IJMS
Vol 49, No 2, February 2024

Original Article

Epstein-Barr Virus Promotes Tumorigenicity and Worsens Hodgkin Lymphoma Prognosis by Activating JAK/STAT and NF-κB Signaling Pathways

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Received: 19 November 2022
Revised: 25 April 2023
Accepted: 30 March 2023

What's Known

- Epstein-Barr virus (EBV) activates JAK/STAT and NF-κB pathways in patients with EBV-positive Hodgkin lymphoma (HL).
- The role of EBV in the prognosis of classical HL remains ambiguous and controversial.

What's New

- EBV promotes the expression of pro-apoptotic and survival immortalization proteins of NF-κB and JAK2/STAT1 pathways. EBV-positive is associated with poor clinicopathological and survival outcomes in HL patients.
- Therapeutic targeting of NF-κB and JAK2/STAT1 pathways could potentially contribute to the treatment of patients with EBV-associated CHL.

Abstract

Background: Epstein-Barr virus (EBV) is detected in 40% of patients with Hodgkin lymphoma (HL). During latency, EBV induces epigenetic alterations to the host genome and decreases the expression of pro-apoptotic proteins. The present study aimed to evaluate the expression levels of mRNA molecules and the end product of proteins for the JAK/STAT and NF-κB pathways, and their association with clinicopathological and prognostic parameters in patients with EBV-positive and -negative classical Hodgkin lymphoma (CHL).

Methods: A prospective cohort study was conducted from 2017 to 2022 at the Faculty of Medicine, Zagazig University Hospital (Zagazig, Egypt). Biopsy samples of 64 patients with CHL were divided into EBV-positive and EBV-negative groups. The expression levels of mRNA molecules (JAK2, STAT1, IRF-1, PD-L1, IFN-γ, NF-κB, Bcl-xL, COX-2) and the end product of proteins (PD-L1, Bcl-xL, COX-2) were determined and compared with clinicopathological and prognostic parameters. Data were analyzed using the Chi square test and Kaplan-Meier estimate.

Results: EBV-positive CHL patients were significantly associated with positive expression of mRNAs molecules ($P<0.001$) and the end product of proteins ($P<0.001$) for the JAK/STAT and NF-κB pathways, B-symptoms ($P=0.022$), extra-nodal involvement ($P=0.017$), and advanced stage of CHL ($P=0.018$). These patients were more susceptible to cancer progression, higher incidence of relapse ($P=0.008$), poor disease-free survival rate ($P=0.013$), poor overall survival rate ($P=0.028$), and higher mortality rate ($P=0.015$).

Conclusion: Through the activation of JAK/STAT and NF-κB signaling pathways, EBV-positive CHL is associated with poor clinicopathological parameters, higher incidence of disease progression, relapse, and poor overall survival.

A preprint of this manuscript is available on research square (doi: 10.21203/rs.3.rs-1857436/v1).

Please cite this article as: Alabiad MA, Said WMM, Saad RHF, Balata R, Mahmoud AA, Metwally EA, Shalaby AM, Samy W, Yehia AM, Yahia AIO, Alorini M, Abdelrahman DI. Epstein-Barr Virus Promotes Tumorigenicity and Worsens Hodgkin Lymphoma Prognosis by Activating JAK/STAT and NF-κB Signaling Pathways. Iran J Med Sci. 2024;49(2):88-100. doi: 10.30476/IJMS.2023.97287.2896.

Keywords ● Hodgkin disease ● Epstein-Barr virus infections ● Janus kinases ● NF kappa B

Introduction

Hodgkin lymphoma (HL) is an uncommon B-cell lymphoid malignancy that affects 10% to 15% of all lymphoma patients. In

the early and confined stages of cancer, regular chemotherapy and radiation treatment can cure about 80% to 90% of HL patients. Large multinucleated Reed-Sternberg cells within the inflammatory background are the main feature of HL.[1] Several risk factors are associated with the development of HL such as autoimmune diseases, low socioeconomic status, familial congenital and acquired immunodeficiency, a positive family history of HL or other lymphoid neoplasms, and those with latent Epstein-Barr virus (EBV) infection.[2]

Understanding how EBV activates cellular signal transduction pathways could aid in the development of new therapeutic drugs for EBV-associated HL. Latent membrane protein 1 (LMP1) is the main oncogenic protein of EVB and has transmembrane spanning domains that allow LMP1 complexes to cluster and construct binding sites for cellular signaling adaptor proteins, resulting in constitutive activation of several pathways. EBV tumorigenicity is linked to six different types of cell signaling pathways necessary for the growth and survival of malignant cells, namely nuclear factor kappa B (NF-κB), Janus kinase/signal transducer, and transcription activator (JAK/STAT), phosphoinositide-3-kinase/protein kinase B (PI3K/Akt), mitogen-activated protein kinase (MAPK), transforming growth factor beta (TGF-β), and Wnt/β-catenin. Of these, PI3K/AKT, MAPK, TGFβ, and Wnt/βcatenin are mainly involved in the mechanism of epithelial-mesenchymal transition (EMT) and tumor angiogenesis of EBV-associated nasopharyngeal carcinoma.[3]

NF-κB and JAK/STAT are the main signaling pathways involved in EBV-associated lymphoma. LMP1 promotes tumorigenesis by activating different mechanisms that result in the overexpression of different survival-promoting proteins such as programmed death ligand 1 (PD-L1) immune checkpoint, and B-cell lymphoma extra-large (Bcl-xL) and cyclooxygenase-2 (COX-2) anti-apoptotic proteins. LMP1 stimulates the production of activator protein 1 (AP-1) transcription factor, which is a direct promoter of PD-L1. LMP1 mediates its immortalization signaling through active secretion of interferon-gamma (IFN-γ). When IFN binds to its receptor, it causes a rapid development and significant increase of the IFN-γ receptor (IFNGR) heterotetrameric complex on tumor cell membranes composed of IFNGR1 and IFNGR2, which then activates the JAK/STAT and NF-κB pathways.[4]

