In vitro assessment of the efficiency of the PIM-1 kinase pharmacological inhibitor as a potential treatment for Burkitt’s lymphoma

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Abstract. Burkitt’s lymphoma is an aggressive form of lymphoma affecting B lymphocytes. It occurs endemically in Africa and sporadically in the rest of the world. Due to the high proliferation rate of this tumor, intensive multi-drug treatment is required; however, the risk of tumor syndrome lysis is high. Overexpression of the proto-oncogene proviral integration of the Moloney murine leukemia virus (PIM-1) kinase is associated with the development of hematological abnormalities, including Burkitt’s lymphoma (BL). PIM-1 primarily exerts anti-apoptotic activities through BAD phosphorylation. The aim of the present study was to investigate the in vitro efficiency of a PIM-1 kinase pharmacological inhibitor (PIM1-1) in BL. The impact of PIM1-1 was evaluated in terms of the viability and apoptosis status of the BL B cell lines, Raji and Daudi, compared with K562 leukemia cells, which highly express PIM-1. Cell viability and apoptotic status were assessed with western blotting, and PIM-1 gene expression was assessed with reverse transcription-quantitative PCR. After 48 h of treatment, PIM1-1 inhibited the Daudi, Raji and K562 cell viability with a half-maximal inhibitory concentration corresponding to 10, 20 and 30 µM PIM1-1, respectively. A significant decrease of ERK phosphorylation was detected in PIM1-1-treated Daudi cells, confirming the antiproliferative effect. The addition of 10 µM PIM1-1 significantly decreased the PIM-1 protein and gene expression in Daudi cells. An inhibition of the pro-apoptotic BAD phosphorylation was observed in the Daudi cells treated with 0.1-1 µM PIM1-1 and 10 µM PIM1-1 decreased BAD phosphorylation in the Raji cells. The apoptotic status of both PIM1-1-treated cells lines were confirmed with the detection of cleaved capase-3. However, no change in cell viability and PIM-1 protein expression was observed in the 10 µM PIM1-1-treated K562 cells. In conclusion, the findings indicated that the PIM1-1 pharmacological inhibitor may have therapeutic potential in BL, but with lower efficiency in leukemia.

Introduction

Burkitt’s lymphoma (BL) is an aggressive B cell non-Hodgkin lymphoma (NHL), occurring in three distinct clinical and epidemiological variants: Sporadic, endemic and immunodeficiency-associated forms. The hallmark of BL is the chromosomal translocation t(8;14), which causes the upregulation of the c-MYC protein transcription factor as well as uncontrolled B cell proliferation, accounting for the rapid growth rate of BL tumor cells (1). c-MYC is stabilized through the phosphorylation function of the human proto-oncogene

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proviral integration of the Moloney murine leukemia virus (PIM-1) serine/threonine kinase, underpinning the critical regulatory role of PIM-1 in the c-MYC-driven BL tumor cell development (2).

The PIM-1 serine/threonine kinase is a highly conserved protein. PIM-1 is expressed in normal lymphoid and myeloid hematopoietic cells and in various human tissues, such as prostate, testis and oral epithelial cells (3). PIM-1 has a constitutive kinase activity that critically regulates cell migration, cell cycle, cell proliferation, cell survival and exerts anti-apoptotic activities (4). Elevated PIM-1 expression predicts a poor outcome in hematological malignancies, including acute myeloid leukemia, B cell chronic lymphocytic leukemia, diffuse large B cell lymphoma and BL (5). PIM-1-overexpression contributes to tumorigenesis through three pathways, including inhibiting apoptosis (i.e., programmed cell death), stimulating cell proliferation and promoting genomic instability (6). For instance, PIM-1 tumorigenic activity has a synergizing effect with c-MYC in lymphomagenesis via the phosphorylation and inactivation of the pro-apoptotic BAD protein, which inhibits the cleavage of the key pro-apoptotic executioner caspase-3 (7).

Several studies reported that highly expressed PIM-1 is co-located with c-MYC in the nucleus of BL B cells, which strongly accelerates c-MYC-driven lymphomagenesis (8,9).

