proteins have been discovered with thermogenic properties that suggest involvement in the control of metabolic efficiency. Human uncoupling protein 2 (UCP2) is expressed in many tissues with greatest expression in skeletal muscle and tissues of the immune system (1). In rodents, UCP2 is involved in the regulation of energy metabolism. For instance, in adipose tissue of obesity-resistant mice, UCP2 expression is increased 2-fold when compared with obesity-prone animals (1). Uncoupling protein 3 (UCP3) has significant amino acid homology to UCP2 (71%), and expression of UCP3 in humans is much greater in skeletal muscle than in any other tissue (2–4). Several studies have shown that these uncoupling proteins are regulated by dietary alterations (2,5–7), thyroid hormones (4,8,9) and agonists of the β-3 adrenergic receptor (1,2,4), supporting the hypothesis that UCP2 and UCP3

*To whom correspondence should be addressed. Tel: +1 602 200 5345; Fax: +1 602 200 5335; Email: ken.walder@mailexcite.com
+These authors contributed equally to this work

could play an important role in energy metabolism and body weight regulation.

UCP2 and *UCP3* were both mapped to chromosome 11q13 in humans (1,10). The genes were found to be located on the same BAC and P1 clones, indicating that they are probably clustered (10). In Pima Indians, no significant evidence of linkage to obesity or energy metabolism was found in the region where the *UCP2–UCP3* gene cluster maps (11,12). In addition, an amino acid polymorphism within UCP2 was not significantly associated with body mass index (BMI), fat mass or weight gain in Danish Caucasians (13). Significant linkages, however, were reported between resting metabolic rate and markers flanking *UCP2–UCP3* in French Canadians (14).

The aim of this study was to determine whether genetic variations at *UCP2–UCP3* contribute to variation in energy metabolism and obesity in Pima Indians, a population prone to obesity and diabetes (15).

RESULTS

Initially, 82 full-blooded, unrelated, non-diabetic Pima Indians for whom we had measures of energy metabolism were typed for the three polymorphisms at *UCP2* and *UCP3*. Subject characteristics are shown in Table 1 and association results are shown in Table 2. For *UCP2*, both variants were biallelic with heterozygosities of 0.41 and 0.45, respectively, so tests of association included data from all three genotypes. For *UCP3*, the rare CC homozygotes were omitted from analysis since only two individuals had this genotype. Including these two subjects with the heterozygotes did not alter the results.

Sleeping metabolic rate and 24 h energy expenditure were significantly associated with *UCP2* variants. The average sleeping metabolic rate of subjects heterozygous for the Ala/Val polymorphism of exon 4 of *UCP2* was 153 kcal/day higher than Ala/Ala homozygotes and 52 kcal/day higher than Val/Val homozygotes ($P = 0.007$), adjusted for fat-free mass and fat mass. The average sleeping metabolic rate in individuals heterozygous for the insertion/deletion polymorphism in the 3′-untranslated region of exon 8 of *UCP2* was similarly higher than for del/del and ins/ins homozygotes: 135 and 54 kcal/day, respectively ($P = 0.016$). Average 24 h energy expenditure was also higher in the ins/del heterozygotes (+65 kcal/day versus del homozygotes, +35 kcal/day versus ins homozygotes; $P = 0.038$). No significant association was found for the C→T silent polymorphism in exon 3 of *UCP3* with metabolic rate.

Table 1. Characteristics of full-blooded Pima Indians in this study given as means ± SD [range]

Trait	Subjects for energy metabolism association	Subjects for BMI association
n (male/female)	82 (52/30)	790 (466/324)
Age (years)	30 ± 7 [18–56]	37 ± 11 [20–75]
Body weight (kg)	102 ± 21 [52–149]	117 ± 18 [79–167]
Height (cm)	168 ± 8 [149–187]	164 ± 9 [137–187]
24 h energy expenditure (kcal/day)	2349 ± 360 [1523–3342]	–
Sleeping metabolic rate (kcal/day)	1670 ± 284 [994–2425]	–
BMI (kg/m^2)	37.1 ± 8.3 [19.8–56.4]	36.8 ± 7.8 [21.1–73.8]

Table 2. Results of tests for association of metabolic rate[a] with *UCP2* and *UCP3* genotypes (mean ± SEM)