Apoptosis is a form of programmed cell death to remove damaged or unnecessary cells. Many tumors have the capacity to evade apoptosis. The main apoptotic pathways are extrinsic (death receptor-mediated) and intrinsic (mitochondrial-mediated). The extrinsic pathway is activated when TNF receptor 1 or FAS is activated by specific death ligands. The intrinsic apoptotic pathway was extensively studied and therapeutically targeted, as it significantly affects the etiology and resistance of various hematologic malignancies to treatment. The intrinsic pathway is activated by various cellular stresses such as DNA damage, hypoxia, and oxidative stress.[5]

Bcl-xL belongs to the Bcl-2 family of anti-apoptotic proteins. It is a transmembrane protein found in mitochondria and a potent inhibitor of Bcl-2-associated X protein (BAX). It safeguards the integrity of the mitochondrial outer membrane from degradation and subsequent inhibition of the cytoplasmic release of cytochrome to prevent an apoptotic cascade of cytochrome, Apaf-1, and caspase-9 complex, which induces apoptosome activation of caspase-3. The latter stimulates cytoplasmic endonucleases (CAD/ICAD) and proteases, resulting in chromosomal DNA breakdown, chromatin condensation, cytoskeletal remodeling, and cell disintegration. Upregulation of Bcl-xL expression in tumor cells can cause resistance to chemotherapy by enhancing the survival signals of tumor cells.[6]

COX-2 is an enzyme associated with inflammation and a pathway catalyzing the synthesis of prostaglandins (PGs) from arachidonic acid. COX-2 is a cytoplasmic protein not detectable in most normal tissues and is activated by inflammatory and mitogenic stimuli. The COX-2/PGE2 pathway inhibits the two major apoptosis pathways by suppressing signaling through death receptors and mitochondria. Moreover, it increases the expression of anti-apoptotic protein Bcl-2 by activating the Ras-MAPK/ERK pathway. COX-2 is an important angiogenic stress response protein involved in the carcinogenesis and progression of several solid and hematologic malignancies.[7] Given that lymphoma is the sixth most common form of cancer, it is important to develop novel therapeutic drugs to achieve prolonged remission and improve survival.[8] A possible candidate might be non-steroidal anti-inflammatory drugs (NSAIDs), a classic inhibitor of COX-2. Several epidemiological studies revealed that people who regularly take NSAIDs have a lower risk of HL than non- or infrequent users.

Tumor cells express PD-L1 as an adaptive immune response to avoid immunosurveillance. Several types of host cells (e.g., fibroblasts, T-cells, dendritic cells, and macrophages) in the tumor microenvironment (TME) increase the

expression of PD-L1 to further evade antitumor immune response. Therefore, PD-L1 acts as a pro-tumorigenic factor in cancer cells by binding to its receptors and activating proliferative and survival signaling pathways. Inhibition of the PD-L1/PD-1 signaling pathway by antibodies reactivates exhausted immune cells in TME and eradicates cancer cells.[9]

Following the success of immunotherapy drugs to treat refractory solid tumors, immune checkpoint inhibitors have attracted attention as a promising method to treat relapsed lymphoma. PD-L1 modulates the intensity and duration of T-cell activation. Several antibodies blocking PD-1/PD-L1 have been developed as potential drugs to treat a wide range of solid tumors such as bladder, kidney, melanoma, and lung cancers.[10]

Given the above, the present study aimed to evaluate the expression of mRNA molecules and the end product of proteins for the JAK/STAT and NF-κB pathways. To determine their validity as a potential immunotherapeutic target, their expression levels were then compared with clinicopathological and prognostic parameters in patients with EBV-positive and EBV-negative classical Hodgkin lymphoma (CHL).

Patients and Methods

A prospective cohort study was conducted from 2017 to 2022 at the Faculty of Medicine, Zagazig University Hospital (Zagazig, Egypt). The study included 64 CHL patients admitted to the Department of General Surgery for surgical biopsy followed by histopathological diagnosis by the Department of Pathology. These patients were then referred to the Department of Clinical Oncology and Medical Oncology to receive a standard treatment regimen based on the CHL stage and according to the recommended guidelines. Patients were followed up every three months during the first two years and then every six months in the following three years. Clinical follow-up included physical examination, CT scan, and PET-CT scan.

A small amount of each biopsy sample was frozen in liquid nitrogen to detect Epstein-Barr nuclear antigen 1 (EBNA-1) using nested polymerase chain reaction (PCR). Moreover, quantitative real-time PCR (qRT-PCR) was used to detect the mRNA expression levels of JAK2, STAT1, IRF-1, PD-L1, IFN-γ, NF-κB, Bcl-xL, and COX-2. The remainder of each sample was fixed in 10% formalin and stained using hematoxylin and eosin (H&E) for PD-L1, COX-2, and Bcl-xL immunohistochemistry. After light microscopic examination, all samples were categorized into histologic subtypes according to the World Health Organization classification of lymphoid neoplasms.[11]

The study was carried out in accordance with the World Medical Association Declaration of Helsinki 2000 (fifth revision) guidelines for human studies. In addition, the study was approved by the Ethics Committee of Zagazig University (number: ZU-IRB#9901). Biopsy samples were taken after obtaining consent from patients or their legal representatives.

Treatment

Patients were treated in accordance with the National Comprehensive Cancer Network (NCCN) 2022 clinical practice guidelines.[12] Patients received chemotherapy alone or combined-modality therapy depending on the CHL stage and risk factors. Most patients received a standard dose of ABVD (adriamycin, bleomycin, vinblastine, and dacarbazine) every two weeks (per four-week cycle) with dose modification or delay depending on toxicity. Other regimens, such as DHAP (dexamethasone, cisplatin, cytarabine) or ICE (ifosfamide, carboplatin, etoposide) were administered to most patients with lymphoma during the first relapse or refractory to initial therapies.[13] Megavoltage radiation therapy was performed, and a total dose ranging from 30 to 36 Gy was administered in daily fractions of 1.8 to 2.0 Gy.[14]

The bulky disease is denoted as tumors in the chest with a width of at least one-third of the chest diameter or tumors in other areas that are at least 10 cm (about 4 inches) in diameter. Ann Arbor staging system was used to classify HL.[15] The term “B symptoms” refers to symptoms of unexplained fever, excessive night sweats, or loss of more than 10% of body weight within the preceding six months. Progression-free survival (PFS) is defined as the time (in months) from the start of treatment until disease progression or death. Disease-free survival (DFS) is defined as the period during which the patient lives without signs or symptoms of the disease, measured from the end of treatment to the date of relapse or death. Overall survival (OS) refers to the length of time (in months) from diagnosis or last follow-up to the date of death.

