Diabetologia (2001) 44: 629–636

Diabetologia

Common variants in the β2-(Gln27Glu) and β3-(Trp64Arg) - adrenoceptor genes are associated with elevated serum NEFA concentrations and Type II diabetes

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Abstract

Aims/hypothesis. Higher NEFA concentrations predict Type II (non-insulin-dependent) diabetes mellitus but it is not known whether higher NEFA concentrations are genetically determined or reflect coexisting obesity. To address this question we studied whether common variants in two genes encoding for key regulators of lipolysis, the β_2- and β_3- adrenoceptors (B2AR and B3AR) are associated with NEFA concentrations and Type II diabetes.
Methods. A total of 1054 Swedish subjects with varying degrees of glucose tolerance were genotyped for the *Gln27Glu* variant in the *B2AR* and for the *Trp64Arg* variant in the *B3AR* genes using PCR-RFLP.
Results. The B2AR Gln27 allele was more frequent in 219 Type II diabetic patients than in 237 non-diabetic subjects (59.8 % vs 52.3 %; OR = 1.72, $p = 0.02$) while there was no significant difference in the frequency of the B3AR Arg64 allele. Subjects homozygous for the protective alleles (Glu27 and Trp64) had, however, a lower prevalence of diabetes than subjects with other genotype combinations (OR = 0.58, $p = 0.03$). Among sibling pairs discordant for the B2AR Gln27Glu polymorphism, siblings with an excess of the Gln27 allele had higher fasting insulin ($n = 217$; $p = 0.02$) and NEFA concentrations (107 sex-matched pairs; $p = 0.01$) than siblings with an excess of the Glu27 allele. Among sibling pairs discordant for the B3AR Trp64Arg variant, siblings with the Arg64 allele had higher 2 h glucose ($n = 48$; $p = 0.01$) and NEFA concentrations (16 pairs matched for sex; $p < 0.04$) than siblings with the Trp64Trp64 genotype.
Conclusions/interpretation. Common variants in the β_2- and β_3- adrenoceptor genes are associated with increased fasting insulin and NEFA concentrations and could increase susceptibility to Type II diabetes. [Diabetologia (2001) 44: 629–636]

Keywords Non-esterified fatty acids, Type II diabetes, lipolysis, β2-adrenoreceptor, β3-adrenoreceptor, insulin.

It is assumed that Type II diabetes is a consequence of the interaction between inherited and environmental factors [1]. Increased levels of non-esterified fatty acids (NEFAs) confer an increased risk of Type II (non-insulin-dependent) diabetes mellitus [2–3]. This could partially be mediated by activation of the glucose-fatty acid cycle leading to skeletal muscle insulin resistance [4] or the impairment of beta-cell function (the so-called lipotoxicity hypothesis) [5]. It is not known, however, whether altered lipolysis and higher NEFA concentrations are genetically determined or result from obesity in Type II diabetes mellitus. Lipolysis in fat cells is stimulated by catecholamines, which bind to specific receptors, including the β_2- and β_3- adrenergic receptors [6]. The human β_2- adrenoreceptor is the predominant receptor subtype involved in adipose tissue lipolysis; it stimulates

Received: 30 November 2000 and in revised from: 15 January 2001

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Abbreviations: B2AR, β2-adrenoreceptor; B3AR, β3 adrenoreceptor; PCR, polymerase chain reaction; OSD, observed sum of differences.

both lipolysis and fat tissue blood flow [7]. It has long been known that the β3-adrenoreceptor plays an important part in lipolysis and resting energy expenditure in rodents, but its role in humans has been questioned [8–9]. Evidence that the β_3 adrenoreceptor is expressed in visceral fat makes it a prime candidate for the regulation of lipolysis in humans as well [10]. The β_2-adrenoreceptor gene (*B2AR*) displays high genetic variability and common polymorphisms at codon 16 (Arg16Gly), 27 (Gln27Glu) and the mutation at codon 164 (Thr164Ile) could result in altered receptor function [11]. It has been suggested that the Glu27 variant is resistant to agonist-promoted down regulation [12]. Although this variant has been associated with obesity [13–15] and Type II diabetes [16–17], the findings have not been replicated in all studies [18–19]. In addition, a *C* to *T* nucleotide substitution at nucleotide –47 has been described in the 5'leader cistron (LC) of the *B2AR* gene (5'LCArg19-Cys), which is in linkage disequilibrium with the codon 16 and 27 polymorphisms [20].

