Uniparental disomy of chromosome 16 unmasks recessive mutations of *FA2H*/SPG35 in 4 families

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ABSTRACT

Objective: Identifying an intriguing mechanism for unmasking recessive hereditary spastic paraplegias.

Method: Herein, we describe 4 novel homozygous *FA2H* mutations in 4 nonconsanguineous families detected by whole-exome sequencing or a targeted gene panel analysis providing high coverage of all known hereditary spastic paraplegia genes.

Results: Segregation analysis revealed in all cases only one parent as a heterozygous mutation carrier whereas the other parent did not carry *FA2H* mutations. A macro deletion within *FA2H*, which could have caused a hemizygous genotype, was excluded by multiplex ligation-dependent probe amplification in all cases. Finally, a microsatellite array revealed uniparental disomy (UPD) in all 4 families leading to homozygous *FA2H* mutations. UPD was confirmed by microarray analyses and methylation profiling.

Conclusion: UPD has rarely been described as causative mechanism in neurodegenerative diseases. Of note, we identified this mode of inheritance in 4 families with the rare diagnosis of spastic paraplegia type 35 (SPG35). Since UPD seems to be a relevant factor in SPG35 and probably additional autosomal recessive diseases, we recommend segregation analysis especially in nonconsanguineous homozygous index cases to unravel UPD as mutational mechanism. This finding may bear major repercussion for genetic counseling, given the markedly reduced risk of recurrence for affected families. ***Neurology*® 2016;87:186–191**

GLOSSARY

DMR = differentially methylated region; ***FA2H*** = fatty acid 2-hydroxylase; **HSP** = hereditary spastic paraplegia; **MLPA** = multiplex ligation-dependent probe amplification; **SNP** = single nucleotide polymorphism; **SPG35** = spastic paraplegia type 35; **UPD** = uniparental disomy.

Hereditary spastic paraplegias (HSPs) are a clinically and genetically heterogeneous group of neurodegenerative disorders of the spinal cord affecting mainly the long axons of the corticospinal tract. Spastic paraplegia type 35 (SPG35) is caused by mutations in the fatty acid 2-hydroxylase (*FA2H*) gene located on chromosome 16q23 and is clinically characterized by early-onset spastic tetraparesis, mental retardation, cerebellar ataxia, and extrapyramidal involvement. *FA2H* was first described in 2008 as a rare leukodystrophy gene causing spasticity and dystonia.[1] It is an NADPH (nicotinamide adenine dinucleotide phosphate)-dependent monooxygenase essential for maintenance of the neuronal myelin sheath. SPG35 follows an autosomal recessive trait. To date, approximately 19 families totaling 51 patients have been reported in the literature.

UNIPARENTAL DISOMY Uniparental disomy (UPD) as a concept aroused in the 1980s when analyzing material from spontaneous abortions, establishing the concept of a random union of a disomic gamete with a gamete

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nullisomic for the homolog. This situation was named *uniparental* because both members of such a pair arise from only one parent.[2] UPD is the result of a defective chromosomal segregation in mitosis or meiosis.[3] The potential of UPD to unmask recessive alleles has been described for several diseases (see ref. 4 for review). Two cases have been reported in autosomal recessive spastic ataxia type Charlevoix-Saguenay.[5] In HSP, just one case with SPG18 was found in a whole-exome approach.[6] UPD is a "hot topic" in genetics because of outcome prediction problems in prenatal diagnosis[3] and for the counseling of families on recurrence risk.

METHODS

Patients. Families were seen in the outpatient clinic for HSP at the University Hospital Tübingen, Germany (F1, F2, F3), and at the Donostia University Hospital, Spain (F4).

Standard protocol approvals, registrations, and patient consents. Approval from local ethical committees on human experimentation was received, and written informed consent was obtained from all patients (or guardians of patients) participating in the study. Clinical and genetic data are summarized in the table.