Epstein-Barr Virus Detection

EBV was detected by extracting viral DNA from fresh CHL biopsy samples using QIAamp, Qiagen one-step RT-PCR kit (QIAGEN, USA), according to the manufacturer’s instructions. EBNA-1 gene in all samples was targeted with two sets of outer and inner primers using nested PCR technique.[16]

Real-time Polymerase Chain Reaction

The total RNA was extracted using TRIzol reagent (Invitrogen Inc., Carlsbad, CA, United States). Reverse transcription of miRNA was performed using a transcription kit (Shanghai GeneChem Co., Ltd., Shanghai, China) with RNA U6 as an internal control. mRNA was detected using a reverse transcription kit (Takara Biotechnology Ltd., Dalian, Liaoning, China) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence as an internal reference for the normalization of RT-PCR. SYBR Green quantitative PCR analysis was performed using a 7500 real-time fluorescence quantitative PCR system. The expression levels of target genes were then estimated using the $2^{-\Delta\Delta CT}$ method.[17, 18] Primers STAT1 (#HP210040), JAK2 (#HP208201), IRF-1 (HP205934), PD-L1 (#HP210654), IFN-γ (#HP200586), NF-κB (#HP207409), Bcl-xL (#HP234144), COX-2 (#HP200900), GAPDH (#HP205798), and β-catenin (#KN208947) were purchased from OriGene Technologies (Beijing, China). The sequence of primers is presented in table 1.

Immunohistochemistry

The primary antibodies used were rabbit monoclonal PD-L1 (clone: CAL10, 1:100 dilution) purchased from Biocare Medical (Concord, CA, USA), and rabbit monoclonal COX-2 (clone: SP21, 1:100 dilution) and rabbit monoclonal Bcl-xL (clone: 7D9, 1:200 dilution); both purchased from Invitrogen (Thermo Fisher Scientific, USA).

The tissue slides were deparaffinized in a 56 °C oven for 15 min, 3-5 µm thick paraffin-embedded tissue sections were fixed on positively charged slides, and then washed in xylene for 30 min. The slides were hydrated in descending alcohol series (concentrations of 95%, 85%, and 75%) for five min. The samples were then washed with phosphate-buffered saline (PBS) for five min.[19] Antigen retrieval was performed by microwaving the samples for 20 min in a ready-to-use Dako target retrieval solution (PH=6.0). Using a lint-free tissue (gauze pad), the residual liquid around the sample was carefully removed to keep the reagent within the defined area.[20-24] To inhibit endogenous peroxidase, tissue sections were treated with 3% hydrogen peroxide, incubated for five min, and then carefully rinsed with distilled water.

All primary antibodies were antigen retrieved by microwaving a 10 nM citrate buffer (pH=6.0) for 15 min. All primary monoclonal antibodies were incubated in the tissue sections at room temperature for two hours. The tissue specimens were washed in PBS and incubated with biotin-conjugated secondary antibody for one hour. They were then immersed in biotinylated anti-mouse immunoglobulin and incubated at room temperature for 15 min and subsequently washed in the buffer.[16, 23, 25-27] Streptavidin-HRP was added to the tissue slides and washed after 15 min. They were then treated with diaminobenzidine (DAB) substrate, incubated for 5 to 10 min, and then gently washed with distilled water. The slides were submerged in Mayer's hematoxylin solution and incubated for two to five min, depending on the hematoxylin strength. DPX was used as mounting medium, and the tissue slides were carefully mounted

Table 1: The sequence of primers used for the real-time polymerase chain reaction

Primer	Sequences
EBNA-1 inner	Forward: 5`-AGATGACCCAGGAGAAGGCCCAAGC-3` Reverse: 5`-CAAAGGGGAGACGACTCAATGGTGT-3`
EBNA-1 outer	Forward: 5`-GTAGAAGGCCATTTTTCCAC-3` Reverse: 5`-CTCCATCGTCAAAGCTGCA-3`
JAK2	Forward: 5′- CCAGATGGAAACTGTTCGCTCAG-3′ Reverse: 5′- GAGGTTGGTACATCAGAAACACC-3′
STAT1	Forward: 5′-ATGGCAGTCTGGCGGCTGAATT-3` Reverse: 5′-CCAAACCAGGCTGGCACAATTG-3`
IRF-1	Forward: 5′-GAGGAGGTGAAAGACCAGAGCA-3` Reverse: 5′-TAGCATCTCGGCTGGACTTCGA-3`
PD-L1	Forward: 5′-TGCCGACTACAAGCGAATTACTG-3' Reverse: 5′-CTGCTTGTCCAGATGACTTCGG-3'
IFN-γ	Forward: 5′-GAGTGTGGAGACCATCAAGGAAG-3' Reverse: 5′-TGCTTTGCGTTGGACATTCAAGTC-3'
NF-κB	Forward: 5′-GCAGCACTACTTCTTGACCACC-3' Reverse: 5'-TCTGCTCCTGAGCATTGACGTC-3'
Bcl-xL	Forward: 5'-GCCACTTACCTGAATGACCACC-3' Reverse: 5'-AACCAGCGGTTGAAGCGTTCCT-3'
COX-2	Forward: 5'-CGGTGAAACTCTGGCTAGACAG-3' Reverse: 5'-GCAAACCGTAGATGCTCAGGGA-3'
GAPDH	Forward: 5'-CAACGGATTTGGTCG-TATTGG-3' Reverse: 5'GCAACAATATCCACTTTAC-CAGAGTTAA3'

with a coverslip after clearing in three changes of xylene.

