Identification and Functional Characterization of Novel Mutations in the Melanocortin-4 Receptor

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Keywords

Obesity · Melanocortin-4 receptor · Mutation analysis

Summary

Objective: Melanocortin-4 receptor (MC4R) deficiency is the most common cause of monogenic obesity. In the present study, we screened the *MC4R* gene for mutations in a population of overweight and obese children and adolescents. **Method:** Cross-sectional mutation analysis of 112 overweight/obese children and adolescents and 121 lean individuals. **Results:** We identified 11 sequence variations, 5 of which were present in our control population or had been previously reported as polymorphisms. The remaining 6 variations are disease-causing mutations including 2 novel ones: a I186V mutation and a F280L mutation. The 4 previously described mutations (D90N, M200V, P260Q, Q307X) were identified in single probands. Using confocal imaging, we demonstrated that F280L and P260Q cause intracellular retention of the mutant receptor. No difference in cell surface expression could be detected for the I186V mutation. Using a cAMP responsive luciferase vector, we demonstrated that the receptor with I186V is unable to activate its intracellular signaling pathway while the P260Q mutation causes reduced activation of the receptor. **Conclusion:** We detected MC4R deficiency in 6 patients from our cohort, amounting to a prevalence of 5.3%. Two novel mutations were identified. We also confirmed that intracellular retention is a common pathogenic effect of *MC4R* mutations.

Introduction

Obesity is one of the most important health problems in today's world. It is caused mainly by an interaction between environmental and genetic factors. However, several studies have shown that obesity can also be caused by a mutation in a single gene. These monogenic forms of obesity have been found to be caused by mutations in the genes encoding leptin [1], leptin receptor [2], prohormone convertase 1 [3], proopiomelanocortin [4], melanocortin-3 receptor [5, 6], melanocortin-4 receptor (MC4R) [7, 8] and single minded 1 [9]. MC4R deficiency has proven to be the most common cause of monogenic obesity, with a frequency of about 2.5% in the obese population [10].

The MC4R is a 7 transmembrane receptor expressed in the hypothalamus [11, 12]. This receptor is activated by α -melanocyte stimulating hormone (α -MSH), its endogenous agonist, and is antagonized by agouti-related peptide (AgRP) [11–13]. Leptin stimulates the production of proopiomelanocortin, the precursor of α -MSH and inhibits the expression of AgRP, thus generating an anorexigenic signal. The crucial role of the MC4R in food intake regulation was demonstrated through generation of a knock-out mouse. Huszar et al. [14] showed that MC4R knock-out mice display a maturity-onset obesity phenotype with hyperphagia, increased linear growth, hyperinsulinemia and hyperglycemia. In 1998, two groups simultaneously reported the first human mutations in *MC4R*: they found a deletion and insertion which completely abolished receptor function. Proband carrying these mutations had severe early-onset obesity and hyperphagia without other complications [7, 8]. Since then, MC4R has been widely stud-

ied in several populations of obese adults and children. Mutation frequencies vary considerably between populations, ranging from 0.5 to 6% [15–24].

Most mutations were found in the heterozygous state, although some homozygous mutations have also been reported [19, 25–28]. Several *MC4R* mutations have been functionally characterized. They were mostly found to impair proper receptor trafficking to the plasma membrane, and the mutant receptors were therefore found to be retained intracellularly [29–31]. Mutations can also impair receptor function through affecting ligand binding, constitutive activity and agonist-stimulated signaling [32, 33].

Recently, *MC4R* has also been implicated in the etiology of complex obesity. A genome-wide association study identified variants downstream from *MC4R* to be significantly associated with common obesity in Caucasian populations [34]. This finding was subsequently replicated by several other investigators [35–38] and is now accepted as a genuine association signal for complex obesity. These findings demonstrate that *MC4R* is important in the development of both monogenic obesity and common obesity.

Previously, we reported that no mutations were present in a population of Belgian obese adults, adolescents and children [39]. In the current study, we present data from a mutation analysis in a second group of overweight and obese children and adolescents.

