

## ORIGINAL ARTICLE

Four novel *UCP3* gene variants associated with childhood obesity: effect on fatty acid oxidation and on prevention of triglyceride storageCV Musa<sup>1,2</sup>, A Mancini<sup>3</sup>, A Alfieri<sup>1,4</sup>, G Labruna<sup>1,3</sup>, G Valerio<sup>4</sup>, A Franzese<sup>5</sup>, F Pasanisi<sup>6</sup>, MR Licenziati<sup>7</sup>, L Sacchetti<sup>1</sup> and P Buono<sup>1,3,4</sup>

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**Objective:** The objective of the study was to look for uncoupling protein 3 (*UCP3*) gene variants in early-onset severe childhood obesity and to determine their effect on long-chain fatty acid oxidation and triglyceride storage.

**Methods and results:** We identified four novel mutations in the *UCP3* gene (V56M, A111V, V192I and Q252X) in 200 children with severe, early-onset obesity (body mass index-standard deviation score >2.5; onset: <4 years) living in Southern Italy. We evaluated the role of wild-type (wt) and mutant *UCP3* proteins in palmitate oxidation and in triglyceride storage in human embryonic kidney cells (HEK293). Palmitate oxidation was ~60% lower ( $P<0.05$ ;  $P<0.01$ ) and triglyceride storage was higher in HEK293 cells expressing the four *UCP3* mutants than in cells expressing wt *UCP3*. Moreover, mutants V56M and Q252X exerted a dominant-negative effect on wt protein activity ( $P<0.01$  and  $P<0.05$ , respectively). Telmisartan, an angiotensin II receptor antagonist used in the management of hypertension, significantly ( $P<0.05$ ) increased palmitate oxidation in HEK293 cells expressing wt and mutant proteins ( $P<0.05$ ;  $P<0.01$ ), including the dominant-negative mutants.

**Conclusions:** These data indicate that protein *UCP3* affects long-chain fatty acid metabolism and can prevent cytosolic triglyceride storage. Our results also suggest that telmisartan, which increases fatty acid oxidation in rat skeletal muscle, also improves *UCP3* wt and mutant protein activity, including the dominant-negative *UCP3* mutants.

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**Keywords:** *UCP3* variants; childhood obesity; palmitate oxidation; telmisartan; Oil Red O; dominant negative

**Context:** Human uncoupling protein 3 (*UCP3*) is the muscle-specific mitochondrial transmembrane carrier that uncouples oxidative adenosine-5'-triphosphate (ATP) phosphorylation.

## Introduction

Human uncoupling protein 3 (*UCP3*) is a member of a family of mitochondrial inner membrane anion carrier proteins that uncouples the oxidative phosphorylation from adenosine-5'-triphosphate synthesis.<sup>1,2</sup> The *UCP3* gene consists of

seven exons, six of which encode a transmembrane region. It encodes two forms of transcripts: a full-length messenger (*UCP3L*) and a short isoform (*UCP3S*) that lacks the sixth transmembrane domain; the two messengers are equally expressed in skeletal muscle.<sup>3</sup> The *UCP3* protein is more abundant in glycolytic, type 2 human muscle fibers than in oxidative, type 1 human muscle fibers. It is also expressed, although at lower levels, in cardiac muscle and white adipose tissue.<sup>4,5</sup> Several lines of evidence suggest that *UCP3* is related to cellular fatty acid metabolism rather than to mitochondrial uncoupling of oxidative phosphorylation. In fact, *UCP3* messenger expression in skeletal muscle is rapidly upregulated during fasting, acute exercise and high dietary intake of fat,<sup>6–9</sup> and declines in situations in which fat oxidative capacity is improved, such as after endurance training or weight reduction, and in type 1 muscle fibers that are characterized by a high rate of fat oxidation.<sup>10,11</sup> The

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UCP3 gene has recently been proposed as a candidate gene for obesity.<sup>12</sup>

In the present study, we looked for UCP3 variants in a cohort of severe obese children (body mass index-standard deviation score >2.5) with early-onset obesity (mean age 4 years) living in Southern Italy. We found four novel mutations in the UCP3 gene, all in the heterozygous state. We conducted a functional analysis of wild-type (wt) and mutant UCP3 proteins to assess their role in long-chain fatty acid  $\beta$ -oxidation and triglyceride storage.

We also investigated the association between the -55C/T polymorphism in the UCP3 gene promoter and BMI in our cohort, because only recent studies found an association between the UCP3 -55 C/T polymorphism and BMI in some populations.

Telmisartan and valsartan are two angiotensin II receptor blockers frequently used to ameliorate hypertension in patients who are prone to visceral obesity, metabolic syndrome and diabetes.<sup>13</sup> Recently, telmisartan, but not valsartan, was found to improve long-chain fatty acid oxidation in rat skeletal muscle<sup>14</sup> and to reduce lipid accumulation in liver.<sup>13</sup> It also ameliorates hypertension, improves glucose and lipid metabolism and protects against visceral fat accumulation. In this paper, we also tested the effects of telmisartan treatment on UCP3 wt and mutant protein activity in HEK293 cells.

## Subject and methods

### Subjects

Between 2003 and 2005, 200 obese children (107 girls (53.5%) and 93 boys (46.5%); 1.5–10 years of age) were recruited by the outpatient clinic of the Department of Pediatrics, 'Federico II' University of Naples and by the Department of Pediatrics, A. Cardarelli Hospital, Naples, Italy. All children were Caucasian and lived in the Campania region (Southern Italy). Inclusion criteria were obesity classified as BMI (weight/height<sup>2</sup>) >95th centile, obesity onset <10 years of age and absence of any syndromic or endocrine form of obesity. As controls, 100 (54 males and 46 females) normal-weight healthy individuals (BMI <25 kg m<sup>-2</sup>; aged 24.2  $\pm$  3.4 years), previously enrolled by us,<sup>15</sup> underwent genetic testing for obesity.

Written informed consent was obtained from participants and/or their parents. The study was approved by the ethics committee of the School of Medicine, University of Naples 'Federico II' and was conducted in accordance with the principles of the Helsinki II Declaration.

