Case Report

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Characterization of a rare variant (c.2635-2A>G) of the *MSH2* gene in a family with Lynch syndrome

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Filomena Cariola[1], Vittoria Disciglio[1], Anna M. Valentini[2], Claudio Lotesoriere[3], Candida Fasano[1], Giovanna Forte[1], Luciana Russo[1], Antonio Di Carlo[1], Floranna Guglielmi[1], Andrea Manghisi[1], Ivan Lolli[3], Maria L. Caruso[2] and Cristiano Simone[1]

Abstract

Introduction: Lynch syndrome is caused by germline mutations in one of the mismatch repair genes (*MLH1, MSH2, MSH6*, and *PMS2)* or in the *EPCAM* gene. Lynch syndrome is defined on the basis of clinical, pathological, and genetic findings. Accordingly, the identification of predisposing genes allows for accurate risk assessment and tailored screening protocols.

Case Description: Here, we report a family case with three family members manifesting the Lynch syndrome phenotype, all of which harbor the rare variant c.2635-2A>G affecting the splice site consensus sequence of intron 15 of the *MSH2* gene. This mutation was previously described only in one family with Lynch syndrome, in which mismatch repair protein expression in tumor tissues was not assessed. In this study, we report for the first time the molecular characterization of the *MSH2* c.2635-2A>G variant through in silico prediction analysis, microsatellite instability, and mismatch repair protein expression experiments on tumor tissues of Lynch syndrome patients. The potential effect of the splice site variant was revealed by three splicing prediction bioinformatics tools, which suggested the generation of a new cryptic splicing site. The potential pathogenic role of this variant was also revealed by the presence of microsatellite instability and the absence of MSH2/MSH6 heterodimer protein expression in the tumor cells of cancer tissues of the affected family members.

Conclusions: We provide compelling evidence in favor of the pathogenic role of the *MSH2* variant c.2635-2A>G, which could induce an alteration of the canonical splice site and consequently an aberrant form of the protein product (MSH2).

Keywords
Lynch syndrome, MSH2, splicing variant, familial cancer

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Introduction

Lynch syndrome (LS; previously referred as hereditary non-polyposis colorectal cancer, HNPCC) is the most common hereditary cause of colorectal cancer (CRC), accounting for 2% to 4% of all CRC syndromes.[1] LS is associated with an increased lifetime risk of developing tumors in the colon (10%–82%), endometrium (15%–60%), ovary (1%–20%), and stomach (6%–13%). Increased frequency of other malignancies, including carcinoma of the small intestine, pancreas and biliary tract,

[1]Medical Genetics, National Institute of Gastroenterology, IRCCS "S. De Bellis," Castellana Grotte, Bari, Italy
[2]Department of Pathology, National Institute of Gastroenterology, IRCCS "S. De Bellis," Castellana Grotte, Bari, Italy
[3]Department of Oncology, National Institute of Gastroenterology, IRCCS "S. De Bellis," Research Hospital, Castellana Grotte, Bari, Italy

Corresponding author:
Cristiano Simone, Medical Genetics, National Institute of Gastroenterology, IRCCS "S. de Bellis," Via Turi 27, Castellana Grotte, Bari 70013, Italy.
Email: cristiano.simone@irccsdebellis.it; cristianosimone73@gmail.com