A common Trp64Arg polymorphism in the first intracellular loop of the β_3-adrenoreceptor gene (*B3AR*) was described in three studies [21–23] as being associated with increased capacity to gain weight, abdominal obesity, insulin resistance and an earlier onset of Type II diabetes. These findings have been replicated in several [24–27] but not all studies [28–31]. There are several problems with association studies and negative replication studies are thus to be expected [32]. The choice of an appropriate control group presents a problem in most association studies and can only be overcome by the use of family-based controls [33].

Interaction between two susceptibility loci was shown to significantly increase the NPL score for the Type II (non-insulin-dependent) diabetes locus on chromosome 2 [34] and the Arg64 variant in the β3-adrenoreceptor gene has been shown to be a factor predisposing to weight gain in carriers of a promoter variant in the uncoupling protein 1 (*UCP1*) gene [35–36]. Epistasis could also be predicted between related genes like the genes encoding for the β-adrenergic receptors. To our knowledge no studies have evaluated the interaction between common variants in the β_2- and β_3- adrenergic receptor genes and the putative role of such interaction for Type II diabetes.

To address these questions we studied the prevalence of two common variants in the β_2- (Gln27Glu) and β_3- (Trp64Arg) adrenergic receptor genes and their effect on clinical characteristics such as serum NEFA and insulin concentrations as well as on prevalence of Type II diabetes using a family-based genotype-discordant sibling-pair approach and a case-control association study.

Subjects and methods

Subjects. A total of 1054 Caucasian subjects from southern Sweden were genotyped for the Gln27Glu variant in the *B2AR* gene and for the Trp64Arg variant in the *B3AR* gene. Of these, 817 (356 non-diabetic subjects and 461 diabetes patients) were siblings from 253 families with Type II diabetes. All these subjects had a first-degree family member with diabetes. As control subjects we also included 237 unrelated non-diabetic spouses without a family history of diabetes (males/females = 109/128, age = 60.9 ± 10.3 years, BMI = 28.5 ± 4.4 kg/m^2). For comparison between allele and genotype frequencies between diabetes patients and the non-diabetic control subjects, 219 unrelated diabetes patients (males/females = 110/109, age = 60.9 ± 10.4 years, BMI = 28.5 ± 4.4 kg/m^2) from the diabetes families were selected at random and compared with the control subjects.

Before participating in the study, the purpose, nature and potential risks were explained and informed written voluntary consent was obtained from each subject. The study protocol was approved by the ethics committee of Lund University.

Study Design. In the sibling pair analysis we tested the hypothesis that the phenotypic difference between siblings discordant for the Gln27Glu variant in *B2AR* or the Trp64Arg variant in *B3AR* would differ significantly from zero. For the Gln27Glu variant in the *B2AR,* we advanced the hypothesis that there will be a dose-dependent effect on the number of Gln27 alleles on the phenotype when comparing the Gln27Gln27 homozygote and Gln27Glu27 heterozygote siblings with the Glu27-Glu27 homozygote siblings, or the Gln27Gln27 homozygote siblings with the Gln27Glu27 heterozygote siblings. In other words, we always compared a sibling with a greater number of Gln27 alleles with a sibling with fewer Gln27 alleles. A total of 365 sibling pairs discordant for this polymorphism from 117 different families were identified and included in the study. Of them, 190 sibling pairs had the same gender (sex-matched). Complete clinical data were available from 209 sibling pairs and 107 sex-matched sibling pairs. The same approach was applied to 108 sibling pairs (53 sex-matched sibling pairs) from 37 families discordant for the Trp64Arg variant in the *B3AR* gene. Complete clinical data were available from 48 sibling pairs (16 sibling pairs matched for sex). Only the sex-matched sibling pairs were included for the analysis of differences in WHR, and triglyceride, HDL-cholesterol and NEFA concentrations. For estimation of differences in prevalence of diabetes between genotype discordant sibling pairs, only one sibling pair from each family was used.