Next-generation sequencing. Mutations in the *FA2H* gene were uncovered by whole-exome sequencing (F1, F4) or disease-specific gene panel diagnostics (F2, F3). HSP panel diagnostics screened >90 known spasticity genes following targeted enrichment by Agilent in-solution or HaloPlex technology. Genes included in the panel approach are provided on request. Whole-exome sequencing was performed using the SureSelect Human All Exon 50 Mb kit (Agilent, Santa Clara, CA) and HiSeq2000 instruments (Illumina, San Diego, CA; for technical background, compare ref. 7).

Multiplex ligation-dependent probe amplification assay. We designed a multiplex ligation-dependent probe amplification (MLPA) assay targeting all coding exons of the *FA2H* gene and the 5′ UTR (8 probes in total; available on request) to screen for *FA2H* deletions. These target probes were applied in combination with the SALSA MLPA P300 Human reference probemix (MRC Holland) according to the manufacturer's instructions.

Testing for UPD16. The following polymorphic microsatellite markers on chromosome 16 were analyzed by PCR for index patients and both parents: D16S3024, D16S3131, D16S511, and D16S413. PCR amplicons were evaluated by fragment analysis (Beckman Coulter CEQ8000).

Microarray analyses. High-resolution chromosome analysis (single nucleotide polymorphism [SNP] array analysis) was performed using the Human CytoScan 750K Array (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Genotypes and mendelian errors were detected using the Affymetrix Chromosome Analysis Software (version 3.0.0.42) and UPDtool.[8]

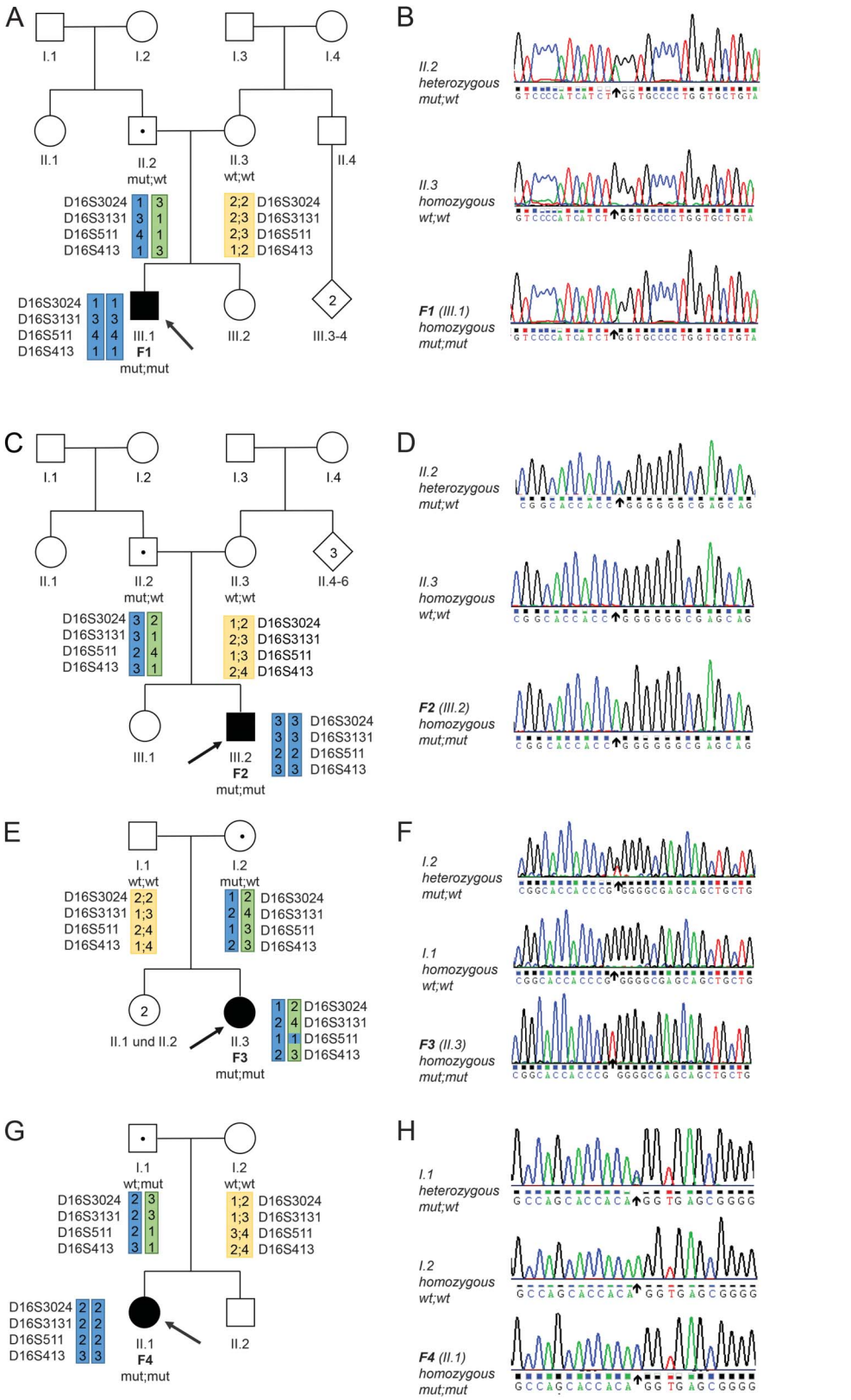
Methylation assay. Approximately 1 μg DNA was subjected to sodium bisulfite treatment using the EZ Methylation-Gold Kit (ZYMO, Orange, CA). Converted DNA was used as a template