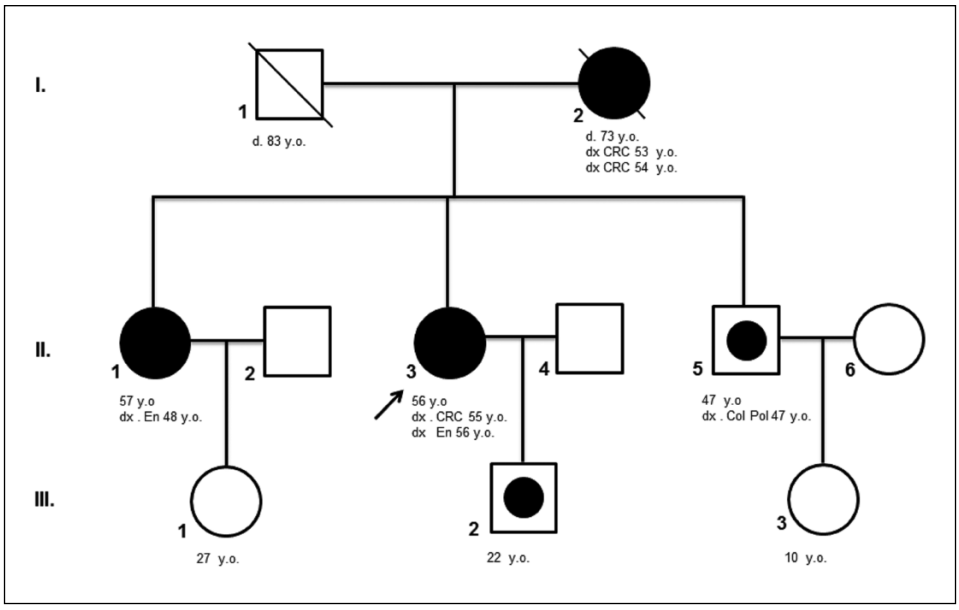

Figure 1. Pedigree of the family. The squares indicate males; the circles represent females. The slashed symbols indicate deceased individuals; unfilled symbols indicate unaffected individuals. The black arrow indicates the index case. The black dotted circles indicate individuals carrying the *MSH2* gene mutation. y.o. indicates the age at presentation and d. indicates deceased individuals. The numbers next to the cancer diagnoses (dx) denote age at onset of colorectal cancer (CRC), colon polyposis (Col. Pol.), and endometrial cancer (En).

has also been documented. Furthermore, LS may occasionally be associated with non-cancerous growths (polyps) in the colon. Individuals with LS tend to develop cancer at an early age, often <45 years.[1,2]

LS is inherited in an autosomal dominant pattern and is caused by heterozygous germline mutations in one of the major DNA mismatch repair (MMR) genes: *MSH2, MLH1, MSH6*, and *PMS2*. A deletion involving the *EPCAM* gene occurs in a proportion of patients without an MMR gene mutation, causing a heritable form of epigenetic silencing of the *MSH2* gene. Rarely, germline deletions encompassing both the *EPCAM* and the *MSH2* genes are reported in LS patients.[3] Carriers with mutations in these genes have a higher lifetime cancer risk due to a lower ability to repair DNA damage in microsatellite regions during cell division, showing tumor-specific variations in length or size of these loci (microsatellite instability, MSI), a very frequent LS molecular hallmark.[4] LS diagnosis is based on clinical, pathological and genetic findings, and the identification of causative genetic alterations is important for accurate risk assessment and personalized screening protocols.[3] In the era of precision medicine, advanced technologies, such as next generation sequencing, have led to the identification of numerous genetic variants. Accurately interpreting variants is challenging, and deciphering the underlying clinical significance is important to ascertain their role in disease risk and clinical phenotype. In addition, the characterization of rare variants in representative families might provide further insight into genotype–phenotype association.

Here, we report a family case with three members affected by LS-related cancers in which an *MSH2* gene mutation (c.2635-2A>G) involving the intron 15 splice acceptor site was identified. This variant has been previously reported only in one family case with LS.[5] With the aim of establishing the molecular consequences of the identified variant we (a) investigated the global population allele frequency to exclude its polymorphic nature; (b) performed an *in silico* analysis to predict its effect on RNA splicing; and (c) assessed MSI and MMR protein expression in patients' tumor tissues to evaluate a putative loss of function of the MSH2 protein.

Case description

In the present study, we report a family with a vertical transmission of cancer through two generations, whose members showed clinical features of LS according to Amsterdam II criteria and revised Bethesda guidelines (Figure 1). The index case—the first person in the family in which the genetic alteration predisposing to LS was detected—is a 56-year-old female (Figure 1 (II.3)). The first colonoscopy and computed tomography (CT) examination performed at the age of 55 years revealed a tumor mass of about 4 cm protruding into the lumen of the ascending colon. The patient underwent surgical treatment with right colectomy and complete resection of neoplastic lesions. Histologic examination of the surgically resected tumor showed a moderate differentiated adenocarcinoma (G2T3N0M0) with deep and ulcerated mucinous area, and