Phenotypic characterization. Studies were carried out at 08:00 h after a 10-h overnight fast. Subjects were asked not to smoke, not to take their morning medication and not to exercise strenuously the day before the test. Height and weight were measured with subjects in light clothing and no shoes. Body mass index (BMI) was calculated and expressed in kilograms per meter squared (kg/m^2). Waist was measured with a soft tape midway between the lowest rib and the iliac crest and the hip circumference at the widest part of the gluteal region and the waist-to-hip ratio (WHR) was calculated as a measure of central adiposity. Systolic and diastolic blood pressure were measured with the subject in a supine position after a 15 min rest in the right arm twice and the mean was calculated. If the fasting blood glucose concentration was below 10 mmol/l an oral glucose tolerance test (OGTT) was done. During the OGTT, subjects ingested 75 g of glucose in a volume of 300 ml and venous samples for measurement of blood glucose and serum insulin were drawn at –5, 0 and 120 min. Ve-

Table 1. Frequency of the Gln27Glu polymorphism in the β_2-adrenoceptor and of the Trp64Arg polymorphism in the β_3-adrenoceptor genes and their genotype combinations in non-diabetic subjects and Type II diabetes patients

Non-diabetic subjects (n = 237)		Diabetes patients (n = 219)
Gln27Glu polymorphism in the β_2- adrenoceptor gene		
Gln27Gln27 n (%)	71 (30.0)	79 (36.1)
Gln27Glu27 n (%)	106 (44.7)	104 (47.5)
Glu27Glu27 n (%)	60 (25.3)	36 (16.4) [a]
Trp64Arg polymorphism in the β_3 -adrenoceptor gene		
Arg64Arg64 n (%)	1 (0.4)	0 (0)
Trp64Arg64 n (%)	31 (13.1)	35 (16.0)
Trp64Trp64 n (%)	205 (86.5)	184 (84.0)
Allele frequencies		
Gln27 (%)	52.3	59.8 [b]
Arg64 (%)	7.0	8.0
Genotype combinations of *Trp64Arg* and *Gln27Glu*		
Gln27Gln27 + Arg64Arg64 n (%)	1 (0.4)	0 (0)
Gln27Gln27 + Trp64Arg64 n (%)	13 (5.5)	16 (7.3)
Gln27Gln27 + Trp64Trp64 n (%)	57 (24.0)	63 (28.8)
Gln27Glu27 + Arg64Arg64 n (%)	0 (0)	0 (0)
Gln27Glu27 + Trp64Arg64 n (%)	12 (5.1)	15 (6.8)
Gln27Glu27 + Trp64Trp64 n (%)	94 (39.7)	89 (40.6)
Glu27Glu27 + Arg64Arg64 n (%)	0 (0)	0 (0)
Glu27Glu27 + Trp64Arg64 n (%)	6 (2.5)	4 (1.8)
Glu27Glu27 + Trp64Trp64 n (%)	54 (22.8)	32 (14.6) [c]

Subjects with the Gln27Gln27 and Gln27Glu27 compared with subjects with Glu27Glu27 genotype [a] $p = 0.02$, [b] $p = 0.02$. [c] $p = 0.03$ for subjects with the genotype combination Glu27-Glu27 + Trp64Trp64 compared with subjects having all other genotype combinations

nous fasting blood samples were also drawn for the measurement of serum concentrations of NEFA, total cholesterol, HDL-cholesterol and triglycerides. The new World Health Organisation criteria were used for the diagnosis of diabetes, that is patients were considered to have diabetes if their fasting blood glucose was greater than or equal to 6.1 mmol/l or 2 h glucose during an OGTT of more than 11.1 mmol/l or if the subjects received anti-diabetic medication.

Assays. Blood glucose during the OGTT was measured using a photometric method based on the glucose-dehydrogenase method using the HemoCue Blood Glucose Analyser (HemoCue AB, Ängelholm, Sweden). Serum was separated and kept at –20 °C until analysis. NEFA concentration was measured by an enzymatic colourimetric method using a commercial kit (Wako Chemicals GmbH, Neuss, Germany). Insulin concentrations were measured in samples from subjects not treated by insulin by a specific radioimmunoassay (DAKO Diagnostics, UK). Total cholesterol, HDL-cholesterol and triglyceride concentrations were analysed with commercially available kits (Technicon DAX 48, Bayer, Sweden).

