RESEARCH ARTICLE

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Interpretation of Farnesoid X Receptor Immunohistochemical Expression in Discriminating Hepatocellular Carcinoma from Its Non-Neoplastic Mimics as an Adjunct to Glypican 3

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Abstract

Aims: Differentiating hepatocellular carcinoma (HCC) and non-neoplastic lesions may be challenging. Immunohistochemistry (IHC) can help in the comparative morphologic evaluation of HCC and its mimics. Farnesoid X receptor (*FXR*) is a nuclear metabolic receptor essential for bile salts homeostasis and other biological functions of liver cells. Preliminary studies have shown that *FXR* can be useful for diagnosing HCC. This study aimed to assess the role of Farnesoid X receptor (*FXR*) combined with Glypican 3 (*GPC3*) in differentiation between HCC and non-neoplastic hepatic lesions. **Material and methods:** Immunohistochemistry of *GPC3* and *FXR* was performed in 38 cases of primary hepatic lesions using an automated immunohistochemical stainer. The study included 17 primary HCC cases and 21 non-neoplastic hepatic lesions (5 cases were focal nodular hyperplasia, 7 were regenerative nodules and 9 were dysplastic nodules). **Results:** The percentage of positive *GPC3* and low or negative *FXR* expression was significantly higher in HCC cases than non-neoplastic hepatic lesions (P value <0.001). The sensitivity and specificity of *GPC3* in differentiating HCC from non-neoplastic hepatic lesions were 70.6% and 85.7%, respectively, while the sensitivity and specificity of *FXR* were 58.8% and 100%, respectively. **Conclusion:** The present work revealed that *FXR* could be combined with *GPC3* in distinguishing between HCC and non-neoplastic hepatic lesions with improved specificity rather than using an individual marker.

Keywords: Glypican 3- Farnesoid X receptor- Hepatocellular carcinoma- immunohistochemistry

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Introduction

Hepatocellular carcinoma (HCC) is responsible for 80–90% of cases of primary hepatic cancer and is the third most frequent cause of cancer deaths globally (Zhuo et al., 2019). It is the fourth most frequent cancer in Egypt. The cause for the rise in incidence may be due to the development in screening programs and diagnostic tools, raising the survival rate of cirrhotic patients that increases the risk of developing HCC, and increasing the cases of hepatitis C virus in Egypt as well as its complications (Rashed et al., 2020).

In 2001, the European Association for the Study of the Liver (EASL) approved noninvasive diagnosis of HCC based on serological tests and advanced imaging procedures; ever since, new innovational modalities have further evolved to optimize the reproducibility of these measures (Schraml et al., 2015). Nonetheless, HCC not only confuses with peculiar groups of non-neoplastic lesions at the imaging spectrum but also evolves in the background of such lesions on many occasions (Jemal et al., 2011). The American Association for the Study of Liver Diseases (AASLD) and the EASL delineated a specific category of such group to necessitate pathological evaluation for conclusive decision (López Panqueva, 2015).

Despite significant progress in HCC treatment, tumor recurrence and metastasis are unavoidable. Early detection and treatment of HCC can significantly increase the effectiveness of treatment and extend patient life (Sun et al., 2017).

Glypican3 (*GPC3*) is a member of a family of heparan sulfate proteoglycans; it was gradually promoted as a legitimate marker for HCC diagnosis due to its restricted expression in the neoplastic setting. Additionally, studies have further advocated for its value as a candid

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for therapeutic targeting, privileged by a scarcity of its expression in other normal healthy tissues (Guo et al., 2020). Furthermore, *GPC3* efficacy in HCC detection was consistent in tissue cores and resection specimens (Li et al., 2013). Nevertheless, testing a single marker never met the clinical application needs in the HCC context, even with *GPC3* (Guo et al., 2020).

Accordingly, searching for additional appropriate supporting markers was always a priority quest, and many markers were proposed for such incentives (Zacharakis et al., 2018). Early detection of HCC in risk patients has a significant value because late diagnosis is the main attribute of high HCC-associated mortality (Meng et al., 2012). Moreover, detecting HCC recurrence after treatment is crucial because of its relatively common incidence and drastically ominous consequences (Toso et al., 2011). Nevertheless, almost all studies that entertained their search are confined to finding other positive HCC identifiers. On the other hand, a search for an exclusion identifier that highlights the preserved normal hepatocellular differentiation rather than its loss was always overlooked.

