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UNRAVELING THE COMPLEXITY OF IMMUNE CHECKPOINT MODULATION IN GASTROINTESTINAL MALIGNANCIES

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Abstract

Gastrointestinal cancers are accountable for the highest mortality rates on a global scale, including gastric adenocarcinomas and hepatocellular carcinoma on a large scale. Tumor microenvironment has a vital role in influencing tumor behavior. Evading immune destruction prevents the immunological killing of cancer cells by T and B lymphocytes, macrophages and natural killer cells in the early stages of cancer progression. To determine the relative expression level of immunological markers and cytokines (CTLA-4, PD-1, PDL-1, LAG-3, IL-7R, and IL-12A) in tumor microenvironment of patients with gastrointestinal malignancies and their correlation with clinicopathological characteristics. Blood samples of gastrointestinal malignant patients were collected, followed by RNA extraction and cDNA synthesis for gene expression. The quantification of immune checkpoints and cytokines was carried out on the DNA using RT-qPCR. There is a significant increase in CTLA-4 expression in all the GIT cancers as compared to healthy controls. A slight increase in LAG-3 expression was observed in esopharyngeal carcinoma patients as compared to other GIT cancers. Downregulation of IL-7R and IL-12A was observed in all GIT cancers, however, gastric cancer shows a notable decrease in expression as compared to other GIT cancers and controls. Genetic expression of targeted immune checkpoints and cytokines is associated with cancer progression and helpful in unveiling the different mechanisms through which tumor microenvironment evades the immune system. This will lead to the development of effective immune checkpoint inhibitors as high-treatment efficacy drugs which activate the immune system, contributing to improved prognosis and therapeutic methods in aggressive tumor.

Keywords: *Cytokines, Gastrointestinal cancers, Gene expression, Immunological markers, Malignancies, Tumor microenvironment*

INTRODUCTION

The diverse nature of tumor microenvironment majorly includes resistance to cell death, immortality of replication machinery, angiogenesis, genomic instability, non-mutational epigenetics and metabolic reprogramming (1). Gastrointestinal cancers contribute for approximately 26% of the cancer cases worldwide and 35% of all cancer related fatalities. A study shows about 60% of gastric cancer patients of Grade III and IV were in northern parts of Peshawar (2). The incidence of esophageal cancer patients is highest in Balochistan province i.e., 4.1 per 100,000 (3). Several risk factors contribute to gastrointestinal cancers i.e., smoking, alcohol consumption, obesity and Hepatitis B or C infection.

Major immune checkpoint pathways activating in colorectal and gastric cancer progression are cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and programmed death 1 (PD-1)/programmed death ligand 1 (PDL-1). CTLA-4, a protein of immunoglobin superfamily found in endocytic vesicles, is transported to cell membrane to colocalize with receptors of T-cells (4). Programmed death 1 (PD-1) is a member of CD28 (B7) costimulatory receptors present on myeloid cells, T-cells, and B-cells. The ligand of this receptor i.e., programmed death ligand-1 (PDL-1) is present on present on leukocytes, nonlymphoid tissues and non-hematopoietic cells. Binding of PD-1 with PDL-1 leads to downregulation of immune

response by inhibiting proliferation of T-cells, production of IL-2 and survival of T-cells (5). Moreover, Lymphocyte activation gene 3 (LAG-3/CD223) is a transmembrane protein of immunoglobin family present on T-cells with greater affinity to bind with MHC-II molecules present on APC and negatively regulates the immune function by reducing proliferation, activation and survival of T-cells (6).

In addition, Interleukin-7R (IL-7R) positively regulates the immune response by maintaining development and survival of B and T lymphocytes. The binding of IL-7R with its ligand IL-7 leads to downstream activation of tyrosine kinases i.e., Janus kinase (JAK1 and JAK3) which phosphorylates signal transducer to promote IL-2 gene expression. It has been studied that (7). The m-RNA levels of IL-7 are high in peripheral blood mononuclear cells (PBMCs) of pancreatic cancer patients. Similarly, interleukin 12A (IL-12A) also activates the natural killer cells, thus increasing the expression of CD69 and CD25. Cytotoxic NK cells secrete IFN-γ to control the growth of tumor and perform apoptosis of cancerous cells, thus playing a critical role in anti-tumor immune response (8-9). Moreover, immune checkpoint inhibitors (ICIs) have proven to be efficacious therapeutics across various cancers. However, with the widespread adoption of immunotherapeutic strategies, clinicians now face significant challenges. These challenges encompass overcoming resistance to ICIs and ensuring accurate evaluations of drug efficacy, particularly in the context of hepatocellular carcinoms.