Genotype	*n*	Sleeping metabolic rate (kcal/day)	24 h Energy expenditure (kcal/day)
UCP2 exon 4			
Ala/Ala	18	1561 ± 38	2286 ± 36
Ala/Val	34	1714 ± 27	2324 ± 26
Val/Val	30	1662 ± 28	2375 ± 27
P-value	0.007	0.084	
UCP2 exon 8			
ins/ins	17	1673 ± 37	2347 ± 34
ins/del	38	1727 ± 25	2382 ± 23
del/del	27	1592 ± 31	2317 ± 27
P-value	0.016	0.038	
UCP3 exon 3			
C/C	2	1606 ± 118	2351 ± 104
C/T	18	1637 ± 40	2378 ± 34
T/T	62	1687 ± 21	2348 ± 18
P-value	0.196	0.682	

[a]Sleeping metabolic rate and 24 h energy expenditure were adjusted for fat-free mass, fat mass and sex. For *UCP2*, all three genotypes were tested resulting in an F test with 2 df. For *UCP3*, the rarer genotype (CC) was omitted (sample size of two), resulting in an F test with 1 df.

Table 3. Results of tests for association of BMI[a] with *UCP2* exon 8 genotype (mean ± SD)

	All subjects	Subjects aged >45
n (male/female)	790 (466/324)	139 (91/48)
UCP2 genotype		
ins/ins	37.4 ± 16.7	38.4 ± 13.4
ins/del	36.0 ± 15.8	33.7 ± 13.6
del/del	36.9 ± 14.1	34.6 ± 14.7
P-value	0.39	0.04

[a]BMI was adjusted for age and sex.

The exon 8 variant of *UCP2* was typed in a further 790 full-blooded Pima Indians, whose characteristics are given in Table 1. The allele frequencies were 0.41 for the insertion, and 0.59 for the deletion, while the genotype frequencies were 16% for ins/ins, 49% for ins/del and 35% for del/del. Concordance with Hardy–Weinberg equilibrium was confirmed for this variant (χ^2 = 0.5, df = 2). There was no difference in allele frequencies between diabetic and non-diabetic groups (χ^2 = 3.1, df = 2, *P* = 0.21).

Overall, no significant association was detected between the exon 8 variant and phenotypic measures of obesity such as BMI, or indicators of body fat distribution (waist circumference, waist-to-thigh ratio). However, when the analysis was confined to individuals over the age of 45 years [*n* = 139, age 54.7 ± 7.2 years, BMI 34.5 ± 6.7 kg/m^2 (mean ± SD)], we found evidence of an association between the genotype at the exon 8 variant of *UCP2* and BMI (adjusted for age and sex; *P* = 0.04). Individuals heterozygous for the ins/del polymorphism, who exhibited the highest sleeping metabolic rate and 24 h energy expenditure, had the lowest BMI, as would be expected (Table 3).

UCP2 mRNA levels were measured in muscle biopsies from 23 randomly chosen Pima Indians to assess the effect of this polymorphism on *UCP2* mRNA processing/stability. Of this group, only one individual was found to be homozygous for the insertion allele, and therefore was excluded from the analysis. Of the remaining 22 subjects, 10 were homozygous for the deletion, while 12 were heterozygotes. There was no significant difference in *UCP2* gene expression between these two groups (ins/del 0.21 ± 0.08 versus del/del 0.27 ± 0.15 arbitrary units; *P* = 0.27).

DISCUSSION

By virtue of their ability to uncouple electron transport from ATP synthesis, the uncoupling proteins may play an important role in the regulation of human energy metabolism. The substantial levels of *UCP2* expression in human adipose tissue (2) and muscle (1–3), and the high levels of *UCP3* expression in human skeletal muscle (2–4) are consistent with this viewpoint. The discovery of significant linkage between resting metabolic rate and *UCP2–UCP3*-linked microsatellite markers (14) suggests that variation in one or both of these genes can have an effect on metabolic rate.

In this study, we detected significant association of *UCP2* variants with metabolic rate during sleep and total energy expenditure over 24 h, and, to a lesser extent, with BMI in individuals >45 years of age. Total energy expenditure is comprised of the resting metabolic rate, the thermic effect of food and the energy cost of physical activity. Sixty to seventy percent of total energy expenditure is related to resting metabolic rate, which itself can be partitioned into the energy expended for sustaining life (~90%) (in this study estimated by sleeping metabolic rate) and energy expended to maintain the conscious state without physical activity (~10%). The major determinants of an individual's sleeping metabolic rate are the amount of fat-free mass and fat mass. Moreover, skeletal muscle metabolism is a major determinant of basal and sleeping metabolic rates (16). The expression of *UCP2* and *UCP3* in skeletal muscle tissue and the dominant role of sleeping metabolic rate in total energy expenditure are consistent with the potential effect of *UCP2–UCP3* genetic variation on metabolic rate and, therefore, body weight. The magnitude of the *UCP2* effect, however, appears to be small in this study. After adjustment for fat-free mass and fat mass, *UCP2* variants account for only 5–11% of the remaining variation in sleeping metabolic rate.