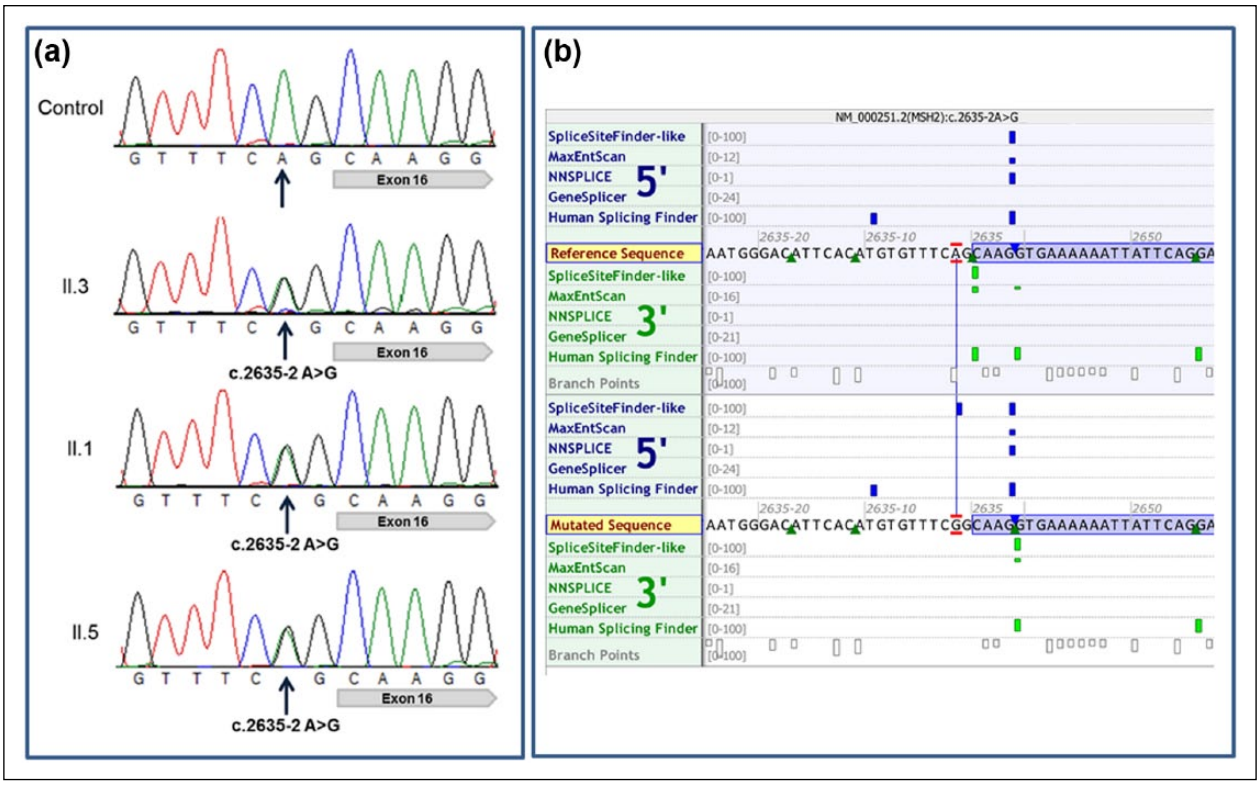

Figure 2. Sequence electropherogram and in silico analysis of the c.2635-2A>G splice-site mutation in the *MSH2* gene. (a) Sanger sequencing of the *MSH2* gene showing the missense mutation c.2635-2A>G in all affected family members in addition to a wild-type sequence. (b) Screenshot from the Alamut software. Splicing effect window around the *MSH2* c.2635-2A>G variant. The top box represents the wild-type sequence with an A at position c.2635-2; the bottom box represents the mutated sequence with a G at position c.2635-2. The dark blue bars represent predicted donor splice sites. The green bars represent predicted acceptor splice sites. The diagram shows the abolition of the canonical acceptor splice site and the creation of a new cryptic acceptor splice site at position c.2639.

wall infiltration of the intestine segment reaching the perivisceral adipose tissues. The lesion was also characterized by vascular permeation. No lymph node involvement was observed. The patient started follow-up and after three months, magnetic resonance and CT examinations revealed an endoluminal uterine lesion of about 10×6×8.5 cm, which was treated with a total laparoscopic hysterectomy. Histologic examination of the surgically resected tumor showed endometrial adenocarcinoma (G2T1AN0M0) with myometrium and uterine adnexal involvement.