MATERIALS AND METHODS

SAMPLE COLLECTION AND DATA GROUPING / CHARACTERISTICS

80 cancer patients who fulfilled the study criteria were enrolled and EDTA Blood samples of 5ml each were collected from the oncology department of Jinnah Hospital, Lahore with an informed consent. Moreover. 56 healthy persons were also enrolled as controls in the study. Samples were transported to the Molecular Diagnostics Laboratory, FCCU for further processing. 20 patients had been lost to follow up, while the remaining 60 patients were divided as: Group 1: Healthy individuals taken as control sample (Ctrl), Group 2: Patients diagnosed with esopharyngeal carcinoma, Group 3: Patients diagnosed with gastric cancer, Group 4: Patients diagnosed with hepatobiliary carcinoma, Group 5: Patients diagnosed with colorectal cancer.

STUDY DESIGN

This was a cross-sectional analytical study. Patients aged between 20 to 80 years, histologically confirmed GIT cancer patients suffering from hepatocellular carcinoma, gastric cancer, esophageal cancer, rectal cancer, gall bladder cancer, ECOG performance status of 0 to 1, having no evidence of gastric lymphomas and gastrointestinal stromal tumors (GIST), were included in the study. Whereas, vulnerable population such as children, prisoners, psychiatric patients, pregnant and lactating females, history of malignancy other than GIT malignancy patients treated with multiple cancers, were excluded.

RNA EXTRACTION AND cDNA SYNTHESIS

RNA extraction from blood was performed using (Invitrogen TRIzol reagent: Catalog# 15596026, USA) according to the manufacturer's instructions for protocol. The quality and quantity of RNA was analyzed by using NanoDrop 2000/2000c Spectrophotometer (Thermoscientific, USA). The samples having 260/230 and 260/280 absorbance ratio of 1.5-1.8 were selected for further cDNA processing using (M-MuLV) Reverse Transcriptase Kit (Catalogue # K1622, Thermoscientific, USA) as per manufacturer's instruction for protocol.

PRIMER DESIGNING AND OPTIMIZATION

Primers were designed using the NCBI primer designing tool. A set of primers (forward and reversed) was designed for each of the immunological marker i.e., CTLA-4, PD-1, PDL-1, LAG-3, IL-7R and IL-12 A. Primers were optimized using gradient PCR thermocycler (Bio-Rad T100-Thermocycler, USA) to determine the best Tm. For confirmation of primers, In-Silico PCR was performed using UCSC Genome Browser.

MARKERS	PRIMER SEQUENCE	
CTLA-4	Forward	5′ CTACCTGGGCATAGGCAACG 3′
	Reverse	5′ CCCCGAACTAACTGCTGCAA 3′
PD-1	Forward	5′ CGTGGCCTATCCACTCCTCA 3′
	Reverse	5′ ATCCCTTGTCCCAGCCACTC 3′
PDL-1	Forward	5′ AAATGGAACCTGGCGAAAGC 3′
	Reverse	5′ GATGAGCCCCTCAGGCATTT 3′
LAG-3	Forward	5′ GAGGCTTCTTGGAGCAGCAGT 3′
	Reverse	5′ ATGTCCCTGGCTCACCTGTCT 3′
IL-7R	Forward	5′ TCGCTCTGTTGGTCATCTTGGC 3′
	Reverse	5′ CCACCCTATGAATCTGGCAGTC 3′
IL-12 A	Forward	5′ CAGCACAGTGGAGGCCTGTTT 3′
	Reverse	5′ AGGCCAGGCAACTCCCATTAG 3′
GAPDH	Forward	5′GTCTCCTCTGACTTCAACAGCG 3′
	Reverse	5′ACCACCCTGTTGCTGTAGCCAA 3′

REAL TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-QPCR)