It was surprising to observe higher sleeping metabolic rate for *UCP2* heterozygotes. Given that the *UCP2* variants reported here were not associated with severe loss of function, one could speculate that increased metabolic rate might be due to different, but synergistic, biochemical properties of the two alleles.

Despite the association of the exon 8 variant of *UCP2* with sleeping metabolic rate, no significant association was found with BMI in the larger population of Pima Indians (*n* = 790). Only when the analysis was restricted to Pima Indians aged >45 years was a significant association found with BMI. This suggests that genetic variation in *UCP2* can affect metabolic rate, but that this effect may take many years to translate into a difference in body composition.

The location of this polymorphism in the 3′-untranslated region of exon 8 of the *UCP2* gene suggests possible involvement in mRNA processing or in the stability of the transcript. However, we found no significant difference in mRNA levels in skeletal muscle of Pima Indians according to the genotype at this site. This result does not preclude a possible effect of the insertion/deletion polymorphism on post-transcriptional modification or translation of *UCP2* mRNA, which potentially could result in altered levels and/or activity of the mature protein. It is also possible that this polymorphism is simply acting as a marker for another genetic variant in this region which affects energy metabolism.

In conclusion, genetic variation at the *UCP2* locus on human chromosome 11q13 was significantly associated with sleeping metabolic rate and 24 h energy expenditure in Pima Indians, and also with BMI in older Pima Indians.

MATERIALS AND METHODS

Subjects

In a first phase of this study, 82 full-blooded, non-diabetic Pima Indians (one per nuclear family) were randomly selected for association studies with energy metabolism. Subjects were genotyped at three polymorphic sites in *UCP2* and *UCP3*. A further 790 full-blooded Pima Indians were genotyped for association analysis with BMI at the exon 8 *UCP2* variant. Glucose tolerance was assessed by oral glucose tolerance test

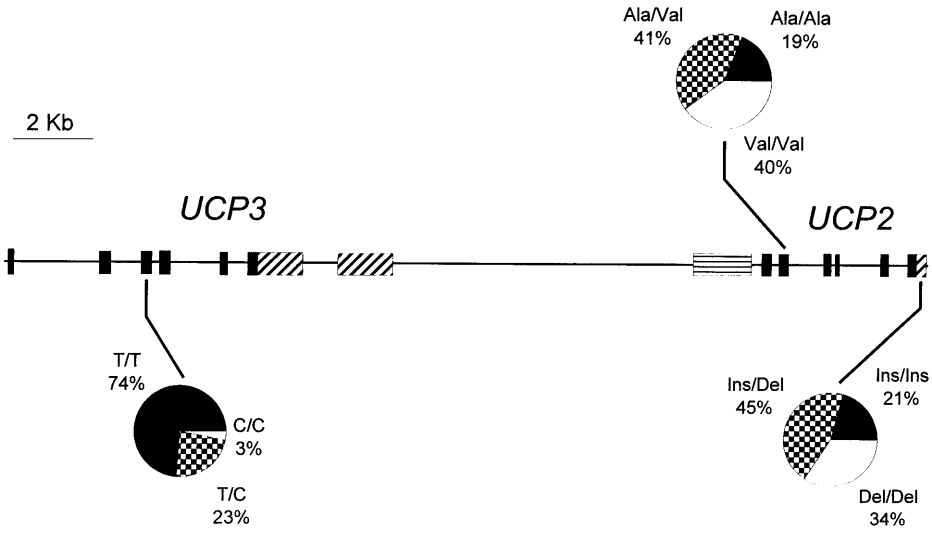

Figure 1. Chromosomal organization of human *UCP2–UCP3*. Exons are indicated by black bars. Untranslated regions are indicated by diagonal lines. *UCP3* has two alternative spliced forms (Solanes *et al.*, 1997). The *UCP2* boundary locations of exons 1 and 2 were not determined precisely so this region is indicated by horizontal lines. Genotypic frequencies for variant sites are given in pie charts.

according to World Health Organization (WHO) criteria (19). Characteristics of the two groups are given in Table 1.