Immunohistochemical Evaluations

Hodgkin Reed-Sternberg (HRS) tumor cells and peritumoral cells were independently evaluated. Tumor cell positivity was defined as staining of >5% of tumor cells, whereas microenvironment positivity was defined as staining of >20% of the entire cell population, regardless of the staining intensity.[16] COX-2 was evaluated by dividing the number of positively stained HRS cells by the total number. The percentage was recorded in five representative fields at ×400, considering that more than 10% of HRS cells express COX-2 as positive.[28] Bcl-xL was labeled as brown granular cytoplasmic staining of HRS cells, and apoptotic index (AI) was calculated as the percentage of HRS-positive cells in 10 randomly selected fields without sclerosis or fibrosis at ×400. HRS-negative was defined, when <5% of these cells were positive.[29]

Statistical Analysis

Data were analyzed using GraphPad Prism 7.0 software (GraphPad Software, Boston, MA, USA).

Table 2: Association between clinicopathological parameters and EBV-positive and -negative patients

Clinicopathological parameters and outcomes		Total	EBV negative (n=30)	EBV positive (n=34)	P value
		n (%)			
Age group	<45 years	44 (68.8)	19 (43.2)	25 (56.8)	0.380
	≥45 years	20 (31.3)	11 (55)	9 (45)	
Sex	Male	34 (53.1)	16 (47.1)	18 (52.9)	0.975
	Female	30 (46.9)	14 (46.7)	16 (53.3)	
Types of Hodgkin lymphoma	Lymphocytic rich	6 (9.4)	1 (16.7)	5 (83.3)	0.286
	Mixed cellularity	19 (29.7)	9 (47.4)	10 (52.6)	
	Nodular sclerosis	39 (60.9)	20 (51.3)	19 (48.7)	
PD-L1 IHC	Negative	36 (56.2)	26 (72.2)	10 (27.7)	<0.001
	Positive	28 (43.7)	4 (14.2)	24 (87.5)	
COX-2 IHC	Negative	39 (60.9)	23 (58.9)	16 (41.0)	0.015
	Positive	25 (39.1)	7 (28.0)	18 (85.7)	
Bcl-xL IHC	Negative	37 (57.8)	12 (32.4)	25 (66.7)	0.006
	Positive	27 (42.2)	18 (67.6)	9 (33.3)	
B-symptoms	Absent	42 (65.6)			
	Present	22 (34.4)			
Bulky	Absent	50 (78.1)			
	Present	14 (21.9)			
Extra-nodal involvement	Absent	49 (76.6)			
	Present	15 (23.4)			
Stage	Early (I-II)	37 (57.8)			
	Advanced (III-IV)	27 (42.2)			
Response to 1st line chemotherapy	Complete remission	37 (57.8)	22 (60.0)	15 (55.9)	0.018
	Partial remission	15 (23.4)	6 (26.7)	9 (20.6)	
	Stable disease	8 (12.5)	1 (6.7)	8 (17.6)	
	Progressive disease	4 (6.3)	1 (6.7)	2 (5.9)	
2nd line chemotherapy	No	37 (57.8)			
	Yes	27 (42.2)			
Response to 2nd line chemotherapy	Complete remission	26 (40.6)	16 (100.0)	10 (93.3)	0.87
	Progressive disease	1 (1.6)	0 (0.0)	1 (6.7)	
	Not involved	37 (57.8)	16 (100.0)	10 (93.3)	
Progressive disease	Mean DFS (months)		52.1	43.4	0.013
	Median DFS		NR	43.0	
	5-year DFS		76.7%	44.1%	
	Mean OS (months)		56.1	53.7	0.028
	Median OS		NR	57	
	5-year OS		76.7%	47.1%	
Relapse	Absent	38 (59.4)	23 (76.7)	15 (44.1)	0.008
	Present	26 (40.6)	7 (23.3)	19 (55.9)	
Mortality	Alive	39 (60.9)	23 (58.9)	16 (41.1)	0.015
	Died	25 (39.1)	7 (28)	18 (72)	

Chi square test; 95% confidence interval; P<0.05 statistically significant. DFS: Disease-free survival; OS: Overall survival; IHC: Immunohistochemistry; NR: Not reached

Table 3: The mRNA expression of JAK2/STAT1 and NF-κB signaling pathways in patients with EBV-positive and EBV-negative Hodgkin lymphoma

Parameters*	EBV-negative (n=30)	EBV-positive (n=34)	P value
JAK2	0.9±0.2	5.2±0.3	<0.001
STAT1	2.8±0.4	13.7±1.9	<0.001
IRF-1	11.6±1.7	48.8±3.1	<0.001
PD-L1	4.4±0.5	25.3±0.8	<0.001
IFN-γ	2.7±1.1	7.6±1.9	<0.001
NF-κB	4.1±0.2	8.3±0.8	<0.001
Bcl-xL	5.8±0.3	14.7±1.1	<0.001
COX-2	9.2±3.4	29.7±9.5	<0.001

*Using quantitative real-time polymerase chain reaction

The Chi square test was used to analyze the expression levels and their association with predictive clinical-pathological parameters. The Kaplan-Meier method was used to estimate the OS and DFS curves and log-rank tests to analyze the differences. P<0.05 was considered statistically significant.

Results

The clinicopathological characteristics of the patients are presented in table 2. Of the 64 CHL patients, 30 (46.9%) were EBV-negative and 34 (53.1%) EBV-positive. Among the EBV-positive patients, 57.8% had stage I-II and 42.2% stage III-IV disease. The response to first-line treatment showed complete remission in 57.8% of all patients, partial remission in 23.4%, stable disease in 12.5%, and progressive disease in 6.3%. Second-line chemotherapy was administered to 42.2% of the patients, of which 40.6% showed complete remission and 1.6% had progressive disease. Of all the patients, 39.1% died due to the disease.

