

# Mutations in DNMT1 cause autosomal dominant cerebellar ataxia, deafness and narcolepsy

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**Autosomal dominant cerebellar ataxia, deafness and narcolepsy (ADCA-DN) is characterized by late onset (30–40 years old) cerebellar ataxia, sensory neuronal deafness, narcolepsy–cataplexy and dementia. We performed exome sequencing in five individuals from three ADCA-DN kindreds and identified *DNMT1* as the only gene with mutations found in all five affected individuals. Sanger sequencing confirmed the *de novo* mutation p.Ala570Val in one family, and showed co-segregation of p.Val606Phe and p.Ala570Val, with the ADCA-DN phenotype, in two other kindreds. An additional ADCA-DN kindred with a p.GLY605Ala mutation was subsequently identified. Narcolepsy and deafness were the first symptoms to appear in all pedigrees, followed by ataxia. *DNMT1* is a widely expressed DNA methyltransferase maintaining methylation patterns in development, and mediating transcriptional repression by direct binding to *HDAC2*. It is also highly expressed in immune cells and required for the differentiation of CD4+ into T regulatory cells. Mutations in exon 20 of this gene were recently reported to cause hereditary sensory neuropathy with dementia and hearing loss (HSAN1). Our mutations are all located in exon 21 and in very close spatial proximity, suggesting distinct phenotypes depending on mutation location within this gene.**

## INTRODUCTION

Autosomal dominant cerebellar ataxia, deafness and narcolepsy (ADCA-DN, MIM 604121) is a polymorphic disorder first described in 1995 in a Swedish pedigree in which five affected individuals were identified and studied (1). The disease is characterized by late onset (age 30–40 years) narcolepsy–cataplexy, sensory neuronal deafness, cerebellar ataxia, dementia and, more variably, psychosis, optic atrophy and other symptoms. Studies of cerebrospinal fluid (CSF) hypocretin-1 have shown low or undetectable levels,

suggesting loss of hypocretin-producing cells as the cause of the narcolepsy in these subjects (2).

We identified three additional kindreds: (i) a large multigenerational autosomal dominant pedigree from the USA, with 13 affected individuals, including 6 living; (ii) a sporadic occurrence of the disease in a 50-year-old Italian patient with unaffected elderly parents, suggesting the activity of a *de novo* mutation; (iii) a multiplex Italian pedigree with 4 known affected, and a similar disease course. The sporadic Italian case was notable, as narcolepsy–cataplexy was the first symptom (age 42), followed by hearing loss, memory

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**Table 1.** Symptoms in the four probands with approximate age of onset when documented

| Phenotypes                   | Italian proband 1 | US proband      | Italian proband 2 | Swedish proband |
|------------------------------|-------------------|-----------------|-------------------|-----------------|
| Mutation                     | Ala570Val         | Ala570Val       | Gly605Ala         | Val606Phe       |
| Gender                       | M                 | M               | M                 | M               |
| Age                          | 57                | 58              | 47                | 56 (deceased)   |
| Excessive daytime sleepiness | 42 <sup>a</sup>   | 35 <sup>a</sup> | 43 <sup>a</sup>   | 18 <sup>a</sup> |
| Cataplexy onset              | 42 <sup>a</sup>   | 44 <sup>a</sup> | 43 <sup>a</sup>   | 32              |
| Sleep paralysis              | No                | No              | No                | Yes             |
| Hypnagogic hallucination     | No                | No              | Yes               | Yes             |
| REM sleep behavior disorder  | Yes               | Yes             | Yes               | Yes             |
| MSLT (SL, SOREMP)            | 5, 5 (age 47)     | 2, 4 (age 45)   | 10, 4 (age 47)    | 11, 3 (age 29)  |
| Cerebellar ataxia            | 46                | 48              | 47                | 37              |
| Hearing deficit              | 43 <sup>a</sup>   | 48              | 43 <sup>a</sup>   | 32              |
| Memory loss and depression   | 43                | 52              | No                | Yes             |
| Limb lymphedema              | 45                | No              | 47                | No              |
| Sensory neuropathy           | 47                | 55              | 47                | 49              |
| Optic atrophy                | 55                | 57              | 47                | 38              |
| Type II diabetes             | No                | 38              | No                | 47              |
| Dementia                     | No                | 54              | No                | 52              |
| Psychosis                    | 55                | 52              | No                | 47              |
| Hypocretin-1 (pg/ml)         | 123               | Not available   | 93                | 62              |
| HLA-DQB1*06:02               | Negative          | Negative        | Negative          | Negative        |

Yes, present, with age of onset when available. No, absent or not reported at current age or death.