### Physical measurements

A trained dietitian measured the height, weight and waist circumference (recorded to the nearest 0.1 cm, 0.1 kg and 0.1 cm, respectively) of the enrolled children. Waist was measured with a flexible steel tape measure while children

were in the standing position after gentle expiration. BMI percentiles for age and BMI-standard deviation scores were determined based on the Center for Disease Control normative curves.<sup>16</sup> Blood pressure was measured with an aneroid sphygmomanometer on the left arm with the subject supine after 5 min of rest, with an appropriately sized cuff.<sup>17</sup> Systolic (Korotkoff phase I) and diastolic blood pressure (Korotkoff phase V) were measured three times and the average was used for analysis.

### Laboratory measurements

After a 12-h overnight fast, plasma glucose and insulin, and serum triglycerides, total cholesterol and high-density lipoprotein cholesterol were measured in enrolled children. Insulin resistance was calculated with the homeostasis model assessment of insulin resistance (HOMA-IR) index (fasting insulin  $\times$  fasting glucose/22.5), as described by Matthews *et al.*<sup>18</sup> HOMA-IR  $\geq$  2.5 was considered an index of impaired insulin sensitivity. The general characteristics of the obese children are reported in Table 1.

Body composition was evaluated with bioimpedance analysis (STA/BIA; Akern, Florence, Italy) in children carrying a UCP3 mutation and in their matched controls.

### DNA amplification and genotyping

Genomic DNA was obtained from whole blood of obese and non-obese subjects using Nucleon BACC-2 (GE Healthcare Europe-Amersham, Little Chalfont, UK). The UCP3 gene was amplified in a final volume of 50  $\mu$ l containing 50 ng of genomic DNA; 1 U of Taq DNA polymerase (Invitrogen S.r.l.,

**Table 1** Clinical and biochemical characteristics of the severely obese children ( $n=200$ ) genotyped

Parameters	Mean values $\pm$ s.d.	Normal value range
Age (years)	5.5 $\pm$ 3.2	
BMI (kg m <sup>-2</sup> )	26.4 $\pm$ 3.7	
BMI-SDS	3 $\pm$ 0.75	(<2)
Waist-to-hip ratio	0.97 $\pm$ 0.06	(<0.88)
Hip circumference	79.8 $\pm$ 9.2	(<57.1 cm)
SBP	94.5 $\pm$ 13.7	(<111 mm Hg)
DBP	61.5 $\pm$ 7.1	(<71 mm Hg)
Triglycerides	82.5 $\pm$ 41.8	(<103 mg dl <sup>-1</sup> )
Cholesterol	160.6 $\pm$ 31.9	(<180 mg dl <sup>-1</sup> )
LDL cholesterol	95.9 $\pm$ 30.0	(<130 mg dl <sup>-1</sup> )
HDL cholesterol	46.9 $\pm$ 11.1	(> 36 mg dl <sup>-1</sup> )
AST	27.8 $\pm$ 5.4	(10–40 U l <sup>-1</sup> )
ALT	24.8 $\pm$ 10.2	(<40 U l <sup>-1</sup> )
TSH	2.7 $\pm$ 1.2	(0.54–4.53 $\mu$ U ml <sup>-1</sup> )
FT3	4.4 $\pm$ 0.5	(3.0–9.1 pmol l <sup>-1</sup> )
FT4	1.2 $\pm$ 0.2	(0.85–1.75 ng dl <sup>-1</sup> )
HOMA	2.2 $\pm$ 1.4	(<2.5)
Insulin	10.82 $\pm$ 41.8	(<28 $\mu$ U ml <sup>-1</sup> )

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI-SDS, body mass index-standard deviation score; DBP, diastolic blood pressure; FT3, free triiodothyronine; FT4, free thyroxine; HDL, high-density lipoprotein; HOMA, homeostasis model assessment; LDL, low-density lipoprotein; SBP, systolic blood pressure; TSH, thyroid-stimulating hormone. Values are means  $\pm$  s.d.; numbers in parenthesis indicate the normal range corrected for the sample mean age (5.5  $\pm$  3.2 years).

Milan, Italy); 200  $\mu\text{M}$  of each deoxynucleotide triphosphate, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 2.5 mM  $\text{MgCl}_2$ , 0.2 mg  $\text{ml}^{-1}$  bovine serum albumin (BSA) and 200 nM of the specific primers. The primers used for UCP3 gene sequencing are here reported:

Promoter-Fw	5'-GCGTCCACAGCTTAAAGGAG-3'
Promoter-Rev	5'-GAACAAGGAGAAGGGAGAGG-3'
UCP3-F2	5'-ATCACTCCATCAGCCTTCTC-3'
UCP3-F2	5'-TCTTTGTACAGGGTCTGAGG-3'
UCP3-F3	5'-CAGCATGGTTGTCTCAGGC-3'
UCP3-F3	5'-TGCCTCTGAGTCTAGACTTC-3'
UCP3-F4	5'-AGGAGGTCTGAGTGGACATC-3'
UCP3-F4	5'-GTCAAGTGAAGTATCTTTGTTGTG-3'
UCP3-F5	5'-CATTCTCCATTCCATTCC-3'
UCP3-F5	5'-TCCTTCTAAAACCCAGTGGCC-3'
UCP3-F6	5'-TTGGGGACAAACAGTGCATAC-3'
UCP3-F6	5'-GTACTCTTACCCTACATC-3'
UCP3-F7	5'-GAGAGCACGCATCTGTTG-3'
UCP3-F7	5'-TCTGTGCCATGTGTGCGTG-3'

PCR fragments were separated by electrophoresis on a 1.5% agarose gel and purified. The two strands were sequenced (BigDye Terminator v3.1 cycle sequencing method on an ABI-Prism 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA).

#### Cloning of human wt and mutant UCP3 complementary (c)DNAs in a eukaryotic expression vector

Total mRNA from a human osteosarcoma cell line (Saos-2) expressing UCP3 protein was reverse transcribed using oligo (dT). UCP3L and UCP3S cDNAs were amplified in PCR reactions using the same 5'-primer (CTTCCAGGACTATGGTGG) but different 3'-primers: GTTCAAAACGGTGATTCCCG for UCP3L and GAAAGAAGCCCCTGTTCTCTG for UCP3S, respectively.<sup>19</sup> UCP3L and UCP3S cDNAs were inserted into the mammalian expression vector p3xFLAG-CMV-7.1 (Sigma-Aldrich S.r.l., Milan, Italy) downstream from the N-terminal 3 $\times$  FLAG epitope and then sequenced in both directions. QuickChange site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA, USA) was used to generate the four mutants (V56M, A111V, V192I and Q252X) from the cloned wt UCP3L cDNA according to the manufacturer's protocol. Recombinant constructs were purified using a Qiagen column (Qiagen S.p.A., Milan, Italy) and sequenced on both strands.