EBV-positive patients were highly significantly associated with B-symptoms (P=0.022), extra-nodal involvement (P=0.017), advanced stage of CHL (P=0.018), and positive expression of PD-L1 (P<0.001), COX-2 (P=0.015), and Bcl-xL (P=0.0067). They also had a higher risk of cancer progression and relapse after therapy (P=0.008), poor DFS (P=0.013), poor OS (P=0.028), and a higher death rate (P=0.015) (table 2).

The results showed a highly significant association of EBV-positive patients with mRNA expression levels of JAK2, STAT1, IRF-1, PD-L1, IFN-γ, NF-κB, Bcl-xL, and COX-2 for both the JAK2/STAT1 and NF-κB signaling pathways (table 3). Positive expression of PDL1, COX-2, and Bcl-xL was associated with old age, the presence of B-symptoms, bulky tumor, extra-nodal involvement, and advanced CHL stages (P<0.001) (table 4 and figures 1 -3).

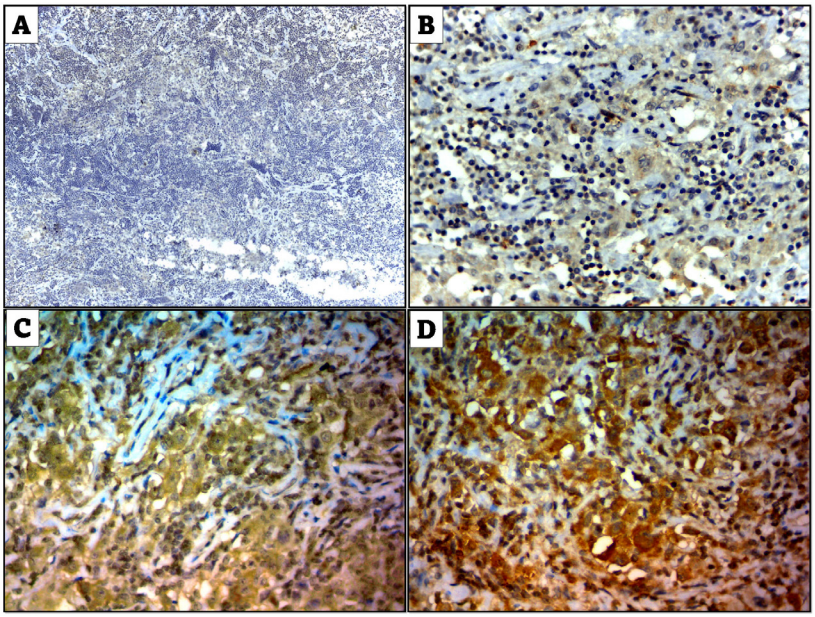

Figure 1: Immunohistochemical expression of PD-L1 in Hodgkin Reed-Sternberg tumor cells and peritumoral microenvironment in the form of brown cytoplasmic and/or membranous staining. Immunohistochemical expression of PD-L1 shows (A) negative expression with less than 5% positive tumor cells (×100), (B) weak expression (×400), (C) moderate expression (×400), and (D) strong expression (×400).

Table 4: Association of clinicopathological features and outcomes with the expression of PDL-1, COX-2, and Bcl-xL (n=64)

