MTNR1B G24E Variant Associates With BMI and Fasting Plasma Glucose in the General Population in Studies of 22,142 Europeans

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OBJECTIVE—Common variants in the melatonin receptor type 1B (*MTNR1B*) locus have been shown to increase fasting plasma glucose (FPG) and the risk of type 2 diabetes. The aims of this study were to evaluate whether nonsynonymous variants in *MTNR1B* associate with monogenic forms of hyperglycemia, type 2 diabetes, or related metabolic traits.

RESEARCH DESIGN AND METHODS—*MTNR1B* was sequenced in 47 probands with clinical maturity-onset diabetes of the young (MODY), in 51 probands with early-onset familial type 2 diabetes, and in 94 control individuals. Six nonsynonymous variants (G24E, L60R, V124I, R138C, R231H, and K243R) were genotyped in up to 22,142 Europeans. Constitutive and melatonin-induced signaling was characterized for the wild-type melatonin receptor type 1B (MT2) and the 24E, 60R, and 124I MT2 mutants in transfected COS-7 cells.

RESULTS—No mutations in *MTNR1B* were MODY specific, and none of the investigated *MTNR1B* variants associated with type 2 diabetes. The common 24E variant associated with increased prevalence of obesity (odds ratio 1.20 [1.08–1.34]; $P = 8.3 \times 10^{-4}$) and increased BMI ($\beta = 0.5$ kg/m^2; $P = 1.2 \times 10^{-5}$) and waist circumference ($\beta = 1.2$ cm; $P = 9 \times 10^{-6}$) in combined Danish and French study samples. 24E also associated with decreased FPG ($\beta = -0.08$ mmol/l; $P = 9.2 \times 10^{-4}$) in the Danish Inter99 population. Slightly decreased constitutive activity was observed for the MT2 24E mutant, while the 124I and 60R mutants displayed considerably decreased or completely disrupted signaling, respectively.

CONCLUSIONS—Nonsynonymous mutations in *MTNR1B* are not a common cause of MODY or type 2 diabetes among Danes. *MTNR1B* 24E associates with increased body mass and decreased FPG. Decreased MT2 signaling does apparently not directly associate with FPG or type 2 diabetes. *Diabetes* 59: 1539–1548, 2010

Genome-wide association (GWA) studies have shown that common variation in the melatonin receptor type 1B (*MTNR1B*) locus increases the level of fasting plasma glucose (FPG) and the risk of type 2 diabetes (1,2). The intronic rs10830963 variant showed the strongest and most independent signal of association (1,3), and subsequent studies have reported that carriers of the rs10830963 risk allele have reduced insulin secretion after both oral and intravenous glucose challenges (3–5). Thus, it has been proposed that this common *MTNR1B* variant increases the risk of type 2 diabetes and impaired fasting glycemia as a result of a defect of the pancreatic β-cells.

The neurohormone melatonin is secreted from the pineal gland during the night and functions as a hormonal message of the photoperiod. Nocturnal pineal melatonin secretion is generated by the central clock in the suprachiasmatic nucleus, which is entrained by the 24-h light-darkness cycle (6). Melatonin is particularly involved in regulation of seasonal and circadian rhythms, such as the sleep-wake cycle, locomotor activity, feeding, core body temperature, immune function, and hormone levels (6,7). Circadian rhythms are highly involved in regulation of a wide range of physiological processes including metabolism (8), and circadian disturbances including shift work and sleep disorders increase the risk of both cardiovascular and metabolic disorders (9,10). Moreover, chronic sleep deficit is an independent risk factor for obesity (11), and both circadian rhythms and melatonin secretion are impaired in type 2 diabetic patients (12). Extrapineal sources of melatonin secretion have also been described, e.g., the retina, the pancreas, and the enteroendocrine cells of the gastrointestinal tract (GI-tract). In these peripheral tissues, melatonin may have autocrine and paracrine properties (13,14). Interestingly, the concentration of melatonin within the gastrointestinal tract is considerably higher than in plasma, presumably due to release from the enteroendocrine cells (15,16). This secretion of melatonin into the gastrointestinal tract is suggested to be related to food intake and digestion, independently of the photoperiodic secretion of melatonin from the pineal gland (16,17).

Melatonin mediates its effect through two high-affinity receptors, the MT1 and MT2 receptors, encoded by *MTNR1A* and *MTNR1B*, respectively (18,19). Both recep-

tors belong to the family of seven transmembrane (7TM) G-protein–coupled receptors. In humans, MT2 is expressed in the suprachiasmatic nucleus and various brain regions yet also in peripheral tissues, including adipose tissue and the gastrointestinal tract (20,21). Recently, *MTNR1B* expression was also found in human pancreatic islets and in β-cells (2,4,22).

It has been shown that common variants within a gene can lead to increased risk of type 2 diabetes while rare coding mutations within the same gene lead to monogenic forms of diabetes, e.g., maturity-onset diabetes of the young (MODY) (23). In addition, coding *MTNR1B* variants have not yet been reported in relation to metabolism. Thus, the aims of this study were to evaluate whether mutations in *MTNR1B* are responsible for genetically unexplained MODY subtypes (MODY-X) and to investigate the influence of nonsynonymous *MTNR1B* variants in relation to type 2 diabetes and related metabolic traits. We also aimed to determine the functional effect of selected *MTNR1B* variants on MT2 receptor signaling.

RESEARCH DESIGN AND METHODS

Detailed characteristics of the screened probands are presented in online appendix Table 1 (available at http://diabetes.diabetesjournals.org/cgi/content/full/db09-1757/DC1). Control individuals ($n = 94$) for the mutation screening were drawn by random from the population-based Inter99 study.

MODY patients. MODY patients ($n = 47$) without mutations in *HNF4A*, *GCK*, or *HNF1A* referred to molecular genetic testing were recruited via the outpatient clinic at Steno Diabetes Center. Inclusion criteria for MODY families were at least one family member with diagnosis of diabetes before the age of 25 years and diabetes in two or more consecutive generations. In addition, the proband should not have been treated with insulin within the first year of diagnosis or should have displayed fasting serum C-peptide >100 pmol/l 1 year after diagnosis. Patients were excluded if they were GAD65 auto-antibody positive.

Early-onset type 2 diabetic patients. Early-onset type 2 diabetic patients ($n = 51$) were recruited at Steno Diabetes Center. All patients were diagnosed before the age of 40 years and had a positive family history of type 2 diabetes in two or more consecutive generations. Patients were excluded if they were GAD65 auto-antibody positive or if fasting serum C-peptide was <100 pmol/l at investigation.