#### Cell culture and UCP3 protein expression

HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units  $\text{ml}^{-1}$  penicillin and 100  $\mu\text{g ml}^{-1}$  streptomycin (Invitrogen S.r.l.) at 37°C with 5%  $\text{CO}_2$ . The plasmids expressing the wt or the mutated UCP3 proteins were transiently transfected in HEK293 cells using Lipofectamine

2000 reagent (Invitrogen S.r.l.) according to the manufacturer's instructions. The pRL CMV vector (Promega Italia S.r.l., Milan, Italy) expressing the *Renilla* luciferase cDNA (RLuc) reporter gene was co-transfected (0.1  $\mu\text{g}$ ) and used as internal control reporter to verify transfection efficiency.

All the experiments were performed at 24 h post-transfection: at this time, we verified that the wt and mutants UCP3 proteins were expressed in appreciable amounts and correctly localized in the mitochondria. We also performed a cell-viability test, using Trypan blue (Sigma-Aldrich S.r.l.) according to the manufacturer's protocol and we observed 100% cell viability at 24 h post-transfection.

#### Preparation of mitochondrial and submitochondrial extracts and western blot

HEK293 cells were transiently transfected with plasmids that express wt or mutant UCP3 proteins. At 24 h after transfection, cells were washed in phosphate-buffered saline (PBS) pH 6.9 (Sigma-Aldrich S.r.l.), harvested and mitochondrial protein extracts were prepared using the Qproteome Mitochondria Isolation Kit (Qiagen S.p.A.) according to the manufacturer's instructions. Submitochondrial protein extracts were prepared from mitochondria freshly isolated as described above. Briefly, mitochondria were resuspended in a hypotonic medium (10 mM KCl, 2 mM HEPES, pH 7.2) and incubated for 20 min on ice to swell mitochondria and break the outer mitochondrial membrane, thereby releasing proteins from the intermembrane space. The swollen mitochondria were subsequently centrifuged at 11 200 r.p.m. and the supernatant (containing the soluble intermembrane space proteins) and the pellet (containing proteins on or associated with the inner mitochondrial membrane and matrix) were collected. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories S.r.l., Segrate, Milan, Italy).

For western blot analysis, 40  $\mu\text{g}$  of mitochondrial and submitochondrial protein fractions were run on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to a nitrocellulose membrane (GE Healthcare Europe-Amersham). Membranes were incubated for 1 h and 30 min at room temperature with specific antibodies and then incubated for 1 h with antibody-horse-radish peroxidase-conjugated anti-mouse Ig (1:3000 Sigma-Aldrich). Immunoreactive bands were visualized with the enhanced chemiluminescence reagents kit (ECL; GE Healthcare Europe-Amersham) according to the manufacturer's instructions. We used antitumor necrosis factor type 1 associated protein, TRAP-1 antibody (1:1000; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-COX-IV mouse monoclonal antibody (1:1000; Santa Cruz Biotechnology Inc.), anti-FLAG antibody (1:5000) and anti-tubulin antibody (1:500; Sigma-Aldrich S.r.l.).<sup>20,21</sup>

#### Palmitate oxidation and telmisartan treatment

Wt and mutant UCP3 proteins were expressed in HEK293 cells to evaluate the role of UCP3 in long-chain fatty acid

oxidation. HEK293 cells were seeded into 24-well plates and transiently transfected with either wt or mutant UCP3-expressing constructs alone or with wt and mutant UCP3-expressing constructs in equal amounts (1:1 ratio), such that the amount of DNA transfected each time was the same (namely, 0.8 µg). The pRL CMV vector was also co-transfected. Palmitate oxidation was measured as reported elsewhere.<sup>22</sup> Briefly, 24 h after transfection, cells were washed with PBS and incubated with 500 µl of preincubation medium (Krebs Ringer Bicarbonate Medium; Sigma-Aldrich S.r.l.) containing 0.5 g l<sup>-1</sup> BSA (fatty acid free; Sigma-Aldrich S.r.l.) for 1 h. After preincubation, the medium was removed and 200 µl of incubation medium (110 µmol l<sup>-1</sup> palmitate, 16.7 Ci ml<sup>-1</sup> [<sup>3</sup>H] palmitate and 0.5 g l<sup>-1</sup> BSA in PBS) were added to each well, which were incubated at 37 °C for 2 h. The incubation medium was transferred to columns containing ~3 ml of Dowex-1 ion-exchange resin (Sigma-Aldrich S.r.l.) previously charged with 1.0 mol l<sup>-1</sup> NaOH and washed with MilliQ water until the eluate had the same pH as the water. Then, each well was washed once with 300 µl of PBS that was collected and applied to the columns. The columns were finally washed with 2 ml of water. The resin binds the nonmetabolized palmitate and allows the tritiated water produced by β-oxidation to pass through. The eluate (2.5 ml) was collected in a scintillation vial. Then, 6 ml of scintillation cocktail (Picofluor 40; Packard Instruments Co Inc., Downers Grove, IL, USA) was added to each vial and the vials were counted in a liquid scintillation counter Tri-CARB 1500 (Packard Instrument Co Inc.). For each sample, counts per min (c.p.m.) were normalized to the luciferase activity determined by the Dual-Luciferase Reporter Assay System (Promega Italia S.r.l.), according to the manufacturer's instructions. The background signal was determined on untransfected control cells.

To evaluate the effects of the angiotensin II antagonist telmisartan on long-chain fatty acid β-oxidation in the presence of wt and mutated UCP3 proteins, HEK293 cells were transfected with wt UCP3L-expressing construct alone or co-transfected with wt and mutant UCP3-expressing constructs in equal amounts (1:1 ratio). At 24 h after transfection, cells were incubated first with 500 µl of preincubation medium for 1 h at 37 °C and then with 200 µl of a medium containing 110 µmol l<sup>-1</sup> palmitate and 0.5 g l<sup>-1</sup> BSA in PBS for 3 h. After the first 30 min, telmisartan (Sigma-Aldrich S.r.l.) was added to the medium at a final concentration of 10 µM,<sup>14</sup> and the incubation was continued for an additional 1 h and 30 min. During the last 1 h of incubation, [<sup>3</sup>H] palmitate (16.7 Ci ml<sup>-1</sup>) was added to the cells. Lastly, palmitate oxidation was measured in the medium, as reported above.