Table Clinical details and variant description of SPG35 cases

	Family 1	Family 2	Family 3	Family 4
Ethnicity	Serbian	German	German	Spanish
Sex	M	M	F	F
Consanguinity	–	–	–	–
Age at examination, y	12	9	6	18
Age at onset, y	3	4	4.5	4
Age at wheelchair use, y	10	7	NA	11
Cognitive deficits	Moderate to severe	No deficits	Mild	Mild
Tetraspasticity	+	+	+	+
Limb ataxia	UL/LL	UL/LL NA (weakness)	None	UL/LL
Extrapyramidal involvement	Rigidity	Rigidity	–	Dystonia
Birth weight	Normal	Normal	Normal	Normal
Mutation	chr16:74760209, c.527G>A, p.Trp176* (hom), upd(16)pat (isodisomy)	chr16:74808523, c.131C>A, p.Pro44Gln (hom), upd(16)pat (isodisomy)	chr16:74808521, c.133G>T, p.Gly45Trp (hom), upd(16)mat (heterodisomy with segmental isodisomy)	chr16:74718989, c.785A>C, p.Lys262Thr (hom), upd(16)pat (isodisomy)
MAF EVS	–	–	–	–
MAF ExAC, %	–	–	–	0.00083 (all)
Conservation amino acid	NA	High	High	High
Physicochemical differences	NA	Moderate (Grantham distance: 76)	Large (Grantham distance: 184)	Moderate (Grantham distance: 78)
PolyPhen-2 (HumDiv)	NA	1.000	1.000	0.999

Abbreviations: EVS = Exome Variant Server; ExAC = Exome Aggregation Consortium; LL = lower limb; MAF = minor allele frequency; NA = not applicable; UL = upper limb.
Symbols: – = sign/symptom absent; + = sign/symptom present.
Age at onset = beginning of gait disturbance. Variant coordinates refer to GRCh37 and the reference sequence NM_024306.4, respectively. Variant classification criteria are derived from the ACMG (American College of Medical Genetics and Genomics) criteria.[9]