Material and Methods

Subjects

112 unrelated overweight and obese children and adolescents (51 boys and 61 girls; adolescence defined as age ≥ 12 years; table 1) consulting the Child Obesity Clinics from the Antwerp University Hospital and the Virga Jesse Hospital, Hasselt, both in Belgium, were recruited. The patient population has a mixed ethnicity. The percentile lines that pass through the BMI of 25 and 30 at 18 years of age on the Flemish age- and sex-specific BMI growth curves are used as cut-off for the diagnosis of overweight and obesity in our cohort [40]. For patients carrying *MC4R* mutations, parents and other family members, if available, were contacted for inclusion in the study. A control population of 121 lean subjects was also examined for mutations in *MC4R* (inclusion criterium: BMI between 18.5 and 25 kg/m²). All control subjects were recruited among personnel from Antwerp University Hospital and the Department of Medical Genetics. All control subject were of Caucasian origin (table 1). All participants gave their written informed consent. The study protocol was approved by the local ethics committee.

Anthropometry

Height was measured to the nearest 0.1 cm. Weight was measured on a digital scale to the nearest 0.1 kg. BMI was calculated for all patients, and Z-scores were based on the Flemish growth curves [40].

Mutation Analysis of *MC4R*

The *MC4R* gene was amplified by PCR on genomic DNA isolated from whole blood. The entire coding region of *MC4R* (999 bp) was screened for mutations using denaturing high performance liquid chromatography (DHPLC) or high-resolution melting curve analysis (HRM). The control population was screened with both methods. DHPLC analysis was carried

Table 1. Characteristics of the study populations^a

	Children	Adolescents	Control subjects
N	71	41	121
Age, years	8.7 $\pm$ 0.3	14.0 $\pm$ 0.2	38.1 $\pm$ 0.9
Male/female	34/37	17/24	14/107
Weight, kg	50.3 $\pm$ 2.0	94.3 $\pm$ 2.7	63.9 $\pm$ 0.7
Height, m	1.37 $\pm$ 0.02	1.67 $\pm$ 0.01	1.68 $\pm$ 0.01
BMI, kg/m ²	26.1 $\pm$ 0.5	33.8 $\pm$ 0.7	22.6 $\pm$ 0.1
BMI Z-score ^b	2.5 $\pm$ 0.1	2.5 $\pm$ 0.1	–

^aMean value $\pm$ standard error of mean is given.

^bBMI Z-score was based on data from the Flemish Growth Charts [40].

out as described previously [39]. Briefly, *MC4R* was divided into 3 amplicons which were all analyzed on a WAVE Nucleic Acid Fragment Analysis System (Transgenomic Inc, Crewe, UK). Samples with aberrant chromatograms compared to wild type were sequenced to confirm and identify nucleotide changes. For HRM, the coding region of *MC4R* was divided into 4 amplicons. HRM was performed using the Lightcycler LC480 Real-Time PCR System (Roche, Penzberg, Germany) with incorporation of LCGreen+ fluorescent dye (Idaho Technology Inc., Salt Lake City, UT, USA) into the PCR product. Samples with melting curves deviating from wild type were sequenced. Sequencing was performed with ABI BigDye Terminator v1.1 Cycle Sequencing kits on an ABI Prism Genetic Analyzer 3130xl (Applied Biosystems Inc, Foster City, CA, USA). Primer sequences for DHPLC, HRM and sequencing are available upon request.

Subcellular Localization of Mutant Receptor

Wild-type *MC4R* was amplified directly from genomic DNA of a control individual using primers which add a *NheI* (5'-ATC GAT GCT AGC TCC TGC CAG CAT GGT GAA CTC C-3') and a *HindIII* (5'-GTA ACT AAG CTT ATA TCT GCT AGA CAA GTC-3') restriction site upstream and downstream of *MC4R* respectively. PCR was performed using iProof High-Fidelity DNA polymerase (Bio-Rad, Hercules, CA, USA), according to the manufacturer's protocol. Using *NheI* and *HindIII*, wild-type *MC4R* was cloned into the mammalian expression vector pEGFP-N1 (Clontech, Mountain View, CA, USA) which adds a C-terminal EGFP tag to *MC4R*. Mutant receptors carrying I186V, P260Q or F280L were created with in vitro mutagenesis using a Quikchange Site Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). All cloned products were sequenced using a standard sequencing protocol as described above.