#### *Oil Red O staining*

Intracellular triglyceride accumulation was determined by Oil Red O staining. Briefly, HEK293 cells were seeded in poly-D-lysine eight-well culture slides (VWR International S.r.l.,

Milan, Italy), and transiently transfected with either wt or mutant UCP3-expressing plasmids alone or with wt and mutant UCP3 constructs in a 1:1 ratio, such that the amount of DNA transfected each time was the same (namely, 0.4 µg). At 24 h after transfection, cells were treated with 500 µM and 1 mM palmitate (Sigma-Aldrich, S.r.l.) complexed with BSA for 24 h. Then, cells were washed twice with PBS, fixed in a 10% formalin-containing PBS solution for 15 min and stained with Oil Red O working solution (5 mg Oil Red O ml<sup>-1</sup> isopropanol) for 15 min at room temperature. Cells were counterstained with hematoxylin and then covered with a coverslip. The stained lipids were viewed and photographed using a phase-contrast microscope (Leica Microsystems S.r.l., Milan, Italy) at ×40 magnification. The number of Oil Red O-stained lipid droplets/number of cells were counted. At least five randomly chosen fields were counted for each sample.

#### *Statistical analysis*

Allele frequencies were calculated by allele counting, and the deviation from Hardy–Weinberg equilibrium was evaluated by  $\chi^2$  analysis. The difference between metabolic and anthropometric variables in the two groups, wt and heterozygous mutation carriers, was evaluated by one-way analysis of variance. The statistical analysis was performed with SPSS software, version 10 (IBM, Chicago, IL, USA). The data relative to functional analysis are shown as mean ± s.d. and were analyzed with the Student's *t*-test. Differences were considered statistically significant at a *P*-value of <0.05.

## **Results**

#### *Clinical, biochemical and genetic features of study participants*

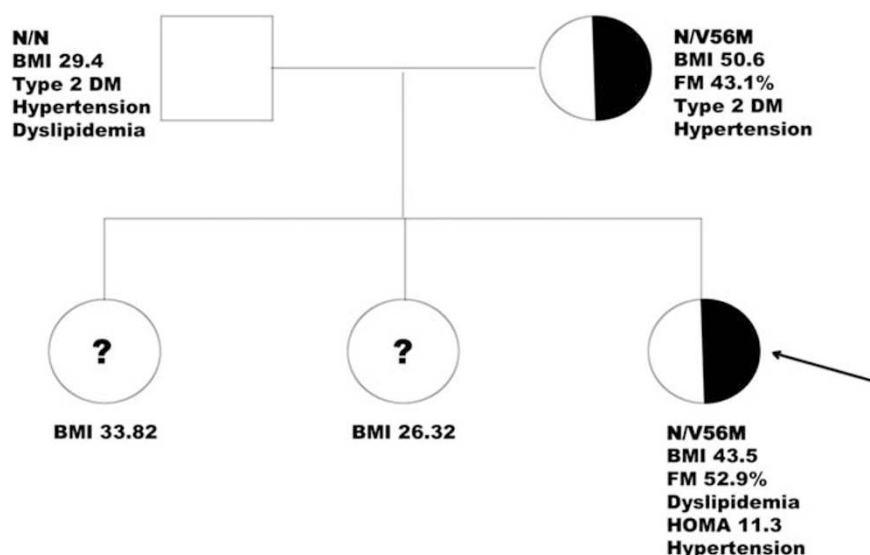
All clinical and biochemical parameters were within reference intervals for the mean age of the sample (Table 1). The 200 obese children had only high BMI-standard deviation score (mean 3) and waist-to-hip ratio (mean 0.97) values as expected in a sample with an average age of 5.5 years and early-onset obesity <4 years. Clinical (BMI, diastolic and systolic blood pressure) and biochemical characteristics (serum total cholesterol, triglycerides, glucose, aspartate aminotransferase and alanine aminotransferase) of the control normal-weight young subjects were in the reference range for the mean age of the sample (24.2 years).<sup>15</sup>

To determine whether *UCP3* gene variants contribute to the early-onset of obesity, we genotyped the cohort of severely obese children and 100 normal-weight non-diabetic subjects living in Southern Italy. We found three novel missense (V56M, A111V and V192I), one non-sense (Q252X, which generates a truncated protein) and two silent (S101S and A122A) mutations in the obese children and one polymorphism (V9V) in two normal-weight and two obese children. We also found a nucleotide change (10372 C/T) in intron 4 in one obese child (Table 2). All mutations are in the heterozygous state; mutations A111V, V192I and Q252X

**Table 2** Mutations and polymorphisms detected in the *UCP3* gene in severely obese children ( $n=200$ ) and non-obese controls ( $n=100$ ) living in Southern Italy

Region	Nucleotide change	Amino-acid change	Obese, n (%)	Control group, n (%)
<i>UCP3</i> variants				
5'-UTR	-55 C/C; C/T; T/T	—	143 (75.6); 44 (23.3); 2 (1)	65 (73.6); 22 (25.3); 1 (1.1)
Exon 2	8990 G/A	V9V	2 (1)	2 (2)
Exon 3	9666 G/A	V56M	3 (1.5)	—
Exon 3	9832 C/T	A111V	1 (0.5)	—
Exon 3	9576 C/T	S101S	1 (0.5)	—
Exon 4	10 099 C/T	A122A	1 (0.5)	—
Exon 5	11 449 G/A	V192I	1 (0.5)	—
Exon 6	12 105 C/T	Q252X	1 (0.5)	—
Intron 4	10 372 C/T	—	1 (0.5)	—

Abbreviations: UCP3, uncoupling protein 3; UTR, untranslated region.



**Figure 1** Pedigree of the family with the V56M mutation. The arrow indicates the female proband carrying the V56M mutation. Status for BMI ( $\text{kg m}^{-2}$ ), % fat mass (% FM), type 2 diabetes mellitus (type 2 DM), blood pressure, dyslipidemia and HOMA are indicated.

were found in three unrelated probands; mutation V56M was found in two male siblings and in an unrelated girl (Table 2). We also analyzed the -55C/T polymorphism in the promoter region of the *UCP3* gene in the obese and control groups. The genotype distribution for *UCP3* -55C/T (CC, CT, TT) was in Hardy-Weinberg equilibrium. Genotype and allele frequencies did not differ between obese and non-obese subjects (Table 2).

To exclude the involvement of other obesity gene variants in the increased fat mass in our obese subjects, we genotyped them for POMC, MC4R and UCP1 variants, but found no mutations.