Farnesoid X receptor (*FXR*) is a nuclear metabolic receptor essential for bile salts homeostasis and other biological functions of liver cells. Moreover, it has a hepatoprotective impact through eliciting anti-inflammatory/ tissue regeneration signals; thus, its function is mandatory to maintain several differentiation qualities of hepatocytes; thereby, it was anticipated that downregulation of *FXR* is an early event in hepatocellular carcinogenesis (Takahashi et al., 2018; Sun et al., 2021). The association of *FXR* in liver cancer has been examined in several in vivo and in vitro studies. Therefore, more studies should be performed to determine the exact role of *FXR* in liver cancer (Wang et al., 2020; Girisa et al., 2021).

Considering these above-mentioned valuable underpinnings, we designed our study to test *GPC3* and *FXR* expression in both neoplastic and non-neoplastic hepatic lesions to exploit the potential diagnostic value of this combination, in addition to spotting any observations that might reflect useful insights in hepatocellular carcinogenesis.

Materials and Methods

Case selection

This retrospective study included 38 cases of formalin-fixed paraffin-embedded tissue blocks of hepatocellular carcinoma cases (HCC) and other non-neoplastic hepatic lesions which were diagnosed as a result of hepatectomy, lobectomy, or liver transplantation that were collected from the pathology lab of Ain Shams University Hospital, from the period between 2018 and 2021. The available clinicopathological data of the cases were obtained from the pathology reports.

A total of 17 cases were diagnosed as primary HCC in the liver, and 21 cases were diagnosed as non-neoplastic hepatic lesions, whereas 5 cases were diagnosed as focal nodular hyperplasia (FNH), 7 cases were diagnosed as regenerative nodules and 9 cases were diagnosed as dysplastic nodules. A routine histological examination was performed with hematoxylin and eosin staining. Diagnosis of the cases mentioned above was reviewed by two expert pathologists in hepatic pathology independently. A third pathologist resolves disagreements between the two evaluators to make a final decision. Histopathologic grading of HCC cases was assessed according to the WHO classification (Nagtegaal et al., 2019).

Exclusion criteria

Exclusion criteria included: (1) cases of inaccurate histological diagnosis. (2) Patients receiving preoperative chemotherapy or radiotherapy. (3) Cases diagnosed by core biopsies.

Immunohistochemistry

Immunohistochemistry was performed on 4 µm formalin-fixed paraffin-embedded tissue sections in an automated immunohistochemical stainer (Bond, Leica Biosystems) with Bond Polymer Refine Detection System. The protocol in immunostainer was: Antigen retrieval with citrate buffer pH 6.0 for 20 minutes. Then the slides were incubated with rabbit polyclonal anti-*FXR* (H-130, sc13063, Santa Cruz Biochemicals, Santa Cruz, CA, USA, dilution 1:100) and rabbit monoclonal Anti-*GPC3* (SP86, ab95363, ABCAM Cambridge, Cambridge, UK, dilution1: 100); for 30 min at room temperature. After performing polymeric detection using the ImmPRESS kit (Vector Laboratories, Burlingame, USA), the antigen-antibody complex was exposed through the application of chromogen 3.30-diaminobenzidine (DAB, Vector Laboratories, Burlingame, USA).

All sections were then counterstained with hematoxylin. The manufacturer provided a positive slide. Negative control sections were prepared with the omission of the primary antibody. All sections were evaluated by counting five hundred cells from ten random fields in each case by two pathologists, using the double-blind method.

GPC3 was expressed in a cytoplasmic and/or membranous pattern, and the tumor cells were considered positive if more than 10% of the tumor cells within the section were positively stained. *GPC3*-positive stained cells were evaluated as follows: *GPC3*-negative (<10%) and *GPC3*-positive (overexpression) (>10%) (Karaoğullarindan et al., 2022).

While *FXR* cytoplasmic and nuclear staining was considered positive. Specimens were considered "positive" for *FXR* when more than 5% of the tumor cells within the section were positively stained and scored according to the percentage of positive tumor cells as 0: negative staining- 0-4% of cells positive; 1: 5-24% of cells positive; 2: 25-49% of cells positive; 3: 50-100% of cells positive, and its intensity as 0: negative staining, 1: mild staining; 2: intermediate staining; 3: intense staining. Finally, the expression of *FXR* was classified as low; if the total score was 0 - 2 and high; if the total score was ≥3 (Giaginis et al., 2017).