Genotyping. The *B2AR* Gln27Glu and the *B3AR* Trp64Arg polymorphisms were genotyped by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) methods. A 169 bp fragment containing the Gln27Glu polymorphism was amplified with primers β_2-27-MM-F (5'-CCGGACCACGACGTCACCCAG) and β_2-27-R, (5'-CCAGTGAAGTGATGAAGTAGTT) of which the former contains a nucleotide mismatch (underlined) to create a *BstNI* recognition site in case of the Glu27 allele. The primers were designed according to the published β_2-adrenergic receptor cDNA sequence [37]. PCR reactions were done with 50 ng of genomic DNA in a total volume of 20 µl containing 10 pmol of each primer, 4 nmol dNTPs, 0.5 U Taq polymerase (Pharmacia, Sweden) in 1x $(NH_4)_2SO_4$-buffer (16 mmol/l $(NH_4)_2SO_4$; 67 mmol/l TRIS pH 8.8; 0.01 % Tween), with 3 % formamide and 1.5 mmol/l $MgCl_2$. PCR conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation (94 °C for 30 s), annealing (60 °C for 30 s) and extension (72 °C for 30 s), with the final extension at 72 °C for 10 min. The PCR products were digested with 1 U of *BstNI* (New England Biolabs, Beverly, Mass., USA) for 2 h at 60 °C using the buffers recommended by the manufacturer. The fragments were separated on 4.5 % multipurpose agarose gel (Appligene, Illkirch, France) with ethidium bromide and visualized under ultraviolet light.

The Trp64Arg polymorphism was genotyped by PCR amplification as described previously [23], with the following changes; the PCR reactions were done in a total volume of 20 µl, the $MgCl_2$ concentration was 3.0 mmol/l, 6 % formamide was added to each reaction, and annealing temperature was 63 °C. The PCR products were digested with 3U *BstNI* (New England Biolabs, Beverly, Mass., USA) using conditions recommended by the manufacturer. The digested samples were separated on a 4 % multipurpose agarose gel (Appligene, Illkirch, France).

Statistical analysis. The significance of frequency differences was tested by chi-squared (χ^2) test analysis using a BMDP software statistical package (Biomedical Data Processing version 7.0, 1992, Los Angeles, Calif., USA). The significance of frequency differences of diabetes between genotype discordant sibling pairs was estimated using one sibling pair from each family by McNemar test of symmetry. The phenotypic differences in continuous variables between genotype discordant sibling pairs were computed using a modified permutation test for paired replicates [38–39].

For computation of phenotypic differences, the value in the sibling with an excess of Gln27 alleles was subtracted from the value in the sibling with more Glu27 alleles and the value in siblings heterozygote for the Trp64Arg64 genotype subtracted from the Trp64Trp64 homozygote siblings.

If the observed sum of differences was in the 5 % region of rejection, the difference between the pairs was considered significant. Because of computational limitations, the two-tailed p value was estimated using a very large (10^7) random sample from all the possible permutations. All statistical tests were two-sided and a p value less than 0.05 was considered statistically significant. The significance of phenotypic differences found in the permutation test between genotype discordant sibling pairs was also estimated using one sibling pair from each family by paired t-test. Log_{10} transformed means where used for skewed data (2-h glucose, fasting insulin, 2-h insulin, triglycerides and NEFA concentrations). Data on variables showing significant differences between sexes (WHR, triglyceride, HDL-cholesterol and NEFA concentrations were analysed for sex-matched sibling pairs only.

Results

Allele and genotype frequencies. The frequency of the *B2AR* Gln27Glu and *B3AR* Trp64Arg variants were in Hardy Weinberg equilibrium (Table 1). The

Table 2. Clinical characteristics of sibling pairs discordant for the *Gln27Glu* polymorphism in the β_2- adrenoceptor gene