<sup>a</sup>First symptoms leading to consultation.

problems and depression (age 43), lower limb lymphedema (age 45), cerebellar ataxia (age 46), peripheral sensory neuropathy (age 47) and optic atrophy (age 55). The CSF hypocretin-1 level was low (Table 1), and CSF tau protein was high (614 pg/ml, normal values  $141 \pm 127$  pg/ml), whereas 14-3-3 levels were normal. A multiple sleep latency test (MSLT) was also positive. As in some members of the original Swedish pedigree (1), HLA-DQB1\*06:02 was negative in each of the two new Italian pedigrees, and in all but one affected of the US kindred. In all these cases, narcolepsy–cataplexy was an early symptom, although there is variability in onset age and occurrence of symptoms, as reported for the Swedish family (1) (see Table 1 for representative data on the probands, including CSF hypocretin-1 levels when available).

To identify the cause of ADCA-DN, we performed exome sequencing in five affected members of three pedigrees. Following identification of *DNMT1* as causing the disorder, we also sequenced the *DNMT1* gene in all available samples from all four kindreds.

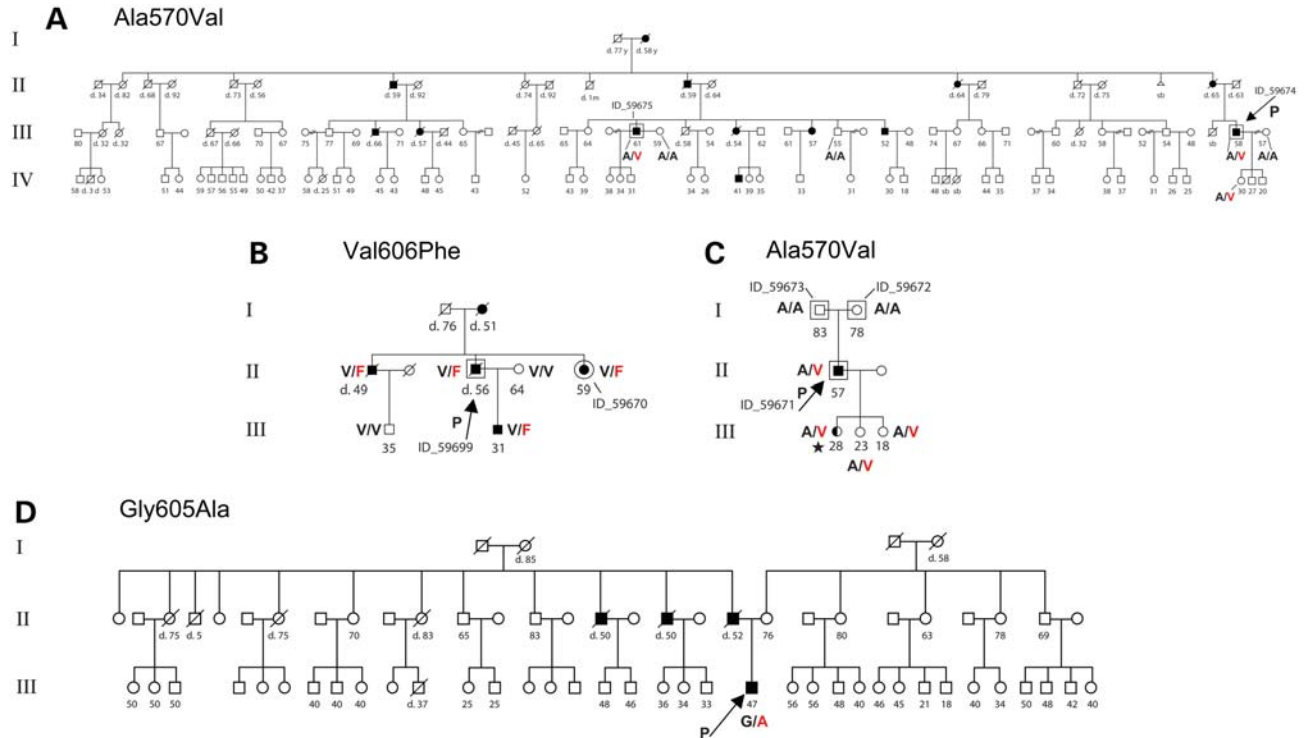
## RESULTS

Exome capture and sequencing were conducted in the sporadic Italian case plus his unaffected parents, and in two affected individuals for each of the two multiplex pedigrees from Sweden and the USA (Fig. 1). For each sample, 0.33 lanes of a flowcell were sequenced on a HiSeq 2000 System, generating, on average, 8.77 Gb of sequence per individual. 96.5% of the reads could be mapped to the reference genome (hg19) with, on average, 80.2% mapping on the exome target. The average read depth was 108 with between 88 and 91.5% of the target regions covered at least 20 times. Single-nucleotide missense, nonsense, splice-site, stoploss and frameshift variants and small insertions and deletions (indels) were identified by an

analysis pipeline using Burrows-Wheeler alignment (BWA) (v 0.5.9) for read alignments, and with SAMtools (v 0.1.7) for variant calling (see Supplementary Material, Table S1). We filtered these variants to exclude those present in dbSNP-132 with an average heterozygosity greater than 0.02, in 507 control exomes from patients with other unrelated diseases, and in the 1000 Genomes data (May 2011 release).