Epidemiological studies. Genetic epidemiological studies were performed in the Danish population-based Inter99 study ($n = 6,002$) (24,25), the Danish ADDITION screening cohort of individuals at high risk of developing type 2 diabetes ($n = 8,576$) (26), individuals recruited at Steno Diabetes Center ($n = 2,293$), the French population–based D.E.S.I.R. study ($n = 4,626$) (27,28), and in a sample of adults from the French Super Obese (FSO) study (29) ($n = 645$). Detailed descriptions and clinical characteristics of the study populations are found in appendix Tables 2 and 3.

Case-control studies and quantitative trait analyses. Case-control studies of type 2 diabetes ($n = 3,617$ case and $n = 4,975$ control subjects) included all unrelated type 2 diabetic patients ($n = 328$) and glucose-tolerant control individuals ($n = 4,460$) from the Inter99 study, type 2 diabetic patients from the Danish ADDITION intervention study ($n = 1,617$), and individuals recruited at the outpatient clinic at Steno Diabetes Center ($n = 1,672$ case and $n = 515$ control subjects). Normal glucose tolerance and type 2 diabetes were defined according to the World Health Organization 1999 criteria (30), and all control individuals had normal fasting glycaemia and were normal glucose tolerant following a 75-g oral glucose tolerance test (OGTT).

Patients treated for type 2 diabetes were not included in obesity case-control studies or in quantitative trait analyses. Danish case-control studies of obesity included up to 6,828 individuals, comprising 2,608 normal-weight (BMI <25 kg/m²) and 971 obese (BMI ≥30 kg/m²) individuals from the Inter99 study, up to 2,881 obese individuals from the Danish ADDITION screening cohort, and 272 normal-weight and 96 obese individuals from Steno Diabetes Center. French case-control studies of obesity ($n = 3,773$) included individuals from the population-based D.E.S.I.R. study (normal weight, $n = 2,738$; obese, $n = 390$) and obese individuals from the FSO study ($n = 645$). Quantitative analyses of BMI and waist circumference were performed in individuals from the Danish population-based Inter99 study sample ($n = 5,885$). Potential associations with BMI and waist circumference for G24E, L60R, and V124I were evaluated in the French population–based D.E.S.I.R. study sample ($n = 4,626$) and in nonpopulation-based study samples: the

Danish ADDITION screening cohort ($n = 8,576$), samples collected at Steno Diabetes Center ($n = 649$), and adults from the FSO study ($n = 645$). Analyses of biochemical variables obtained during an OGTT were performed in individuals from the Danish population-based Inter99 study. All study participants were unrelated and Danish or French by self-report. Informed written consent was obtained from all individuals before participation. The studies were approved by the regional ethical committees and were in accordance with the principles of the Declaration of Helsinki II.

Sequencing. *MTNR1B* was divided into four segments covering the two exons, exon-intron boundaries, and untranslated regions (UTRs). The segments were amplified by standard PCR and sequenced directly by the Sanger sequencing method. All primers were designed with Primer3 (http://frodo.wi.mit.edu/). The sequences were analyzed on a 3130XL genetic analyzer (Applied Biosystems), and mutations were detected with SeqScape v2.5 (Applied Biosystems). Details of primers and PCR conditions are provided in online appendix Table 4. DNA was purified from leukocytes obtained from blood samples.

Genotyping. Genotyping was performed by KASPa® (KBioscience, Hoddesdon).Genotype success rates were >96%, and concordance between 513 duplicate samples was >99%. Genotypes obeyed Hardy-Weinberg equilibrium in all study populations ($P > 0.01$). For rare variants with minor allele frequencies (MAFs) <0.5% (L60R, V124I, and R138C), mutation carriers and noncalled samples, selected by visual inspection of the genotype plots, were evaluated by direct sequencing according to the mutation detection protocol. No additional carriers were found by resequencing of 384 randomly selected samples. DNA was purified from leukocytes obtained from blood samples.

Statistical analyses. The statistical analyses were performed using RGui, version 2.8.1 (http://www.r-project.org). A P value of <0.05 was considered significant. Results are reported uncorrected for multiple testing. Statistical analyses were calculated assuming an additive (rs8192552 [G24E]) or dominant (L60R, V124I, rs8192553 [R231H], rs61746674 [R138C], and rs61747139 [K243R]) genetic model. Logistic regression was applied to test for differences in genotype distribution in case-control studies, and linear regression was applied to test quantitative variables for differences between genotype groups. Effect sizes are given as actual values or percentage if the trait was logarithmically transformed. The combined Danish and French case-control studies of obesity and quantitative analyses of BMI and waist circumference were performed assuming a fixed effect while adjusting for nationality (Danish or French) as a factor. All analyses were adjusted for sex and age. Conditional analyses were performed by comparing a model including one variant with an alternative model including two variants adjusted for age, sex, and BMI. In the type 2 diabetes case-control studies, we had >80% statistical power to detect an odds ratio (OR) of 1.65, 1.45, and 1.20, assuming MAFs of 0.5, 1, and 5%, respectively.

MT2 receptor signaling. Mutations were constructed for G24E, L60R, and V124I by PCR using the overlap extension method as previously described (31). The PCR products were digested, purified, and cloned into the pCMV-Tag(2B) vector. All mutations were verified by restriction endonuclease mapping and subsequent DNA sequence analysis using an ABI 310 automated sequencer. COS-7 cells were grown in Dulbecco's modified Eagle's medium 1885 supplemented with 10% FCS, 2 mmol/l glutamine, and 0.01 mg/ml gentamicin. Cells were transfected with 10 μg cDNA of wild-type or mutant MT2 receptors and 10 μg cDNA of a Gα∆6qi4myr (32) using the calcium phosphate precipitation method with chloroquine addition. The chimeric G-protein allows the Gαi-coupled receptors to signal through the signal transduction pathways known for the Gαq-coupled receptors (32).

One day after transfection, COS-7 cells were incubated for 24 h with 5 μCi of [³H]-*myo*-inositol (PT6-271; Amersham) in 1 ml medium, washed twice in buffer (20 mmol/l HEPES [pH 7.4]), and were subsequently incubated in 0.5 ml buffer supplemented with 10 mmol/l LiCl at 37°C for 30 min. After stimulation with various concentrations of melatonin for 45 min at 37°C, cells were extracted with 10% ice-cold formic acid followed by incubation on ice for 30 min. The generated [³H]-inositol phosphate was purified on Bio-Rad AG 1-X8 anion-exchange resins. All experiments were done in duplicates and repeated three times. Comparisons at each concentration point of melatonin between wild-type and mutant receptors were made by unpaired t tests.

