High Frequency of ASXL1 and IDH Mutations in Young Acute Myeloid Leukemia Egyptian Patients

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Abstract

Background: Prognostication of AML patients depends on association of genetic and epigenetic abnormalities. We aimed to evaluate the frequency and prognostic significance of Additional Sex comb’s Like1 (ASXL1), Isocitrate Dehydrogenase (IDH) and CEBPA mutations in AML assessing their association with different cytogenetic risk category. Methods: We used High Resolution Melting (HRM) technology that detects small differences in PCR amplified sequences by direct melting using EvaGreen saturating dye to analyze epigenetic mutations in 70 denovo AML patients. Results: Median age of AML patients was 39.5 years (18-75). ASXL1, IDH and CBL mutations were detected in 14 (20%), 10 (14%) and 5 (7%) patients, respectively. Mean age of ASXL1 and IDH mutants vs. wild type was 35.9±14.6 years and 42.9±14.4 years (p=0.114) and 46.7±15.2 years vs. 40.6±14.5 years (p=0.290), respectively. AML cytogenetic risk groups included low (25/70, 36%), intermediate (33/70, 47%) and high-risk (12/70, 17%). Nine/14 (64%) ASXL1 and 8/10 (80%) IDH mutants were classified as intermediate risk and 9 ASXL1 positive (64%) were adolescent and young adults (AYA). Overall survival (OS) of mutant ASXL1 vs. wild type was 1.1 years (95% CI 0.83-1.4) vs. 1.9 years (95% CI 0.71-7.51), respectively (p=0.056). OS of mutant IDH vs. wild type was 1.25 years (95% CI 0.85-1.6) vs. 1.8 years (95% CI 1.2-6.7), respectively (p=0.020). In intermediate risk cytogenetic group, ASXL1 and IDH mutants had shorter OS than wild type; 1.1 years (95% CI 0.97-1.2) vs. 2.1 years (95% CI 0.14-10.8) (p=0.002) and 1.8 years (95% CI 0.69-3.15) vs. 2.3 years (95% CI 1.1-5.5) (p=0.05), respectively. Conclusion: ASXL1 and IDH mutations occur at a high incidence among young Egyptian AML patients with intermediate risk cytogenetics and confer a poorer outcome. Integration of mutations into risk profiling may predict outcome and impact therapeutic approach of young AML patient with uncertain prognosis.

Keywords: Acute myeloid leukemia- ASXL1- IDH- survival- intermediate risk cytogenetics

Introduction

Acute Myeloid Leukemia (AML) is a disease of elderly driven by many genetic and epigenetic aberrations that variably impact outcome. Recently, understanding AML pathogenesis has been clarified by newer molecular techniques (Döhner et al., 2015; Watts et al., 2018). Age is a strong prognostic factor in AML and unfavorable genetic profiles increase proportionately with advanced age (Creutzig et al., 2018). However, apart from age, genetic mutations can also affect outcome (Kuwatsuka et al., 2018).

In Egypt, the median age of incidence for AML is around 40 years (Ibrahim et al., 2014; El Gammal et al., 2019; Khaled et al., 2019); an age relatively younger than western reports where the median age of diagnosis is 68 years (Schnittger et al., 2013; Howlader et al., 2021). About 55% of newly diagnosed AML patients carry chromosomal abnormalities in addition to many genetic mutations as fms like tyrosine kinase 3 (FLT3-ITD), Nucleophosmin (NPM1), CCAAT/enhancer binding protein alpha (CEBP A) and Runt-related transcription factor 1 (RUNX1) mutations (Patriarca et al., 2015; Saultz et al., 2016). The European Leukemia Net (ELN) guidelines stratify AML into three prognostic risk categories (Favorable, Intermediate and Adverse) by combining the presence of karyotypic aberrations with genetic mutations (Döhner et al., 2017). As more mutations are discovered, molecular pathways in AML continue to be unraveled (Rocquain et al., 2010; De Kouchkovsky et al., 2016). However, molecular profiles across different age groups were different with increasing mutational burden with age (Creutzig et al., 2018). Genes regulating epigenetic modifications and chromatin structure as Additional Sex

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comb’s Like1 (ASXL1), Isocitrate Dehydrogenase (IDH) and Casitas B-lineage Lymphoma (CBL) have emerged as critical for AML pathogenesis and their alterations represent important prognostic markers in AML (Gallipoli et al., 2015). The incidence of alterations in these genes was mainly reported in elderly AML (Creutzig et al., 2018). Therefore, we sought to study their incidence in our young AML patients including a group of adolescent and young adults (AYA) in an attempt to identify disease outcome according to genetic mutations in a different age category.