CFX 96 qPCR system by Bio-Rad was used to perform RT-qPCR as per standard protocol, to observe the expression analysis of mRNA CTLA-4, PD-1, PDL-1, LAG-3, IL-7R and IL-12 A. A total reaction mixture of 11µl per reaction was used consisting of optimized concentrations of SYBR green mix, cDNA Template, Forward primer and Reverse primer. GAPDH (glyceraldehyde-3-phosphate dehydrogenase), a housekeeping gene used for the normalization of biomarker expression levels. A SYBR green reaction mix without a template RNA was used as an internal negative control to observe any handling error or contamination. Blood of 40 healthy patients was collected and their cDNA was made. This cDNA was used as a negative control for the calibration of inflammatory markers in patients. cDNA extracted from the blood of GIT malignant patients as positive control.

RT-QPCR DATA ANALYSIS

For the analysis of data obtained from the RT-qPCR results, relative fold change expression of the inflammatory markers was measured. The relative expression of markers was calculated with reference to the control samples by applying the following formula:

$$\text{Relative Fold Change} = \frac{\text{Normalized Diseased Sample*}}{\text{Normalized Control Sample**}}$$

$$\text{Normalized Diseased Sample}^{*} = \frac{\text{Each Cq of diseased sample with desired Gene}}{\text{Each Cq of diseased sample with GAPDH}}$$

$$\text{Normalized Control Sample}^{**} = \frac{\text{Average Cq of control samples with desired Gene}}{\text{Average Cq of control samples with GAPDH}}$$

STATISTICAL ANALYSIS

Graph Pad Prism Software (version 8) was used. For demographics, frequencies were plotted, and results were expressed as mean ± SD. Significant relation between multiple variables were assessed by non-parametric Kruskal Wallis test with 95% confidence interval. All the experiments were repeated twice, and consistent reproducibility was seen. A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

DEMOGRAPHIC FEATURES OF STUDY POPULATION

In this study 60 patients of gastrointestinal malignancies were enrolled along with 56 healthy individuals taken as control. There percentage of diseased patients was 40% females and 60% males with reference range of ages between 23 to 65 years. Similarly, there were 40% healthy females and 60% healthy males belonging to ages 19 to 30 years. The gender-wise mean age of healthy and diseased patients was also calculated along with their standard deviation (Table I).

Table I. Gender-wise demographic features of study population

Parameter	Groups	Gender	Age Range (Years)	Mean±(SD)
Age (Years)	Control	M= (60%)	19-30	24±2.27
		F= (40%)	22-25	24±2.35
	Disease	M= (60%)	23-65	52±10.85
		F= (40%)	32-52	41±7.50

CLINICAL PARAMETERS OF STUDY POPULATION

In this study the average hemoglobin in controls was found to be 13.98. Healthy individuals have total leukocyte count, an average of 6.58 and platelet count of 226. Patients diagnosed with esopharyngeal carcinoma showed an average hemoglobin of 12.45, total leukocyte count of 8.2 and platelet count 234. Parameters of patients diagnosed with gastric, hepatobiliary and colorectal cancer (Table II).

Table II. Clinical parameters of patients diagnosed with different types of cancers

Parameter		Hemoglobin (g/dL)	Total leukocyte count ($x10^9$/L)	Platelet count ($x10^9$/L)
Normal Range		11.6-16.6	3.4-9.6	135-371
		Mean±SD	**Mean±SD**	**Mean ±SD**
	Control	13.98±1.14	6.58±1.40	226.33±66.41
Cancer Type	Esopharyngeal	12.45±1.76	8.2±3.09	234±68.08
	Gastric	11.98±1.71	7.1±3.09	267.63±92.37
	Hepatobiliary	12.86±1.311	7.68±3.23	229.6±84.58
	Colorectal	9.9±1.56	7.27±1.95	255.11±131.95

