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Epstein-Barr virus lytic cycle involvement in diffuse large B cell lymphoma

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Abstract

Epstein-Barr virus (EBV)-mediated B cell transformation is achieved predominantly through the action of latent proteins, but recent evidence suggests that lytic EBV replication has also a certain pathogenic role in lymphomagenesis, at least in the early phases of cell transformation. Particularly, in diffuse large B cell lymphoma (DLBCL), the EBV lytic cycle is by and large unexplored, so to disclose lytic cell contribution to lymphomagenesis, our aim was to evaluate viral early and late lytic gene expression in relation to several immune response markers in a series of EBV+ DLBCL from Argentina. An unexpected number of cells expressed lytic transcripts, being transcribed at the BZLF1, BHRF1, and BLLF1 locus, by real-time quantitative polymerase chain reaction. This lytic antigen expression was confirmed by immunohistochemical staining for BMRF1 early lytic protein, and a positive correlation between lytic and latent genes was confirmed, revealing a close link between their expressions in EBV+ DLBCL pathogenesis. Remarkably, BZLF1 displayed a negative correlation with CD4 cell counts, and this could be in part justified by the restriction of antigen presentation previously reported. The direct correlation for the late lytic gene BLLF1 and IFNγ in this series could represent a specific response directed towards this antigen. Interleukin 10 transcripts also displayed a positive correlation with lytic expression, indicating that regulatory mechanisms could be also involved on EBV-associated DLBCL pathogenesis in our series. Complete lytic reactivation in EBV-positive tumours could potentially kill EBV-positive malignant cells, providing a tool to promote tumour cell killing mediated by EBV as a complementary treatment strategy.

KEYWORDS

diffuse large B-cell lymphoma, Epstein-Barr virus, lytic cycle

1 | INTRODUCTION

Diffuse large B cell lymphoma (DLBCL), the most common group of malignant lymphomas, accounts for 30% of adult non-Hodgkin lymphomas. In 2016, the revision of the WHO classification scheme[1] changed the previous entity Epstein-Barr virus-positive (EBV+) DLBCL of older persons to EBV+ DLBCL "not otherwise specified" because it has been increasingly recognized in younger patients.[2-4]

Epstein-Barr virus presents both lytic and latent cycles of infection. Viral latency is characterized by the expression of a limited subset of viral genes that encode the viral proteins required for EBV immortalization of primary B cells. In fact, all EBV-associated malignancies have a predominantly latent pattern of viral gene expression.[5] However, a small number of lytic infected cells were described in biopsies of EBV-associated lymphoproliferative disorders in immunosuppressed individuals.[6] Typically, EBV-driven lymphomagenesis is thought to primarily involve latent cycle, but there is increasing evidence that lytic gene products and tegument proteins contribute to the development and maintenance of malignancies through the induction of growth factors and oncogenic cytokine production.[7] The contribution of early lytic viral gene expression to malignant transformation in the absence of late viral antigens was demonstrated in severe combined immunodeficiency and humanized mice, suggesting that lytic infection is either

Abbreviations: DLBCL, diffuse large B cell lymphoma; EBV+, Epstein-Barr virus-positive; GrB, granzyme B; IHC, immunohistochemistry; qPCR, quantitative polymerase chain reaction; TGFβ, transforming growth factor β

abortive or that cells expressing late viral proteins are rapidly eliminated.[7,8] It seems plausible that lytic infected cells promote EBV-induced lymphomas through paracrine mechanisms and/or immunosuppressive factors, including activation of immunosuppressive cytokines, such as interleukin 10 (IL-10) and transforming growth factor (TGFβ),[8] and the evasion of inflammatory response by attenuation of IFNγ.[9] In humanized mice, the development of an effective specific T cell response acts to eliminate cells with lytic viral infection.[8] BZLF1 early lytic protein was expressed in a few tumour cells in several endemic Burkitt lymphoma cases, indicating entry into the lytic cycle.[10] In addition, BZLF1 expression was described in 1 sample from a series of 118 non-Hodgkin B-cell lymphomas.[11] Despite these evidences, the exact role of EBV lytic activity has remained elusive and further investigation is required.