RESULTS

Mutation detection. Table 1 and Fig. 1 show a summary of all variants identified in the mutation screening. No novel *MTNR1B* mutations were found in the 47 MODY-X probands. One novel nonsynonymous mutation, V124I, was found in an early-onset type 2 diabetic patient. One novel synonymous variant, I48I, and one novel 3′UTR

TABLE 1
MAFs of *MTNR1B* variants identified from sequencing of Danish probands from MODY families and families with early-onset type 2 diabetes

			MAFs			
rs	Variant	Position	MODY ($n = 47$)	Early-onset type 2 diabetes ($n = 51$)	Controls ($n = 94$)	Genotyping ($n = 9,912$)
Novel	c.38C>T, p.13A>V	92,342,570 (exon 1)	0	0	0.005	–
Novel	c.38C>A, p.13A>E	92,342,570 (exon 1)	0	0	0.005	–
rs8192552	c.71G>A, p.24G>E	92,342,610 (exon 1)	0.21	0.14	0.14	0.092
Novel	c.144C>T, p.48I>I	92,342,683 (exon 1)	0	0.008	0	–
rs6483210	Exon 2 − 18C>T	92,354,243 (intron 1)	0.03	0.02	0.04	–
Novel	c.370G>A, p.124V>I	92,354,407 (exon 2)	0	0.008	0	0.004
rs61746674	c.412C>T, p.138R>C	92,354,449 (exon 2)	0	0.02	0	0.001
rs8192553	c.692G>A, p.231R>H	92,354,729 (exon 2)	0	0.008	0.005	0.006
rs61747139	c.728A>G, p.243K>R	92,354,765 (exon 2)	0.01	0.02	0.04	0.033
Novel	Exon 2 + 38T>C	92,355,164 (3′UTR)	0	0.008	0.005	–
Novel	Exon 2 + 107G>T	92,355,233 (3′UTR	0	0	0.005	–
rs1562444	Exon 2 + 370G>A	92,355,497 (3′UTR)	0.59	0.55	0.49	–
rs12792653	Exon 2 + 437G>A	92,355,564 (3′UTR)	0.59	0.55	0.48	–
Novel*	c.179T>G, p.60L>R	92,342,718 (exon 1)	0	0	0	0.00055

Variant locations are displayed as base numbers counting from the p-arm telomere of chromosome 11 (according to the UCSC Genome Browser on Human Mar. 2006 Assembly: http://genome.ucsc.edu/cgi-bin/hgGateway). *L60R was not identified in this screening but identified in a French population recently (ref. 2).

variant, exon 2 + 38T>C, as well as seven known variants (dbSNP build 129), were also observed in the screened probands. Four known variants—rs8192552 (G24E), rs61746674 (R138C), rs8192553 (R231H), and rs61747139 (K243R)—changed an amino acid in the protein. In addition, three novel mutations, A13V, A13E, and exon 2 + 107G>T, were identified in three unrelated glucose-tolerant individuals but were not observed in any of the diabetic probands. The previously described L60R mutation was not observed in this screening but was included in the present study because of a reported prevalence of 0.4% in the French population (2). Since no mutations were familyspecific, we did not test for cosegregation in the families.

Epidemiological studies. Six nonsynonymous variants (L60R, V124I, rs8192552 [G24E], rs61746674 [R138C], rs8192553 [R231H], and rs61747139 [K243R]) were initially genotyped in the population-based Inter99 population ($n = 6,002$), screen-detected type 2 diabetic individuals from the Danish ADDITION study ($n = 1,617$), and additional nondiabetic individuals ($n = 621$) and type 2 diabetic patients ($n = 1,672$) recruited from the Steno outpatient clinic. MAFs in the Danish population ($n = 9,912$) are shown in Table 1.

Case-control studies of type 2 diabetes and obesity. Potential associations with susceptibility to type 2 diabetes were evaluated in Danish case-control studies ($n = 8,592$) (Table 2). The investigated *MTNR1B* nonsynonymous variants did not associate with type 2 diabetes (Table 2) and also did not after the analyses were adjusted for BMI (data not shown).

Potential associations with susceptibility to obesity were evaluated in Danish (up to 6,828 subjects) and French ($n = 3,773$) case-control studies. Codons 24E and 124I were initially associated with increased risk of obesity (G24E per allele OR 1.27 [95% CI 1.08–1.48], $P = 0.003$; V124I 2.17 [1.01–4.67], $P = 0.05$) in a Danish case-control study ($n = 4,837$). No obese individuals were carriers of L60R, and no associations with obesity were observed for R138C, R231H, or K243R (Table 3). Following genotyping of additional Danish obese case subjects ($n = 1,991$), the

association between G24E and obesity remained significant in Danish analyses (1.21 [1.05–1.39], $P = 0.008$) (Table 3). In a French case-control study, 24E similarly associated with increased risk of obesity (1.21 [1.00–1.46], $P = 0.05$) (Table 3), and in combined analyses of Danish and French individuals the association was substantiated (1.20 [1.08–1.34], $P = 8 \times 10^{-4}$) (Table 3). When these analyses were restricted to the population-based samples Inter99 and D.E.S.I.R., results were similar (Inter99 1.37 [1.15–1.64], $P = 5 \times 10^{-4}$; D.E.S.I.R. 1.48 [1.13–1.93], $P = 0.004$; and combined 1.40 [1.21–1.43], $P = 9 \times 10^{-6}$). Following additional genotyping, L60R and V124I did not associate with obesity in Danish, French, or combined study samples (Table 3).

Quantitative analyses of BMI and waist circumference. Quantitative analyses of BMI and waist circumference were performed in the Danish population–based Inter99 study sample ($n = 5,885$) (Table 4). Associations for G24E, L60R, and V124I were evaluated in the French D.E.S.I.R. population-based study sample ($n = 4,626$) (Table 4), as well as in nonpopulation-based samples, i.e., Danish ADDITION screening cohort ($n = 8,576$), samples collected at Steno Diabetes Center ($n = 649$), and FSO adults ($n = 645$) (online appendix Table 7).

Codon 24E associated with a dose-dependent increase in BMI ($\beta = 0.5$ kg/m^2 [0.2–0.7], $P = 0.002$) and waist circumference ($\beta = 1.1$ cm [0.4–1.8], $P = 0.003$) in the Danish Inter99 population (Table 4). In the French D.E.S.I.R. population-based study sample, similar associations were observed between 24E and increased BMI ($\beta = 0.5$ kg/m2 [0.2–0.7], $P = 0.001$) and waist circumference ($\beta = 1.3$ cm [0.6–2.0], $P = 4 \times 10^{-4}$) (Table 4). In combined Danish and French population–based analyses, the findings were substantiated (BMI $\beta = 0.5$ kg/m^2 [0.3–0.7], $P = 1 \times 10^{-5}$; waist $\beta = 1.2$ cm [0.7–1.7], $P = 9 \times 10^{-6}$) (Table 4). However, G24E did not show any significant associations with BMI and waist circumference in non–population-based study samples, i.e., the Danish ADDITION screening cohort, the samples collected at Steno Diabetes Center, and FSO adults (online appendix Table 7).