Current anticancer therapies available for BL are associated with life-threatening side effects because of tumor lysis syndrome, especially in older patients with poorer outcomes compared with younger patients (10). Chemoresistance has also been detected in tumor cells overexpressing PIM-1, such as BL B cells (11). PIM-1 kinase is important in BL tumorigenesis and chemoresistance and could be a promising therapeutic target for patients diagnosed with BL. There is a need for more specific and less toxic molecular PIM-1-targeted therapy options for patients with BL. The aim of the present study was to determine the pro-apoptotic effect of a PIM-1 kinase pharmacological inhibitor (PIM1-1) in two BL B cell lines, Daudi and Raji cells, compared with the K562 leukemia cell line that has high levels of PIM-1.

Materials and methods

Cell culture and treatment. The BL (Raji and Daudi) and leukemia (K562) cell lines were purchased from the American Type Culture Collection and authenticated by the supplier. The cells were cultured in complete medium consisting of RPMI-1640 with 2 mM L-glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin and supplemented with 10% fetal calf serum, (all Gibco; Thermo Fisher Scientific Inc.). The cells were seeded in T-75 cm² flasks and maintained in a humidified incubator under standard conditions (37°C; 5% CO₂).

PIM-1 (cat. no. 18/144326; 10 mM stock solution; Tocris Bioscience) was solubilized in DMSO and diluted in RPMI-1640. The cells were exposed to various concentrations (0.1, 1, 10, 20, 30 and 40 µM) of PIM-1 and incubated for 48 h. DMSO 0.4% and 1 µM of the protein kinase inhibitor staurosporine (STS) were used as negative and positive controls, respectively.

Cell viability. B cell lines (Raji and Daudi) and leukemic cell line (K562) (10⁴) were seeded in 100 µl complete RPMI-1640 medium per well of 96-well plates. The cell treatment was applied as aforementioned and was performed in triplicate. The number of viable cells were determined using the CellTiter-Glo® Luminescent Assay kit (Promega Corporation) and based on the quantification of ATP generated from metabolic reactions, which was indicative of active cells. The amount of ATP produced was proportional to the number of viable cells. The inhibitory concentration (IC)-50 of PIM-1 that resulted in a 50% decrease in the viable cell number was also calculated.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from the untreated and treated cells (1.5x10⁵/cm²) using the PureLink™ RNA Mini kit (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. High-quality RNA (1 µg) was reverse transcribed to cDNA using an Applied Biosystems™ High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The cDNA was used as the template for the quantitative PCR reaction. RT-qPCR was performed through real-time monitoring of the increase in fluorescence of the SYBR® Green dye (cat. no. 4309155; Qiagen GmbH), using the 7900 Fast Real-Time PCR system (Thermo Fisher Scientific, Inc.). The primer pair sequences (Invitrogen; Thermo Fisher Scientific) were used as previously described (12). The thermocycling conditions were as follows: Heat denaturing at 95°C for 10 min, then by 40 cycles of denaturing at 95°C for 10 sec followed by annealing at 57°C for 20 sec and finally extension at 72°C for 30 sec. The fold-change of PIM-1 RNA expression levels measured by the cycle threshold (Cq) values were calculated and normalized to the expression levels of the housekeeping gene GAPDH, according to 2^-ΔΔCq method (13) as follows: Fold-change=2^-ΔΔCq with ΔΔCq=ΔCq (PIM-1_treated-GAPDH_treated)-ΔCq (PIM-1_control-GAPDH_control).