ASXL1 gene plays a central role as an epigenetic regulator that participates in modulation of the transcription of genes involved in differentiation or proliferation as it affects histone methylation (Rahmani et al., 2019). Mutations of ASXL1 exon 12 have been demonstrated as relatively new molecular aberrations (Asada et al., 2019) reported in about 6% to 30% among AML patients (Schnittger et al., 2013; Paschka et al., 2015; Kakosaiou et al., 2018) and show a worse prognosis and inferior overall survival (OS) (Sasaki et al., 2020).

ASXL1 mutations are considered as early founder mutations in AML pathogenesis beside their inclusion among the high-risk genetic category in the last ELN recommendations (Kakosaiou et al., 2018). Association between ASXL1 mutations and different karyotype abnormalities are quite diverse (Paschka et al., 2015).

Mutations of IDH1 genes in AML were reported around 20% and were found more frequently in elderly individuals (Medeiros et al., 2017). IDH mutations are commonly found within the intermediate-risk cytogenetic group especially with normal karyotype (Inoue et al., 2016; Papaemmanuil et al., 2016; ELNahass et al., 2020). AML with IDH1 mutations is characterized by abnormal histone and DNA methylation which may result in a blocked cell differentiation (Inoue et al., 2016).

Novel mutations in CBL gene have also been found in AML such as several gain-of-function mutations (Nadeau et al., 2017). CBL gene (localized on human chromosome 11q23) is a negative regulator of activated FLT3 receptor tyrosine kinase. CBL gene mutations have been found in approximately 5% of de novo and secondary AML (Liyasova et al., 2015).

The incidence of epigenetic mutations in younger AML patients has not been estimated. Genomic analysis for newly diagnosed young AML patients plays a crucial role in their categorization especially for the intermediate risk category that represents half of newly diagnosed AML. Approximately 45% of AML patients show a normal karyotype at diagnosis and are classified as intermediate risk (Bollé et al., 2015; Lagunas-Rangel et al., 2017). The optimal therapeutic strategies for this subgroup are still largely debatable and a considerable heterogeneity is found in this population regarding outcome and survival. Identifying a high risk subgroup among young intermediate risk AML patients according to epigenetic markers is an additional integrated molecular risk stratification based on detection of acquired mutations (Mahmoud et al., 2016). Molecular profiling provides further classification, and prognostication which may help in more specific selection for therapeutic interventions (Wertheim et al., 2015; Papaemmanuil et al., 2016; Sun et al., 2018; Waitkus et al., 2018).

This study was undertaken to assess the incidence and frequency of ASXL1, IDH1 and CBL mutations in de novo young AML patients to evaluate their association with cytogenetic risk category and assess their impact on disease outcome.

Materials and Methods

Patients and samples

Seventy de novo AML patients with median age 39.5 years (18-75), along with fifty healthy controls were enrolled in this study. Patients presented to the Medical Oncology and Hematology Unit at the National Cancer Institute (NCI), Cairo University between 2016 and 2019. AML diagnosis was based on morphology, cytochemistry, immunophenotyping, cytogenetics and routine molecular detection of NPM1, CEBPA, C-KIT and FLT3-ITD by Polymerase Chain Reaction (PCR). Diagnosis was established according to the World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues (Arber et al., 2016). Cytomorphology was based on May-Grunwald-Giemsa stain. Karyotypes by short term cultures were analyzed by G-banding according to International System for Human Cytogenomic Nomenclature (ISHCN) (McGowan-Jordan, 2016). Immunophenotyping was performed by Coulter EPICS XL-MCL (Coulter Corporation, Hialeah) (Kern et al., 2004). Patients were risk classified according to the combination between karyotypic aberrations and genetic mutations according to the ELN classification (Döhner et al., 2017).