Variable	Gln^{27}	Glu^{27}	OSD
n sibling pairs (men/women)	209 (108/101)	209 (92/117)	
n sex-matched sibling pairs (men/women)	107 (49/58)	107 (49/58)	
Age (years)	58 ± 13	58 ± 13	82.3
BMI (kg/m^2)	27.9 ± 4.4	28.0 ± 4.7	–5.96
WHR	0.91 ± 0.09	0.91 ± 0.09	–0.39
Systolic BP (mm/Hg)	141 ± 20	140 ± 19	589
Diastolic BP (mm/Hg)	78 ± 9	78 ± 9	–220
FB- glucose (mmol/l)	8.2 ± 3.3	8.1 ± 3.4	128
2-h B glucose (mmol/l)	10.1 ± 5.3	9.9 ± 4.8	6.54
FS- insulin (mU/l)	12.1 ± 10	9.2 ± 8.1	302 [a]
2-h insulin (mU/l)	46 ± 45	50 ± 51	–301
FS-Triglycerides (mmol/l)	1.8 ± 1.2	1.6 ± 0.9	17.7
FS-cholesterol (mmol/l)	5.9 ± 1.1	5.9 ± 1.3	–18.8
HDL-cholesterol (mmol/l)	1.2 ± 0.4	1.2 ± 0.4	–3.7
Fs-NEFA (μmol/l)	810 ± 340	730 ± 320	9180 [b]
All sibling pairs DM, IGT, NGT (*n*)	113, 39, 57	104, 46, 59	
Sex-matched sibling pairs DM, IGT, NGT (*n*)	67, 20, 20	53, 28, 26	

The Gln^{27} column shows data for siblings with the $Gln^{27}Gln^{27}$ or $Gln^{27}Glu^{27}$ genotypes and the Glu^{27} column shows data for their genotype discordant pairs (with $Gln^{27}Glu^{27}$ or $Glu^{27}Glu^{27}$ genotypes). Data on WHR, FS-Triglycerides, HDL-cholesterol and Fs-NEFA for sex-matched sibling pairs only.
Data are means ± SD. [a] p value = 0.02, [b] p value = 0.01 (permutation tests); OSD = observed sum of differences

Gln27 allele of the *B2AR* gene was more frequent in diabetic subjects than in non-diabetic subjects (59.8 % vs 52.3 %; OR = 1.72, $p < 0.02$). Likewise, the Gln27Gln genotype was more common in the diabetic patients than the Glu27Glu genotype (36.1 % vs 16.4 %, $p < 0.02$).

No significant difference between diabetic and non-diabetic subjects was seen in the *B3AR* Arg64 allele (8.0 % vs 7.0 %). Among the non-diabetic subjects, 22.8 % had the genotype combination Glu27-Glu27 + Trp64Trp64 compared with 14.6 % of the diabetes patients ($p = 0.02$). Phenotypic characteristics of 209 sibling pairs discordant for the Gln27Glu genotype with complete clinical data are shown in Table 2. Fasting insulin ($p = 0.02$) and NEFA ($p = 0.01$) concentrations were higher in siblings with an excess of the Gln27 allele compared with siblings with an excess of the Glu27 allele. If only one sibling pair from each family was considered ($n = 117$ sibling pairs, data not shown), fasting insulin concentrations (11.1 ± 9.1 vs 9.3 ± 10.3 mU/l, $p = 0.05$) but not NEFA concentrations (770 ± 300 vs 740 ± 300 μmol/l, p = ns) were higher in siblings with an excess of the Gln27 allele. If the analysis was restricted to non-diabetic sibling pairs discordant for the Gln27Glu genotype the siblings with an excess of the Gln27 allele showed a trend towards higher fasting insulin (8.3 ± 6.5 vs 6.8 ± 4.0 mU/l; $n = 46$; $p = 0.10$) and NEFA (710 ± 370 vs 640 ± 200 μmol/l; $n = 36$; $p = 0.14$) concentrations compared with siblings with an excess of the Glu27 allele.

Comparison of sibling pairs with different Trp64Arg genotypes. Phenotypic characteristics of 48 (16 matched for sex) sibling pairs discordant for the *Trp64Arg* genotype in *B3AR* with complete clinical data are shown in Table 3. The siblings with the Trp64Arg64 genotype had higher 2-h glucose ($p = 0.01$), and higher fasting NEFA concentration ($p = 0.04$) than siblings with the Trp64Trp64 genotype. If only one sibling pair from each family was considered (data not shown), 35 sibling pairs were available. In this subgroup there was no difference in 2-h glucose concentrations (9.6 ± 6.2 vs.9.2 ± 4.9 mmol/l, p = ns) but in the 12 sex-matched sibling pairs available, NEFA concentrations were still increased in siblings with the Trp64Arg64 genotype (900 ± 390 vs 720 ± 180 μmol/l; $p = 0.03$). There were only 9 non-diabetic sibling pairs from 9 different families discordant for the *B3AR* Trp64Arg variant. In these sibling pairs (5 men and 4 women with Trp64Arg64 genotype and 2 men and 7 women with Trp64Trp64 genotype), the siblings with the Trp64Arg64 genotype had significantly higher NEFA concentrations than siblings with the Trp64Trp64 genotype (910 ± 410 vs 640 ± 150 μmol/l, $p = 0.01$).