Using this strategy, we found that the two affected siblings of the Swedish family shared 77 private variants, whereas the two affected cousins of the US pedigrees shared 30. In the Italian proband, we identified two missense variants which were determined to have occurred *de novo*. Only a single gene, *DNMT1* (MIM 126375) was mutated in all five affected individuals. This gene also fulfilled the criterion that the mutation in the Italian case occurred *de novo*. Sanger sequencing confirmed the *de novo* mutation p.Ala570Val (RefSeq NM\_001130823.1: c.1709G>A) in the Italian case, and demonstrated co-segregation of the disease with the corresponding mutations, p.Val606Phe (NM\_001130823.1: c.1816C>A) in four of four available affected individuals from the Swedish pedigree, and p.Ala570Val (NM\_001130823.1: c.1709G>A) in the two available case samples from the US pedigree.

Upon completion of exome sequencing, an additional ADCA-DN family with four known affected members was identified (Fig. 1). Sanger sequencing of exon 21 of *DNMT1* in the proband identified a third mutation, p.Gly605Ala (RefSeq NM\_001130823.1: c.1814C>G) in the same area of *DNMT1*. Interestingly, all three mutations are located in very close spatial proximity within the *DNMT1* protein (Fig. 2). Functional consequences of these protein variants (all changing an amino acid conserved across all species examined) were evaluated with PolyPhen2 and SIFT, and variants were predicted to be damaging. These results establish *DNMT1* as the causal gene for ADCA-DN.



**Figure 1.** *DNMT1* mutations in four kindreds with autosomal dominant ataxia, deafness and narcolepsy (ADCA-DN). (A) US pedigree. (B) Swedish pedigree previously reported by Melberg *et al.* (1). (C) Italian kindred with *de novo* mutation. (D) Second Italian pedigree. The amino acid changes are noted for each subject for whom we tested for the mutation in the genomic DNA sequence, either whole-exome sequencing (subjects in boxes, for details on exome sequencing results see Supplementary Material, Table S1) or Sanger sequencing. Age of each subject at the time of study, or age at death, is indicated next to each symbol. Black symbol indicates affected status, white indicates unaffected at the time of study. The asterisk denotes a subject with narcolepsy only, as documented by sleepiness and a positive MSLT with three sleep onset REM periods. For details on phenotypes and typical ages of onset for various symptoms, please refer to Table 1.

## DISCUSSION

*DNMT1* is a widely expressed DNA methyltransferase maintaining methylation patterns in development, and mediating transcriptional repression by direct binding to *HDAC2* (MIM 605164) (3). Preferential substrates are hemimethylated CpG islands suggesting enzyme activity is critical in the repression of transcriptional activity in various postmitotic cells.