UCP2–UCP3 polymorphisms

A search for variants of *UCP2* and *UCP3* was performed in 10 unrelated Hispanic individuals by direct sequencing of exons. Screening this limited number of individuals may fail to detect some rarer polymorphisms; however, we feel that this number is sufficient to identify most variants which are sufficiently polymorphic to warrant association studies. Two sites in *UCP2*, a C→T substitution in exon 4 resulting in an ala→val substitution at codon 55, and a 45 bp ins/del in the 3′-untranscribed region of exon 8, were found to be sufficiently polymorphic to warrant association studies. The 45 bp ins/del occurs 158 bp 3′ of the stop codon. It is shown in upper case as follows: CCCTCTTTCCCCACCTCTTCCTTCCGCCCTTTA-CCTACCACCTTCCCTCTTTCAACATTCT. Primers flanking the ins/del were designed to produce products of 457 or 412 bp, with or without the insertion. Sequences of these primers were hUCP2e8f (5′-CAG TGA GGG AAG TGG GAG G-3′) and hUCP2e8r (5′-GGG GCA GGA CGA AGA TTC-3′). Only one variant of *UCP3*, a C→T silent substitution in exon 3 at codon 99 (Tyr), was considered sufficiently polymorphic for informative association analysis. Both the *UCP2* exon 4 and *UCP3* exon 3 polymorphisms were typed by direct sequencing. The genomic organization and locations of the analysed variants are shown in Figure 1.

Determination of *UCP2* gene expression

The location of the *UCP2* exon 8 polymorphism suggests that it may affect the processing and/or stability of the *UCP2* mRNA transcript. We therefore measured the expression of *UCP2* in a subset of 23 individuals. Percutaneous muscle biopsies were taken from the vastus lateralis muscle after an overnight fast. *UCP2* gene expression was measured by reverse transcription PCR (RT–PCR), with β-actin co-amplified as an internal control. The linear phases of the *UCP2* and β-actin PCRs were determined empirically. The PCR products were resolved on 1% agarose gels containing ethidium bromide and photographed under UV transillumination. The relative concentrations of the PCR products were determined by scanning densitometry (BioImage 3.3, Sun Sparc Station 5). Each experiment was performed in triplicate and the mean value was used for analysis. Levels of mRNA were expressed as the ratio of signal intensity for *UCP2* relative to β-actin, corrected for the size of the PCR product.

Phenotypic measurements

Some individuals in the study had multiple measurements of energy metabolism over years. For these subjects, values at the lowest body weight were used in the analyses. This selection should increase the chances of finding potential effects of variants on energy metabolism since, in response to weight gain, an initial low metabolic rate tends to become normalized for the new body size and body composition (17). However, such selection criteria may not be optimal for finding effects on obesity. Twenty-four hour energy expenditure, an average measure of metabolic rate over a full day, was measured in a respiratory chamber (18). Sleeping metabolic rate, a measure of the basal metabolism necessary to support life, was calculated between 11 p.m. and 5 a.m. [using all 15 min periods during which spontaneous physical activity was detected <1.5% of the time (18)]. All energy expenditure results were adjusted for differences in fat-free mass, fat mass, age and sex.

A larger group of 790 full-blooded Pima Indians was then genotyped for the exon 8 polymorphism at *UCP2*. BMI was calculated as weight (kg)/height^2 (m). In individuals with multiple measurements over time, values at the highest body weight, regardless of diabetic status, were used in the analysis as this measure is likely to represent the true susceptibility for obesity. BMI was adjusted for age and sex, and log transformed to approximate a normal distribution.

Statistical analysis

All analyses were performed using the procedures of the SAS Institute (Cary, NC). Associations were determined using multiple regression models based on the least squares method. The effects of these variants on energy metabolism and obesity were estimated by analyses of covariance, while simultaneously adjusting for relevant confounding factors such as age, body composition and sex. Probability values of <0.05 were considered significant.

ACKNOWLEDGEMENTS

We thank the members of the Gila River Indian Community for participating in these studies. Bonnie Cooper and Kim Cray assisted in determining genotypes for the *UCP2* variants. Staff members of the Diabetes and Arthritis Epidemiology Section collected the pedigree data and DNA.