With regard to her family history, her mother developed two primary colon cancers at 53 and 54 years of age, and died at the age of 73 for other reasons. Her sister (Figure 1 (II.1)) developed an endometrial cancer at 48 years of age, which was surgically treated. Her brother (Figure 1 (II.5)) had two colonic polyps at 47 years of age that were endoscopically removed. After genetic counseling, the siblings provided written informed consent for genetic analysis. This study adhered to the ethical guidelines set out in the "Declaration of Helsinki, 2013."[6] Genomic DNA was extracted from peripheral blood of the index case (Figure 1 (II.3)) and her relatives (Figure 1 (II.1) and (II.5)) using the Salting Out kit (Cat. 208011/20, Nuclear Laser Medicine srl, Italy). Sanger sequencing of exons and exon-intron boundaries of the *MSH2* (NCBI reference sequence: NG_007110.2; NM_000251.2), and *MLH1* (NCBI reference sequence: NG_007109.2; NM_000249.3) genes was performed as previously described[7] using an Applied Biosystems® 3130 Genetic Analyzer (ThermoFisher). The *MLH1* and *MSH2* genetic test was first performed on DNA samples of the index case and subsequently the identified variant was investigated on germline DNA of her siblings. Mutations and polymorphisms were confirmed (a) in the index case on DNA samples isolated from a new blood draw; and (b) in her relatives on independently amplified polymerase chain reaction products. *MLH1* gene mutation analysis was negative. A heterozygous *MSH2* gene mutation (c.2635-2A>G), located at the intron 15 splicing acceptor site, was identified in all affected family members (Figure 2(a)). This mutation was also identified in the index case's healthy son (III.2), who was referred to screening and surveillance protocols according to National Comprehensive Cancer Network guidelines.[1] The global population frequency of the identified *MSH2* gene variant was retrieved

from the 1000 genome (http://www.internationalgenome.org/1000-genomes-browsers/), dbSNP150 (https://www.ncbi.nlm.nih.gov/projects/SNP/), Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org/) and NHLBI Exome Sequencing Project (ESP; http://evs.gs.washington.edu/EVS) databases. The identified variant was found to be rare since it was not listed in the above-mentioned databases. Moreover, the HGMD (http://www.hgmd.cf.ac.uk/ac/index.php), LOVD (http://www.lovd.nl/3.0/home), InSiGHT (https://www.insight-database.org/genes) and Clinvar (https://www.ncbi.nlm.nih.gov/clinvar/) databases were interrogated to assess its pathogenicity. This analysis revealed that the *MSH2* c.2635-2A>G variant was previously reported only in one family with LS, which was not described clinically or at the molecular level (MMR protein immunohistochemical (IHC) analysis).[5] In the ClinVar database, two different variants affecting the canonical splice site of *MSH2* intron 15 (c.2635-2A>G, c.2635-2A>T) have been reported as "likely pathogenic" in association with the hereditary cancer predisposing syndrome, according to the American College of Medical Genetics and Genomics and the Association of Molecular Pathology variant classification scheme.[8] In order to ascertain the molecular consequences of the *MSH2* c.2635-2A>G mutation we performed (a) an in silico prediction of the mutational effect; (b) a microsatellite instability analysis; and (c) an MMR protein expression analysis.

To evaluate the effect of the *MSH2* gene variant on RNA splicing, five splice site prediction algorithms integrated in the Alamut Visual Version 2.10 (Interactive Biosoftware; Rouen, France) were interrogated simultaneously: Splice Site Finder (SSF), MaxEntScan (MES), Splice Site Prediction by Neural Network (NNS), Gene Splicer (GS) and Human Splicing Finder (HSF). Default thresholds of each tool were used for the analysis. A variation of more than 10% in at least two algorithms was considered as having an effect on the splicing process. Three algorithms (SSF, MES and HSF) of the Alamut Software suggested that variant c.2635-2 A>G could result in a splice defect due to loss of the canonical site and creation of a cryptic acceptor site at position c.2639 of the *MSH2* gene (Figure 2(b) and Supplementary Table 1). An MSI analysis was carried out to assess the putative instability phenotype of patients' tumors related to the *MSH2* genetic alteration. For this purpose, genomic DNA was extracted from formalin-fixed, paraffin-embedded (FFPE) normal and colon cancer tissues of the index case, and from FFPE normal and endometrial cancer tissues of her sister using the QIAmp DNA FFPE Tissue kit (Qiagen, Hilden, Germany). The paired samples of DNA from FFPE normal and tumor samples were analyzed for MSI using the standard panel of microsatellites (D2S123, D5S346, D17S250, BAT25, BAT26) proposed by the National Cancer Institute, as previously described.[9] The analyzed tumor samples showed alterations of all microsatellite markers (MSI-high, data not shown).