Western blotting. The untreated and treated Daudi, Raji and K562 cells were lysed in Nonidet P40 (NP40) Cell Lysis Buffer composed of 50 mM Tris (pH 7.4), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM NaVO₄, 1% NP40 and 0.2% NaN₃ (all Invitrogen; Thermo Fisher Scientific, Inc.). The lysates were separated from the cell debris through centrifugation at 20,000 x g for 15 min at 4°C and the supernatant was collected. The protein concentrations were quantified using the Qubit™ Protein Assay kit (Thermo Fisher Scientific, Inc.). Total proteins (80 µg per lane) were separated using 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane as described previously (12). The membranes were blocked for 1 h with 3% BSA at room temperature and probed, overnight at 4°C, with mouse or rabbit primary monoclonal antibodies (1:1,000) directed against phosphorylated (p)-BAD (Ser112; cat. no. 5284), total BAD (cat. no. 9239), pro-caspase-3 (cat. no. 14220) and cleaved caspase-3 (cat. no. 9664) procured from Cell Signaling Technology, Inc., p-ERK1 (Tyr204 of ERK1, cat. no. sc-7383), total ERK1 (cat. no. sc-271269), PIM-1 (cat. no. sc-13513) obtained from Santa Cruz Biotechnology, Inc. and GAPDH (cat. no. ab8245) from Abcam. The membranes were washed three times in Tris-buffered saline containing 0.1% Tween-20 (pH 7.4) and incubated with either goat anti-mouse (1:5,000; cat. no. 170-5047; Bio-Rad...
Inc.) or anti-rabbit (1:5,000; cat. no. 170-6515; Bio-Rad Inc.) secondary antibodies conjugated to horseradish peroxidase or with either infrared fluorescent IRDye® 680RD-conjugated goat anti-rabbit (1:10,000; cat. no. 926-68071; Li-COR Biosciences) or IRDye® 800RD-conjugated goat anti-mouse (1:10,000; cat. no. 926-32210; Li-COR Biosciences) secondary antibodies, for 1 h at room temperature. The bands were visualized by incubating the horseradish peroxidase-stained membranes with enhanced chemiluminescence reagents (Clarity™ Western ECL Substrate; Bio-Rad Laboratories, Inc.) and scanned with c-DiGit (Li-COR Biosciences). The IRDye stained membranes were scanned using a LI-COR Odyssey® CLX Scanner. The protein expression level was quantified using ImageJ software v.1.46r (http://rsbweb.nih.gov/ij/index.html).

**Statistical analysis.** All the data are expressed as mean ± standard deviation and each experiment was independently repeated three times. For comparison between the groups, one-way ANOVA with Tukey's HSD post hoc test was used. SPSS Statistics 64-bit MS Windows v.22.0.0.0 (IBM, Corp.) was used to analyze the data, and for gene expression levels, the Applied Biosystems™ QuantStudio™ 6 Flex system (Thermo Fisher Scientific, Inc.) was used. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**PIM1-1 inhibits Raji, Daudi and K562 cell viability.** To study the inhibitory effect of PIM1-1 on Raji, Daudi and K562 cell viability, the cells were incubated for 48 h with different concentrations of PIM1-1 (range, 0.1-40 µM), with 0.4% DMSO (negative control) and 1 µM STS (positive control). The untreated and treated cells were subjected to a luminescent cell viability assay using CellTiter-Glo™. As shown in Fig. 1, a slight increase of Raji and Daudi cell viability was observed at low concentrations of PIM1-1 at 0.1 and 1 µM; indicating a slightly higher amount of ATP generated by treated cells compared with the ATP amount generated by untreated cells. At higher concentrations, PIM1-1 significantly decreased the Daudi cell viability by ~50% at 10 µM (P<0.0028) and by 98% at 40 µM (P<0.0001). In the Raji cells, PIM1-1 significantly decreased cell viability by 50% (P=0.0021) at 20 µM and by ~85% (P<0.0001) at 40 µM. However, for K562 cells, a 48.8% decrease in viability was measured at 30 µM PIM1-1 (P=0.015) and 44.8% at 40 µM (P=0.0051). Increasing concentrations of PIM1-1 decreased the Raji, Daudi and K562 cell viability in a dose-dependent manner. As estimated from the sigmoidal dose curve with a variable slope, the IC50 for the Daudi, Raji and K562 cells were at 10, 20 and 30 µM of PIM1-1, respectively (data not shown). The PIM-1 kinase pharmacological inhibitor PIM1-1 inhibited the cell growth of the BL B cell lines (Raji and Daudi), and the growth of leukemic cell line K562 was the least affected (Fig. 1).