Comparison of sibling pairs discordant for Gln27Glu in B2AR but concordant for the Trp64Arg polymorphism in B3AR genes. To exclude the possibility that the effect of the Gln27 allele in *B2AR* on fasting insulin concentration was due to the coincidental influence of the Trp64Arg polymorphism in *B3AR,* we examined also separately sibling pairs discordant for Gln27Glu in the *B2AR* gene but concordant for the Trp64Arg polymorphism in the *B3AR* gene. Siblings with more Gln27 alleles had still higher fasting insulin ($p = 0.001$) and NEFA ($p = 0.04$) concentrations compared with siblings with more Glu27 alleles (Table 4).

Table 3. Clinical characteristics of sibling pairs discordant for the Trp64Arg polymorphism in the β_3-adrenoceptor gene

Variable	Trp^{64}	Arg^{64}	OSD
n sibling pairs (men/women)	48 (24/24)	48 (18/30)	
n sex-matched sibling pairs (men/women)	16 (5/11)	16 (5/11)	
Age (years)	58 ± 13	60 ± 15	–83.5
BMI (kg/m^2)	26.5.0 ± 5.4	25.5 ± 3.7	94.5
WHR	0.86 ± 0.07	0.89 ± 0.08	–0.23
Systolic BP (mm/Hg)	144 ± 25	143 ± 19	–95
Diastolic BP (mm/Hg)	82 ± 11	77 ± 10	186
FB- glucose (mmol/l)	8.3 ± 4.5	8.5 ± 3.4	–31.7
2-h B glucose (mmol/l)	8.9 ± 5.0	13.9 ± 7.6	–125 [a]
FS- insulin (mU/l)	9.6 ± 5.0	9.9 ± 9.7	–8.4
2-h insulin (mU/l)	42 ± 28	36 ± 36	148
FS-Triglycerides (mmol/l)	1.3 ± 0.6	1.8 ± 1.0	–4.1
FS-cholesterol (mmol/l)	5.8 ± 1.2	6.3 ± 1.2	–14.8
HDL-cholesterol (mmol/l)	1.5 ± 0.4	1.4 ± 0.5	0.24
Fs-NEFA (μmol/l)	760 ± 190	1050 ± 420	–4050 [b]
DM, IGT, NGT (*n*)	19, 7, 22	32, 4, 12	
Sex-matched sibling pairs DM, IGT, NGT (*n*)	11, 1, 4	9, 3, 4	

The Trp^{64} column shows data for the siblings with the $Trp^{64}Trp^{64}$ genotype and the Arg^{64} column shows data for their genotype discordant pairs (with $Trp^{64}Arg^{64}$ genotype). Data on WHR, FS-Triglycerides, HDL-cholesterol and Fs-NEFA for sex-matched sibling pairs only. Data are means ± SD. [a] p value = 0.01, [b] p value = 0.04 (permutation test); OSD, observed sum of differences

Table 4. Clinical characteristics of sibling pairs discordant for the *B2AR* Gln27Glu polymorphism and concordant for the *B3AR* Trp64Arg polymorphism