COS-7 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were incubated at 37 °C in humidified air containing 5% CO₂. One day prior to transfection, 1 $\times$ 10⁴ COS-7 cells were grown on collagen-coated (0.48 mg/ml collagen; BD Biosciences, Franklin Lakes, NJ, USA) coverslips (Knittel Gläser, Braunschweig, Germany) in 24-well plates. Transfection was done using Eugene 6 transfection reagent according to manufacturer's protocol (Roche): 200 ng of wild-type or mutant *MC4R* expression vector was transfected at a 3:1 ratio to Eugene 6 transfection reagent. 24 h after transfection, plasma membranes were fluorescently stained with 1 μ g/ml tetramethyl-rhodamine wheat germ agglutinin conjugate (Invitrogen). After staining, cells were fixed with 4% formaldehyde and mounted on microscope slides. Vectashield Mounting medium (Vector Laboratories, Burlingame, CA, USA) was used to preserve fluorescence. Cells were visualized using a Zeiss LSM 510 confocal microscope equipped with argon (excitation, 488 nm) and helium-neon (excitation, 543 and 633 nm) lasers.

Table 2. Clinical phenotype of probands carrying *MC4R* mutations

Mutation	Age, years	Sex	Height, m	Weight, kg	BMI, kg/m ²	BMI Z-score ^a
D90N	3.7	boy	1.035	29.0	27.1	4.33
I186V	7.3	girl	1.353	53.0	29.0	3.18
M200V	10.4	boy	1.398	50.0	25.6	2.13
P260Q	10.9	girl	1.540	78.4	33.1	2.61
F280L	12.2	girl	1.630	96.3	36.3	2.87
Q307X	14.8	girl	1.775	112.4	35.8	2.82

^aBMI Z-score was based on data from the Flemish Growth Charts [40].

Intracellular Signaling of Mutant Receptor

Wild-type *MC4R* cloned in the mammalian expression vector pcDNA3 was kindly provided by Dr. G. Yeo (Cambridge, UK). Mutant *MC4Rs* were created with a Quikchange Site Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. All cloned products were sequenced using a standard sequencing protocol as described above. HEK293T cells were maintained in DMEM supplemented with 10% FCS, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen). Cells were incubated at 37 °C in humidified air containing 5% CO₂. Intracellular signaling capacity of mutant receptors was determined using a cAMP responsive luciferase construct (pCRE-Luc; Stratagene). One day before transfection, 1.5×10^4 cells were seeded on a 96-well plate. Cells were transfected with 25 ng wild-type or mutant *MC4R* expression vector, 25 ng pCRE-Luc and 2.5 ng internal control plasmid pRL-TK, which constitutively expresses Renilla luciferase. Transfections were carried out using Fugene 6 transfection reagent according to the manufacturer's protocol (Roche). 24 h after transfection, cells were stimulated with varying concentrations of α -MSH (10^{-5} to 10^{-10} mol/l; Bachem, Well-am-Rein, Germany) diluted in culture medium. Cells were allowed to incubate for 3 h in the presence of α -MSH. Then the cells were lysed and Firefly and Renilla luciferases were sequentially activated and quenched using the Dual-Glo Luciferase assay System (Promega, Madison, WI, USA). Luminescence was detected with a Victor² Multilabel Plate Reader (Perkin Elmer, Waltham, MA, USA). For all experimental constructs, the assay was repeated four times. The ratio of firefly-to-renilla luciferase luminescence was calculated to correct data for transfection efficiency. Data for experimental constructs were all normalized to the firefly-to-renilla ratio for wild-type *MC4R* stimulated with 10^{-5} mol/l α -MSH (set at 100%).

Results

Screening of *MC4R* in our patient and control population revealed 11 sequence variations. Three of these were found in both our patient and control population (V103I, I198I and I251L). G324G was only identified in a control individual while T112M was only found in an obese individual, but this variant has previously been reported as a polymorphism [22, 41]. V103I and I251L were also previously described as polymorphisms; their minor alleles lead to a slightly reduced body weight [42, 43].

The mutations D90N, I186V, M200V, P260Q, F280L and Q307X were each found heterozygously in single, obese probands, but not in our control population. D90N and M200V have been described previously [17, 44]. During the

Fig. 1. Pedigree of family with *MC4R* F280L mutation.