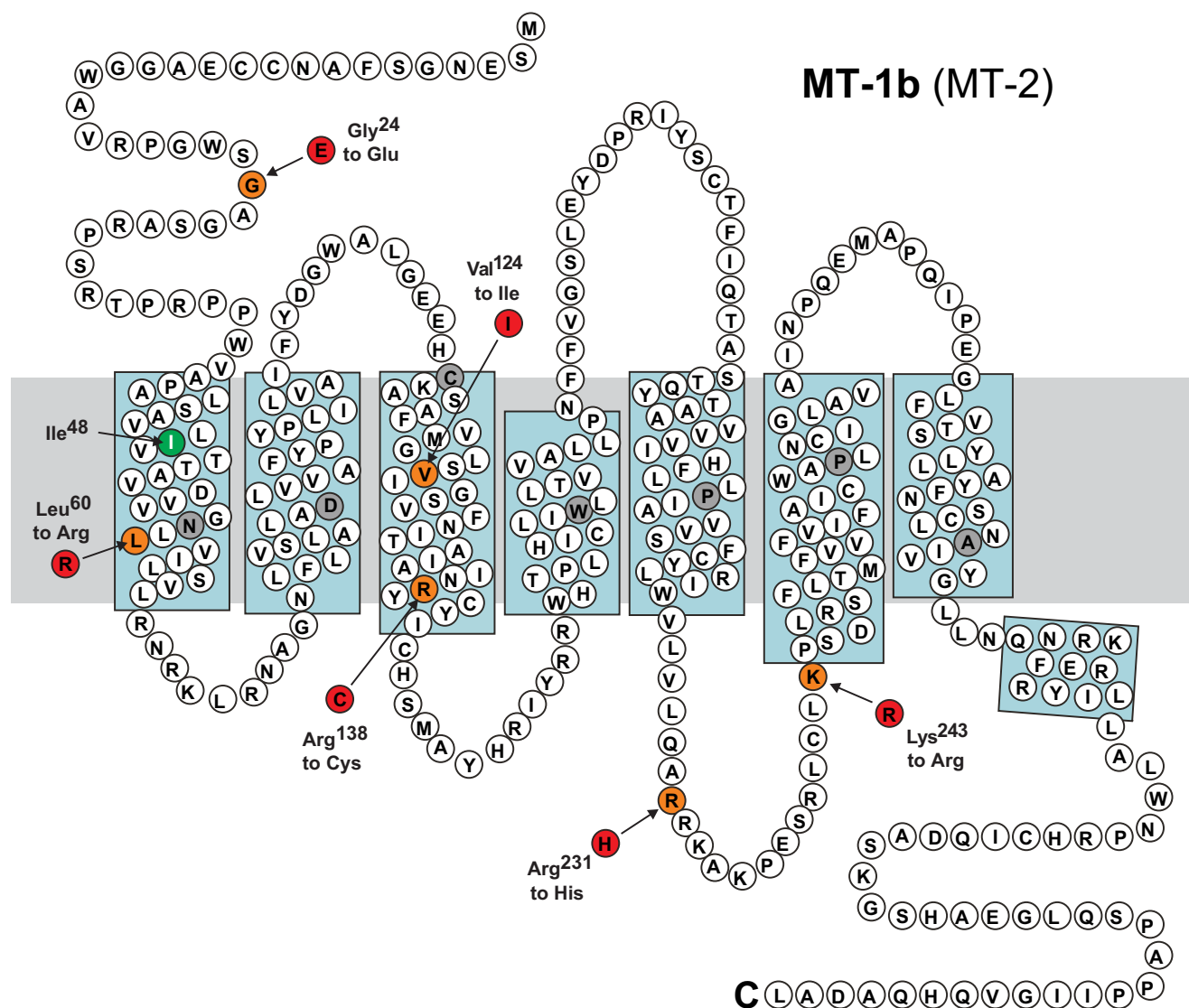L60R was found in only four individuals from the Inter99

FIG. 1. Capital letters are abbreviations of amino acids. Orange-colored circles represent the wildtype amino acid in the mutated position, and red-colored circles represent the mutant amino acid. Green-colored circles represent silent mutations. Gray-colored circles are the highly conserved residues in the 7TM rhodopsine family. The gray bar represents the cell membrane, and blue squares indicate α-helices.

population. Codon 60R was borderline associated with decreased BMI ($\beta = -4.4$ kg/m^2 [-8.7 to 0.0], $P = 0.05$) in the Danish Inter99 population (Table 4). No significant associations with BMI and waist circumference were observed in the French population–based D.E.S.I.R. cohort (Table 4), in the combined Danish and French analyses (Table 4), or in any of the selected cohorts (online appendix Table 7).

V124I did not associate with BMI or waist circumference in Inter99, in the D.E.S.I.R. cohort, or in combined Danish and French analyses (Table 4). Sporadic associations with increased BMI were found in the Danish ADDITION screening cohort of individuals in high risk of undiagnosed type 2 diabetes and in the French FSO study (online appendix Table 7). R138C, R231H, and K243R did not show any significant association with

TABLE 2
Danish case-control studies of type 2 diabetes for six *MTNR1B* variants in 3,617 case and 4,975 control individuals

	Normal glucose tolerance	Type 2 diabetes	OR (95%CI)	P
G24E (rs8192552)	4,019/831/33	2,935/598/32	1.00 (0.87–1.15)	0.97
L60R	4,891/3/0	3,548/2/0	2.58 (0.32–21.07)	0.38
V124I	4,883/35/0	3,484/30/0	1.27 (0.69–2.35)	0.44
R138C (rs61746674)	4,898/9/0	3,525/5/0	0.91 (0.23–3.63)	0.89
R231H (rs8192553)	4,853/53/1	3,498/41/0	0.98 (0.58–1.63)	0.92
K243R (rs61747139)	4,572/331/4	3,310/214/5	0.95 (0.76–1.19)	0.67

Data are presented as wildtype/heterozygous/homozygous unless otherwise indicated. ORs and *P* values were calculated assuming a dominant (L60R, V124I, R138C, R231H, and K243R) or additive (G24E) genetic model adjusted for age and sex.