In Argentina, EBV presence in DLBCL was described in both paediatric and adult patients, displaying latency II and III patterns, together with BZLF1 lytic gene expression in a lesser extent.[3,12] To disclose lytic cell contribution to DLBCL lymphomagenesis, our aim was to evaluate viral early and late lytic gene expression in relation to several immune response markers.

2 | METHODS

2.1 | Biopsy samples

The studied series included 13 EBV+ DLBCL cases from a previously described larger cohort of 102 DLBCL biopsies obtained from 2 medical centres of Buenos Aires, Argentina.[3,12] This study has the approval of the Institutional Review Board and the Ethics Board of both centres and is also in accordance with the Helsinki Declaration of 1975, as revised in 1983. A written informed consent was obtained from all the included patients.

2.2 | Cell culture and induction of Epstein-Barr virus lytic replication

BL cell lines, namely, Raji (EBV type I) and P3HR1 (EBV type II), were maintained in RPMI media (Gibco) supplemented with 10% (*v*/*v*) fetal bovine serum (Natocor), 100 units/mL gentamicin, and 2 mM L-glutamine (Gibco) at 37°C with 5% CO_2. For EBV lytic induction, cells were seeded in log phase growth at 5×10^5 cells/mL. After 24 hours, the cells were concentrated to 2×10^6 cells/mL and treated with 12-*O*-tetradecanoylphorbol-13-acetate 50 ng/mL (Sigma). The cells were harvested 72 hour post induction. After harvest, cells were washed as above and stored at −70°C prior to RNA isolation as positive controls for gene expression studies or immediately employed to prepare a cell block from cell suspension to be used as positive control for immunohistochemistry (IHC).

2.3 | RNA extraction followed by real-time quantitative polymerase chain reaction for Epstein-Barr virus lytic transcripts and cytokines

Total RNA was purified from each of the formalin-fixed paraffin-embedded biopsy samples by using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion, Texas, USA), and 2 μg of total RNA was reverse transcribed by using Superscript II reverse transcription kit (Invitrogen) according to the manufacturer's instructions. RNA from lytic-induced cell lines used as calibrator was extracted by MasterPure™ RNA Purification Kit (Epicenter, Madison, WI, USA) according to the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) for immediate-early BZLF1, early BHRF1, late BLLF1 viral genes, and the endogenous HPRT gene was performed and validated as previously described[3,12] by using a StepOne real-time (RT) detection system (Applied Biosystems, Foster City, CA). Quantitative polymerase chain reactions with specific primers for target cytokine (IL-10, TGFβ1, and IFNγ) genes were performed and validated as for the EBV genes. Primer sequences are listed in Table S1. All primers (Invitrogen) were designed by using the Primer Express package (Applied Biosystems) and were directed against highly conserved regions, using mRNA sequences from public database and checked to span introns to avoid DNA detection. In addition, to remove contaminated DNA in total RNA, a DNAse step prior RT reaction was included for all RNA samples. Every run contained at least 2 "no template" controls in which nuclease-free H_2O was substituted for the template to check for amplicon contamination and no RT to detect possible DNA contamination. The normalized transcription values were calculated by the Pfaffl method.[13] The mean value of the Ct of an equivalent quantity of RNA input from lytic-induced P3HR1or Raji cell line was used as calibrator when appropriate.

2.4 | Immunohistochemistry

Immunohistochemical analysis of the early lytic EBV protein BMRF1 was performed by using citrate antigen retrieval and BMRF1 G3-E31 antibody (1:200, Abcam, Cambridge, UK). Sections were incubated with primary antibody ON at 4°C by using the manufacturer's blocking and detection protocols in the Universal Vectastain Elite ABC (Vector). Bound antibody was detected by diamino-benzidine chromogen (Dako), and tissues were counter-stained with haematoxylin. Immunohistochemistry staining for tumour microenvironment immunophenotyping was performed on formalin-fixed paraffin-embedded tissue sections with a panel of antibodies: CD4 for T helper (Leica, Newcastle, UK), CD8 for cytotoxic LT (Dako), Foxp3 for T regulatory (abcam, Cambridge, UK) granzyme B (GrB) for activated cytotoxic cells (clone GB7, AbD Serotec, Oxford, UK), PD-1 (CD279) for exhausted T-cells (AbDSerotec), and IL-10 (Abcam) for antiinflammatory cytokine productive cell markers, as previously described.[3,12] The counting of microenvironment CD4, CD8, Foxp3, GrB, PD-1, and IL-10 positive cells was performed as follows: numbers of total or immunopositive cells per high-power field were counted by 2 independent observers blinded to the characteristics of each subject, in 10 fields selected on the basis of the best-preserved tissue areas.