To elucidate whether MMR protein expression was affected in tumors, we performed an IHC analysis of MLH1, PMS2, MSH2, and MSH6 proteins on the FFPE samples used in MSI analysis, as previously described.[4] IHC staining revealed that MLH1 expression in tumor cells in colorectal (Figure 3(a)) and endometrial (Figure 3(b)) cancers was preserved, and showed strong and diffuse immunoreactivity, as well as PMS2 protein expression (data not shown). Conversely, IHC staining for MSH2 did not show immunoreactivity in the tumor cells of colorectal (Figure 3(c)) and endometrial (Figure 3(d)) cancers. Consistently, IHC analysis revealed the loss of MSH6 protein expression in these tumor samples (data not shown). Taken together, our data suggest that MSH2 and MSH6 protein loss was due to the *MSH2* gene mutation.

Conclusion

This study provides the first molecular characterization of the *MSH2* c.2635-2A>G variant, which was identified in three family members with a clinical manifestation of LS. This variant affects the splice acceptor site of *MSH2* intron 15. This mutation has been previously reported in one LS family case, together with other well-known causative mutations in *MLH1* and *MSH2* genes identified in 18 unrelated LS families; however, the putative loss of MSH2/MSH6 heterodimer proteins was not investigated.[5] We performed an in silico prediction analysis, a global population frequency analysis, and MSI and MMR protein expression experiments on patients' tumor tissues in order to ascertain the potentially causative role of the identified variant in LS manifestation.

To assess the putative effect of the identified acceptor site variant on the splicing process, we used the Alamut software. Bioinformatics results suggested that the identified mutation could significantly affect the molecular splicing mechanism. In fact, a potential deleterious effect on the RNA splicing process was predicted due to the loss of the natural acceptor splice site of *MSH2* intron 15 and the creation of a new cryptic acceptor site four nucleotides downstream. This result suggests that the *MSH2* c.2635-2A>G variant might cause an abnormal exon joining with the generation of a non-functional MSH2 protein. With the use of next-generation sequencing technologies in clinical practice, the detection of novel, previously uncharacterized sequence variants has greatly increased. Global population variant frequency is important for clinical interpretation; in fact, minor allele frequency in the global population is one of the key factors for clinical classification of a given sequence.[8] The frequency of the identified variant was assessed by interrogating different population databases. This analysis revealed that the *MSH2* c.2635-2A>G variant was not listed in the dbSNP 150, 1000 Genome, ExAC and ESP databases, suggesting that it is most likely to be pathogenic.

We then analyzed the effect of the *MSH2* c.2635-2A>G variant on MMR protein expression in colon and endometrial