TABLE 3
Case-control studies of obesity for six *MTNR1B* variants in Danish (up to $n = 6,828$) and French ($n = 3,773$) individuals

	Danish				French				Combined ($n = 10,601$)	
	Normal weight	Obesity	OR (95%CI)	P	Normal weight	Obesity	OR (95%CI)	P	OR (95%CI)	P
G24E*	2,365/442/17	3,168/678/33	1.21 (1.05–1.39)	0.0081	2,276/353/14	859/159/8	1.21 (1.00–1.46)	0.046	1.20 (1.08–1.34)	8.3×10^{-4}
L60R*	2,822/3/0	3,868/1/0	0.12 (0.01–1.4)	0.091	2,708/8/0	1,019/4/0	1.29 (0.39–4.33)	0.68	0.66 (0.21–2.06)	0.48
V124I*	2,824/12/0	3,833/31/0	1.74 (0.83–3.67)	0.14	2,691/3/0	1,016/2/0	1.78 (0.29–10.81)	0.53	1.74 (0.91–3.35)	0.095
R138C	2,822/6/0	1,906/3/0	0.89 (0.21–3.82)	0.87						
R231H	2,809/26/1	1,888/20/0	1.0 (0.53–1.9)	0.99						
K243R	2,647/185/2	1,786/124/3	0.99 (0.77–1.27)	0.93						

Data are presented as wild-type/heterozygous/homozygous unless otherwise indicated. ORs and P values were calculated assuming a dominant (L60R, V124I, R138C, R231H, and K243R) or additive (G24E) genetic model adjusted for age and sex. Nationality has been applied as an adjusting factor in the combined analyses. *Additional obese cases ($n = 1,991$) were genotyped for G24E, L60R, and V124I. Normal weight was defined as BMI < 25 kg/m^2. Obesity was defined as BMI ≥ 30 kg/m^2.

BMI or waist circumference in the Danish Inter99 population (Table 4).

Quantitative analyses of biochemical variables obtained from an OGTT. Analyses of anthropometric measurements and biochemical variables during an OGTT were performed in the Danish population–based Inter99 (Table 5). Codon 24E associated with a decrease in FPG (per allele $\beta = -0.06$ mmol/l [-0.1 to -0.01], $P = 0.02$) (Table 5). This association was strengthened when analyses were adjusted for the difference in BMI ($\beta = -0.08$ mmol/l [-0.13 to -0.03], $P = 9 \times 10^{-4}$), but no significant interactions with BMI were observed ($P = 0.2$). Conditional analyses of *MTNR1B* intronic variant rs10830963, previously reported to increase FPG (1–4), and rs8192552 (G24E) were performed. The linkage disequilibrium between the two variants were D$' = 0.99$, $r^2 = 0.04$ (online appendix Table 5). After inclusion of rs10830963 in the model, rs8192552 (G24E) still had an effect on FPG ($P = 0.02$) (online appendix Table 6).

Four carriers of codon 60R had decreased insulinogenic index ($\beta = -83\%$ [-152 to -13], $P = 0.02$) (Table 5), which remained nominally significant when analyses were adjusted for the difference in BMI ($\beta = -72\%$ [-140 to -5], $P = 0.04$).

243R was nominally associated with decreased FPG ($\beta = -0.08$ mmol/l [-0.16 to -0.01], $P = 0.04$) (Table 5). Conditional analyses of rs10830963 and rs61747139 (K243R) in linkage disequilibrium (D$' = 0.99$; $r^2 = 0.01$) were performed (online appendix Table 5). After inclusion of rs10830963 in the model, the effect of rs61747139 (K243R) disappeared ($P = 0.2$) (online appendix Table 6). No associations with anthropometric or biochemical measurements were observed for V124I, R138C, or R231H.

Analyses of MT2 receptor signaling. The wild-type MT2 receptor displayed a relatively high degree of constitutive signaling activity and a clear dose-related increase in signaling in response to melatonin (Fig. 2). The dose-response curve for melatonin in the 24E mutant form of the MT2 receptor was similar to that of the wild-type receptor with respect to potency and efficacy; however, the ligand-independent constitutive signaling was slightly decreased (Fig. 2). Functional characterization of the 60R mutant receptor revealed that both constitutive and melatonin-induced signaling were completely disrupted for this mutant (Fig. 2). The 124I mutant receptor had decreased constitutive activity and maximal efficacy compared with the wild-type MT2 receptor.

DISCUSSION

In the present study, we found no MODY-specific mutations in *MTNR1B*. We describe novel associations of G24E with measures of obesity and FPG. None of the investigated *MTNR1B* variants were associated with type 2 diabetes. Decreased MT2 Gαi-coupled signaling, as observed to various extents for 24E, 60R, and 124I mutant receptors, did not correlate strongly with type 2 diabetes or FPG.

The common 24E variant (MAF 9.2% in the Danish population) increased the risk of obesity and BMI in Danish and French individuals but also decreased FPG by 0.08 mmol/l per allele, as estimated in the Danish population. Conditional analyses showed that this effect on FPG could only be partly explained by linkage to the previously reported intronic rs10830963 variant. An apparent paradox is that FPG is decreased while BMI and waist circumference are increased in carriers of 24E, and we hypothesize that these dual findings could be due to differential regulation in specific target tissues. The effect on FPG may reflect the functionality of pancreatic MT2 receptors as previously suggested for the signal obtained in the GWA studies (4). It has also been suggested based on findings in animal and in vitro experiments that melatonin may act on body weight regulation through activation of central (brain) or peripheral (gastrointestinal tract and adipocyte) receptors (7,33), and the effect we observed on BMI might therefore reflect the function of MT2 receptors in these tissues. When the association between 24E and waist circumference was adjusted for BMI in the Inter99 and D.E.S.I.R. cohorts, no significant associations were observed (data not shown), pointing toward an effect of the genotype on overall fat distribution rather than on specific abdominal obesity. Investigations in populations with detailed measures of body composition would consequently be of interest for future studies.

G24E (rs8192552) has not been reported in large GWA studies of obesity (34–36). The variant has not been genotyped in HapMap, and its coverage by GWA studies is thus unclear. It may therefore be that rs8192552 is not adequately captured by variants analyzed in previous GWA studies. Alternatively, heterogeneity among the investigated GWA populations could be an explanation for the lack of strongly significant association with BMI or obesity in these studies.