Variable	Gln^{27}	Glu^{27}	OSD
n (men/women)	187 (91/96)	187 (88/99)	
n sex-matched sibling pairs (men/women)	103 (48/55)	103 (48/55)	
Age (years)	58 ± 12	58 ± 12	192
BMI (kg/m^2)	27.7 ± 4.6	28.3 ± 5.0	–6.74
WHR	0.91 ± 0.09	0.91 ± 0.09	0.046
Systolic BP (mm/Hg)	141 ± 19	139 ± 19	599
Diastolic BP (mm/Hg)	78 ± 9	78 ± 9	249
FB- glucose (mmol/l)	8.4 ± 3.3	8.1 ± 3.5	125
2-h B glucose (mmol/l)	10.2 ± 5.3	9.7 ± 4.1	16.6
FS- insulin (mU/l)	12.3 ± 10.7	9.3 ± 9.0	562 [a]
2-h insulin (mU/l)	46 ± 47	51 ± 53	–426
FS-Triglycerides (mmol/l)	1.8 ± 1.2	1.6 ± 0.9	25.9
FS-cholesterol (mmol/l)	5.9 ± 1.1	5.8 ± 1.2	3.58
HDL-cholesterol (mmol/l)	1.1 ± 0.3	1.2 ± 0.4	–2.7
Fs-NEFA (μmol/l)	840 ± 490	720 ± 310	11600 [b]
DM, IGT, NGT (*n*)	107, 35, 45	94, 45, 48	
Sex-matched sibling pairs DM, IGT, NGT (*n*)	63, 20, 20	52, 28, 23	

The Gln^{27} column shows data for siblings with the $Gln^{27}Gln^{27}$ or $Gln^{27}Glu^{27}$ genotypes and the Glu^{27} column shows data for their genotype discordant pairs (with $Gln^{27}Glu^{27}$ or $Glu^{27}Glu^{27}$ genotypes). Data on WHR, FS-Triglycerides, HDL-cholesterol and Fs-NEFA for sex-matched sibling pairs only. Data are means ± SD. [a] p value = 0.001, [b] p value = 0.04 (permutation test). OSD, observed sum of differences

Comparison of sibling pairs discordant for Trp64Arg in B3AR but concordant for the Gln27Glu polymorphism in B2AR genes. The same approach was applied to compare sibling pairs discordant for the Trp64Arg polymorphism in the *B3AR* gene but concordant for the polymorphism in *B2AR* (Table 6). Siblings with the Trp64Arg64 genotype had still higher 2-h glucose concentrations ($p = 0.04$) while the difference in NEFA concentrations did not reach significance ($p = 0.06$).

Discussion

This study using a family-based approach suggests that common variants in the *B2AR* and *B3AR* genes are associated with higher NEFA, insulin and glucose concentrations. Whereas the Arg64 allele in *B3AR* seemed to influence NEFA and glucose concentrations more, the Gln27 allele in *B2AR* had a greater effect on insulin concentrations. We also observed a more frequent prevalence of diabetes in subjects

Table 5. Clinical characteristics of sibling pairs discordant for the *B3AR* Trp64Arg polymorphism and concordant for the *B2AR* Gln27Glu polymorphism

Variable	Trp^{64}	Arg^{64}	OSD
n (men/women)	22 (8/14)	22 (8/14)	
n sex-matched sibling pairs (men/women)	12 (3/9)	12 (3/9)	
Age (years)	59 ± 9	61 ± 15	–30.2
BMI (kg/m^2)	26.5 ± 4.0	26.3 ± 4.7	3.28
WHR	0.87 ± 0.08	0.87 ± 0.06	0.176
Systolic BP (mm/Hg)	145 ± 26	144 ± 17	30.0
Diastolic BP (mm/Hg)	81 ± 12	77 ± 10	34.9
FB- glucose (mmol/l)	10.1 ± 5.6	8.8 ± 3.2	29.7
2-h B glucose (mmol/l)	9.3 ± 6.1	16.5 ± 6.8	–64.7 [a]
FS- insulin (mU/l)	8.8 ± 4.0	11.2 ± 14	–32.7
2-h insulin (mU/l)	32 ± 15	29 ± 22	23.6
FS-Triglycerides (mmol/l)	1.2 ± 0.7	1.4 ± 0.9	–5.5
FS-cholesterol (mmol/l)	6.1 ± 1.0	6.1 ± 1.0	–1.31
HDL-cholesterol (mmol/l)	1.4 ± 0.4	1.4 ± 0.3	0.22
Fs-NEFA (μmol/l)	740 ± 110	1040 ± 420	–7080
DM, IGT, NGT (*n*)	12, 4, 6	18, 1, 3	
Sex-matched sibling pairs DM, IGT, NGT (*n*)	63, 20, 20	52, 28, 23	

The Trp^{64} column shows data for the siblings with the $Trp^{64}Trp^{6}$ genotypes and the Arg^{64} column shows data for their genotype discordant pairs (with $Trp^{64}Arg^{64}$ genotypes). Data on WHR, FS-Triglycerides, HDL-cholesterol and Fs-NEFA for sex-matched sibling pairs only. Data are means ± SD. [a] p value = 0.01; OSD, observed sum of differences

with the allele Gln27 in *B2AR*. Subjects who were homozygote for the low risk alleles Glu27 in the *B2AR* and Trp64 in the *B3AR* genes also had a lower prevalence of diabetes than subjects with other genotype combinations. The Arg64 allele in *B3AR* has been associated with impaired catecholamine-induced lipolysis [40–42] and the Glu27 allele of the *B2AR* gene with resistance to agonist-induced down regulation [11]. How this would influence NEFA concentrations is still not clear.