Statistical analysis

The Chi-square test assessed the value of *FXR* and GPC 3 proteins expression in discriminating HCC from benign hepatic focal lesions. A P value of < 0.05 (two-

tailed) was considered statistically significant, and a P value of < 0.01 (two-tailed) was considered very significant. SPSS software 20.0 (IBM SPSS, New York, NY, USA) and Microsoft® Excel 2007 were used for all analyses. The diagnostic value of each immunoprofile was analyzed for sensitivity, specificity, positive predictive value, and negative predictive value.

Results

Clinic-pathological results

The study included 38 specimens of hepatic lesions; 17 (44.7%) cases were diagnosed as primary HCC and 21 (55.3%) were diagnosed as non-neoplastic hepatic lesions. Eleven of the HCC cases (64.7%) were males, and 6 were females (35.3%) with an average age of 59±13.31 years. While 9 of the non-neoplastic hepatic lesions were males (42.9%) and 12 were females (57.1%) with an average age of 47.05±12.11 years.

Seventeen cases of HCC include two (11.8%) out of 17 cases of HCC were well differentiated, while 14(82.4%) cases were moderately differentiated, and one (5.9%) case was poorly differentiated HCC. Regarding stage 7 (41.2%) out of 17 cases of HCC were T1, while 5 (29.4%) cases were T2 and 5(29.4%) cases were T3. Histopathologically, HCC nodules showed thick trabeculae of more than three cells thick without portal tracts inside the nodules, in which well-differentiated cases showed mild nuclear atypia. Moderately and poorly differentiated cases showed moderate to marked nuclear atypia (irregular nuclear membrane, prominent nucleoli, and multinucleation) (Figure 1, A and B).

Twenty-one cases of non-neoplastic lesions, including 5 (13%) cases, were diagnosed as focal nodular hyperplasia (FNH), seven (18.4%) cases were diagnosed as regenerative nodules and nine (23.7%) cases were diagnosed as dysplastic nodules (DNs) in which 4 cases were low-grade dysplastic nodules (LGDNs) and 5 cases were high-grade dysplastic nodules (HGDNs). Histopathologically, focal nodular hyperplasia cases showed bland hepatocytes surrounded with fibrous septa with mild chronic Inflammation. Regenerative nodules showed bland hepatocytes containing portal tracts with mild chronic inflammation and bile ductular proliferation inside the nodules. LGDNs showed preserved hepatic plates at two cells thick with mild nuclear atypia. HGDNs showed hepatocyte plates of two to three cells thick with moderate nuclear atypia and basophilic cytoplasm, portal tracts seen inside the nodules (Figure 2, A, B and C).

Table 1. Relation between HCC Cases and Non-Neoplastic Hepatic Lesions Regarding GPC3 and FXR Expressions

		HCC cases (N=17)	non-neoplastic hepatic lesions (N=21)	X^2*	P value
Glypican 3 expression	Negative	5 (29.4%)	18 (85.7%)	12.47	<0.001 HS**
	Positive	12 (70.6%)	3 (14.3%)		
FXR expression	Low	10 (58.8%)	0 (0.0%)	16.77	<0.001 HS**
	High	7 (41.2%)	21 (100.0%)		