Clinicopathological features and outcomes		PDL1			Cox-2			Bcl-xL		
		Negative (n=36)	Positive (n=28)	P value	Negative (n=39)	Positive (n=25)	P value	Negative (n=37)	Positive (n=27)	P value
Age(years)	<45 (n=42)	32 (76.1%)	10 (23.8%)	<0.001	33 (75.0%)	11 (25.0%)	0.001	23 (52.3%)	21 (47.7%)	0.015
	≥45 (n=22)	4 (18.1%)	18 (81.8%)		6 (30.0%)	14 (70.0%)		4 (20.0%)	16 (80.0%)	
Sex	Male (n=34)	11 (32.3%)	13 (38.2%)	0.193	20 (58.8%)	14 (41.2%)	0.712	19 (55.9%)	15 (44.1%)	0.018
	Female (n=30)	25 (83.8%)	15 (50.0%)		19 (63.3%)	11 (36.7%)		8 (26.7%)	22 (73.3%)	
Type of Hodgkin lymphoma	Lymphocytic rich (n=6)	1 (16.6%)	5 (83.3%)	0.117	2 (33.3%)	4 (66.7%)	0.346	2 (33.3%)	4 (66.7%)	0.716
	Mixed cellularity (n=19)	11 (57.8%)	8 (42.1%)		12 (63.2%)	7 (36.8%)		7 (36.8%)	12 (63.2%)	
	Nodular sclerosis (n=39)	24 (61.5%)	15 (38.4%)		25 (64.1%)	14 (35.9%)		18 (46.2%)	21 (53.8%)	
EBV	Negative (n=30)	26 (86.6%)	4 (13.3%)	<0.001	23 (76.6%)	7 (23.3%)	0.015	18 (60.0%)	12 (40.0%)	0.0067
	Positive (n=34)	10 (29.4%)	24 (70.5%)		16 (47.0%)	18 (52.9%)		9 (26.5%)	25 (73.5%)	
B-symptoms	Absent (n=42)	34 (80.9%)	8 (19.0%)	<0.001	38 (90.5%)	4 (9.5%)	<0.001	25 (59.5%)	17 (40.5%)	<0.001
	Present (n=22)	2 (9.1%)	20 (90.9%)		1 (4.5%)	21 (95.5%)		2 (9.1%)	20 (90.9%)	
Bulky	Absent (n=50)	35 (70.0%)	15 (30.0%)	<0.001	38 (76.0%)	12 (24.0%)	<0.001	26 (52.0%)	24 (48.0%)	0.003
	Present (n=14)	1 (7.1%)	13 (92.8%)		1 (7.1%)	13 (92.9%)		1 (7.1%)	13 (92.9%)	
Extra-nodal involvement	Absent (n=49)	35 (71.4%)	14 (10.2%)	<0.001	36 (73.5%)	13 (26.5%)	<0.001	26 (52.0%)	23 (46.9%)	0.001
	Present (n=15)	1 (6.6%)	14 (93.3%)		3 (20.0%)	12 (80.0%)		1 (7.1%)	14 (93.3%)	
Stage	Early I-II (n=37)	37 (100.0%)	0 (0.0%)	<0.001	35 (94.6%)	2 (5.4%)	<0.001	24 (64.9%)	13 (35.1%)	<0.001
	Advanced III-IV (n=27)	11 (40.7%)	16 (59.3%)		4 (14.8%)	23 (85.2%)		3 (11.1%)	24 (88.9%)	
Response to 1st line chemotherapy	Complete remission (n=37)	27 (72.9%)	10 (27.0%)	0.001	29 (74.4%)	8 (32.0%)	0.001	22 (81.5%)	15 (40.5%)	0.003
	Partial remission (n=15)	8 (53.3%)	7 (46.6%)		8 (20.5%)	7 (28.0%)		5 (18.5%)	10 (27.0%)	
	Stable disease (n=8)	1 (12.5%)	7 (87.5%)		2 (5.1%)	6 (24.0%)		0 (0.0%)	8 (21.6%)	
	Progressive disease (n=4)	0 (0.0%)	4 (100.0%)		0 (0.0%)	4 (16.0%)		0 (0.0%)	4 (10.8%)	
Response to 2nd line chemotherapy	Complete remission (n=26)	14 (100.0%)	12 (92.3%)	0.481	10 (100.0%)	16 (94.1%)	>0.999	21 (95.5%)	5 (100.0%)	>0.999
	Progressive disease (n=1)	0 (0.0%)	1 (7.7%)		0 (0.0%)	1 (5.9%)		0 (0.0%)	1 (4.5%)	
Relapse	Absent (n=38)	29 (76.3%)	9 (23.6%)	<0.001	30 (78.9%)	8 (21.05%)	0.003	27 (71.05%)	11 (28.9%)	0.0095
	Present (n=26)	7 (26.9%)	19 (73.1%)		9 (34.6%)	17 (65.3%)		10 (38.4%)	16 (61.5%)	
Mortality	Alive (n=39)	29 (74.3%)	10 (25.6%)	<0.001	30 (76.9%)	9 (23.1.0%)	0.001	28 (71.7%)	11 (28.2%)	0.046
	Died (n=25)	7 (28.0%)	18 (72.0%)		9 (36%)	16 (64.0%)		9 (36%)	16 (64%)	
DFS	Mean (months; 95% CI)	53.7	38.3	<0.001	54.02	37.36	<0.001	51.1	42.5	0.014
	Median	NR	39		NR	30		NR	40	
	5-year	81.60%	26.90%		76.90%	32%		73.00%	40.70%	
OS	Mean (months; 95% CI)	56.9	51.8	<0.001	57.8	50.2	<0.001	56.5	52.5	0.004
	Median	NR	54		NR	55		NR	55	
	Five-year	81.60%	30.80%		76.90%	36.00%		75.70%	40.70%	

Chi square test; 95% confidence interval; P<0.05 statistically significant; DFS: Disease-free survival; OS: Overall survival; NR: Not reached

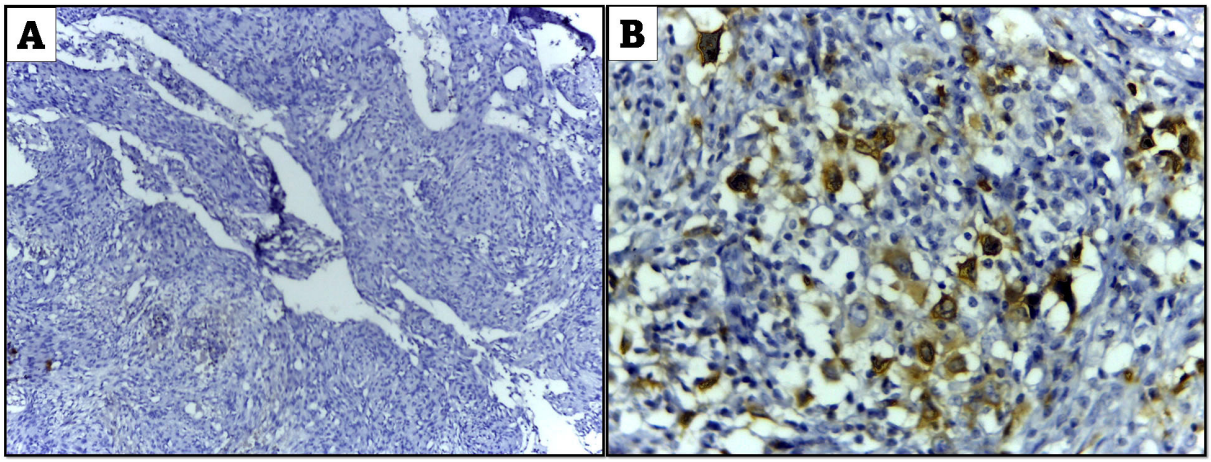

Figure 2: COX-2 immunohistochemical expression in Hodgkin Reed-Sternberg tumor cells indicated by brown cytoplasmic staining. Immunohistochemical expression of COX-2 shows (A) negative expression with less than 10% positive tumor cells (×100) and (B) positive expression with more than 10% positive tumor cells (×400).