Higher NEFA concentrations have been shown to predict subsequent Type II diabetes [2–3]. All subjects in our study were examined in the fasting state under standardized conditions but we only used fasting NEFA concentrations and it can, of course, be asked how representative fasting NEFA concentrations of diurnal NEFA excursions are. During the day the NEFA concentrations are generally suppressed by meal-induced insulin secretion to reach peak concentrations early in the morning [43–44]. Previous studies have shown a strong correlation between fasting NEFA concentrations and NEFA concentrations measured during the day [44] and fasting NEFA concentrations and suppression of NEFA concentrations by low dose insulin [45]. This was also seen in this study, in which fasting and 2-h NEFA concentrations during OGTT correlated significantly in 422 subject (men, $r = 0.43$, $p < 0.000001$; women, $r = 0.40$, $p < 0.000001$).

There are several potential mechanisms by which higher NEFA concentrations could promote Type II diabetes. In the liver, NEFA stimulates hepatic glucose production [46–47] and interferes with insulin extraction [48]. This could partly explain the higher insulin concentrations seen in carriers of the Gln27 allele. In the muscle, higher NEFA concentrations can by substrate competition lead to impaired insulin-stimulated glucose metabolism and insulin resistance [49]. In the pancreas, prolonged exposure to increased NEFA concentrations is believed to have a role in the development of beta-cell dysfunction characteristic of Type II diabetes [50–52].

Several reports have failed to replicate the original reports of an association between the Arg64 allele in *B3AR* and diabetes, insulin resistance and weight gain [28–29, 53]. Most genetic association studies are viewed as problematic because they have been plagued with lack of reproducibility. There are several reasons for these shortcomings: case-control studies are often unable to detect subtle differences in phenotypes or show stratification bias between cases and controls. The only way to overcome this problem is to use a family-based design.

Taken together the data from such a family-based study show that common variants in the *B2AR* and *B3AR* genes are associated with higher NEFA, insulin and glucose concentrations and Type II diabetes. The limited number of genotype discordant sibling pairs studied, however, and the fact that we carried out multiple comparisons could dilute the significance of some results. On the other hand, most of the variables studied are strongly interrelated, e. g. increased NEFA concentrations are known to predict Type II diabetes. Therefore a Bonferroni correction might seem too conservative.

Given that these alleles are relatively common in the population they are likely to confer a considerable risk for Type II diabetes in the population. In fact, the population risks (PAR) for subjects carrying the Gln27 allele in the *B2AR* was 0.42, which means

that this variant could explain up to 40% of the increased risk of Type II diabetes in the Swedish population. The PAR for subjects with the *B3AR Arg64* allele was not significantly different between diabetic and control subjects, however the combination of Glu27 in the *B2AR* and Trp64 in the *B3AR* variants seems to lower the risk of diabetes by 50%.

In conclusion, we provide evidence that common variants in the *B2AR* and *B3AR* receptor genes are associated with higher NEFA concentrations and could increase the risk for Type II diabetes.

Acknowledgements. This work was supported by grants from the Swedish Medical Research Council, The Sigrid Juselius Foundation, the Swedish Diabetes Research Foundation, the Juvenile Diabetes Foundation (JDF-Wallenberg grant K 98–990–12812–01A), the Albert Påhlssons Foundation, Malmö University Hospital, The Ernhold Lundström Foundation, the Diabetes Association in Malmö, the Anna-Lisa and Sven-Eric Lundgren Foundation, the Swedish Foundation for the Study of Diabetes and an EEC-paradigm BMH-4-CT95–0662 grant. We are indebted to the study subjects for their participation and Y. Wessman and M Åberg for their expert technical assistance.

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