The parents of the 200 obese children were invited to undergo genotyping to determine the mode of transmission of mutations in families, but only the parents of the girl carrying mutation V56M consented to genotyping. As shown in Figure 1, the mother, who was severely obese (BMI 50.6), carried mutation V56M in the heterozygous

state, similar to her daughter. Furthermore, she had waist circumference of 114 cm (normal 80 cm) and was affected by type 2 diabetes and hypertension. Mutation V56M was absent from the father, who was overweight (BMI 29.4) and also affected by type 2 diabetes, hypertension and dyslipidemia. Their daughter was severely obese (BMI 43.5); of her two sisters, one was overweight (BMI 26.3) and the other was obese (BMI 33.8), but they were not available for genotyping. Interestingly, the three children carrying mutation V56M had a much higher percentage of fat mass (~50.0%) than the children carrying other *UCP3* gene mutations (between 36 and 45%). Furthermore, the girl carrying mutation V56M (see Figure 1) had elevated systolic blood pressure (130 mm Hg), low levels of high-density lipoprotein cholesterol ( $39 \text{ mg dl}^{-1}$ ), high levels of low-density lipoprotein cholesterol ( $113.4 \text{ mg dl}^{-1}$ ) and a high HOMA index (11.3). Hence, this girl had three components of the metabolic syndrome, as did her parents, plus insulin-resistance.



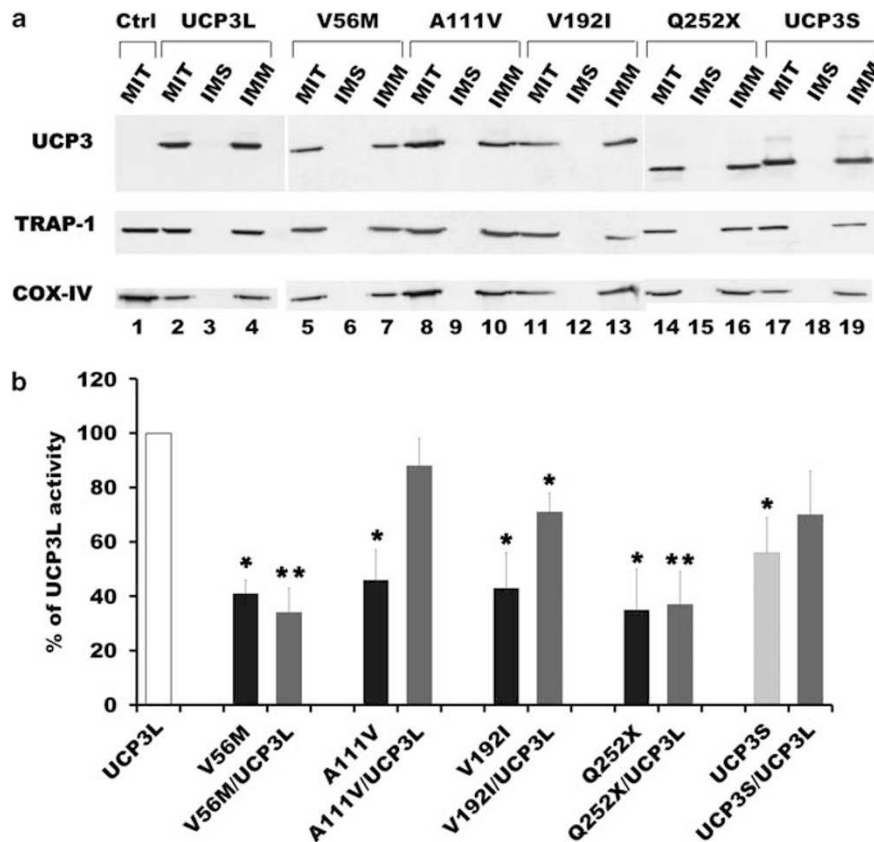
*Involvement of UCP3 wt and mutant proteins in long-chain fatty acid metabolism*

We investigated the effects of wt UCP3 proteins and V56M, A111V, V192I and Q252X mutant proteins on long-chain fatty acid oxidation and triglyceride storage in HEK293 cells. HEK293 cells are, at present, the most widely used cell line for *in vitro* studies in which expression plasmids are transfected in order to produce proteins (also channel proteins) and to study their activity. Wild-type long and short UCP3 isoforms and mutant proteins were expressed in HEK293 cells that lacked endogenous UCP3 protein in the mitochondria. First, we evaluated the correct targeting of wt and mutant proteins in the inner membrane and matrix (IMM) using mitochondrial and sub-mitochondrial protein fractions from HEK293-expressing wt or mutated UCP3 proteins. Both wt UCP3L and UCP3S isoforms were correctly localized in the IMM, and were absent in the intermembrane space (Figure 2a, lanes 2–4 and 17–19, respectively).

Similarly, all UCP3 mutant proteins were correctly localized in IMM, and were absent in the intermembrane space (Figure 2a, lanes 7, 10, 13, 16 and lanes 6, 9, 12, 15, respectively).

We next evaluated the  $\beta$ -oxidation capacity of palmitate, a long-chain fatty acid, in HEK293 cells expressing wt or mutant UCP3 proteins and treated with  $^3\text{H}$ -labeled palmitate. Palmitate  $\beta$ -oxidation capacity was evaluated by measuring tritiated water produced by cells and it was expressed as a percentage of UCP3L activity, taken as 100%. The UCP3S isoform retained 55% of UCP3L activity (Figure 2b); moreover, palmitate oxidation was significantly reduced in HEK293 cells expressing the mutated proteins. In particular, V56M and Q252X mutants retained only 40 and 35% of UCP3L activity, respectively. A111V and V192I retained  $\sim 45\%$  of UCP3L activity (Figure 2b).