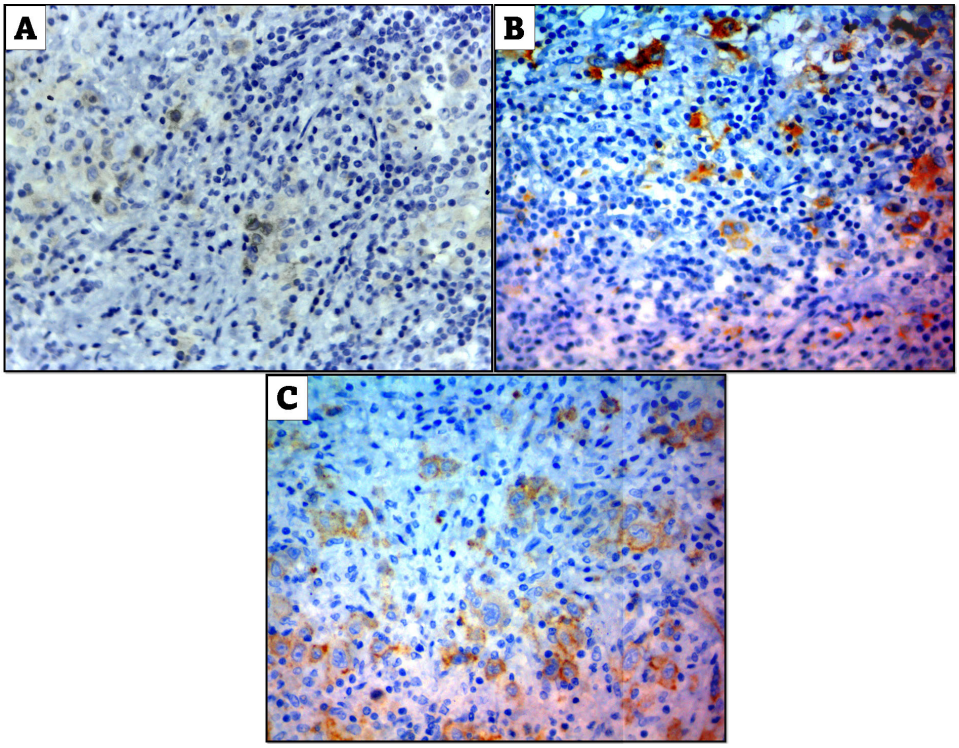

Figure 3: Bcl-xL immunohistochemical expression in Hodgkin Reed-Sternberg tumor cells. The expression of Bcl-xL appears as granular brown cytoplasmic staining of tumor cells. Immunohistochemical expression of Bcl-xL shows (A) negative expression with less than 5% positive tumor cells and (B, C) positive expression with more than 5% positive tumor cells (×400).

Patients with positive expression of PDL-1, COX-2, and Bcl-xL had a higher risk of cancer progression, relapse after therapy (P<0.001, P=0.003, and P=0.0095, respectively), poor DFS (P<0.001, P<0.001, and P=0.014, respectively), poor OS rate (P<0.001, P<0.001, and P=0.004, respectively), and higher mortality rate (P<0.001, P=0.003, P=0.0095, respectively) (figure 4).

Discussion

HL is a malignant lymphoid tumor that accounts for less than 1% of all malignancies diagnosed annually worldwide. Despite significant therapeutic advances in lymphoma treatment, disease progression after standard chemoradiotherapy occurs in up to 40% of non-HL patients and 15% of those with HL. Given the high rate of treatment failure, there is a need for new targeted therapies to achieve prolonged remission and higher survival rates.[8]

In the present study, the results showed a highly significant association between EBV-positivity and positive expression of mRNAs in both JAK/STAT and NF-κB pathways.