G24 is conserved in rhesus and mouse but not in elephant, opossum, or platypus. Functionally, we observed that the MT2 24E mutant had a somewhat decreased

TABLE 4
BMI and waist circumference in Danish ($n = 5,885$) and French ($n = 4,626$) population–based individuals stratified according to six *MTNR1B* genotypes

	Danish Inter99				French D.E.S.I.R.				Combined ($n = 10,510$)	
	N	Age (years)	BMI (kg/m^2)	Waist (cm)	N	Age (years)	BMI (kg/m^2)	Waist (cm)	BMI (kg/m^2)	Waist (cm)
G24E (rs8192552)										
Wild type	4,736	46 ± 8	26.1 ± 4.5	86 ± 13	3,874	47 ± 10	24.6 ± 3.8	82.8 ± 11.5		
Heterozygous	995	46 ± 8	26.6 ± 4.7	88 ± 13	657	46 ± 10	25.0 ± 4.2	84.3 ± 12.5		
Homozygous	36	45 ± 10	27.1 ± 5.2	86 ± 14	26	46 ± 8	25.4 ± 4.3	84.4 ± 10.8		
Effect size			0.5 kg/m^2	1.1 cm			0.5 kg/m^2	1.3cm	0.5 kg/m^2	1.2 cm
95% CI			0.2 to 0.7	0.4–1.8			0.2–0.7	0.6–2.0	0.3–0.7	0.7–1.7
P_{add}			0.0019	0.0029			0.0011	3.7×10^{-4}	1.2×10^{-5}	9.4×10^{-6}
L60R										
Wild type	5,764	46 ± 8	26.2 ± 4.5	86 ± 13	4,641	47 ± 10	24.6 ± 3.7	83.0 ± 11.6		
Heterozygous	4	48 ± 9	21.9 ± 3.9	80 ± 13	15	47 ± 10	25.0 ± 4.1	86.9 ± 11.0		
Effect size			−4.4 kg/m^2	−7.1 cm			0.1 kg/m^2	0.8 cm	−0.9 kg/m^2	−1.0 cm
95% CI			−8.7 to 0.0	−18.2 to 4.1			−1.8 to 2.0	−4.2 to 5.9	−2.8 to 1.0	−6.0 to 4.0
P_{dom}			0.051	0.21			0.92	0.75	0.36	0.69
V124I										
Wild type	5,767	46 ± 8	26.2 ± 4.5	87 ± 13	4,621	47 ± 10	24.6 ± 3.7	83 ± 12		
Heterozygous	41	46 ± 8	27.0 ± 4.1	89 ± 12	9	47 ± 9	25.3 ± 3.4	84 ± 11		
Effect size			0.8 kg/m^2	1.5 cm			0.8 kg/m^2	1.9 cm	0.8 kg/m^2	1.6 cm
95% CI			−0.6 to 2.1	−20.0 to 50.0			−1.6 to 3.1	−4.1 to 8.0	−0.4 to 1.9	−1.3 to 4.5
P_{dom}			0.28	0.41			0.52	0.53	0.19	0.29
R138C (rs61746674)										
Wild type	5,781	46 ± 8	26.2 ± 4.5	87 ± 13						
Heterozygous	12	45 ± 8	25.3 ± 4.0	82 ± 10						
Effect size			−0.9 kg/m^2	−4.9 cm						
95% CI			−3.5 to 1.6	−11.3 to 1.6						
P_{dom}			0.47	0.14						
R231H (rs8192553)										
Wild type	5,733	46 ± 8	26.2 ± 4.5	86.5 ± 13.2						
Heterozygous	61	47 ± 7	26.4 ± 4.0	86.2 ± 13.9						
Homozygous	1	55	23.8	74						
Effect size			0.2 kg/m^2	0.0 cm						
95% CI			−0.9 to 1.3	−2.9 to 2.8						
P_{dom}			0.74	1.0						
K243R (rs61747139)										
Wild type	5,418	46 ± 8	26.2 ± 4.5	86.5 ± 13.2						
Heterozygous	379	46 ± 7	26.2 ± 4.6	86.8 ± 13.4						
Homozygous	3	48 ± 12	24.6 ± 1.6	82.0 ± 2.7						
Effect size			0.0 kg/m^2	0.4 cm						
95% CI			−0.5 to 0.4	−0.8 to 1.5						
P_{dom}			0.89	0.55						

Data are means ± SD stratified according to genotypes of *MTNR1B* G24E, L60R, and V124I. Effect sizes and *P* values are calculated assuming an additive (P_{add}) or dominant (P_{dom}) genetic model of inheritance adjusted for age and sex. Nationality has been applied as an adjusting factor in the combined analyses.

constitutive activity but otherwise responded similarly to the wild-type receptor upon melatonin stimulation, as observed in cells cotransfected with a promiscuous G-protein commonly used to study signaling properties of Gαi-coupled receptors (32). The MT2 receptor is believed to signal mainly through Gαi but has been shown to be able to activate a number of intracellular signaling pathways (37,38). The association of the G24E variant with BMI and FPG observed in the present study will justify an in-depth cell biological and molecular pharmacological analysis of this variant in particular; such studies are, however, hampered by lack of appropriate in vitro cell systems that diligently reflect the cell biology of tissues where MT2 is normally expressed. Nevertheless, the normal maximal signaling efficacy observed for the 24E mutant indicates that the amino acid substitution does not lead to misfolding or lack of cell surface expression, which

otherwise is a common molecular phenotype for 7TM receptor variants (39). Given that the 24E variant is located in the extracellular part of the receptor, it is plausible that receptor interactions with other ligands or proteins may be modified by the amino acid substitution. Finally, because the variant is common, other yet unidentified regulatory variants in linkage disequilibrium could also be responsible for the observed associations.

L60R was very rare among Danes (MAF 0.056%) where a tendency toward decreased BMI was observed. However, this finding could not be confirmed in a French population, although the variant had a higher frequency (MAF 0.16%) among French individuals. In the Danish Inter99 population, four 60R carriers had on average 83% decreased glucose-stimulated insulin response measured by the insulinogenic index, but still no effect on FPG was observed. It is an important limitation that we have very few 60R

TABLE 5
Biochemical measurements obtained from an oral glucose tolerance test in 5,885 non-diabetic or treatment-naïve individuals from the Danish Inter99 study stratified according to 6 MTNR1B genotypes