To confirm the decrease of cell proliferation, the level of p-ERK in the untreated cells and cells treated with DMSO, STS or (0.1, 1.0 or 10 µM) PIM1-1 were assessed using western blotting. In the Raji cells, any addition of PIM1-1 slightly decreased p-ERK1 compared with the untreated cells (Fig. 2). However, a significant decrease in the level of p-ERK1 was observed in the Daudi cells treated with 10 µM of PIM1-1 compared with the untreated cells. Used as a positive control, the protein kinase inhibitor STS, when added to Raji and Daudi cells, resulted in a significant decrease of the level of p-ERK1 compared with the p-ERK1 expression level detected in the untreated cells (Fig. 2). No change was observed in the level of p-ERK1 in the K562 cells in all the conditions used. Hence, various antiproliferative effects of PIM1-1 on the BL B cell lines Raji and Daudi were confirmed by the variation of the decrease in the levels of p-ERK1 measured in each PIM1-1-treated B cell line. At the low concentrations at which the PIM1-1 was tested (0.1-10 µM), the leukemic cell growth based on p-ERK1 level remained unchanged.

**PIM1-1 induces the downregulation of PIM-1 and BAD phosphorylation in the Daudi and Raji cell lines.** PIM-1 negatively regulates its own expression (14). PIM-1 kinase inhibitor efficiency was assessed based on the PIM-1 kinase expression level. At the protein level, using western blotting, PIM1-1 tested between 0.1 and 10 µM did not significantly decrease PIM-1 protein expression in PIM1-1-treated Raji cells (Fig. 3). However, at 1 and 10 µM, PIM1-1 significantly decreased PIM-1 protein expression level by 70% (P<0.01) in Daudi cells, compared with the expression level detected in untreated Daudi cells (Fig. 3A). For the K562 cells, PIM1-1 did not change the PIM-1 protein expression level compared with untreated cells (Fig. 3). STS significantly inhibited PIM-1 protein expression level by 85% in both the Raji and Daudi cells, but not in K562 cells (Fig. 3A). As expected, DMSO did not affect the PIM-1 expression level in all types of cells compared with untreated cells (Fig. 3).

PIM-1 kinase phosphorylates the pro-apoptotic protein BAD and promotes BAD inactivation, which supports cell survival (7,15). The level of p-BAD was evaluated to confirm the variation of PIM-1 kinase expression observed in the PIM1-1-treated cells. Notably, a significant decrease in p-BAD was observed in the Raji cells treated with 10 µM PIM1-1 compared with the level detected in the untreated cells (Fig. 3). A significant decrease and quasi-disappearance of p-BAD was observed in Daudi cells treated with 1 and
10 µM of PIM-1 compared with the untreated cells (Fig. 3). As expected, STS decreased p-BAD levels in both Raji and Daudi cells, but DMSO did not affect p-BAD in the three cell lines (Fig. 3). In all the conditions applied, the level of p-BAD in the K562 cells did not change, compared with the untreated cells (Fig. 3).

To verify the downregulation of the PIM-1 protein expression observed in PIM1-1-treated Daudi cells, PIM-1 mRNA expression level was examined in Daudi cells treated with 10 µM PIM1-1. A significant decrease (~70%; P=0.0003) of the PIM-1 mRNA expression level was observed in PIM1-1-treated Daudi cells compared with the untreated and DMSO-treated cells (Fig. 4).

Detection of cleaved caspase-3 in PIM1-1-treated Raji and Daudi cells. At the functional level, the blockade of PIM-1 kinase activity principally results in the induction of apoptosis (16). After Raji and Daudi cell treatment with various concentrations (range, 0.1-10 µM) of PIM1-1, the protein extracts were subjected to western blotting for the detection of cleaved caspase-3, a hallmark of apoptosis (17). A weak detection of cleaved caspase-3 was observed in the PIM1-1-treated Raji cells; however, cleaved caspase-3 was observed in the Daudi cells treated with 10 µM PIM1-1 (Fig. 5). As expected, no cleavage of pro-caspase-3 was observed in the DMSO-treated cells but some cleavage occurred in STS-treated cells (Fig. 5).