Because all mutations were found in the heterozygous state, we tested the possibility that mutated proteins can



**Figure 2** Sublocalization (a) and activity (b) of wt and mutant UCP3 proteins. (a) Western blot of mitochondrial (MIT) and submitochondrial (intermembrane space (IMS) and IMM) protein extracts (40  $\mu\text{g}$ ) obtained from untransfected HEK293 cells (lane 1, Ctrl) and from HEK293 cells expressing wt UCP3L (lanes 2–4) and V56M (lanes 5–7), A111V (lanes 8–10), V192I (lanes 11–13) and Q252X (lanes 14–16) mutant proteins. Protein extracts from cells expressing wt UCP3S (lanes 17–19) are also shown. A specific anti-FLAG monoclonal antibody was used to reveal wt and mutant UCP3 proteins. Anti-Trap-1 and anti-COX-IV antibodies were used as control for IMM localization. (b) Activity of wt and mutant UCP3 proteins calculated as percentage of  $^3\text{H}$ -labeled palmitate oxidation. Percentage of palmitate oxidation capacity of wt UCP3 isoforms (UCP3L, white bar and UCP3S, light gray bar) and of V56M, A111V, V192I and Q252X mutant proteins (black bars) in HEK293 cells. We assigned an arbitrary value of 100% to UCP3L isoform activity. Palmitate  $\beta$ -oxidation capacity was also assayed in HEK293 cells coexpressing UCP3L isoform and mutant proteins in equal amounts (V56M/UCP3L, A111V/UCP3L, V192I/UCP3L, Q252X/UCP3L and UCP3S/UCP3L, gray bars). Data represent the means  $\pm$  s.d. of four different experiments. \* $P < 0.05$  and \*\* $P < 0.01$  represent statistical differences vs UCP3L.

exert a dominant-negative effect on wt UCP3L activity. We choose to refer all successive analyses to long isoform of UCP3 (UCP3L) activity in that the UCP3L protein is the only isoform detectable in the human skeletal muscle also using such large amounts of protein mitochondrial extracts as 15 mg.<sup>5</sup> To this aim, we co-transfected equal amounts of UCP3L-expressing construct with constructs expressing V56M, A111V, V192I or Q252X in HEK293 cells, and evaluated the dominant-negative effect of mutated proteins on UCP3L activity by determining palmitate  $\beta$ -oxidation capacity. V56M and Q252X mutants exerted a dominant-negative effect on UCP3L activity, whereas A111V and V192I activity were rescued by UCP3L co-transfection (Figure 2b). Also, the UCP3S isoform activity was only partially rescued in co-transfected cells mimicking a slight dominant-negative effect on UCP3L activity (Figure 2b).

Interestingly, the V56M mutant protein was associated with higher BMI, percentage of fat mass and HOMA and insulin values in obese children carrying UCP3 mutations. To evaluate the role of long and short wt UCP3 isoforms in the prevention of triglyceride storage, we treated HEK293 cells expressing wt UCP3 isoforms (long and short) or mutant proteins with 500  $\mu$ M or 1 mM palmitate and evaluated triglyceride storage by Oil Red O staining. Similar results were obtained with either palmitate concentration. The number of Oil Red O-positive spots was significantly lower in cells expressing the UCP3L isoform than in untransfected cells (Control (Ctrl); Figures 3a and b, compare UCP3L with Ctrl). As expected, neither the UCP3S isoform nor the four mutant proteins prevented triglyceride storage (Figures 3a and b), although at different extent, as shown by the higher number of Oil Red O-positive spots compared with UCP3L-expressing cells. Again, as expected, UCP3L co-transfection partially rescued the activity of the A111V and V192I mutant proteins as well as UCP3S isoform but did not affect the activity of the V56M and Q252X dominant-negative mutant proteins (Figure 3b). Interestingly, subjects carrying V56M or Q252X dominant-negative mutations had the highest plasma non-esterified fatty acid values, mild liver steatosis and higher fat mass and lower free fat mass values (data not shown).

#### *Telmisartan improved palmitate oxidation capacity in HEK293 cells coexpressing UCP3L and mutant proteins*

Telmisartan, 10  $\mu$ M, increases fatty acid oxidation in skeletal muscle by activating the peroxisome proliferator-activated receptor- $\gamma$  pathway.<sup>14</sup> Therefore, we evaluated whether telmisartan improves palmitate  $\beta$ -oxidation capacity in cells coexpressing the UCP3L isoform and mutated UCP3 proteins. HEK293 cells were transiently transfected with UCP3L-expressing construct alone or co-transfected with constructs expressing the UCP3L and V56M, A111V, A192I and Q252X mutant proteins in equal amounts in order to mimic the heterozygous state of probands. Telmisartan, 10  $\mu$ M, was added to the culture for 3 h and long-chain fatty acid

$\beta$ -oxidation capacity was evaluated in the presence of tritiated palmitate. Palmitate oxidation capacity was calculated as percentage with respect to UCP3L-expressing cells in the absence of telmisartan taken as 100% (Figure 4, UCP3L). We found that 10  $\mu$ M telmisartan increased  $\beta$ -oxidation capacity in cells expressing UCP3L, by  $\sim$ 40% with respect to untreated cells.  $\beta$ -Oxidation capacity was also significantly higher in telmisartan-treated cells coexpressing UCP3L and all mutant proteins than in the untreated counterpart cells (Figure 4, compare gray with black bars). Interestingly, telmisartan increased  $\beta$ -oxidation capacity by approximately two- to three-fold in cells coexpressing UCP3L and the dominant-negative mutants Q252X and V56M.