*Chi square test (FE: Fisher Exact); **p-value<0.001 is highly significant

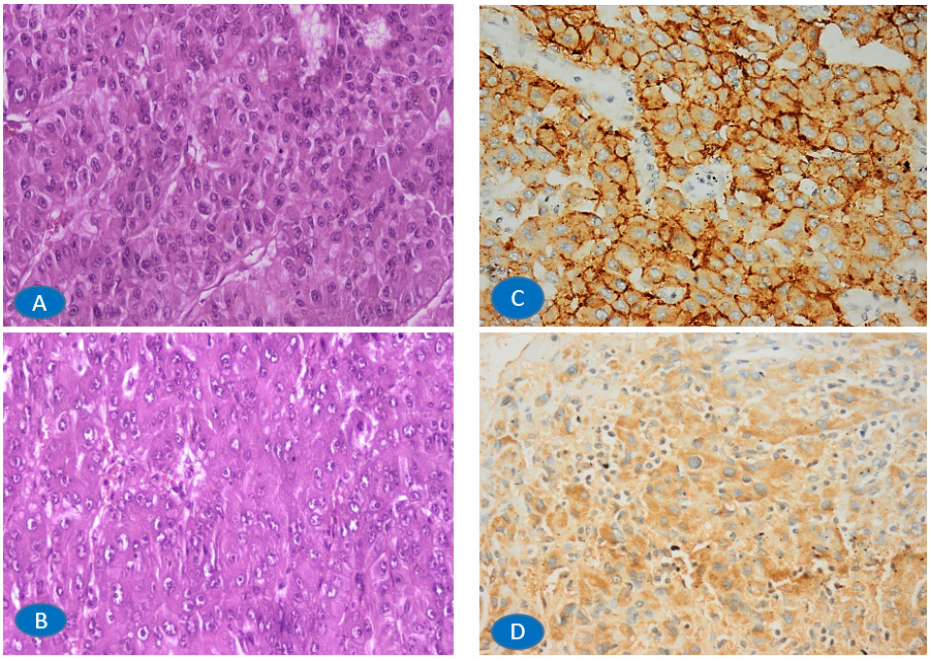

Figure 1. Photomicrograph of HCC Cases. Histopathological features of moderately differentiated HCC, 200x (A) and poorly differentiated HCC, 200x (B). Immunohistochemical expression for Glypican 3 in moderately differentiated HCC showing diffuse cytoplasmic and membranous expression, x400 (C). Immunohistochemical expression for FXR in poorly differentiated HCC showing high expression, x400 (D).

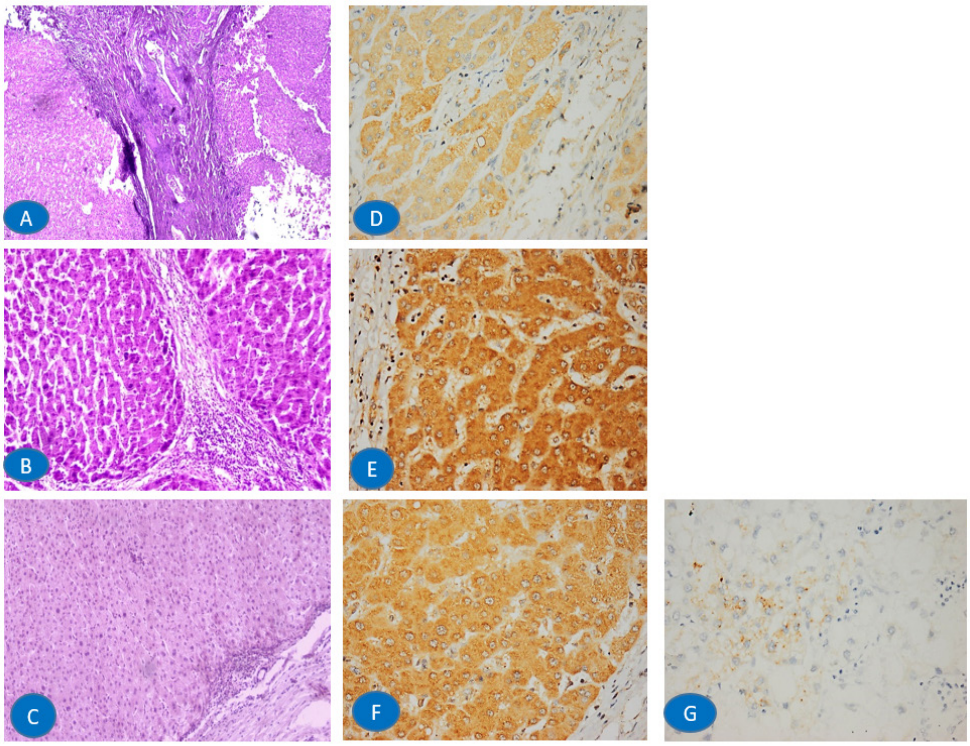

Figure 2. Photomicrograph of Non-Neoplastic Hepatic Lesions. Histopathological features of focal nodular hyperplasia, 100x (A) regenerative nodule, 100x (B) and dysplastic nodule, 100x (C). Immunohistochemical expression for Farnesoid X receptor (FXR) showing high expression in focal nodular hyperplasia, x400 (D). FXR high expression in regenerative nodule, 400x (E). FXR high expression in low grade dysplastic nodule, 400x (F). High grade dysplastic nodule showing focal positive Glypican 3 (GPC3) expression, x400 (G).