Filled symbols are individuals with obesity. The first line below the symbols indicates the genotype status for F280L: N, normal allele; M, mutant allele; ??, unknown genotype. The second line shows BMI of the individual at investigation.

course of this study, P260Q and Q307X were also described [45, 46]. Characteristics of the probands are given in table 2.

For all mutation carriers, we tried to contact parents and other family members to determine whether the mutation co-segregates with obesity. We could only include family members for the probands carrying D90N and F280L.

The mother of the proband with D90N was also obese (BMI = 43.5 kg/m²) and was found to carry the same mutation. No other family members were available, but familial anamnesis reports overweight in both the mother's and the father's family. The father, who was not willing to participate in the study, was also reported to be morbidly obese (BMI = 43.6 kg/m²).

When studying the family of the proband with F280L, we clearly demonstrated that this mutation co-segregates with obesity (fig. 1). We further found that the mutation was inher-

Fig. 2. Localization of wild-type and mutant MC4R.

Images were made with a Zeiss LSM 510 confocal microscope equipped with argon (excitation, 488 nm) and helium-neon (excitation, 543 and 633 nm) lasers. The first panel shows EGFP fluorescence (excitation 488 nm). The second panel visualizes fluorescence of the tetramethyl-rhodamine wheat-germ agglutinin conjugate (TMR-WGA) which binds to the plasma membrane (PM) (excitation 543 nm). A merged view is shown in the third panel.

A Empty pEGFP-N1 expression vector: EGFP is expressed ubiquitously throughout the cell, without co-localization with the PM **B** MC4R wild type: co-localization of EGFP and TMR-WGA fluorescence indicates the localization of the wild-type MC4R on the PM. **C** MC4R I186V: co-localization of green and red fluorescence indicates that this mutant receptor is present on the PM. **D** MC4R P260Q: no co-localization of EGFP and TMR-WGA is present on the merged view, indicating that this mutant MC4R is not expressed on the PM. **E** MC4R F280L: the mutation causes intracellular retention of the receptor since no co-localization of EGFP and TMR-WGA is seen.

ited from the mother. Furthermore, the maternal grandmother, uncle and great-uncle also harbored this mutation. All mutation carriers were severely obese. The lean younger sister of the proband was found to carry wild-type *MC4R*. The obese father (BMI = 31.9 kg/m²) was not included in the family analysis.

Functional characterization was performed for the two newly identified mutations (I186V and F280L) and for P260Q since the experiments performed here had not been previously described for this mutation. The other mutations were not included in the functional analysis since they had been previously characterized [44, 45, 47, 48].

Fig. 3. Intracellular signaling of wild-type and mutant MC4R. HEK293 cells were transfected with pcDNA3 with wild-type MC4R, MC4R with I186V or P260Q, in combination with a pCRE-Luc reporter and pRL-TK as an internal control. After stimulation with different concentrations of α -MSH, luciferase luminescence was measured. Relative ratios are shown (compared to wild type). Error bars represent standard deviation.

The 3 mutant constructs were created in a mammalian pEGFP-N1 expression vector to study cellular localization of these mutants. Images of transfected cells were made with a confocal microscope. Wild-type MC4R is located on the plasma membrane (fig. 2B). Confocal imaging of P260Q and F280L showed that both mutations are retained intracellularly (fig. 2D–E). For I186V, we demonstrate here that the mutation does not alter membrane expression of the protein. Co-localization of EGFP and tetramethyl-rhodamine wheat-germ agglutinin conjugate (TMR-WGA) is clearly present (fig. 2C).

For the I186V mutation, we also performed a luciferase assay to determine the ability of the mutant receptor to stimulate cAMP production in response to α -MSH. We also included the P260Q mutation in this assay to compare our results with those obtained by Stutzmann et al. [45] during the course of our study. For this experiment, both mutant constructs were created in a mammalian pcDNA3 expression vector. Measurement of luciferase as an indirect measure of intracellular cAMP showed complete loss of function for the I186V mutation (fig. 3). At maximal stimulation, this mutant receptor only showed 1.7% of activation compared to the wild-type receptor. P260Q caused a severe reduction in intracellular signaling capacity, as demonstrated by a maximal activation of 7.9% compared to wild type (fig. 3).