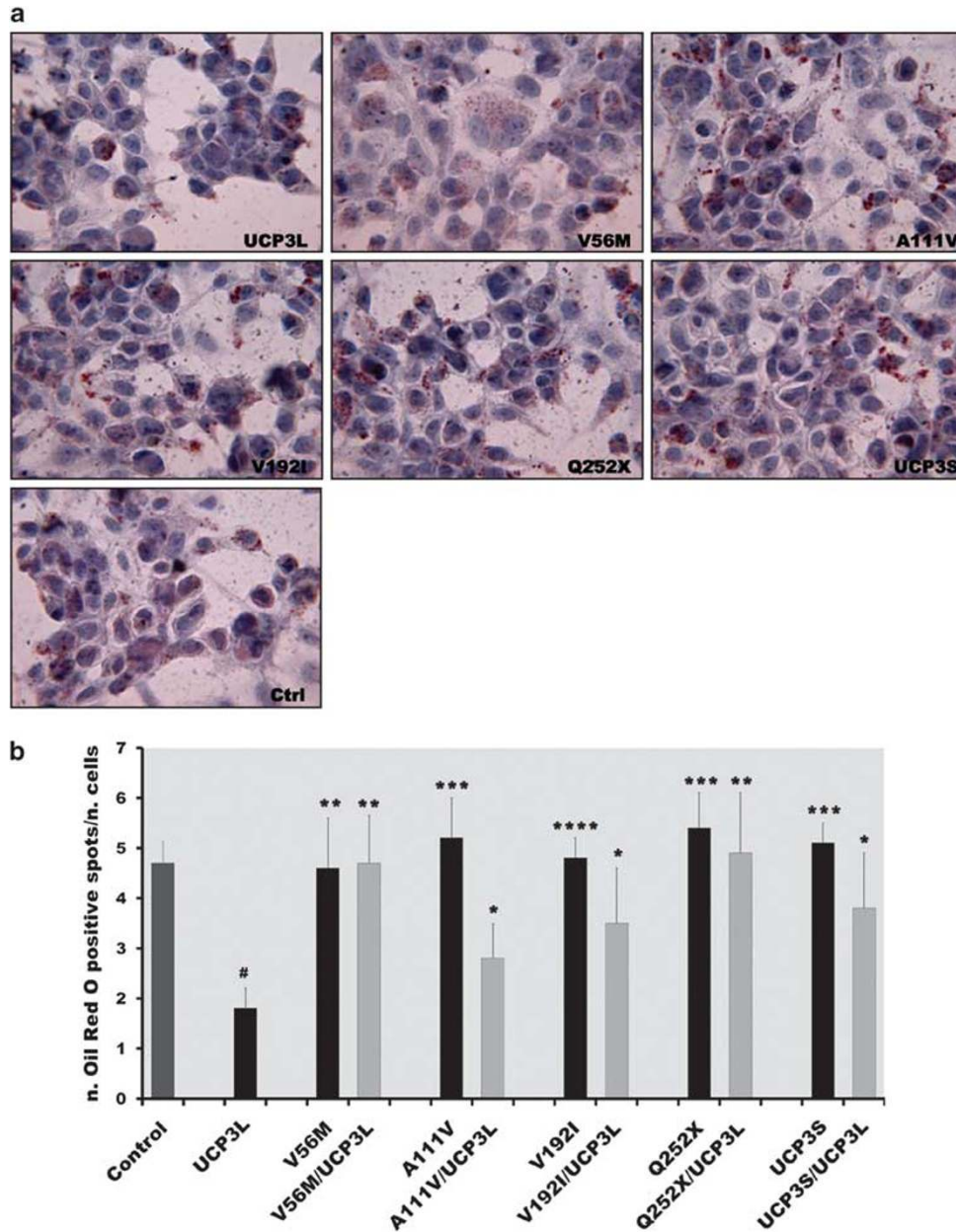
## Discussion

Different functional roles have been postulated for UCP3: UCP3 has been implicated in fatty acid metabolism in conditions of excess mitochondrial fatty acid supply;<sup>23,24</sup> UCP3 is involved in body energy balance. In fact, mice overexpressing human UCP3 have a lower body weight than wt mice.<sup>25–28</sup> Furthermore, observational studies in humans showed that UCP3 protein expression was reduced by 40% after weight loss in type 2 diabetic patients,<sup>11</sup> and UCP3 protein expression was negatively correlated with BMI in non-diabetic obese subjects.<sup>29</sup>

In humans, UCP3 expression is restricted to skeletal muscle. Because skeletal muscle is responsible for most of the daily energy expenditure, and a reduction in energy expenditure is a risk factor for the development of obesity,<sup>30</sup> UCP3 has been indicated as an obese susceptibility gene. Furthermore, the *UCP3* gene was mapped on chromosome 11q13, in a region that has been linked to obesity and hyperinsulinemia.<sup>31</sup>

Several *UCP3* gene variants have been implicated in obesity in humans.<sup>32–34</sup> The most extensively studied *UCP3* variant is the  $-55C/T$  polymorphism in the promoter region. The association of this polymorphism with overweight is controversial. In fact, it was associated with elevated UCP3 mRNA expression in male non-diabetic Pima Indians,<sup>35</sup> with an increased BMI in a French population,<sup>36</sup> with an increased hip-to-waist ratio in women of Asian origin<sup>37</sup> and with BMI and diabetes mellitus in a German population.<sup>38</sup> Conversely, the  $-55C/T$  polymorphism was associated with a lower BMI in a UK population<sup>39</sup> and in US Caucasian and Spanish populations,<sup>40,41</sup> whereas no association was found between  $-55C/T$  and BMI or percentage of body fat in Danish obese and control subjects.<sup>42,43</sup> In our cohort, we found no association between  $-55C/T$  and BMI, which is in agreement with Dalgaard and Berentzen.<sup>42,43</sup>

Only few studies have been reported so far on the positive association between UCP3 mutations and obese phenotype, but no functional analyses were performed in eukaryotic cells.<sup>32–34</sup> Hence, the functional analysis of the wt and



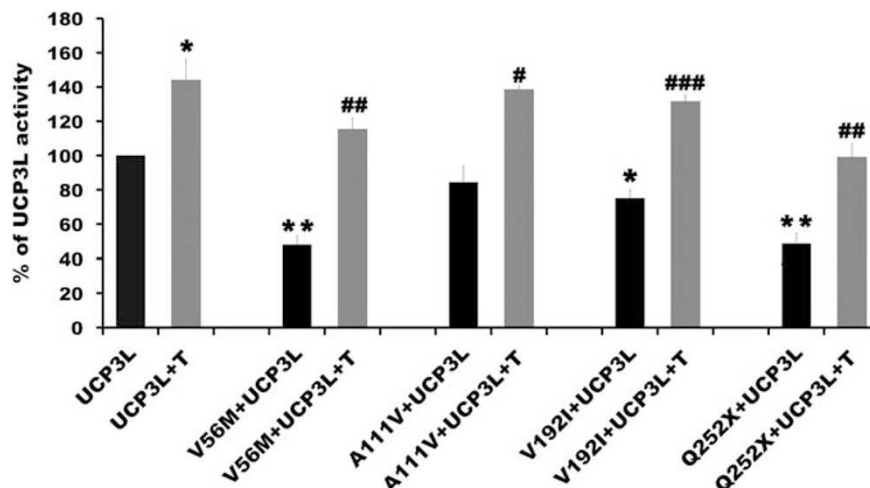
**Figure 3** Triglyceride storage of wt and mutant UCP3 proteins. (a) Oil Red O staining of HEK293 cells expressing wt UCP3L and UCP3S isoforms and mutant V56M, A111V, V192I and Q252X UCP3 proteins treated with 1 mM palmitate. Red points indicate triglyceride depots;  $\times 40$  magnification. Ctrl indicates HEK293 cells not expressing UCP3 protein. (b) The number of Oil Red O-positive spots/number of cells is reported. Control (heavy gray bar) represents number of Oil Red O-spots/number of cells in HEK293 not expressing UCP3 protein. Black bars represent Oil Red O-spots/number of cells in HEK293 expressing wt UCP3L or UCP3S isoforms or V56M, A111V, V192I and Q252X mutant proteins; light gray bars represent Oil Red O-spots/number of cells in HEK293 coexpressing UCP3L isoform and UCP3S isoform or mutant proteins in equal amounts (UCP3S/UCP3L, V56M/UCP3L, A111V/UCP3L, V192I/UCP3L and Q252X/UCP3L). Data represent the means  $\pm$  s.d. of five different fields. # $P < 0.001$  vs Control; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ , \*\*\*\* $P < 0.001$  vs UCP3L-expressing cells.

mutant UCP3 proteins identified in our severely obese children is the first attempt made in eukaryotic cells to unravel the role of UCP3 in handling long-chain fatty acids.