BIOPSY PROFILE OF GASTROINTESTINAL PATIENTS

10% gastrointestinal malignant patients were of esopharyngeal carcinoma, 5% with moderately differentiated esophageal squamous cell carcinoma (ESCC) and the other 5% having metastatic squamous cell carcinoma of oropharynx. Out of 20% gastric cancer patients. 10% of the patients were of poorly differentiated adenocarcinoma of stomach and other 10% with poorly differentiated signet ring cell adenocarcinoma. There were 20% hepatobiliary carcinoma patients. Biopsy profiles showed that 5% were having metastatic adenocarcinoma of bile duct i.e., cholangiocarcinoma. 5% of the patients were suffering from well differentiated adenocarcinoma of gall bladder. 10% of the patients were suffering from hepatocellular carcinoma, 5% were having metastatic well differentiated histologic grade and the other 5% were with moderately differentiated adenocarcinoma. There were 40% colon and rectal cancer patients. 5% with poorly differentiated adenocarcinoma. 25% of the patients were diagnosed with moderately differentiated adenocarcinoma. 10% with well differentiated adenocarcinoma, two having the mucinous nature.

CHEMOTHERAPEUTIC REGIMES

Esopharyngeal cancer patients were given combinations of carboplatin and paclitaxel. Patients diagnosed with gastric cancer were given carboplatin and paclitaxel as chemotherapeutic drugs. Some of the patients were also treated with 5' fluorouracil and nivolumab. Patients diagnosed with hepatobiliary carcinoma were given gemcitabine, oxaliplatin, zoldria, capcitabine and pembrolizumab as chemotherapeutic drugs. Colorectal cancer patients were treated using cizumab and FOLFOX which is a combination of folinic acid, fuorouracil, oxaliplatin (Table III).

Table III. The chemotherapeutic regimes of different gastrointestinal cancer patients

Cancer Type	Chemotherapy Regimen					
	Carbo	Pacli	Gem	Oxali	FOLFOX	Nivo
Esopharyngeal Cancer	✓	✓				
Gastric Cancer	✓	✓			✓	✓
Hepatobiliary Cancer			✓	✓		
Colorectal Cancer				✓	✓	

Chemotherapeutic regimens i.e., carboplatin (Carbo), paclitaxel (Pacli), gemcitabine (Gem), oxaliplatin (Oxali), nivolumab (Nivo) and FOLFOX (Folinic acid, Fluorouracil, Oxaliplatin) in diseased sample population.

CONTRASTING EXPRESSION OF CTLA-4 AND PD-1/PDL-1 IN GIT CANCER

Relative fold change calculation from RT-qPCR results of different gastrointestinal cancers were plotted against healthy controls for CTLA-4, PD-1 and PDL-1. The results illustrated in Fig. 1A) show significant increase in CTLA-4 expression in all GIT cancers as compared to healthy individuals depicting suppression of immune response during cancer progression ($p<0.0023$). Among all GIT cancers, the highest CTLA-4 expression was observed in hepatobiliary cancer patients. Fig. 1B) and 1C) show that there was no change in PD-1 and PDL-1 expression profile of different GIT cancers contrary to CTLA-4. Only patients diagnosed with esopharyngeal carcinoma showed a slight increased PDL-1 expression as compared to other GIT cancer patients.