Figure 1 Uniparental disomy in families with spastic paraplegia type 35



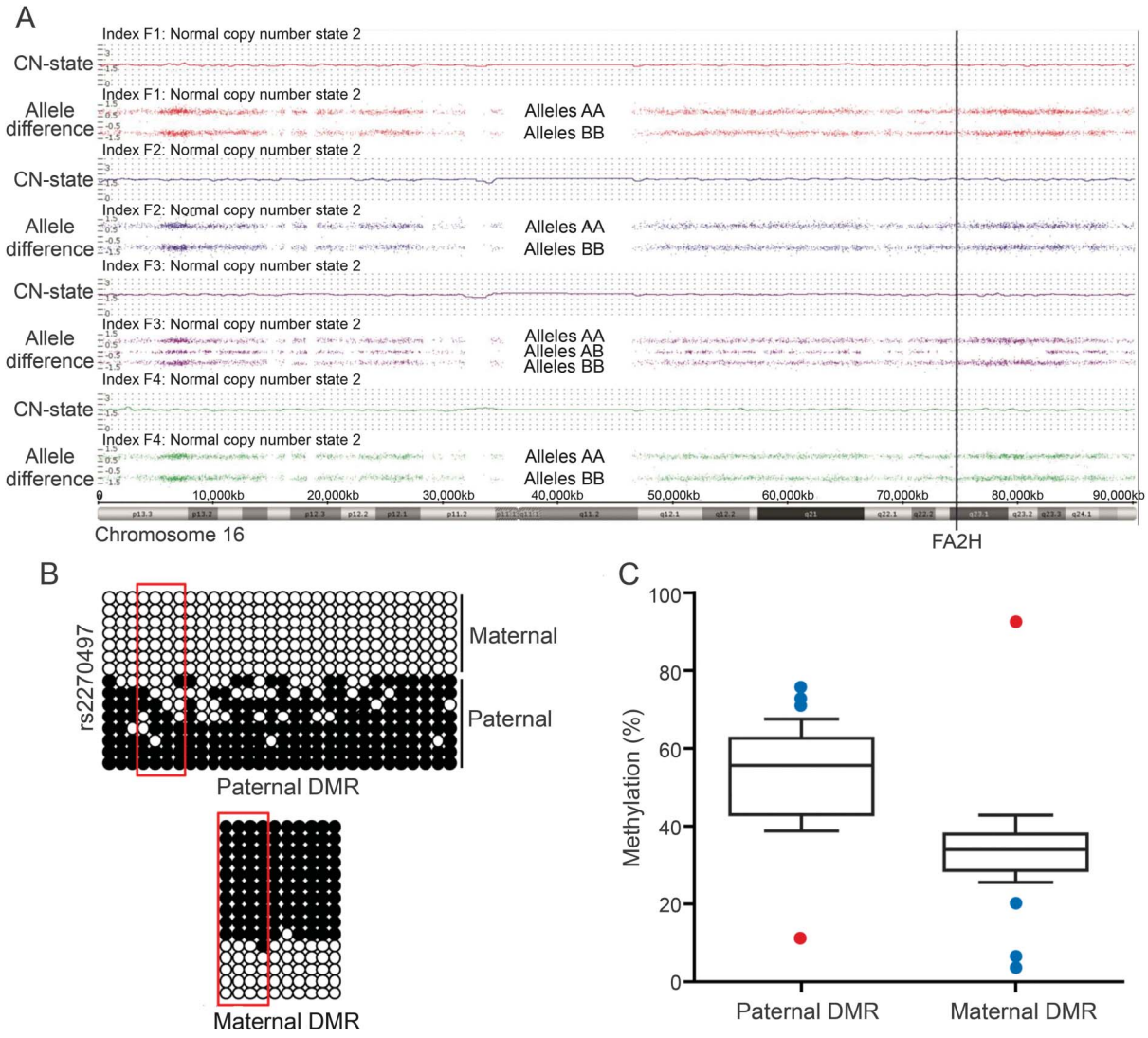
The blue/green boxes show the 2 different alleles of the repetitive DNA motif in the tested microsatellite (binned). The maternal alleles in F1 (A), F2 (C), and F4 (G) or the paternal alleles in F3 (E) cannot be assigned to distinct haplotypes (yellow boxes). Chromatograms of the index patients and their parents are shown for F1 (B), F2 (D), F3 (F), and F4 (H). The arrow indicates the position of the base substitution. mut = mutation; wt = wild-type.

for PCR, with the amplicons cloned into pGEM-T Easy Vector (Promega), transfected into JM109 and individual colonies sequencing using standard T7 sequence primer.