2.5 | Statistical analysis

Statistical analysis was performed by using GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA, USA). Categorical variables were analyzed by using Fisher exact test. Mann-Whitney test was used to compare the means between groups. Correlations between data

were determined by employing Spearman rank correlation index. All tests were 2-tailed, and a *P* less than .05 was considered statistically significant.

3 | RESULTS AND DISCUSSION

The EBV lytic cycle involves the sequential expression of immediate early proteins, typically transactivators of viral early gene expression; early proteins, essential components of the viral DNA replication complex; and late proteins, mainly structural proteins of the virion.[14] Those lytic proteins represent an antigenic-rich challenge for the host immune system.[15] It has been largely demonstrated that EBV-mediated B cell growth transformation is predominantly achieved through the collective action of latent proteins. Even though latency programs predominate in EBV-driven tumours, recent evidence suggests that lytic EBV replication has certain pathogenic role, at least in the early phases of cell transformation.[7,8] Abate et al observed RNAseq in an endemic BL cohort, the expression of lytic genes suggesting a noncanonical latency program of the virus with a subset of viral episomes initiating lytic reactivation.[16] Particularly, in DLBCL, the EBV lytic cycle is by and large unexplored, but recently, Morishima et al described higher IgG antibody titres against lytic antigens, which were considered as a marker of EBV reactivation, in a series of 13 EBV+ DLBCL patients older than 50 years.[17] Moreover, Strong et al also reported high immediate early lytic expression in DLBCL cases but without other lytic genes.[11]

In this study, EBV lytic cycle was analyzed in a series of 13 EBV+ DLBCL cases (5 paediatric and 8 adult) by means of qPCR and immunohistochemical staining. In addition, correlation of lytic expression with latent expression and several immune markers was assessed. Additionally to the type II and III viral latency expressions previously described in our series,[3,12] an unexpected number of cells expressed lytic transcripts, most notably being transcribed at the BZLF1, BHRF1, and BLLF1 locus, as demonstrated by RT-qPCR. Consistent with previous results from other studies in B cell malignancies, our findings also suggested a diversity of latency-associated gene expression probably because of noncanonical programs with a subset of cells exhibiting expression of lytic genes.[7,8,11,16,18,19] Furthermore, lytic antigen expression was confirmed by immunohistochemical staining for BMRF1 early lytic protein, a DNA polymerase factor essential for lytic virus replication,[20] observed in 9/13 (70%) of our EBV+ DLBCL cases (Figure 1). This was in agreement with similar observations found in other B-cell lymphomas,[16,19] where it was proposed that lytically infected B-cells secrete factors that may promote tumourigenesis, including growth and angiogenesis factors and immunosuppressive cytokines.