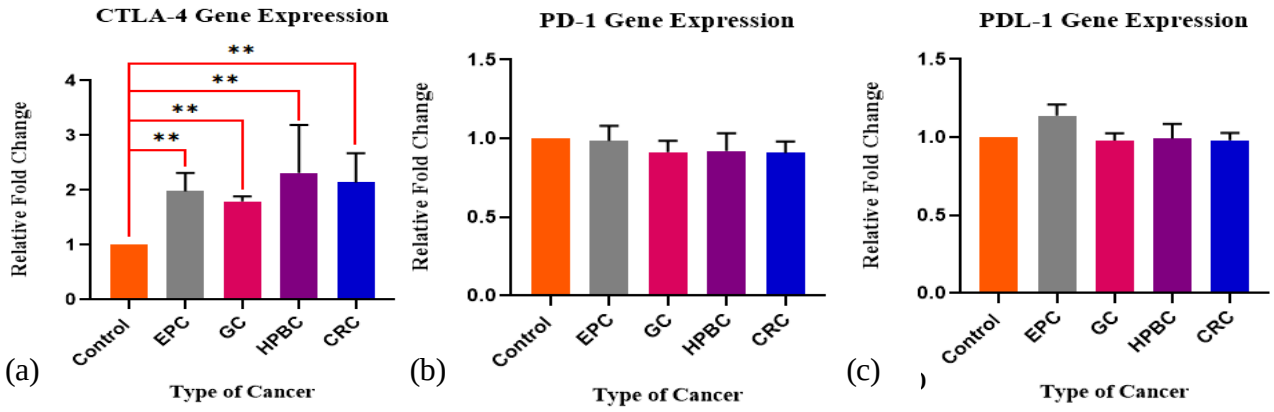

Fig. 1 (a, b & c). Contrasting expression of CTLA-4 as compared to PD-1/PDL-1 in (EPC) Esopharyngeal Carcinoma, (GC) Gastric Cancer,(HPBC) Hepatobiliary Carcinoma, (CRC) Colorectal Cancer. (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ns not significant $p > 0.05$).

INSIGNIFICANT EXPRESSION OF LAG-3 WITH LOW EXPRESSION OF IL-7R AND IL-12A IN TUMOR MICROENVIRONMENT MODULATION

Relative fold change calculation from RT-qPCR results of different gastrointestinal cancers were plotted against healthy controls for LAG-3. The results illustrated in Fig. 2 (a) show no significant change in expression of LAG-3 in all GIT cancers as compared to healthy individuals depicting reduced T-cell activation and suppression of immune response during cancer progression. However, a slight increase in LAG-3 expression was observed in esopharyngeal carcinoma patients compared to other GIT cancers ($p<0.1570$). Relative fold change calculation from RT-qPCR results of different gastrointestinal cancers were plotted against healthy controls for IL-7R and IL-12A. The results illustrated in Fig. 2 (b) and (c) show that downregulation of IL-7R and IL-12A was observed in all GIT cancers with a notable decrease in expression as compared to other GIT cancer and control ($p<0.0365$).

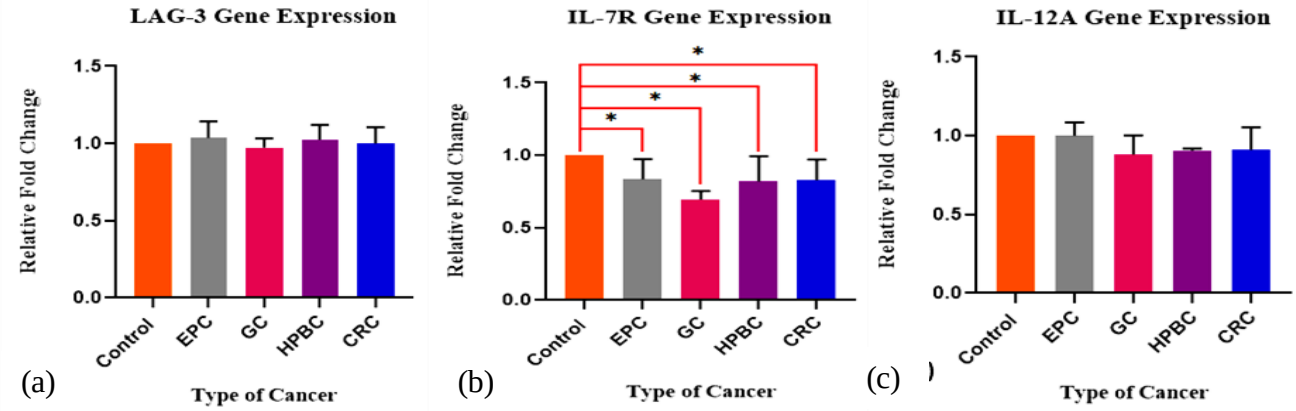

Fig. 2 (a). Insignificant expression of LAG-3 in (EPC) Esopharyngeal Carcinoma, (GC) Gastric Cancer, (HPBC) Hepatobiliary Carcinoma, (CRC) Colorectal Cancer (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ns not significant $p > 0.05$).
(b & c). Low expression of IL-7R Aand IL-12A in (EPC) Esopharyngeal Carcinoma, (GC) Gastric Cancer, (HPBC) Hepatobiliary Carcinoma, (CRC) Colorectal Cancer (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ns not significant $p > 0.05$).