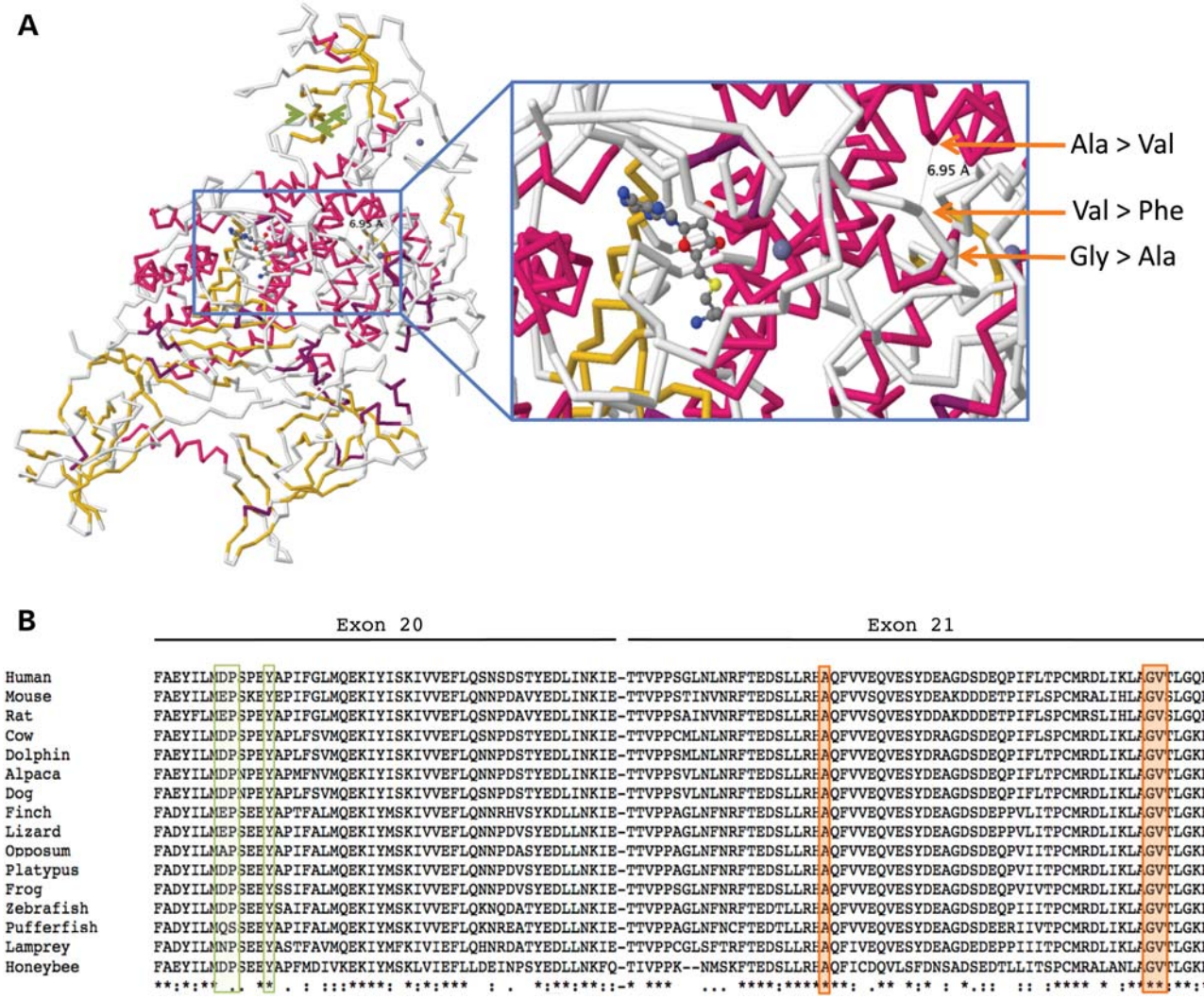
Our three mutations are localized in exon 21 of the protein and substitute amino acids that are highly conserved across all vertebrates (Fig. 2). Based on recent crystallographic studies, as for the previously published mutations reported in hereditary sensory and autonomic neuropathy type I HSN1 (4), our mutations are located in the replication foci targeting sequence motif (RFTS), within the N-terminal region of the protein. It is notable that our mutations are flanked by p.Asp564, an amino acid critical to the interaction of the RTFS domain with the catalytic domain, and by a series of three phenylalanines (Phe644, Phe647, Phe648 in the RTFS-CXXC linker) indispensable to the narrowing of the entrance of the DNA-binding pocket and to anchoring the RTFS domain to the DNA-binding pocket (5). Remarkably, crystallographic structure indicates that the wild-type p.Gly605, p.Val606 and p.Ala570 are sterically facing each other in a hinge covering the DNA-binding groove of the protein (Fig. 2). It is thus likely that our mutations affect DNA-binding recognition or the interaction with other proteins in