In our experimental system, the UCP3 short isoform is localized in the IMM and shows a slight dominant-negative

effect on UCP3 long isoform activity. Further experiments are required to validate the functional activity of the short isoform of UCP3, also in muscle cells. Similarly, it will be necessary to define *in vivo* the expression and the localization of the Q252X mutant protein, which lacks the sixth transmembrane domain.





**Figure 4** Effects of telmisartan treatment on palmitate oxidation activity of wt and mutant UCP3 proteins. Oxidation of  $^3\text{H}$ -labeled palmitate in HEK293 cells expressing either UCP3L isoform or UCP3L and mutant UCP3 proteins in equal amounts (V56M/UCP3L, A111V/UCP3L, V192I/UCP3L and Q252X/UCP3L), in the absence (black bars) or presence (gray bars; +T) of telmisartan treatment. Data represent the means  $\pm$  s.d. of four different experiments reported as a percentage of the value obtained for UCP3L-expressing cells in the absence of telmisartan treatment (UCP3L black bar) to which we assigned an arbitrary value of 100%. \* $P < 0.05$  and \*\* $P < 0.005$  vs UCP3L; # $P < 0.05$ , ## $P < 0.005$  and ### $P < 0.001$  vs corresponding black bar (-T).

The crystallographic structure of the UCP3 protein is not available, and hence we are not able to correlate the mutations identified with the UCP3 protein structure. Regarding the structure, we can only speculate on the type of amino acid substitution and/or on UCP3 domains in which the changed amino acids are located. All the data reported in our paper regarding the domains and the transmembrane structures of the UCP3 protein were obtained from the UniProt database. V56M is a mutation that consists of a substitution of a non-polar amino acid in an amino acid that is also non-polar. V56 amino acid, highly conserved in eukaryotes, falls in a domain (Ith solute carrier=solcar repeat) involved in transporting fatty acid anions from the mitochondrial matrix into the intermembrane space. V192I is a substitution of a non-polar amino acid in a hydrophobic amino acid. V192 amino acid, highly conserved in eukaryotes, falls in the fourth transmembrane domain included in the II solcar repeat. Despite the fact that these two variants are located in important regions involved in the transport of anions of fatty acids, they affect differently the activity of UCP3L.

Similarly, we do not know if the Q252X variant is essentially the same as making cells homozygous for UCP3S. What we know is that in our experimental system, both the Q252X mutant and the UCP3 short isoform are localized in the mitochondria associated with the inner membrane (IMM), but they show a different effect on UCP3L isoform activity. In particular, the UCP3S isoform retained 55% of UCP3L activity, whereas the Q252X mutant retained only 35% of UCP3L activity. However, as we mentioned previously, *in vivo*, we never detected the UCP3S isoform in mitochondrial extracts from skeletal muscle biopsies. Moreover, we

have no data *in vivo* regarding the expression of the Q252X mutated protein because muscle biopsies of the subject carrying the Q252X mutation are not available.

UCP3 expression increases glucose metabolism and protects against hyperglycemia.<sup>25,44</sup> Moreover, UCP3 messenger and protein expression was found to be decreased in muscle tissue of pre-diabetic and diabetic subjects.<sup>45,13</sup> Because of the early onset of obesity in our cohort (mean age 4 years), we found no correlation between the HOMA index and the activity of mutated UCP3 protein. However, the HOMA index was elevated in two subjects carrying mutation V56M (11.24 and 3.05, respectively, compared with the mean value of 2.2 in our obese cohort), as were insulin plasma concentrations (53.9 and 14.7, respectively, vs 10.82). We re-examined the female proband carrying mutation V56M 10 years after the first observation when she was 17 years old. She was still obese (BMI 47.6) and reported diet-resistant weight gain. These data suggest a link between V56M and severe human obesity, and extend our knowledge about the role of UCP3 in fatty acid oxidation and in the prevention of triglyceride storage. Interestingly, the highest percentage of fat mass was found among obese subjects carrying the V56M and Q252X dominant-negative mutants.

Telmisartan is both a selective peroxisome proliferator-activated receptor modulator and an angiotensin II receptor blocker.<sup>13,14,46-49</sup> Recently, it was found to be effective in the treatment of hypertension, to improve glucose and lipid metabolism and to protect against diet-induced weight gain and visceral fat accumulation. Telmisartan also increased fatty acid metabolism in murine muscle myotubes by decreasing acetyl CoA carboxylase 2 expression, thereby resulting in inhibition of fatty acid synthesis and stimulation of fatty acid oxidation.<sup>49</sup> Finally, studies conducted in

humans showed that telmisartan positively affected HbA1c, total and low-density lipoprotein cholesterol and hypertension in type 2 diabetes patients.<sup>50–52</sup> Consequently, telmisartan could be used to treat obese, type 2 diabetes with hypertension and hence reduce the risk of cardiovascular diseases.

In conclusion, our data support the notion that protein UCP3 is involved in long-chain fatty acid metabolism in mitochondria and in the prevention of cytosolic triglyceride storage. We also provide evidence that telmisartan improves palmitate oxidation in cells expressing the dominant-negative UCP3 mutant proteins V56M and Q252X. Further experiments are needed in order to test if telmisartan may be useful in subjects in whom fatty acid metabolism is severely impaired.

Our future aim is also to enlarge our cohort study and to investigate if the activity of mutant-negative UCP3 proteins is correlated with dietary fat intake and/or with the degree of daily physical activity.

### Conflict of interest

The authors declare no conflict of interest.

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