Patients with Acute promyelocytic leukemia and history of AML treatment or therapy related were excluded. Median follow up was 22 months (0.03-35.69). Informed consents were provided by all patients and healthy controls. Study was approved by NCI Institutional Review Board according to Helsinki Declaration, (IRB No: IRB00004025).

Methods

ASXL1, IDH1/2 and CBL mutations screening by High-Resolution Melting (HRM) Analysis

DNA was extracted from bone marrow (BM) aspirates or peripheral blood using Puregene® Blood Core Kit A (Qiagen, Germany). Primers were designed, using “primer 3 plus”, to amplify exon 12 of ASXL1, exon 4 of IDH1 and IDH2, and exons 8 and 9 of CBL genes covering most common reported mutations in ASXL1; c.1934dupG “rs750318549”, c.1900_1922del “rs766433101” and c.1934delG, IDH1; R132C “rs121913499” and R132H “rs121913500”, IDH2; R140Q “rs121913502”, R172K “rs121913503” and R172W “rs1057519906”, and CBL; p.(Glus366Lys) “rs397517076”.

Twenty nanogram of DNA was amplified in a final volume of 10 uL containing 1X High Resolution Melting (HRM) PCR Master Mix (Type-it™ HRM™ PCR KIT (Qiagen, Germany) including EvaGreen dsDNA (double-stranded deoxyribonucleic acid) saturating fluorescent binding dye, 0.2mM of each primer and
characteristics and frequency of genetic mutations are provided in Table 1. No mutations in ASXL1, IDH1/2 or CBL genes were detected in the 50 healthy controls recruited in this study.

Median peripheral blood (PBL) blasts of mutant ASXL1 was 74 % (7-85) vs. 58 % (4 - 98) for wild type ASXL1. Ten/12 (83%) mutant ASXL1 patients had PBL blasts ≥ 50% vs. 2/12 (17%) wild type ASXL1 (p =0.051). Seven/10 (70%) mutant IDH had BM blasts ≥ 50% vs. 3/10 (30%) wild type patients (p ≥0.001). Four/5 (80%) mutant CBL patients had BM blasts ≥ 50% vs. 1/5 (20%) wild type patients. From 10 patients with IDH mutations, IDH1 was positive in 2 (20%) while IDH2 mutations in 8 (80%) patients.

Table 1. Clinical and Genetic Characteristics of 70 AML Patients

| Patients | number (percent) |
|----------|------------------|
| Gender   |                  |
| Male     | 32 (45.7%)       |
| Female   | 38 (54.3%)       |
| FAB subtype |              |
| AML M0   | 6 (9%)           |
| AML M1   | 12 (17%)         |
| AML M2   | 25 (35%)         |
| AML M4   | 20 (29%)         |
| AML M5   | 7 (10%)          |
| Cytogenetic risk |        |
| Low      | 25 (36%)         |
| Intermediate | 33 (47%)      |
| High     | 12 (17%)         |
| Genetic Mutations |   |
| ASXL1    | 14 (20%)         |
| IDH      | 10 (14%)         |
| CBL      | 5 (7%)           |
| FLT3-ITD | 12 (17%)         |
| NPM1     | 20 (29%)         |

Results

Median age of 70 newly diagnosed AML patients was 39.5 years (18-75); mean age 41.5±14.6 years. Patients’ characteristics and frequency of genetic mutations are provided in Table 1. No mutations in ASXL1, IDH1/2 or CBL genes were detected in the 50 healthy controls recruited in this study.

Median peripheral blood (PBL) blasts of mutant ASXL1 was 74 % (7-85) vs. 58 % (4 - 98) for wild type ASXL1. Ten/12 (83%) mutant ASXL1 patients had PBL blasts ≥ 50% vs. 2/12 (17%) wild type ASXL1 (p =0.051). Seven/10 (70%) mutant IDH had BM blasts ≥ 50% vs. 3/10 (30%) wild type patients (p ≥0.001). Four/5 (80%) mutant CBL patients had BM blasts ≥ 50% vs. 1/5 (20%) wild type patients. From 10 patients with IDH mutations, IDH1 was positive in 2 (20%) while IDH2 mutations in 8 (80%) patients. NPM1 and FLT3-ITD co-occurred in only
There was a slight male predominance in ASXL1 and IDH mutations occurrence as 8/14 (57%) and 7/10 (70%) were males; respectively in addition 3/5 (60%) mutants CBL were males. Interestingly, the mean age of ASXL1 mutant was lower than wild type counterpart and ASXL1 mutations were found at a higher frequency in AYA. The mean age of different mutational category is provided in Table 3. The incidence of genetic mutations in AYA vs. older AML patients is demonstrated in Table 4.