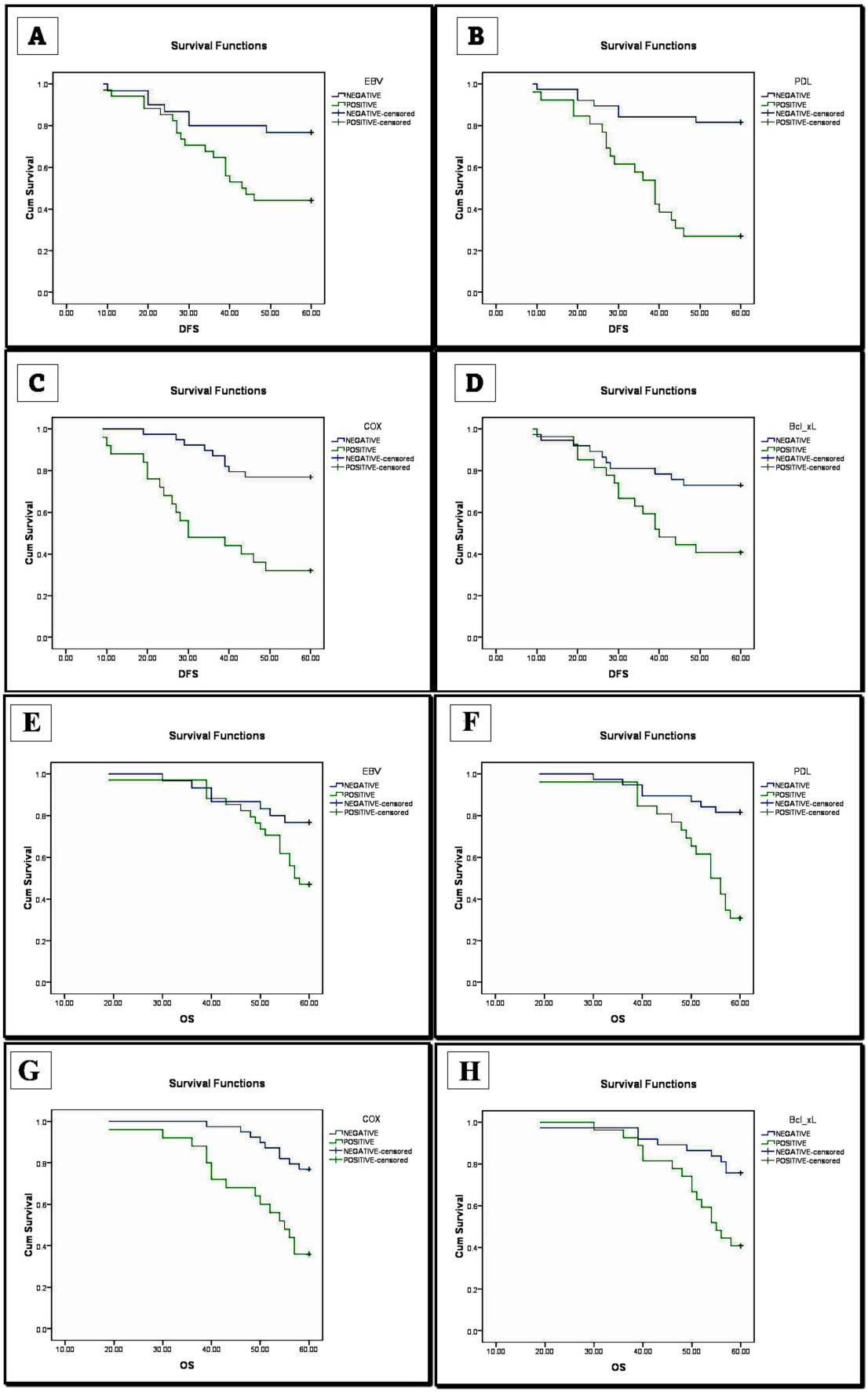

Figure 4: Kaplan-Meier plot of disease-free survival of all patients (N=64) stratified by (A) EBV, (B) PDL-1, (C) COX-2, and (D) Bcl-xL. Kaplan-Meier graph of overall survival of all patients (N=64) stratified by (E) EBV, (F) PDL-1, (G) COX-2, and (H) Bcl-xL.

A highly significant association was found between EBV-positive CHL and the expression of PD-L1, Bcl-xL, and COX-2; poor clinicopathological parameters, the presence of B-symptoms, extra-nodal involvement, advanced stages of the disease, susceptibility to cancer progression, a higher incidence of relapse, poor DFS rate, poor OS rate, and higher mortality rate. In addition, compared to EBV-negative patients, those with EBV-positive CHL were significantly associated with positive expression of COX-2 and Bcl-xL proteins, which could inhibit the two main apoptosis pathways. This indicated that EBV infection increased their expression through the activation of the NF-κB signaling pathway.[6]

In this study, we found that EBV positivity and its associated proteins, including PD-L1, COX-2, and Bcl-xL, were correlated with poor clinic prognostic parameters and were indicative of poor clinical outcomes in patients with CHL, such as B symptoms, extranodal involvement, and advanced stage, higher risk of cancer progression and relapse after therapy, poor DFS, poor OS, and high risk of mortality.

EBV achieves oncogenicity in different human disorders such as nasopharyngeal carcinoma, gastric carcinoma, lymphomas, and lymphoproliferative disorder by activating the six main signaling pathways (NF-κB, PI3K/AKT, JAK/STAT, MAPK, TGF-β, and Wnt/β-catenin). As a result, it enhances epithelial-mesenchymal transition, angiogenesis, and inhibition of apoptosis with subsequent activation of tumor cell survival, proliferation, and metastasis. JAK2/STAT1 and NF-κB pathways play the main role in EBV-induced tumorigenicity in different types of lymphoma.[3] Our results revealed that high mRNAs expression of JAK2, STAT1, IRF-1, PD-L1, IFN-γ, NF-κB, Bcl-xL, and COX-2 of both the JAK2/STAT1 and NF-κB signaling pathways were significantly associated with EBV-positive CHL. This is in line with the results of a study by Moon and colleagues[17] reporting that the activation of JAK2/STAT1/IRF-1 signaling significantly increased the PD-L1 expression levels in EBV-positive tumor cells more than in EBV-negative ones. In another study, Li and colleagues[30] confirmed the association of EBV-LMP1 with tumorigenicity through the activation of NF-κB signaling pathway.

Apoptosis can be regulated by apoptotic-regulating proteins such as Bcl-2 and BAX-family proteins. Increased expression of Bcl-xL, one of the potent anti-apoptotic proteins of the Bcl family, is a possible cause for the blockade of the caspase cascade**.** Furthermore, the COX-2/PGE2 pathway inhibits intrinsic and extrinsic apoptosis pathways by increasing the expression of Bcl protein through the activation of the Ras-MAPK/ERK pathway.[10] Two other studies also reported that patients with EBV-positive CHL were significantly associated with poor clinicopathological and prognostic parameters compared to patients with EBV-negative CHL.[26, 31] Mestre and colleagues showed that COX-2 expression in HRS cells was not only associated with poor clinicopathological parameters but was also a prognostic factor in patients with early CHL.[32] Ohno and colleagues showed that COX-2 expression was inversely related to AI, and the index was lower in COX-2-positive than in COX-2-negative cancer cells.[33] This is indicative of the antitumor potential of non-NSAIDs due to their ability to block the COX-2 pathway and reverse its effect, which is previously noted in lymphomas caused by Kaposi sarcoma, herpesvirus, and EBV. Studies utilizing NSAIDs showed their anticancer potential through inhibition of COX-2 and PGE2 activity, which are thought to play a key role in the etiology of several malignancies.[7] Some studies reported the additional therapeutic value of NSAIDs and selective COX-2 inhibitors as potential chemoprotective agents against EBV-associated diseases and cancer.[34, 35]

Therapeutic targeting of immune checkpoint protein (PDL1) and COX-2 is a potential treatment modality for lymphoma, especially EBV-positive cases. In addition, the determination of PD-1/PD-L1 cell surface expression is a major criterion to identify patients eligible for immune checkpoint blockade therapy.[34, 36] So far, many researchers have indicated the significance of using anti-inflammatory drugs for curing different types of cancer.

The main limitation of our study was the low sample size. In addition, not all signaling pathways of molecules were evaluated. Therefore, large-scale prospective studies with more in-depth molecular assessments are recommended to substantiate our findings.

Conclusion

Through the activation of JAK/STAT and NF-κB signaling pathways, EBV-positive CHL is associated with poor clinicopathological parameters, higher incidence of disease progression, relapse after therapy, and poor overall survival. Therefore, therapeutic targeting of NF-κB, JAK2, STAT1, PD-L1, Bcl-xL, or COX-2 could potentially contribute to the treatment of patients with EBV-associated CHL.

Authors' Contribution

M.A: Study concept, M.A-A: Revising the manuscript. All authors equally contribute to

data collection, data analysis, and writing the manuscript. They all have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest: None declared.

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