DISCUSSION

Studies showed that genetic variations in CTLA-4 pathway result in overexpression in tumor tissue of colon cancer patients promoting immune infiltration of lymphocyte (10). Our results indicated CTLA-4 overexpression in almost all of the gastrointestinal cancers as compared to normal samples. CTLA-4 was also constitutively overexpressed in-vitro SW480 cells. This overexpression was reduced by capecitabine to determine its efficacy (11). There is a contrasting expression of PD-1/PDL-1 in all of the gastrointestinal cancers (12). It may be possible that when CTLA-4 is overexpressed in tumor microenvironment then PD-1/PDL-1 is not expressed. We could not find any evidence supporting this contrary mechanism of both immune checkpoints. Enrolled gastrointestinal cancer patients were taking paclitaxel, nivolumab, carboplatin, cisplatin, FOLFOX as chemotherapeutic drugs resulting in decreased PD-1 expression. An experimental study conducted supported our results that nivolumab decreased the expression of PD-1 receptor in solid tumors of patients diagnosed with colorectal cancer (13). The chemotherapeutic effect of 5-fluorouracil and oxaliplatin (FOLFOX) resulted in a reduced PD-1/PDL-1 pathway leading to a complete cure of colorectal tumor in mice. Blockade of PD-1/PDL-1 pathway induced infiltration of tumor by activated PD-1+ CD8 T cells (14).

No significant change in the expression of LAG-3 in all GIT cancers except esopharyngeal carcinoma patients depicting reduced T-cell activation and suppression of immune response. It was supported by immunohistochemical staining resulting in an increased expression of LAG-3 in surgically resected esophageal squamous cell carcinoma (15). Overexpression of LAG-3 along with FGL-1 was analyzed in hepatocellular carcinoma (HCC) patients serving as a promising immunotherapeutic target (16) . It may be possible that insignificant expression of LAG-3 is due to less infiltration of T lymphocytes in tumor microenvironment at the chemotherapeutic stage. A combination of nivolumab with relatlimab (anti-LAG-3) was considered first-line treatment for chemotherapy of gastric and gastroesophageal adenocarcinomas (17) . Downregulation of IL-7R in all GIT cancers was supported by a decreased IL-7R expression in Helicobacter bilis-induced experimental colitis in mouse models. Anti-IL-7R antibody was used effectively in mice to reduce the proliferation of T-cells (18) . Contrastingly, mRNA sequencing and qRT-PCR results have shown high mRNA levels of IL-7R in pancreatic ductal adenocarcinoma patients as compared to patients diagnosed with acute pancreatitis and chronic pancreatitis showing the decreasing trend of IL-7R expression with increasing tumor size (19).

Our results showed downregulation of IL-12A in all GIT cancers whereas a notable decrease in expression was observed in gastric cancer as compared to other GIT cancers and control samples. This may predict the aggressive nature of tumor and less infiltration of lymphocytes in tumor microenvironment. Our results were supported by an experimental study conducted on colon cancer patients to analyze the significantly lower expression of IL-12A in tumor microenvironment of cancer stage III & IV as compared to patients having cancer in the early stage (20) .

An experimental study showed that overexpression of IL-12A transformed the tumoricidal macrophages to suppress the growth of hepatocellular carcinoma (HCC) within the tumor microenvironment through the downregulation of STAT-3 and VEGF-A (21).

CONCLUSION

Overall, the RT-qPCR result analysis showed that immune checkpoints and cytokines have a crucial role in regulating the immune system during cancer progression, leading to lower survival chances in patients. However, our study had some limitations such as the majority of the enrolled patients had a poor socioeconomic status due to which they did not have initial biopsy reports confirming the grade of cancer. Our study aimed to conduct a comparative analysis of immunological markers and cytokines to study intra-tumoral heterogeneity and overall analysis of tumor microenvironment with reference to surrounding normal tissue. We can extend our experimental study by using liquid biomarkers, but it is only possible when the proper concentration of tumor is known.

Ethical Statement:

This study was conducted after getting approval of the Ethical Review Committee under Ref No: (ERC-112-2022) and Institutional Review Board under Ref No: (IRB-416/11-2022) of Forman Christian College (A Chartered University) and Ethical Review Board of Jinnah Hospital Lahore under Ref No: (ERB133/9/01-12-2022/S1ERB).

Conflict of Interest:

Authors have no conflict of interest.

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