Pyrosequencing was performed to quantitate methylation of each amplicon. Standard primers were used with the exception that the reverse primer was biotin-labeled. PCR products were sequenced using a PyroMark Q96 MD machine.

RESULTS Mutation analysis. Three novel missense variants and one nonsense variant (compare the table) were identified, which were not listed with significant frequencies in public databases (EVS [Exome Variant Server], ExAC [Exome Aggregation Consortium], and dbSNP). The missense variants affect highly conserved amino acids and are predicted to be damaging, e.g., by PolyPhen-2. All variants appeared to be homozygous. Because of their rarity, the matching clinical phenotype, their homozygous appearance, conservation of affected amino acids, *in silico* predictions for missense variants, and predicted loss-of-function for the nonsense mutation, these variants were classified as likely pathogenic (class 4, derived from ACMG [American College of Medical Genetics and Genomics] criteria[9]).

Figure 2 **In-depth characterization of UPD16 in families with spastic paraplegia type 35**



(A) UPD confirmed by microarray analysis. The CN state indicates the normal DNA CN for all index patients. A lack of heterozygosity at the *FA2H* locus is shown in the allele difference plots. Heterozygous alleles in index F3 refer to the heterodisomy, homozygous alleles to the segmental isodisomy. (B) Confirmation of UPD16 by *ZNF597*-imprinted DMR methylation pyrosequencing. Strand-specific methylation profiles for the *ZNF597* DMRs as determined by cloning and direct sequencing. Each circle represents a single CpG dinucleotide on a DNA strand (results for multiple DNA strands are depicted as rows), filled circles indicate a methylated cytosine, and open circles an unmethylated cytosine. Note that the sample used for the PCR of the paternally methylated region was heterozygous for a polymorphism (rs2270497) that allowed for allelic origin of methylation to be ascertained. The boxes indicate the CpG sites quantified by pyrosequencing. (C) The average methylation as determined by pyrosequencing in control samples for both DMRs is consistent with one methylated and unmethylated allele. pUPD16 cases (blue data points) and mUPD16 (red data points) showing reciprocal methylation profiles consistent with parental origin. The control box plots represent the interquartile range for control samples and whiskers span from 5th to 95th percentile. CN = copy number; DMR = differentially methylated region; UPD = uniparental disomy.

Segregation testing and identification of UPD in 4 families. Mutations identified through next-generation sequencing were confirmed by Sanger sequencing. In all families, neither consanguinity nor a founder effect were supported by family history. When sequencing the parents, surprisingly, the respective mutation was found either only in the fathers while the mothers were wild-type (F1, F2, and F4) or the mother was identified as a heterozygous carrier with the father being wild-type (F3) (figure 1). MLPA excluded genomic deletions in all 4 index patients. To trace segregation of the maternal and paternal alleles, we then genotyped 4 polymorphic microsatellite markers spanning regions of 198–258 base pairs on chromosome 16 surrounding the *FA2H* gene. In F1, 2 identical alleles of the *FA2H* gene, both carrying the W176* mutation, were inherited from the father and none of the maternal *FA2H* alleles was passed on to the index case. Likewise, in F2 and F4, 2 identical *FA2H* alleles were paternally inherited, causing homozygosity for the mutations Pro44Gln and Lys262Thr, respectively, in the affected offspring. In F3, the affected child presented with a maternal heterodisomy and additionally a segmental maternal isodisomy (marker D16S511, approximately 6 Mb downstream of the *FA2H* gene). These findings confirm a paternal isodisomic UPD of chromosome 16 in F1, F2, and F4 as well as a maternal heterodisomic UPD with a segmental isodisomic UPD16 in F3 (see figure 1).