	N	Age (years)	Plasma glucose (mmol/l)			Serum insulin (pmol/l)			Homa-IR	Insulinogenic index
			Fasting	30 min	120 min	Fasting	30 min	120 min		
G24E (rs8192552)										
Wild type	4,736	46 ± 8	5.5 ± 0.8	8.7 ± 1.9	6.2 ± 2.1	34 (24–51)	243 (174–349)	155 (96–253)	8.3 (5.6–12.8)	24.3 (16.8–35.9)
Heterozygous	995	46 ± 8	5.5 ± 0.6	8.7 ± 1.8	6.2 ± 2.0	35 (24–53)	263 (181–368)	160 (95–277)	8.3 (5.7–13.1)	25.1 (17.1–38.4)
Homozygous	36	45 ± 10	5.3 ± 0.8	8.1 ± 1.6	6.8 ± 2.3	38 (24–53)	257 (1,799–380)	168 (117–295)	9.2 (5.4–12.1)	28.6 (20.5–39.0)
Effect size			−0.06 mmol/l	0.01 mmol/l	0.04 mmol/l	1%	4%	3%	0%	4%
95% CI			−0.1 to −0.01	−0.1 to 0.1	−0.09 to 0.2	−3 to 5	1 to 8	−2 to 8	0 to 8	0 to 8
P_{add}			0.017*	0.91	0.53	0.62	0.018	0.25	0.96	0.040
L60R										
Wild type	5,764	46 ± 8	5.5 ± 0.8	8.7 ± 1.9	6.2 ± 2.1	34 (24–51)	244 (175–354)	156 (96–256)	8.3 (5.7–12.9)	24.5 (16.8–36.3)
Heterozygous	4	48 ± 9	5.6 ± 0.5	9.1 ± 1.8	5.5 ± 2.6	17 (15–21)	147 (109–173)	95 (79–122)	4.2 (3.6–5.3)	12.1 (9.3–14.3)
Homozygous										
Effect size			0.01 mmol/l	0.3 mmol/l	−0.8 mmol/l	−66%	−67%	−58%	−66%	−83%
95% CI			−0.7 to 0.7	−1.4 to 2.0	−2.8 to 1.2	−124 to −9	−130 to −4	−136 to 20	−127 to −4	−152 to −13
P_{dom}			0.98	0.76	0.45	0.023	0.036	0.15	0.037	0.020*
V124I										
Wild type	5,767	46 ± 8	5.5 ± 0.8	8.7 ± 1.9	6.2 ± 2.1	34 (24–51)	245 (175–354)	156 (96–256)	8.3 (5.7–12.9)	24.5 (16.8–36.5)
Heterozygous	41	46 ± 8	5.6 ± 0.7	9.1 ± 1.7	6.4 ± 2.6	31 (21–44)	235 (145–321)	140 (74–234)	7.7 (5.2–10.6)	22.9 (15.8–30.9)
Homozygous										
Effect size			−0.0 mmol/l	0.4 mmol/l	0.2 mmol/l	7%	14%	15%	7%	13%
95% CI			−0.2 to 0.2	−0.1 to 0.9	−0.4 to 0.8	−25 to 11	−31 to 3	−39 to 11	−27 to 12	−33 to 6
P_{dom}			0.95	0.16	0.54	0.42	0.12	0.26	0.47	0.18
R138C (rs61746674)										
Wild type	5,781	46 ± 8	5.5 ± 0.8	8.7 ± 1.9	6.2 ± 2.1	34 (24–51)	245 (175–353)	156 (96–256)	8.3 (5.7–12.9)	24.5 (16.8–36.4)
Heterozygous	12	45 ± 8	5.5 ± 0.6	8.2 ± 1.7	6.1 ± 1.1	33 (23–38)	240 (197–276)	167 (121–212)	8.0 (5.2–9.0)	25.3 (17.8–32.1)
Effect size			−0.09 mmol/l	−0.6 mmol/l	−0.1 mmol/l	22%	8%	3%	23%	2%
95% CI			−0.5 to 0.3	−1.6 to 0.4	−1.3 to 1.1	−55 to 11	−42 to 27	−42 to 49	−59 to 12	−37 to 40
P_{dom}			0.69	0.26	0.86	0.19	0.66	0.88	0.20	0.92
R231H (rs8192553)										
Wild type	5,733	46 ± 8	5.5 ± 0.8	8.7 ± 1.9	6.2 ± 2.1	34 (24–51)	245 (175–353)	156 (97–256)	8.3 (5.7–12.9)	24.4 (16.8–36.3)
Heterozygous	61	47 ± 7	5.6 ± 0.9	8.8 ± 2.1	6.0 ± 2.6	37 (27–48)	294 (202–376)	151 (63–230)	8.9 (6.2–12.0)	29.1 (18.7–39.5)
Homozygous	1	55	5.2	6.9	4.2	37	212	65	8.6	25.4
Effect size			0.0 mmol/l	0.0 mmol/l	−0.4 mmol/l	2%	9%	24%	2%	3%
95% CI			−0.2 to 0.2	−0.4 to 0.5	−0.9 to 0.2	−13 to 18	−6 to 23	−14 to 20	−14 to 19	−14 to 20
P_{dom}			1.0	0.91	0.17	0.78	0.24	0.88	0.78	0.75
K243R (rs61747139)										
Wild type	5,418	46 ± 8	5.5 ± 0.8	8.7 ± 1.9	6.2 ± 2.1	34 (24–51)	246 (175–353)	156 (96–256)	8.3 (5.6–12.9)	24.5 (16.8–36.3)
Heterozygous	379	46 ± 7	5.5 ± 0.7	8.6 ± 1.8	6.1 ± 2.0	35 (24–50)	232 (177–358)	154 (95–239)	8.3 (5.7–13.0)	24.5 (17.3–36.6)
Homozygous	3	48 ± 12	5.8 ± 1.3	9.2 ± 2.4	8.5 ± 4.8	27 (26–28)	313 (190–318)	146 (146–250)	6.0 (5.8–6.2)	37.0 (36.0–37.9)
Effect size			−0.08 mmol/l	−0.09 mmol/l	−0.1 mmol/l	1%	0%	3%	0%	2%
95% CI			−0.2 to −0.01	−0.3 to 0.1	−0.3 to 0.1	−5 to 8	−6 to 6	−11 to 6	−7 to 7	−5 to 9
P_{dom}			0.035*	0.32	0.24	0.66	0.95	0.51	0.98	0.57