Table 2. Validity of GPC3 Positive Expression for Differentiation between HCC and Non-Neoplastic Hepatic Lesions

GPC3 expression	HCC	non-neoplastic lesions	Sensitivity	Specificity	PPV	NPV
Positive	12	3	70.60%	85.70%	80%	78.30%
Negative	5	18				

Immunohistochemical results

Regarding *GPC3*, HCC showed positivity in 12 (70.6%) out of 17 cases and negativity in 5 (29.4%) cases (Figure 1, C). Non-neoplastic hepatic lesions showed negative reactions in 18 (85.7%) out of 21 cases and focal positive reactions in 3 (14.3%) cases; all three cases were HGDNs (Figure 2, G). The frequency of the difference in *GPC3* expression between the two groups was highly Significant (P value <0.001) (Table 1).

While *FXR* showed low expression in 10 (58.8%) out of 17 HCC cases and showed high expression in 7 (41.2%) cases (Figure 1, D). In contrast, *FXR* low expression wasn't detected in non-neoplastic hepatic lesions and showed high expression in all cases (Figure 2, D, E and F). Moreover, it should be noted that *FXR* subcellular distribution was found only cytoplasmic in all examined non-neoplastic and HCC cases. The frequency of the difference in *FXR* expression between the two groups was highly Significant (P value <0.001) (Table 1).

The sensitivity of *GPC3* positive expression for diagnosing HCC from non-neoplastic hepatic lesions in the study cases was 70.6% and its specificity was 85.7%. The positive predictive value was 80% and the negative predictive value was 78.2% (Table 2).

While the sensitivity of *FXR* low expression for the diagnosis of HCC from a non-neoplastic component in the study cases was 58.8%, and its specificity was 100%. The positive predictive value was 100%, and the negative predictive value was 75% (Table 3). These data revealed that *FXR* immunostaining was a more specific marker (100%) for distinguishing HCC from non-neoplastic hepatic cases.

Discussion

We designed a study to differentiate HCC from non-neoplastic hepatic lesions; the diagnostic yield of 2 markers was used individually or as a panel as a whole.

Table 3. Validity of FXR Low Expression for Differentiation between HCC and Non-Neoplastic Hepatic Lesions

FXR expression	HCC	non-neoplastic lesions	Sensitivity	Specificity	PPV	NPV
Low expression	10	0	58.80%	100.00%	100&%	75.00%
High expression	7	21				

The current study showed that *GPC3* was overexpressed in 70.6% of HCC, while it exhibited focal expression in only 14.3% of non-neoplastic hepatic lesions; they were all HGDNs, with a highly Significant difference between the two groups (P value <0.001). This was in agreement with several studies that reported that *GPC3* was positive in most HCCs, ranging from 52.5 to 100% of HCC cases (Su et al., 2011; El-Shorbagy et al., 2016; Mohamed et al., 2022). In the present study, *GPC3* was overexpressed in 3 cases (60%) out of 5 cases of HGDNs. Gong et al., (2014) also detected positivity for *GPC3* in DNs, predominantly in higher grades.

The sensitivity and specificity of *GPC3* for HCC detection in our study were 70.6% and 85.7%, respectively. These findings were consistent with another previous study which revealed sensitivity and specificity of 75% and 87%, respectively (Guo et al., 2020). Mohamed et al., (2022) also reported the sensitivity and specificity of *GPC3* for HCC detection at 80% and 83.3%, respectively. Although the above studies have confirmed that *GPC3* is a somewhat sensitive marker for diagnosing HCC, the specificity of a single marker does not match the criteria for clinical application. Numerous studies have suggested that a combination of different markers can be used to diagnose HCC. Previous studies used a combination of *GPC3*+HSP70+GS (glutamine synthetase) markers can specifically distinguish between hepatocellular nodules and early liver cancer, improving the specificity of HCC diagnosis to 100% (Tremosini et al., 2012). Other studies have also suggested Some potential marker combinations, such as the combination of GP73, *GPC3*, and CD34, can improve the specificity of HCC diagnosis to 96.6% (Yao et al., 2013).