Discussion

In hematopoietic cells, PIM-1 is involved in the development and function of the cells. The overexpression of PIM-1 kinase is found in the majority of hematological malignancies, including myeloid and lymphoid leukemia and B cell NHL (18). PIM-1 overexpression contributes to malignant cell proliferation and through dysregulation of the cell cycle and inhibition of apoptosis (19,20). Several studies have investigated the effect of PIM-1 downregulation and its impact on cell survival and apoptosis (16,21). The results of the present study established a connection between PIM-1 inhibition using the PIM-1 pharmacological inhibitor, PIM1-1, and the inhibition of cell survival and increased apoptosis, suggesting that targeting PIM-1 kinase is a potentially promising therapeutic approach in BL. Small molecule inhibitors of PIM kinases have been investigated in preclinical and clinical studies to treat hematological and solid cancer types (21-23). The selective PIM1-1 drug investigated in the present study belongs to the pyridone-based family of small molecules inhibitors of PIM-1 kinase which has been shown to exert an inhibitory effect by binding to the ATP-binding site of
the PIM-1 kinase, suggesting an ATP-competitive inhibitory mechanism (24). Targeting the kinase action of PIM-1 is expected to prevent the phosphorylation of the downstream effectors and to block its capacity to activate or inactivate proteins involved in cell cycle progression and apoptosis, such as BAD (7,15).

In the present study, the treatment of the BL B cell lines, Raji and Daudi, with the novel PIM-1 inhibitor PIM1-1 resulted in decreased cell viability. In addition, a significant decrease of ERK-1 phosphorylation was detected in PIM1-1-treated Daudi cells, confirming the PIM1-1 antiproliferative effect in the BL B cell lines. The current study showed that inhibiting PIM-1 kinase in Daudi and Raji cells with PIM1-1 resulted in a decrease in BAD phosphorylation and induction of apoptosis, revealed by caspase-3 cleavage. These data highlighted the promising therapeutic potential of PIM1-1 against BL B cell development.

PIM-1 is involved in cell proliferation through the regulation of cell cycle progression and decreased apoptosis (19,25). The current study showed that increasing concentrations of PIM1-1 decreased the Daudi and Raji cell viability in a dose-dependent manner; however, the leukemia cell line K562 was less affected. Based on the IC50 determination, Daudi cells were more sensitive to PIM1-1 inhibition compared with Raji and K562 cells. This observation can be explained by the differential PIM-1-expression levels between both the BL B cell lines and the leukemia cells, with the Daudi cells expressing less PIM-1 compared with the Raji cells (3). The K562 cells, used as a positive control for their high-level protein expression of PIM-1 as previously reported in (3,12), were the least responsive to the PIM1-1 inhibitor. A previous study has shown that quercetagetin (a PIM-1 inhibitor) inhibits cell viability and decreases the colony formation rate of nasopharyngeal carcinoma cells (25). These results are in line with a previous study in which the inhibition of PIM-1 with K00135 [imidazo (1,2-b) pyridazines] in murine Ba/F3 cells, acute leukemia cells, and primary blasts from patients with acute myeloid leukemia, selectively reduces cell survival and suppressed the colony proliferation of leukemic blasts (26). In the present study, the decrease in BL B cell line
viability caused by PIM-1 was confirmed by decreased p-ERK, a key signaling protein involved in cell survival and proliferation (27,28). A previous study, conducted with prostate cancer cells, reported that PIM-1 knockdown results in a decrease of p-ERK (29). It is not yet clear whether PIM-1 kinase directly or indirectly affects ERK phosphorylation. However, it would be of interest to investigate the impact of the PIM-1 kinase inhibitor on substrates with similar regulatory survival pathways, such as ERK and phosphatidylinositol 3-kinase/protein kinase B (PKB also known as Akt) as well (30,31). In addition, Akt/PKB has been demonstrated to be activated in two acute myeloid leukemia cell lines intrinsically resistant to the pan-PIM kinase inhibitor AZD1208, following to elevation of mitochondrial reactive oxygen species (ROS) (32). Furthermore, ROS have been recently reported to be important indicators of drug resistance (33). An assessment of Akt/PKB phosphorylation levels and ROS generated in the BL B cells and leukemic cells could reveal their resistance potential to any PIM-1 kinase inhibitor, including PIM-1. Altogether, a combination of pharmacological inhibitors targeting survival pathway signaling proteins, ROS production and PIM-1 kinase could enhance the sensitivity of the BL B cells and leukemic cells to cancer therapy.