Data are means ± SD or median (interquartile range) unless otherwise indicated, stratified according to genotypes of six *MTNR1B* variants. Values of serum insulin, HOMA-IR, and insulinogenic index were logarithmically transformed before statistical analyses. Effect sizes and *P* values are calculated assuming an additive (P_{add}) or dominant (P_{dom}) genetic model of inheritance adjusted for age and sex. *Remains significant after adjusting for the difference in BMI. HOMA-IR and insulinogenic index are calculated as described in online-only appendix.
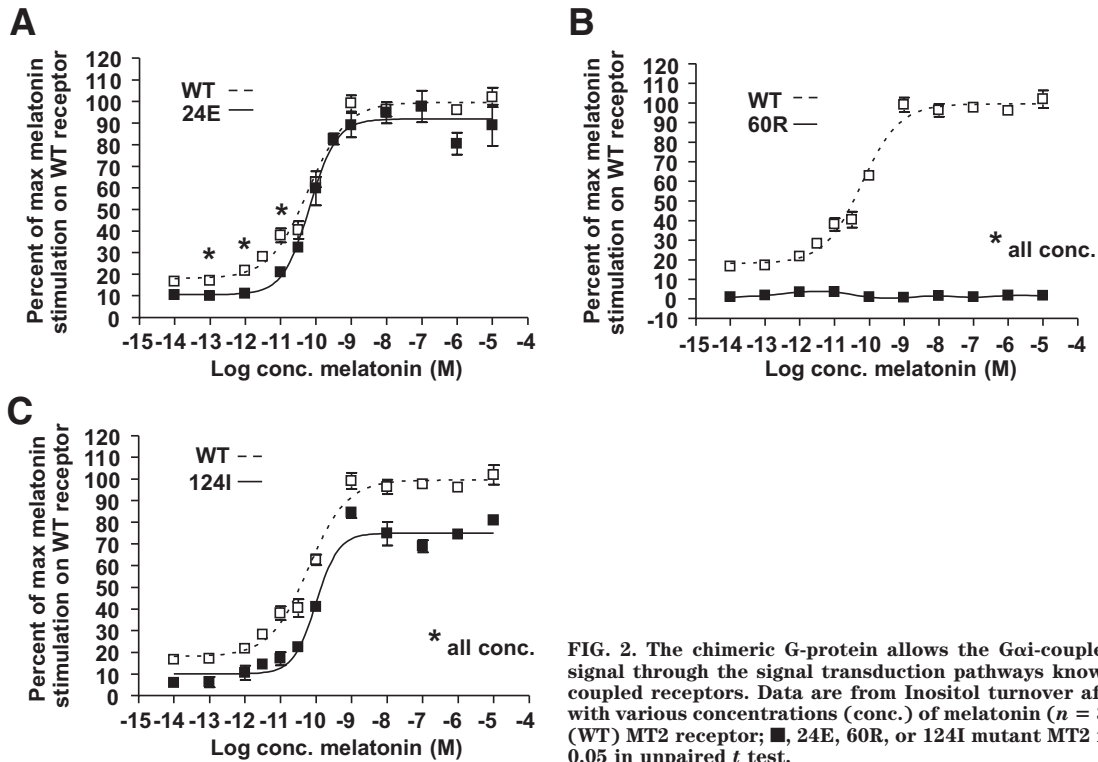
A

B

C

FIG. 2. The chimeric G-protein allows the Gαi-coupled receptors to signal through the signal transduction pathways known for the Gαq-coupled receptors. Data are from Inositol turnover after stimulation with various concentrations (conc.) of melatonin ($n = 3$). □, wild-type (WT) MT2 receptor; ■, 24E, 60R, or 124I mutant MT2 receptors. *$P < 0.05$ in unpaired t test.

carriers. Consequently, we lack statistical power to exclude an effect of L60R, and the results obtained may be spurious. Of particular interest, L60R changes a hydrophobic leucine to a positively charge arginine at position 60, which is highly conserved as an aliphatic hydrophobic residue (82%) in transmembrane segment 1 (TM-I) of 7TM receptors in general (40). It is very likely that the completely eliminated signaling by substitution with the basic arginine residue is caused by disruption of the normal hydrophobic interaction of L60 with the intracellular helix VIII. Although heterozygous knockdown of MT2 signaling revealed no obvious human metabolic phenotype in this study, prospective studies are highly relevant to evaluate whether a phenotype evolves as a function of aging and various environmental pressures. No homozygous mutation carriers have been identified; hence, it is also possible that one functional allele is sufficient to ensure a normal phenotype. Supporting this hypothesis, partial redundancy between the melatonin receptor types has been suggested (37) and both homodimer and MT1/MT2 heterodimer formation is described in vitro, where activation of one binding site is sufficient to induce a conformational change in the dimer (41). In addition, MT2 knockout mice have no obvious metabolic phenotype (42,43).

The initial association between 124I and obesity observed in a Danish case-control study could not be confirmed in combined case-control analyses or in analyses of BMI and waist circumference as quantitative variables in the Danish and French populations. 124I is very rare among French individuals, severely impeding statistical power for replication. We observed, however, an effect of 124I on BMI in the Danish ADDITION screening cohort of individuals in high risk of type 2 diabetes, implying that 124I may increase BMI in selected high-risk groups, although it has no strong effect in the general population. V124 is conserved among mammalian species but not in chicken, lizard, or *Xenopus*, and it has been proposed that

this residue is involved in the binding of melatonin (44) The 124I mutant had impaired MT2 signaling, but because completely disrupted signaling as observed for the 60R mutant does not lead to increased BMI, these results do not support the associations observed for V124I in selected study populations.

We observed various degrees of decreased signaling for the three MT2 mutants, which however, does not seem to associate directly with type 2 diabetes or FPG. This is in line with a recent study by Lyssenko et al. (4) suggesting that increased *MTNR1B* expression in β-cells may be the pathogenic mechanism by which FPG and the risk of type 2 diabetes are increased, supported by the fact that melatonin inhibits insulin secretion. We did, however, observe impaired insulin release for the four 60R carriers, and it is not known at present whether these individuals will develop hyperglycemia or overt type 2 diabetes over time. The associations observed for G24E may not be ascribed to the slightly lower constitutive activity because more severe disrupted signaling, as observed for 60R and 124I mutants, does not lead to comparable phenotypes.

The putative promoter regions of *MTNR1B* and potential distant regulatory regions were not targeted in the present study, and these regions may therefore contain yet undiscovered functional variants of importance for the development of type 2 diabetes and related phenotypes.

Throughout this study, effects on BMI and waist circumference observed in selected study samples somewhat differed from those observed in population-based study samples. This heterogeneity may reflect a true differential effect among individual groups, statistical type II errors due to lack of statistical power in the smaller study samples, or statistical type I errors due to the lack of correction for multiple testing. However, we argue that results from large population-based studies are more reliable in the sense that they are largely free of selection bias, whereas results from selected study samples should

be analyzed and interpreted separately to evaluate whether the variants have a specific effect in a certain subpopulation of interest. When the results were adjusted for for multiple testing (discovery samples, six SNPs and nine independent tests; replication sample, three SNPs and two independent tests), the associations with BMI and waist circumference for G24E in the French D.E.S.I.R. population and in the combined Danish and French study samples remained significant ($P < 0.05$). In the present study, the French D.E.S.I.R. population was used to evaluate the obesity-related discovery results obtained in the Danish Inter99 population for G24E, L60R, and V124I. The two populations are comparable with respect to age and the sampling method. The MAF of L60R and V124I differed among Danish and French individuals, implying that population differences across countries exist for rare variants and thus impeding cross-population replication as a result of drift in statistical power.

In conclusion, we find that nonsynonymous variation in *MTNR1B* is not a common cause of MODY or type 2 diabetes among Danes. *MTNR1B* 24E associates with increased body mass and decreased FPG. MT2 amino acid variants affect receptor signaling, but the observed effects do not seem to associate strongly with FPG or type 2 diabetes.

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