REFERENCES

1. Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Levi-Meyrueis, C., Bouillaud, F. *et al*. (1997) Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nature Genet*., **15**, 269–272.
2. Boss, O., Samec, S., Paoloni-Giacobino, A., Rossier, C., Dulloo, A., Seydoux, J., Muzzin, P. and Giacobino, J.P. (1997) Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression. *FEBS Lett*., **408**, 39–42.
3. Vidal-Puig, A., Solanes, G., Grujic, D., Flier, J.S. and Lowell, B.B. (1997) UCP3: an uncoupling protein homologue expressed preferentially and abundantly in skeletal muscle and brown adipose tissue. *Biochem. Biophys. Res. Commun*., **235**, 79–82.
4. Gong, D.-W., He, Y., Karas, M. and Reitman, M. (1997) Uncoupling protein-3 is a mediator of thermogenesis regulated by thyroid hormone, β3-adrenergic agonists, and leptin. *J. Biol. Chem*., **272**, 24129–24132.
5. Millet, L., Vidal, H., Andreelli, F., Larrouy, D., Riou, J.-P., Ricquier, D., Laville, M. and Langin, D. (1997) Increased uncoupling protein-2 and -3 mRNA expression during fasting in obese and lean humans. *J. Clin. Invest*., **100**, 2665–2670.
6. Matsuda, J., Hosoda, K., Itoh, H., Son, C., Doi, K., Tanaka, T., Fukunaga, Y. *et al*. (1997) Cloning of rat uncoupling protein-3 and uncoupling protein-2 cDNAs: their gene expression in rats fed high-fat diet. *FEBS Lett*., **418**, 200–204.
7. Weigle, D.S., Selfridge, L.E., Schwartz, M.W., Seeley, R.J., Cummings, D.E., Havel, P.J., Kuijper, J.L. and BertrandelRio, H. (1998) Elevated free fatty acids induce uncoupling protein 3 expression in muscle: a potential explanation for the effect of fasting. *Diabetes*, **47**, 298–302.
8. Larkin, S., Mull, E., Miao, W., Pittner, R., Albrandt, K., Moore, C., Young, A. *et al*. (1997) Regulation of the third member of the uncoupling protein family, UCP3, by cold and thyroid hormone. *Biochem. Biophys. Res. Commun*., **240**, 222–227.
9. Masaki, T., Yoshimatsu, H., Kakuma, T., Hidaka, S., Kurokawa, M. and Sakata, T. (1997) Enhanced expression of uncoupling protein 2 gene in white adipose tissue and skeletal muscle following treatment with thyroid hormone. *FEBS Lett*., **418**, 323–326.
10. Solanes, G., Vidal-Puig, A., Grujic, D., Flier, J.S. and Lowell, B.B. (1997) The human uncoupling protein-3 gene: genomic structure, chromosomal localization, and genetic basis for short and long form transcripts. *J. Biol. Chem*., **272**, 25433–25436.
11. Hanson, R.L. and Pima Diabetes Gene Group (1997) Genomic scan for markers linked to type II diabetes in Pima Indians. *Diabetes*., **46** (suppl. 1), 51A (abstract).
12. Norman, R.A., Tataranni, P.A., Pratley, R.E., Thompson, D.B., Prochazka, M., Baier, L.J., Ehm, M.G. *et al*. (1998) Autosomal genomic scan for loci linked to obesity and energy metabolism in Pima Indians. *Am. J. Hum. Genet*., **62**, 659–668.
13. Urhammer, S.A., Dalgaard, L.T., Sorensen, T.I.A., Moller, A.M., Andersen, T., Tybjaerg-Hansen, A., Hansen, T., Clausen, J.O., Vestergaard, H. and Pedersen, O. (1997) Mutational analysis of the coding region of the uncoupling protein 2 gene in obese NIDDM patients: impact of a common amino acid polymorphism on juvenile and maturity onset forms of obesity and insulin resistance. *Diabetologia*, **40**, 1227–1230.
14. Bouchard, C., Perusse, L., Chagnon, Y.C., Warden, C. and Ricquier, D. (1997) Linkage between markers in the vicinity of the uncoupling protein 2 gene and resting metabolic rate in humans. *Hum. Mol. Genet*., **6**, 1887–1889.
15. Knowler, W.C., Pettitt, D.J., Saad, M.F., Charles, M.A., Nelson, R.G., Howard, B.V., Bogardus, C. and Bennett, P.H. (1991) Obesity in the Pima Indians: its magnitude and relationship with diabetes. *Am. J. Clin. Nutr*., **53**, 1543S–1551S.
16. Zurlo, F., Larson, K., Bogardus, C. and Ravussin, E. (1990) Skeletal muscle metabolism is a major determinant of resting energy expenditure. *J. Clin. Invest*., **86**, 1423–1427.
17. Ravussin, E., Lillioja, S., Knowler, W.C., Christin, L., Freymond, D., Abbott, W.G., Boyce, V., Howard, B.V. and Bogardus, C. (1988) Reduced rate of energy expenditure as a risk factor for body-weight gain. *N. Engl. J. Med*., **318**, 467–472.
18. Ravussin, E., Lillioja, S., Anderson, T.E., Christin, L. and Bogardus, C. (1986) Determinants of 24-hour energy expenditure in man: methods and results using a respiratory chamber. *J. Clin. Invest*., **78**, 1568–1578.
19. WHO Study Group (1994) *Prevention of Diabetes Mellitus*. WHO Technical Report Series 727. World Health Organization, Geneva.