Association between ASXL1 mutations and karyotype and other molecular abnormalities

Twelve /14 (85.7%) ASXL1 mutant patients were CN-AML. ASXL1 and FLT3-ITD were mutually exclusive; 13/14 (93%) mutant ASXL1 patients were FLT3-ITD negative. ASXL1 mutation showed an inverse association with NPM1; 8/14 (79%) ASXL1 mutant were NPM1 negative. ASXL1 and CBL mutations co-occurred in one patient.

Mutual exclusivity between genetic markers

All 14 mutant ASXL1 patients were negative for IDH mutations (p=0.088). All mutant CBL patients were negative for core binding factor (CBF) translocations.

Table 2. Correlation between ASXL1, IDH and CBL Mutations and Laboratory Parameters

|                | Hb (g/dl) | TLC (×10^9/L) | Platelets (×10^9/L) | PB blasts (%) | BM blasts (%) |
|----------------|-----------|---------------|---------------------|---------------|---------------|
|                | <8 gm/dl  | 28 (50%)      | 12 (21%)            | 33 (59%)      | 37 (66%)      |
| ASXL1 Wild     | 28 (50%)  | 19 (34%)      | 24(43%)             | 42(75%)       |
| (n=56)         |           |               |                     |               |
| ASXL1 Mutant   | 10 (71%)  | 10 (71%)      | 4 (28%)             | 2 (14%)       |
| (n=14)         |           |               |                     |               |
| IDH Wild       | 31 (51%)  | 40 (67%)      | 20 (33%)            | 14(23%)       |
| (n=60)         |           |               |                     |               |
| IDH Mutant     | 7 (70%)   | 8 (80%)       | 2 (20%)             | 2 (20%)       |
| (n=10)         |           |               |                     |               |
| CBL Wild       | 33 (51%)  | 43 (66%)      | 22 (34%)            | 14(22%)       |
| (n=65)         |           |               |                     |               |
| CBL Mutant     | 4 (80%)   | 4 (80%)       | 1 (20%)             | 2(40%)        |
| (n=5)          |           |               |                     |               |

Table 4. Incidence of Epigenetic Mutations in 70 AML Patients According to Two Ages Cut off 39 and 55 Years

| Age          | ASXL1 Mutant | IDH Mutant | CBL Mutant | All Mutations |
|--------------|--------------|------------|------------|---------------|
| <39 years    | 9 (26%)      | 3 (9%)     | 3 (9%)     | 15 (44%)      |
| ≥39 years    | 5 (14%)      | 7 (19%)    | 2 (6%)     | 14 (39%)      |
| <55 years    | 12 (22%)     | 7 (13%)    | 5 (9%)     | 24 (44%)      |
| ≥55 years    | 2 (13%)      | 3 (19%)    | 0 (0%)     | 5 (31%)       |
and IDH mutations (p<0.001). CBL mutations were significantly associated with FLT3-ITD (3/5, 60%) (p=0.050). The association between ASXL1, IDH and CBL mutations and other molecular and cytogenetic findings is presented in Table 5.

**Association between ASXL1, IDH and CBL mutations and AML cytogenetic risk**

We observed an association between ASXL1 and IDH mutations and cytogenetic risk group (Table 6). Genetic mutations occurred more frequently within the intermediate risk category. No association between CBL mutations and cytogenetic risk groups could be established due to low number of positive CBL mutations.

**Response to therapy and survival in ASXL1 and IDH mutant patients**

At end of induction chemotherapy, 42/70 (60%) patients achieved hematological complete remission (CR). A significant relation was detected between mutant IDH patients and CR rates as only 3/10 mutant IDH (30%) achieved CR at day 28 vs. 7/10 patients (70%) who failed to achieve CR (p=0.036). Median follow up period was 1.85 years (0.3 -5) after exclusion of early deaths. The median OS was 1.97 years. Median OS was significantly superior in wild type ASXL1 and IDH vs. mutant patients (Table 7).