In-depth analysis of UPD16 in SPG35 cases. For a more accurate evaluation of the UPDs, we performed SNP array analyses of all patients and their parents. All patients showed homozygous genotypes at the *FA2H* gene locus. In F1, F2, and F4, homozygosity was spread over the entire chromosome 16. The index patient of F3 showed a homozygous stretch of approximately 10 Mb (72,301,683–82,494,755). An example of the genotyping data is shown in figure 2. The mendelian error check from the Affymetrix Chromosome Analysis Software identified elevated maternal error rates on chromosome 16, indicating a paternal UPD16 in families F1, F2, and F4. The index patient of F3 showed an increased paternal error rate, indicating a maternal UPD. The UPDtool[8] confirmed paternal isodisomy of chromosome 16 for F1, F2, and F4 and maternal partial heterodisomy and isodisomy of chromosome 16 for F3.

To confirm the UPDs, we performed DNA methylation profiling of the 2 reciprocally imprinted differentially methylated regions (DMRs) associated with the *ZNF597* gene at 16q13.3. Bisulfite PCR products incorporated 31 CpG dinucleotides in the amplicon targeting the paternally methylated DMR and 10 CpGs in the maternally methylated DMR (figure 2B). Subsequent pyrosequencing analysis was limited to 4 CpG dinucleotides with the arithmetic mean used as a representative measure of methylation for each index case, parents, and 14 controls. All probands were clearly distinguishable from controls with the pUPD16 cases being relatively hypermethylated at the paternally methylated DMR and unmethylated at the maternal DMR with the opposite profile observed for proband F3 with mUPD16 (figure 2C).

Clinical findings. All 4 children presented with a complicated form of HSP with tetraspasticity and additional symptoms including limb ataxia (3/4), mild cognitive deficits (3/4), and extrapyramidal involvement (3/4). See the table for clinical details.

DISCUSSION

UPD as cause for autosomal recessive diseases and especially HSP appears to be more common than previously thought. When looking from a genome-wide perspective,[10] an estimated rate of segmental UPD is thought to be at 0.578% based on a UPD screening, or 0.026% based on autosomal chromosome pairs investigated in the general population. These data imply the possibility of hidden segmental UPD in the general population.[10] Here, we present 4 cases of SPG35 caused by UPD16.

The high number of UPD16 cases currently identified as cause of SPG35 is surprising. However, trisomy 16 is the most common trisomy in human pregnancies.[3] According to a recent review,[3] supernumerary chromosome 16 is always of maternal origin; therefore, a maternal UPD16 is frequently observed as a result of trisomic rescue. However, paternal UPD16 has also been described. In the absence of a recessive mutation, the outcome was reported to be normal.[11]

The clinical picture of all index patients is in accordance with the phenotypic spectrum previously described for patients with compound heterozygous or homozygous *FA2H* mutation. SPG35 usually begins in early childhood after a normal early development. The initial symptom in the vast majority of SPG35 cases is spastic gait followed by multisystemic affection in accordance with the patients presented here. There is ongoing debate about whether UPD16 is associated with consistent imprinting phenotypes (mUPD16: intrauterine growth restriction with anal atresia[12]; pUPD16: alveolar capillary dysplasia with misalignment of pulmonary veins[13]). Since our index cases do not represent such features, it is unlikely that UPD16 directly contributes to these presentations.

UPD seems to be a rather frequent genetic mechanism for homozygous mutations in *FA2H* in nonconsanguineous families. DNA samples of the parents should be collected more often to perform segregation analysis because the detection of UPD is of major relevance for the risk of recurrence and genetic counseling.

AUTHOR CONTRIBUTIONS

A.S.S. and T.W.R.: drafting and editing of the manuscript, conceptualization of this study, analysis and interpretation of data, acquisition of data. S.B.-W. and K.S.: analysis and interpretation of data, contribution to the manuscript. D.M. and A.P.: acquisition, analysis and interpretation of data, contributing to and revising the manuscript. M.D.-N., K.H., A.S., and M.R.: acquisition, analysis and interpretation of data. S.Z. and O.R.: analysis and interpretation of data, obtaining funding. R.S., P.B., and L.S.: revising the manuscript, conceptualization of this study, study supervision, analysis and interpretation of data, obtaining funding.

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DISCLOSURE

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