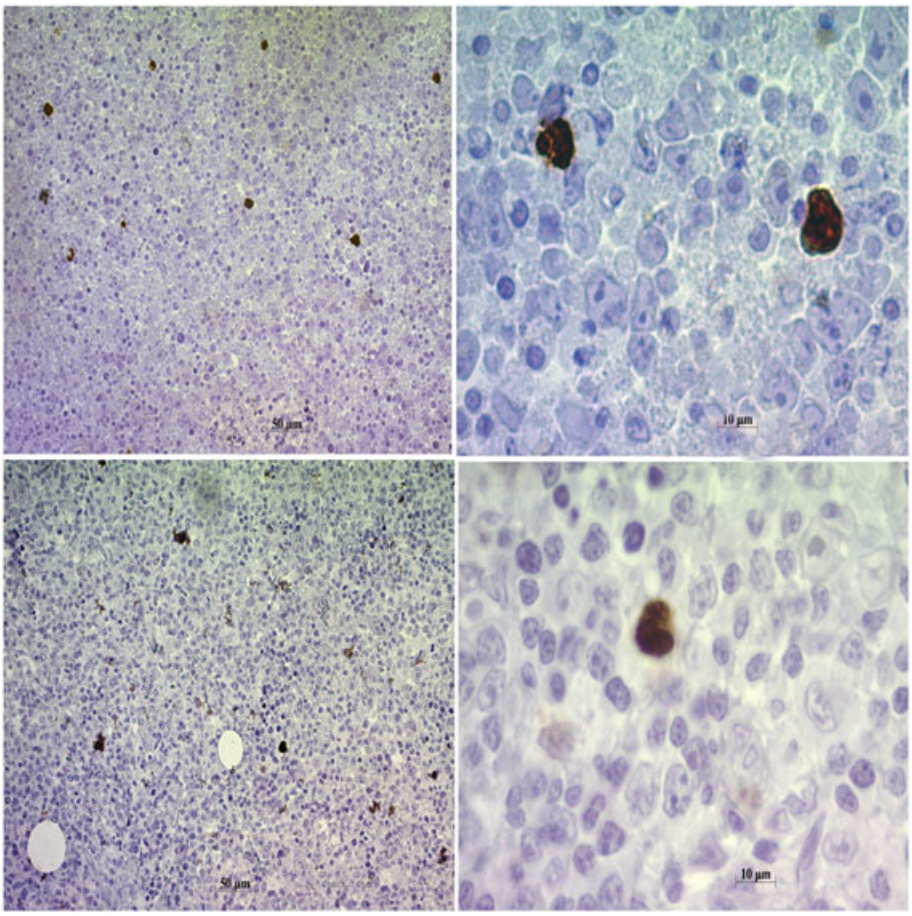

FIGURE 1 Representative expression of Epstein-Barr virus (EBV) early lytic antigen BMRF1 detected in EBV+ diffuse large B cell lymphoma (DLBCL) cases. Nuclear localization of BMRF1 was observed in some neoplastic cells by immunohistochemistry (IHC). Original magnification: ×200 and ×1000. An immunoperoxidase technique in a paraffin section was used. Digital images were obtained with an Axio CamErc 5s (Zeiss) camera and acquired by using digital AxioVision Rel 4.8 image acquisition software [Colour figure can be viewed at wileyonlinelibrary.com]

It has been established that, in a sequential manner, the immediate early genes are activated by cellular transcription factors; then, they activate the promoters of early lytic genes that encode the viral replication proteins. Finally, the late viral genes are expressed.[21] A statistically significant direct correlation among immediate early, early, and late EBV lytic transcripts was demonstrated in this series (Table 1). This fact may indicate that lytic cycle could be complete, given the expression of a packaging gene associated with infectious virion production, in contrast to the lytic-defective viruses described in mice.[7,8] The fact that all lytic antigens studied displayed statistically positive correlation with latent antigens, with the exception of LMP2A (Table 1), could illustrate a close link between lytic and latent gene expressions in DLBCL pathogenesis. In line with this, cells expressing lytic antigens were also described in the context of latency III pattern in lymphoproliferative disorders in immunosuppressed individuals,[6,18] revealing an active role of both EBV lytic and latent cycles in lymphomagenesis process. In our series, early lytic antigen expression was detected in EBV+ DLBCL cases simultaneously with EBV+ cells for latent antigens within the same case. By this, we are not suggesting that latent and lytic antigens are co-expressed in the same cell; on the contrary, each cell displayed either the latent antigen or the lytic one, given that the expression of lytic genes is strictly controlled and repressed in the latent phase. This could be one plausible explanation for the positive correlation between their gene expressions as was previously proposed, that lytic cycle allows horizontal spread of EBV increasing the total number of latently infected cells from which transformed cells arise.[16,19]

However, it is worthwhile to note that the in vitro models not always mimic the patients scenario because in lymphoblastoid cell lines, spontaneous lytic reactivation displayed no correlation with EBV latency types.[22] It can be noticed in Table 1 that neither latency nor lytic gene expression correlated with EBERs + cells in the tumour; thus, EBV gene expression, evaluated by qPCR, could be not exclusively due to EBERs + differential expression between samples.