OS of ASXL1 mutant was 1.1 years (95% CI 0.83-1.4) vs. 1.9 years (95% CI 0.71-7.51) for wild type patients (p=0.056) (Figure 1a). A significant association was established between IDH mutation and survival as median OS of mutant IDH was 1.25 years (95% CI 0.85-1.6) vs. 1.8 years (95% CI 1.2-6.7) for wild type patients (p=0.020) (Figure 1b).

**Discussion**

Results of National population based Cancer Registry and National Cancer Institute in Egypt showed a lower median age of AML patients compared to Western population (Ibrahim et al., 2014; El Gammal et al., 2019). In one Egyptian report including 468 myeloid leukemia, median age was 43 years (Khaled et al., 2019). The median age of AML patients in western countries is around 65 years (Grimwade et al., 2001; Shallis et al., 2019). The prognostic role of many genetic mutations and their association with AML pathophysiology has been largely examined in elderly patients (Papaemmanuil et al., 2016). However, the prevalence of these genetic mutations has not been examined in young AML cohorts. Specific gene mutations as ASXL1 and IDH can further classify patients and affect prognosis (Medinger et al., 2016). This is particularly relevant for intermediate risk AML (Wang et al., 2017). Results regarding frequency of genetic mutations and association with karyotype abnormalities are quite diverse (Schnittger et al., 2013). ASXL1 mutations were reported from 6-30% of AML and were found more common in older patients compared to younger population (Schnittger et al., 2013; Tsai et

| Table 5. Association between ASXL1, IDH and CBL Mutations and Other Molecular and Cytogenetic Findings |
|-------------------------------------------------|
| Mutations | CN-AML | FLT3 +ve | NPM1 +ve | CBF translocations |
| ASXL1 mutant (n=14) | 12/14 (86%) | 1/14 (7%) | 3/14 (21%) | 2/14 (14%) |
| IDH mutant (n=10) | 9/10 (90%) | 2/10 (20%) | 0/10 (0%) | 0/10 (0%) |
| CBL mutant (n=5) | 4/5 (80%) | 3/5 (60%) | 2/5 (40%) | 0/5 (0%) |

| Table 6. Frequency of ASXL1 and IDH Mutations among Different AML Cytogenetic Risk Group |
|----------------------------------------|
| Mutations | Low risk | Intermediate risk | High risk | P |
| Mutant ASXL1 (n=14) | 4 (29%) | 9 (64%) | 1 (7%) | 0.05 |
| Wild ASXL1 (n=56) | 21 (37%) | 24 (43%) | 11 (20%) | |
| Mutant IDH (n=10) | 0 (0%) | 8 (80%) | 2 (20%) | 0.015 |
| Wild IDH (n=60) | 25 (42%) | 25(42%) | 10 (16%) | |