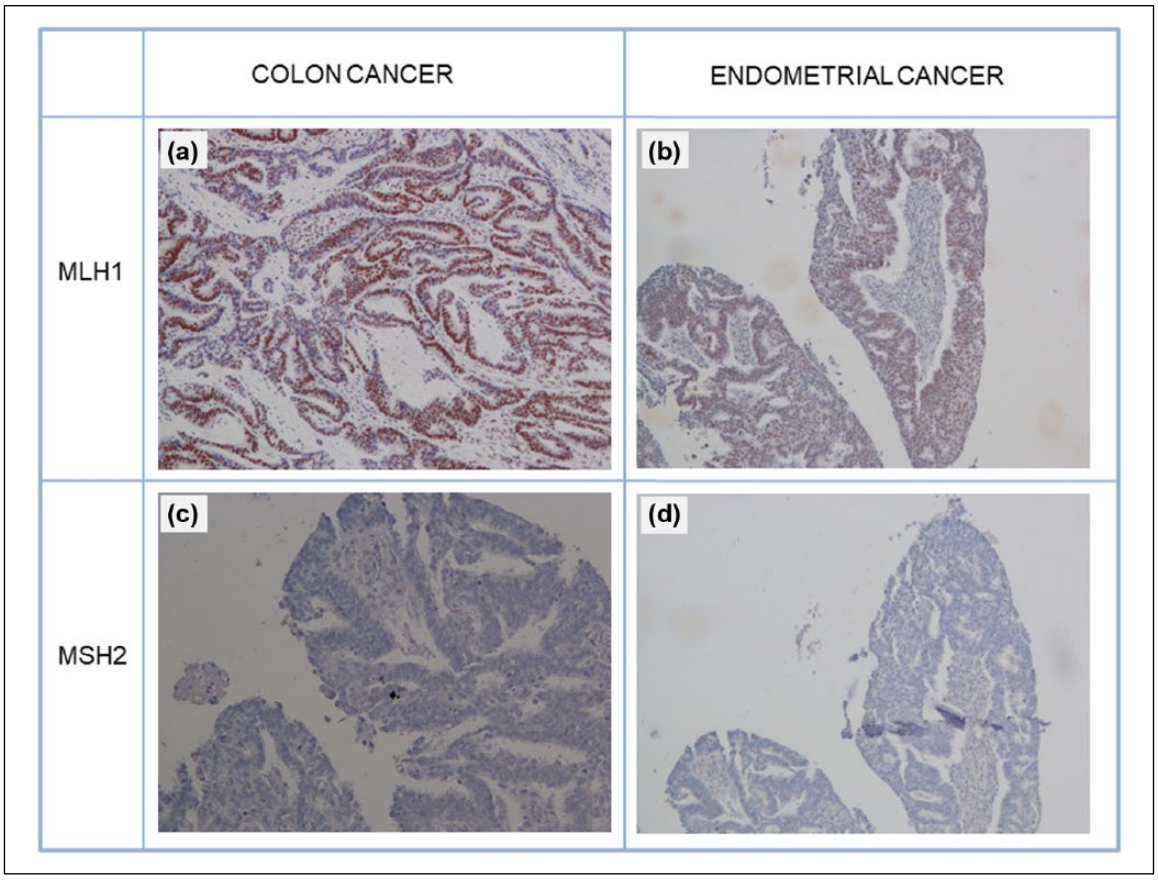

Figure 3. Immunohistochemical analysis showed MLH1 expression in colon (a) and endometrial cancer (b) samples, whereas MSH2 staining was negative in both specimens (colon cancer (c) and endometrial cancer (d)).

tumor tissues of two LS family patients (II.1 and II.3). It is well known that loss of MSH2 function (because of deleterious mutations) manifests as loss of MSH2 and MSH6 IHC detection, whereas loss of MLH1 function (because of deleterious mutations or promoter hypermethylation) is detectable as absent MLH1 and PMS2 expression in IHC staining. Isolated loss of MSH2, MSH6, or PMS2 proteins suggests the presence of germline mutations in the respective genes.[4] Our IHC experiments revealed the absence of MSH2 and MSH6 protein expression in the patients' tumor tissues, whereas normal expression of both MLH1 and PMS2 proteins was observed, suggesting that the *MSH2* c.2635-2A>G variant could produce an aberrant protein. Consistently, these samples showed high microsatellite instability. Other mutations affecting the splicing acceptor site of *MSH2* intron 15 (c.2635-3C>T; c.2635-5T>C) have been previously described in six cases of four unrelated families affected by LS. In these families, a significant reduction of MSH2 mRNA expression was detected in mutation carriers, suggesting a pathogenic role for the variant.[10] Altogether, this experimental evidence supports a pathogenic role for the rare *MSH2* c.2635-2A>G mutation, reinforcing the importance of the molecular characterization of variants with no clear evidence of pathogenicity to offer tailored genetic counseling, management, and surveillance in LS families. Further studies are awaited to elucidate the mechanisms underlying RNA splicing of the *MSH2* c.2635-2A>G variant.

Author contributions

Filomena Cariola and Vittoria Disciglio contributed equally to this work.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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Supplemental Material

Supplemental material for this article is available online.

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