In addition to acting as a survival factor, PIM-1 kinase also plays a role as both a transcription factor and an activator of several transcription factors, including the signal transducer activator transcription-3, which stimulates PIM-1 gene expression (3). In the current study, 10 µM of the PIM1-1 pharmacological inhibitor reduced the PIM-1 mRNA and protein expression levels in the Daudi cells, with no change in the PIM1-1-treated Raji cells compared with the untreated cells. These findings provide evidence for the differential expression of PIM-1 in the Daudi and Raji BL B cell lines, confirming that the Daudi cells are the most responsive to PIM1-1.

PIM1-1 deactivates the pro-apoptotic BAD protein by phosphorylation of Ser112, and changes in the level of this phosphorylation indicates variation of PIM-1 kinase activity (15). BAD is a pro-apoptotic member of a Bcl-2 group that assists with cell death. The phosphorylation of BAD enhances the binding of BAD to 14-3-3 proteins to block the associations between BAD with the anti-apoptotic proteins Bcl2 and Bcl-xL (34). It has been reported that PIM-1 may play a critical role in the control of survival signaling through the inhibition of mitochondrial pro-apoptotic members of the Bcl-2 family, such as BAD (15,35). In the present study, administration of the PIM1-1 inhibitor caused a significant reduction in the BAD phosphorylation level in the Daudi cells from 1 µM of PIM1-1, while the Raji cells showed a reduction on the p-BAD expression level at 10 µM of PIM1-1. These findings are similar to those reported by Chen et al (16) in 2011 who
demonstrated the downregulation of p-BAD in acute myeloid leukemia cells treated with the pan-PIM inhibitor SGI-1776. Forshell et al (9) also reported in 2011 that treating Myc-induced B cell lymphoma with the pan-PIM kinase inhibitor (named as Pim1) caused the dephosphorylation of BAD and the induction of apoptosis. The reduction in PIM-1 expression observed in the current study may have contributed to the decrease in BAD phosphorylation and to the induction of apoptosis, revealed by caspase-3 cleavage. Although executioner caspases, including caspase-3, function in natural cancer cell death (36), PIM-1 phosphorylates the endogenous apoptosis signaling kinase (ASK)1, which suppresses the activation of pro-caspase-3 and maintains cancer cell survival (37). In the present study, inhibition of PIM-1 kinase allowed caspase-3-activation in BL B cell lines, which resulted in strong cleaved caspase-3 expression in Daudi cells and weak expression in the Raji cells after treatment with 1-10 μM PIM1-1. An assessment of ASK1 phosphorylation level using western blotting, expected to decrease following BL B cell treatment with PIM1-1, could confirm apoptosis induction through caspase activation.

In conclusion, the present study demonstrated that the novel PIM1-1 pharmacological inhibitor effectively downregulated PIM-1 expression, markedly decreased cell viability and induced apoptosis, which was revealed by caspase-3 cleavage, in BL B cell lines. These findings provide new evidence for PIM-1 kinase inhibition as a potential therapeutic target for BL treatment. Due to the limited number of cell lines used in the current study, it would be interesting to evaluate the effects of PIM1-1 using other B cell lymphoma cell lines or animal models. Studying the effect of PIM1-1-knockdown on other genes involved in cell proliferation, survival, homing, cell signaling, apoptosis and migration is also required. Future studies should examine the effect of PIM1-1 on primary BL cells obtained from patients and to analyze the chromosomal translocation frequency and c-MYC expression levels, which are the main genetic hallmarks of BL (38). In addition, the synergistic effect between PIM1-1 and current chemotherapies should be evaluated to reduce resistance to chemotherapy and to improve the response to the available therapies, which could provide an effective treatment and improve the survival rate for patients diagnosed with BL.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contribution

IA and AhA conceived the study. IA, SMN and AbA conducted the study. MoA, HAE, SA, KA and MaA conducted the experiments, collected the data, analyzed the data and reviewed the manuscript. Authenticity of all raw data were confirmed by SMN and AbA. MoA, SMN, AbA, AhA and IA wrote the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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