| Table 7. Association between day 28 CR and Survival and ASXL1 and IDH Mutations |
|-------------------------------------------------|
| Mutations | CR Day 28 | P | Survival |
| | CR (n%) | No CR (n%) | No of events | Median survival (years) | P |
| ASXL1 Mutant (n=14) | 7 (50%) | 7 (50%) | 0.393 | 13 | 1.12 | 0.056 |
| ASXL1 Wild (n=56) | 35(63%) | 21 (37%) | 30 | 1.9 |
| IDH Mutant (n=10) | 3 (30%) | 7 (70%) | 0.036 | 10 | 1.25 | 0.02 |
| IDH Wild (n=60) | 39(65%) | 21(35%) | 33 | 1.8 |
In the current study, *ASXL1* was the most frequently mutated gene after *NPM1* with a slightly higher incidence in males and its frequency was comparably higher in our younger patients especially AYA which may denote a different age-related molecular pattern in AML. Regarding laboratory parameters, a significantly higher number of patients in *ASXL1* mutant arm had Hb<8 gm/dl (64%), PBL blasts >50% (86%) and BM blasts >50% (86%) than in the wild type *ASXL1* arm. Most *ASXL1* mutant patients were classified as intermediate risk cyto genetics (9/14, 64%); an incidence higher than previously reported regarding age and cytogenetic risk classification (Schnittger et al., 2013). Variable reports about the incidence and associations with other molecular markers and with biologic characteristics are still reported, mainly because of selected cohorts or different ethnic backgrounds (Schnittger et al., 2013). Among older patients, *ASXL1* mutations were associated with CBFA, wild-type *NPM1*, negative *FLT3-ITD*, mutated *CEBP4*, and inferior CR and OS (Kuwatsuka et al., 2018). In our study, epigenetic mutations were detected in 44% of patient <39 years vs 39% in patients >39 years. Among 29 mutations detected, 5 (31%) mutations (2 *ASXL1*/3 IDH) were encountered in age group >55 years vs. 24 (44%) mutations (12 *ASXL1*/7 IDH/5 CBL) in age group <55 years. In the current work, *ASXL1* mutation was associated with distinct clinical features like male sex, younger age (mean 35.9±14.6), intermediate risk cyto genetics (64% of mutant patients) in addition to adverse prognosis and lower OS. Median survival of *ASXL1* mutant was inferior to wild type patients. Furthermore, AML patients with intermediate risk cyto genetics showed inferior OS for *ASXL1* mutants vs. wild type (p=0.002). *ASXL1* mutation was mutually exclusive with *FLT3-ITD* and *NPM1* mutations and found as independent adverse prognostic factor for OS. We tested *ASXL1* exon 12, which actually comprises >50% of the whole coding region of *ASXL1* using HRM technique which is an alternative and more rapid and cost-effective method of detection in countries of limited resources. All mutations found in *ASXL1* were heterozygous as previously reported (Patel et al., 2012).

Fourteen% of patients were positive for *IDH* mutations with a mean age of 46.7±15.2 years. *IDH1* and 2 mutations were also mutually exclusive as previously reported (Kuwatsuka et al., 2018; ElNahass et al., 2020). This incidence is in agreement with others (Rocquain et al., 2010; Montalban-Bravo et al., 2018) however, mean age of mutation occurrence is still younger than western population. *IDH1* and *IDH2* mutations have been reported in 15-20% and 25-30% of patients with intermediate risk-AML, respectively (Saulitz et al., 2016). Most *IDH* mutations (80%) in our study resided in the intermediate risk cyto genetic category and were higher than reported; where only 20% mutant *IDH* patients were high-risk AML. We have correlated *IDH* mutations with patient characteristics, different laboratory parameters and AML prognostic factors. In the *IDH* mutant arm, a higher number of patients had <8gm/dl (70%), TLC>11x10^9/L (80%) and PBL blasts >50% (80%) vs. wild type arm. *IDH* mutations showed mutual exclusivity with *NPM1*, *FLT3-ITD*, *ASXL1* and *CBL* mutations. All mutant *IDH* patients were negative for CBF translocations. Median survival of *IDH* mutant was inferior to wild type (p=0.020). OS of *IDH* mutant patients in the intermediate risk AML group was inferior to wild type (p=0.05). *IDH* mutations were associated with lower DFS and OS in CN-AML cases with NPM1 mutations and wild-type *FLT3*. DFS and OS of *IDH* mutant patients in the intermediate risk AML group were significantly inferior to wild type. *CBL* mutations were significantly associated with *FLT3-ITD*; however due to low number of mutated patients, conclusions regarding outcome could not be drawn. Regarding the poor outcome of our intermediate risk AML patients with *ASXL1* and *IDH* mutations, the evidence for early allogeneic stem cell transplantation in CR1 in our young patients may be stronger based on these high molecular risk markers.

In conclusion, this study demonstrates an overall high incidence of *ASXL1* and *IDH* mutations in young AML patients and a high incidence of *ASXL1* mutations in AYA. *ASXL1* and *IDH* mutations are frequently occurring in CN-AML and are mutually exclusive with *NPM1* and *FLT3-ITD* and can sub-stratify patients within AML intermediate risk cyto genetic category. Therefore, molecular detection of *ASXL1* and *IDH* mutations could potentially be used in addition to cyto genetics to redefine risk stratification and prognostic stratification of young AML patients with uncertain outcome.

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**Ethics approval and consent to participate**

Informed consents were provided by all patients. Study was approved by NCI Institutional Review Board according to Helsinki Declaration, (IRB No: IRB00004025).

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**Conflict of interest**

The authors declare that they have no competing interests.

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