To characterize immune response in the context of EBV lytic cycle, correlation of BZLF1, BHRF1, and BLLF1 expression with CD4, CD8, Foxp3, GrB, PD1, and IL-10 positive cells was analyzed. Remarkably, BZLF1 displayed a statistically negative correlation with CD4 cell counts ($r = -0.67$, $P = .017$, Spearman correlation test), while BHRF1 just displayed a trend ($r = -0.54$, $P = .07$, Spearman correlation test; Table 2). The CD4+ T cell response against latent viral antigens outnumbers those directed against lytic epitopes in IM patients, while to date, 3 lytic cycle proteins were proved to impede HLA class II-restricted antigen presentation, the BZLF1 transactivator, the viral IL-10 homolog BCRF1, and the viral envelope glycoprotein gp42.[23] Therefore, the disturbance of antigen presentation could be, in part, responsible for the negative correlation between specific lytic antigens and CD4+ cells, but further studies will be necessary to confirm this observation. Besides, we also found that latent antigens EBNA1, 2, and 3C displayed a negative correlation with CD4 cell counts as well (Cohen et al, unpublished observations). In fact, it was demonstrated that latent antigens may also impede CD4 cell response because CD4+ T cell clones against many latent Ag epitopes recognize EBV from lymphoblastoid cell lines poorly, indicating that latent proteins endogenously expressed have limitations to the HLA II presentation pathway.[24] On the other hand, it has been suggested that CD8+ T cell response is driven by direct contact with lytic infected cells, revealing the importance of CD8+ T cells in control of EBV lytic cycle. However, EBV displays several mechanisms to evade CD8+ T cell recognition during lytic replication.[23] Surprisingly, our series did not present a correlation between lytic expression and CD8+ or GrB+ cells (Table 2), although in previous observations, we found that CD8+ and GrB+ cells were higher in EBV+ cases (Cohen et al, unpublished observations), suggesting that cytotoxic response could be involved exclusively in control of latent EBV antigen expression in DLBCL. In line with this, cytotoxic activity in EBV-associated lymphomas was already demonstrated in HL, although this activity was not proven to be successful.[25] Release of IFNγ by NK cells and CD8+ T cells may limit B cell transfor-

TABLE 1 Correlation analysis between lytic and latent gene expressions and with Epstein-Barr virus (EBV)-encoded small RNAs (EBERs) + neoplastic cells from EBV+ diffuse large B cell lymphoma (DLBCL) samples

	BZLF1	BHRF1	BLLF1	EBNA1	EBNA2	EBNA3C	LMP1	LMP2A
BZLF1		0.89 $P = .0001$	0.88 $P < .0001$	0.90 $P < .0001$	0.82 $P = .003$	0.96 $P < .0001$	0.67 $P = .0045$	0.22 $P = .48$
BHRF1	0.89 $P < .0001$		0.81 $P = .001$	0.85 $P = .0002$	0.74 $P = .009$	0.80 $P = .001$	0.78 $P = .0019$	0.18 $P = .55$
BLLF1	0.88 $P < .0001$	0.81 $P = .0008$		0.78 $P = .002$	0.72 $P = .017$	0.85 $P < .0001$	0.57 $P = .044$	0.22 $P = .48$
EBERS[a]	0.38 $P = .20$	0.46 $P = .11$	0.46 $P = .11$	0.54 $P = .055$	0.36 $P = .23$	0.35 $P = .24$	0,34 $P = .25$	0.34 $P = .26$

P as determined by Spearman's rank correlation index. The strength of the correlation is meaningful when absolute *R* value is above 0.6.

[a]DLBCLs were screened for the presence of EBV in the malignant cells by means of EBER ISH. The median of the % EBERs + cells was 60% in the studied group (range 20-90%).

TABLE 2 Correlation analysis of lytic gene expression and microenvironment cell subsets from Epstein-Barr virus-positive (EBV+) diffuse large B cell lymphoma (DLBCL) samples

	CD4	CD8	Foxp3	GrB	PD-1	IL-10
BZLF1	−0.67 $P = .02$	0.14 $P = .69$	0.19 $P = 0.56$	−0.17 $P = .60$	−0.38 $P = .23$	0.1 $P = .82$
BHRF1	−0.54 $P = .07$	−0.036 $P = .92$	0.083 $P = 0.81$	−0.24 $P = .46$	−0.403 $P = .19$	0.48 $P = .23$
BLLF1	−0.44 $P = .15$	0.36 $P = .21$	0.36 $P = 0.28$	0.15 $P = .63$	−0.17 $P = .61$	0.071 $P = .87$