Discussion

MC4R deficiency is widely accepted as the most common cause of monogenic obesity. In children presenting with severe early-onset obesity, screening of *MC4R* is therefore a first step in determining a possible genetic cause of the disease. We screened the coding region of *MC4R* in a population of 112 severely overweight and obese children and adolescents and identified 6 mutation carriers, amounting to an

MC4R mutation frequency of 5.3%. This is in contrast to our previous study in which we did not identify any *MC4R* mutations in a cohort of 123 overweight and obese children and adolescents [39].

However, there are significant differences between the two study populations. In our first study, the study cohort had consisted almost entirely of adolescents (3 children vs. 120 adolescents) while we now studied a population of 71 children (age < 12 years) and 41 adolescents (age $\geq$ 12 years). Furthermore, the mean age of the adolescent sample was also higher in our initial study (mean age = 16.8 years) than in the current cohort (mean age = 14.1 years). The entire cohort now also includes much younger children than those from the previous report (age range = 0.4–19 years vs. 8–20 years). Therefore, we conclude that our population now consists of children with early-onset obesity while our previous report probably contained a large number of patients with pubertal onset of obesity. Furthermore, our first study only included children of Caucasian descent while the second cohort also contains individuals of other ancestries. Finally, the first group of children and adolescents was recruited through an adult outpatient obesity clinic while the second group has been recruited through specific pediatric obesity centers in Antwerp and Hasselt. These differences may explain why we now find *MC4R* mutations at a high frequency in our population while we did not detect any mutation previously. The current frequency is in line with other studies dealing with children with severe early-onset obesity [18, 19, 30].

In total, 11 sequence variations were found in our study. Four of these variations (V103I, T112M, I198I and I251L) are previously reported polymorphisms while another one (G324G) is unreported, but since it is a synonymous mutation which we found in a control subject, we are convinced that this polymorphism is not related to obesity.

On the other hand, several arguments favor the hypothesis that the remaining 6 variations are disease-causing. None of these is found in a control cohort, and they cause either an amino acid substitution or an early stop codon. Furthermore, 4 variations have been previously reported in obese patients, and the disease-causing aspect of some has been confirmed in functional studies.

The D90N mutation was first reported in a single proband by Biebermann and colleagues in 2003 [44]. This mutation was found in a patient with early-onset obesity, but no conclusions on segregation with obesity could be drawn since the mother did not carry the mutation and the father was not available for inclusion. Functional characterization demonstrated that this mutation did not alter cell surface expression or ligand binding. However, no intracellular cAMP accumulation was observed after stimulation with NDP- α -MSH. Co-transfection studies and a sandwich ELISA showed that the D90N mutation exerts its pathogenic effect through a dominant negative influence on the wild-type receptor. This was the first mutation in which a dominant negative effect could

be demonstrated [44]. In the current study, we also detected this mutation in a single proband with early-onset obesity. We were also able to include the obese mother in our analysis and demonstrated that she carried this mutation too. Based on the clinical data, co-segregation with obesity and the functional effects demonstrated by others, we conclude that the D90N mutation is the cause of the early-onset obesity seen in our patient.

Branson and colleagues [17] first described two patients with a M200V mutation in the fifth transmembrane (TM) domain of *MC4R*. These patients were obese and had characteristics of binge eating. Functional description of this mutation showed that no difference from wild type could be demonstrated for cell surface expression, ligand binding or intracellular signaling capacity [47]. We also identified an obese patient carrying this mutation. His father is of normal weight (BMI = 23.9 kg/m²), but his mother has a history of obesity. She has a BMI of 38.5 kg/m² after gastric banding. Since none of the parents were available for genetic screening, no conclusion on segregation with obesity can be drawn. Therefore, we cannot make any definite conclusions on the pathogenic character of this mutation at the moment. However, the fact that this mutation has not been identified in control subjects by us or others [17] leads us to speculate that it may have a negative effect on *MC4R* that is not identifiable through the functional assays currently performed. Further functional testing of this mutation is therefore necessary to further elucidate its effect on *MC4R* functioning.