the *DNMT1*–*HDAC2* complex, creating insufficient CpG methylation and gene silencing in some cases.

Further confirmation of the pathogenicity of the mutation also came from a recent publication reporting that other *DNMT1* mutations (p.Asp490Glu, p.Pro491Tyr, p.Tyr495Cys) all located in exon 20 in a different area of *DNMT1* (Fig. 2) cause hereditary sensory loss with dementia and hearing loss (*HSAN1*, MIM 614116) (4). In *HSAN1*, sensory neuropathy and deafness are the first symptoms to appear (6,7), followed by dementia with cerebellar ataxia noted in only some of the cases (4). Narcolepsy has not been reported in *HSAN1*, although it could have been overlooked as a more minor symptom. In ADCA-DN cases, although hereditary sensory loss is a clinical finding, it is a minor feature occurring later in the course of the disease, first characterized by narcolepsy, then cataplexy and deafness, followed shortly by ataxia (Table 1).

How could distinct mutations within *DNMT1* create diverse late onset phenotypes with preferential targeting of selected population neurons such as hypocretin or Purkinje cells? One possibility may be that gene silencing through CpG methylation by *DNMT1* counterbalances overexpression of some genes, resulting in the accumulation of unwanted cellular aggregates that occur with aging in some neurons of the central and peripheral nervous system. This would explain increased tau protein levels in the CSF and dementia in these patients. Regarding ataxia, previous work has shown





**Figure 2.** (A) Protein structure of mouse *DNMT1* (PDB accession number 3AV5, image generated with Jmol\_S) and locations of newly identified amino acid substitutions (orange arrows) in the RFTS domain. Also shown are positions of amino acid changes reported by Klein *et al.* (4), in the TS domain of the protein (light green arrows). The substrate shown is *S*-adenosyl-L-homocystein. (B) Overview of the degree of conservation of the region containing the three novel mutations (orange boxes) and the two mutations previously reported by Klein *et al.* (4) (light green boxes).

that loss of *DNMT1* activity increases CAG repeat extension in *SCA1* (MIM 164400) during meiosis (8). Our pedigrees were tested for typical *SCA1*, 2, 3,...6 (MIM 146600, 183090, 109150, and 183086) repeats (data not shown) and found to have normal repeat size. Repeat extension may still occur at the somatic level during cerebellar development, or repression of *SCA* genes by methylation near repeats may promote ataxin aggregation.

In isolated cases, narcolepsy/hypocretin deficiency is a sporadic disease tightly associated with HLA-DQB1\*06:02 (~99%) (9). A secondary association with T cell receptor alpha (*TCRA*, MIM 186880) polymorphisms has also been found and independently replicated (10), suggesting an autoimmune mediation of hypocretin cell loss. Recent studies also indicate a role for winter upper airway infections, or H1N1 vaccinations as key environmental triggers (11). In ADCA-DN, one possibility may be that hypocretin cells are unusually susceptible to neurodegeneration induced by the

loss of *DNMT1* and resulting DNA hypomethylation. In this case, the mechanism would not be autoimmune. Another explanation could involve a role for *DNMT1* and DNA methylation in the regulation of various immune cell populations in the context of an autoimmune reaction in ADCA-DN families, although in this case it could occur in the absence of DQB1\*06:02 unlike in sporadic cases. Individuals with *HSAN1* are known to be more susceptible to infections, although this was not the case in our families. Further, *DNMT1* is highly expressed in immune cells, and hypomethylation has been shown to be involved in other autoimmune diseases such as lupus. Finally, *DNMT1* is required for the differentiation of CD4+ into T regulatory cells (through disinhibition of Foxp3 expression) upon TCR stimulation (12). An absence of CD4+ regulatory T cells with specificity toward hypocretin neurons could result in autoimmunity.