P as determined by Spearman rank correlation index. The strength of the correlation is meaningful when absolute *R* value is above 0.6.

mation by EBV lytic cycle in vitro.[15] The direct statistical correlation for the late lytic gene BLLF1 and IFNγ in this series could represent a citotoxic response against this antigen and may be mediated by the CD8+ and/or NK cell populations, in agreement with others. In particular, Abbott et al studied IFNγ production by EBV specific CD8+ cells and found that the relative paucity of late Ag-specific CD8+ T cell responses is particularly striking given the abundant expression of these proteins in lytic-infected cells, and this could be explained by a repressing mechanisms against EBV-specific response.[26] In this regard, we detect that levels of IL-10 transcripts displayed a positive correlation with lytic gene expression, indicating that regulatory and/or immunosuppressive mechanisms could be involved on EBV-associated DLBCL pathogenesis in our series. Unpredictably, this observation did not correlate with the one on IL-10+ cell count. It is conceivable that IL-10 IHC staining could be the result of viral and human IL-10 detection, given that commercially available antibodies targeting this cytokine do not differentiate between them, as reported by others,[27] while qPCR analysis is based upon the use of a specific human IL-10 primer pair that does not include sequences homologous to that of the viral IL-10 gene. Thus, our conclusions are based on the endogenous human IL-10 mRNA expression. An alternative could be to consider that some lymphomas are characterized by oligoclonal B-cell populations, some EBV+ together with EBV-negative. Epstein-Barr virus-infected cells led to abnormal B-cell proliferation, and they could be controlled by the CD8+ T cells, whereas EBV-negative cells could be selected, increasing the EBV-negative clone by which DLBCL may developed, thus escaping from the host immune response.[28] Last of all, we did not detect significant modifications on the rest of the parameters analyzed in the context of BZLF1, BHRF1, and BLLF1 expression, indicating that those cell subpopulations are not specifically involved on EBV-associated DLBCL pathogenesis in our series, as it was previously proposed.[8] The same was observed for TGFβ by qPCR, which expression levels were unaffected by the lytic genes assessed in our cases (Table 3).

In summary, this report shed some light about EBV lytic involvement in EBV+ DLBCL by means of the expression of viral lytic genes and antigens, suggesting that EBV could play an active role in the pathogenesis of this disease. Information about EBV life cycle, its gene expression regulation, and molecular mechanisms of malignant transformation may be essential for a complete understanding of the role of this viral infection in the course of a number of lymphoproliferative diseases. Furthermore, complete lytic reactivation in EBV-positive tumours could potentially kill EBV-positive malignant cells. In consequence, studies designed to disclose EBV lytic cycle contribution to lymphomagenesis and the multiple mechanisms regulating its reactivation could provide a tool to promote tumour cell killing that hopefully leads to the development of minimally toxic, highly specific therapies in EBV-associated malignancies. This is an important point that leads to a better understanding of EBV+ DLBCL pathogenesis and, as a result, the possible application of new treatment strategies.

TABLE 3 Correlation analysis of lytic gene expression and cytokine expression from Epstein-Barr virus-positive (EBV+) diffuse large B cell lymphoma (DLBCL) samples

	IL-10	TGFβ	IFNγ
BZLF1	0.77 $P = .002$	−0.017 $P = .96$	0.51 $P = .078$
BHRF1	0.84 $P < .0001$	0.077 $P = .80$	0.49 $P = .083$
BLLF1	0.65 $P = .017$	0.31 $P = .29$	0.67 $P = .013$

P as determined by Spearman rank correlation index. The strength of the correlation is meaningful when absolute *R* value is above 0.6.

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AUTHOR CONTRIBUTIONS

MC performed experiments, analyzed results, and made the figures; MC and PAC designed the research; AGV contributed with essential information of techniques and helped to made the figures; EDM, FH, FM, and MN performed immunohistochemical staining evaluation; MC and PAC wrote the manuscript; MVP corrected the manuscript.

CONFLICT OF INTEREST

The authors declare no competing financial interests. MC is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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