The nonsense mutation at codon 307 (Q307X) was initially identified in an 8-year-old obese boy [46]. No functional characterization of this mutant was performed at that time. Here, we identified this nonsense mutation in a 14-year-old girl with severe obesity. No family data are available. We did not perform functional assays for this mutation since it has been previously described that truncation of the *MC4R* from codon 306 onwards results in a loss of cell surface expression of the truncated receptor [48]. Furthermore, the di-isoleucine sequence at codons 316/317 was shown to be crucial for cell surface expression of the receptor [49]. Based on these previous data, we conclude that the Q307X nonsense mutation found in our patient and the previously reported case [46] results in a truncated receptor which has lost the ability to be transported to the cell surface.

The P260Q mutation was identified in a 10-year-old patient with severe early-onset obesity. No family data are available for this proband. During the course of our functional experiments, Stutzmann et al. [45] also reported this mutation in two individuals. They reported that this mutation causes a reduced intracellular cAMP accumulation. This result was confirmed in the current study. Here, we also show that this mutation is not expressed on the cell surface by confocal imaging of EGFP-*MC4R* fusion protein. The mutation causes intracellular retention of the receptor, which is consistent with a reduced intracellular signaling capacity as reported by Stutz-

mann et al. [45]. Furthermore, a recent report describes a F261S mutation in a Chinese proband that causes intracellular retention of the mutant receptor [50]. These data also suggest that this region in the 6th TM domain of *MC4R* may be an important region for the cellular localization of the receptor.

We were able to identify 2 previously unreported mutations, F280L and I186V. We found the F280L mutation in a severely obese girl of 12 years. This mutation co-segregated in the family from the mother's side. The proband's lean younger sister was wild type for *MC4R*. The obese father was not willing to participate, but we showed that the disease-causing mutation is inherited from the mother. We developed an EGFP-*MC4R* fusion protein, which allowed us to conclude that the mutant receptor is localized inside the cell instead of on the plasma membrane. Therefore, we hypothesize that the intracellular retention of the mutant receptor causes a reduced inhibition of food intake in this family, ultimately leading to the observed obesity.

A 7-year-old obese girl was found to carry the I186V mutation in the 4th TM domain of *MC4R*. Her mother is of normal weight (BMI = 23.7 kg/m²) while her father is also obese (BMI = 31.8 kg/m²). Parents, however, were not available for genetic screening. Confocal imaging showed that this mutation is expressed on the cell surface similar to wild type. By indirect cAMP measurements, we were able to demonstrate that this mutation causes a complete loss of function of the receptor. However, we cannot conclude whether this is caused by a defect in ligand binding or intracellular signaling as we did not perform binding assays. A G181D mutation in TM4 has been previously reported and was also found to cause a complete loss of function as determined by measurement of intracellular second messenger production and ligand-binding assay [10, 51]. These data strongly suggest that the I186V mutation is pathogenic and is the cause of the obesity seen in the proband.

In conclusion, we identified 6 mutations in patients with early-onset obesity, indicating a mutation frequency of 5.3% in this population of Belgian obese children. Two (I186V and F280L) of the mutations were novel. Functional analysis on these and on a third mutation (P260Q) indicated that 2 of the 3 mutations cause intracellular retention of mutant *MC4R*. This has been shown to be the most common mechanism through which mutations impair *MC4R* signaling [29–31]. It has been shown that 81.3% of childhood obesity-associated mutations in *MC4R* cause intracellular retention of the receptor. This may correlate with an early onset of the obesity seen in the probands carrying these mutations [29]. Furthermore, Tao and Segaloff [52] proposed a classification scheme for *MC4R* mutations which identifies mutations that impair cell surface expression as an important class of pathogenic mutations (class II mutations). For the I186V mutation, a complete loss of intracellular signaling activity could be demonstrated here. This study again stresses the relevance of performing mutation analysis of *MC4R* in obese children.

Acknowledgements

The authors wish to thank Jan Van Daele for technical assistance with confocal imaging. We thank Dr. Jenneke van den Ende and Veerle De Craemer for taking blood samples.

S.B. is a post-doctoral researcher for the Fund for Scientific Research Flanders (FWO-Vlaanderen). D.Z. holds a pre-doctoral specialization scholarship from the Institute for the Promotion of Innovation through

Science and Technology in Flanders (IWT-Vlaanderen). This work was supported by a grant (G0028.05) from the Fund for Scientific Research Flanders (FWO-Vlaanderen) to LVG and WVH.

Disclosure

The authors declare no conflict of interest.

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