Finally, it is also notable that a recent genome-wide association study identified narcolepsy-associated polymorphisms in

the *P2RY11* (MIM 602697) locus in sporadic cases, a gene located in close proximity to (20–100 kb) *DNMT1* (13). In this study, the most highly associated SNP, rs2305795, mapped within the *P2RY11* locus and was associated with decreased expression of *P2RY11* in white blood cells. This previously reported association may be the result of undetected linkage disequilibrium with polymorphisms located within the *DNMT1* locus. Additional fine mapping and sequencing of the *P2RY11-DNMT1* region will be needed to explore this possibility. Alternatively, *DNMT1* regulatory elements may also lie within the *P2RY11* locus, as a region of synteny encompassing *PPAN* (MIM 607793), *P2RY11*, *EIF3S4* (MIM 603913), and *DNMT1* is conserved in vertebrates, suggesting evolutionary pressures (based on the Ancora HCNE browser for the study of highly conserved noncoding elements, <http://ancora.genereg.net/>) (14). The most significantly associated SNP was associated not only with decreased *P2RY11* expression, but also with decreased *DNMT1* expression in white blood cells. Expression of *DNMT1* correlated with that of *P2RY11*, and the genotype effect on *DNMT1* expression was mostly driven by the higher expression of *DNMT1* in CD4+ T cells (Supplementary Material, Fig. S1). Decreased *DNMT1* activity secondary to the *P2RY11* genetic association may thus contribute to increased vulnerability of hypocretin cells even in sporadic, HLA-DQB1\*06:02-associated cases.

## MATERIALS AND METHODS

### Subjects

All subjects gave written informed consent and were investigated by an expert neurologist between 1994 and 2011. Phenotypic details for the Swedish family have been described previously (1,2). For additional details on the phenotype of all probands, see Table 1. Note that all narcolepsy cases had cataplexy and multiple sleep onset REM sleep periods (SOREMP) on the MSLT, a hallmark of narcolepsy. Low CSF hypocretin-1 was documented in three of the four pedigrees.

### Exome sequencing

Genomic DNA was extracted from peripheral blood, following standard protocols. Exome capture was performed by in-solution enrichment of exonic sequences with the SureSelect Human All Exon 50 Mb Kit (Agilent Technologies). Samples were indexed for multiplex-sequencing, using the Multiplexing Sample Preparation Oligonucleotide Kit (Illumina). For each sample, 0.33 lanes of a flowcell were sequenced on a HiSeq 2000 system (Illumina) using 100 bp paired-end reads. Image analysis and base calling were performed with the standard Illumina software with default parameters. We performed read alignment with BWA (v 0.5.9) (15) by using the default parameters with the human genome assembly hg19 (GRCh37) as reference. Single-nucleotide variants and small insertions and deletions (indels) were detected with SAMtools (v 0.1.7) (16). Variant annotation was performed with custom Perl scripts, integrating data from dbSNP (v132) and the UCSC Genome Browser Known Genes track. We filtered variants to exclude those present in

dbSNP-132 with an average heterozygosity >0.02, in 507 control exomes from patients with other unrelated diseases and in the 1000 Genomes data (May 2011 release).

### Sanger sequencing of *DNMT1* variants

Sanger sequencing was performed using BigDye terminator chemistry 3.1 (ABI) on the ABI 3730XL sequencer and sequences were analyzed by using the Sequencher software v4.9. PCR primers were those described in Klein *et al.* (4). Sanger sequencing was performed in all available family members, including those with exome sequence available (Fig. 1). Sequencing of exons 20 and 21 was also performed in 90 non-ADCA-DN samples from various ethnic groups, including Americans, Scandinavians and Italians (mostly sporadic narcolepsy patients), without discovering new polymorphisms.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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*Conflict of Interest statement.* All authors had no conflict of interest.

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