The present study assesses *FXR* expression combined with *GPC3* in differentiating HCC cases from benign hepatic lesions. The results reported that *FXR* expression was downregulated in 58.8% of HCC cases. At the same time, it was highly expressed in all non-neoplastic hepatic lesions, with a highly Significant difference between the two groups (P value <0.001). All cases of HGDNs showed high expression of *FXR*. These results might assume that *FXR* immunostaining could be of diagnostic utility if dysplastic nodules are clinically suspicious, regardless of the expression pattern of *GPC3*.

The present study also suggested that loss of *FXR* expression may contribute to the development and progression of human HCC. This was consistent with previous studies demonstrating that *FXR* expression is down-regulated in several human cancers, such as colon and pancreatic cancer (Lax et al., 2012; Giaginis et al., 2015).

Lv et al., (2018) also reported that loss of *FXR* plays an important role in tumorigenesis of intrahepatic cholangiocarcinoma patients. In another study, *FXR* was found in non-dysplastic tissue, but its expression was diminished as Barrett's esophagus patients progressed to dysplasia and adenocarcinoma (van de Winkel et al., 2011). Moreover, Su et al., (2012) showed that *FXR* expression was dramatically decreased in HCC compared to normal liver tissues. Also, Liu et al., (2012) proved that *FXR* expression was markedly downregulated in human HCC tissues, similar to *FXR*'s tumor suppressor function in animal models.

As was already mentioned, *FXR* expression decreases as liver cancer progresses. However, the exact molecular mechanism of *FXR* downregulation remains unclear currently. Chen et al., (2013) suggested that Inflammation might create a microenvironment that inhibits the expression of *FXR* as the elevated levels of proinflammatory cytokines, such as TNFα, IL-1β, and IL-6 in the majority of human HCC patients, may reduce the *FXR* expression by preventing hepatic nuclear factor 1α (HNF1α) transactivity on the *FXR* gene promoter.

However, Lee et al., (2014) found that *FXR* overexpression in pancreatic cancer tissues with lymphatic metastasis was associated with decreased patient survival, and downregulation of *FXR* was a successful strategy for preventing the progression of pancreatic tumors. Also, Guan et al., (2013) showed that *FXR* overexpression was related to higher histopathological grade, larger tumor size, and lymph node metastasis in esophageal cancer patients.

The sensitivity and specificity of *FXR* for HCC detection in our study were 58.8% and 100%., respectively, revealing that *FXR* immunostaining was a more specific marker than *GPC3* for distinguishing HCC from benign hepatic cases. Therefore, we recommend using a panel of immunohistochemical markers including *FXR* and *GPC3*, to differentiate HCC from non-neoplastic hepatic lesions to improve specificity.

There were some limitations in this study. The main limitation was the small number of available cases of dysplastic nodules to be assessed statistically. In addition, this was a retrospective study with a lack of clinicopathological data for the patients.

In conclusion, in the present work, using *FXR* in combination with *GPC3* in distinguishing between HCC and non-neoplastic hepatic lesions with improved specificity is recommended rather than using an individual marker. Further studies on the prognostic value of lost *FXR* expression in HCC are also recommended.

Abbreviations

HCC, hepatocellular carcinoma; *GPC3*, glypican 3; *FXR*, farnesoid X receptor; EASL, European Association for the Study of the Liver; AASLD, American Association for the Study of Liver Diseases.

Author Contribution Statement

SAR, LSS, and AIAA designed the study, interpreted data, LLS, DEAS, and MMS wrote and revised the manuscript. SAR performed the experiments, analyzed the data, and helped critically revise important intellectual content. SAR, RRA, and DEAS analyzed the data and prepared the figures. All authors read and approve the final manuscript, and follow up on the work.

Acknowledgements

Ethical considerations

The study protocol has been approved by the local ethical committee of Ain Shams University Faculty

of Medicine Research Ethics Committee (REC) FWA 000017585 with IRB approval number: FMASU R 105/2021 and followed the declaration of Helsinki regarding ethical considerations.

Data and materials availability

All data and results associated with our study are presented in the manuscript.

Conflict of interest

We declare no conflict of interest.

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