ORIGINAL ARTICLE

Circulating histone H4 values can relate to disease severity in patients with alcoholic hepatitis and cirrhosis

Los valores circulantes de histona H4 pueden relacionarse con la gravedad de la enfermedad en pacientes con hepatitis alcohólica y cirrosis

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Abstract

Objective: *We aimed to evaluate whether serum histone H4 (sHH4) is associated with alcoholic liver disease (ALD) phenotypes.* **Methods:** *This case–control study included 66 ALD patients and 47 healthy controls (HCs). Patients with ALD were classified into three groups: alcohol-associated steatotic liver, alcoholic hepatitis (AH), and alcoholic cirrhosis (AC). The HIST1H4A kit was used for the enzyme-linked immunosorbent assay of sHH4.* **Results:** *In the AH patients, the median sHH4 value was the highest and the lowest in the HC (3572.32 ng/L vs. 451 ng/L, respectively, p = 0.002). In the AC group, the median sHH4 value was higher in the Child-Pugh classification B (CPC-B) group than in the CPC-A group (p = 0.026). Positive correlations existed between the sHH4 value and the duration of alcohol use and Maddrey's discriminant function scores in patients with AH (rho = 0.886, p = 0.019 for both). In the AC patients, a positive correlation was noted between the sHH4 values and The Model for End-stage Liver Disease Sodium scores (rho = 0.527, p = 0.006).* **Conclusions:** *Increased sHH4 values might be a marker for the severity of AH and AC.*

Keywords: *Alcoholic liver disease. Histone H4. Severity.*

Resumen

Objetivo: *Evaluar si la histona sérica H4 (sHH4) está asociada con fenotipos de enfermedad hepática alcohólica.* **Métodos:** *Estudio de casos y controles que incluyó 66 pacientes con enfermedad hepática alcohólica y 47 controles sanos. Los pacientes con enfermedad hepática alcohólica se clasificaron en tres grupos: hígado esteatósico asociado al alcohol, hepatitis alcohólica (HA) y cirrosis alcohólica (CA). Se utilizó el kit HIST1H4A para el ensayo de inmunoadsorción ligado a enzimas (ELISA) de sHH4.* **Resultados:** *En los pacientes con HA, la mediana del valor de sHH4 fue más alta que en los controles sanos (3572.32 ng/L frente a 451 ng/L; p = 0.002). En el grupo con CA, el valor mediano de sHH4 fue mayor en los pacientes en clase B de Child-Pugh que en aquellos en clase A (p = 0.026). Hubo una correlación positiva entre el valor de sHH4 y la duración del consumo de alcohol y las puntuaciones MDF (Maddrey's Discriminant Function) en los pacientes*

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con HA (rho = 0.886; p = 0.019 para ambos). Se detectó una correlación positiva entre los valores de sHH4 y las puntuaciones MELD-Na (Model for End-stage Liver Disease Sodium) en los pacientes con CA (rho = 0.527; p = 0.006). **Conclusiones:** *Los valores elevados de sHH4 pueden ser un indicador de la gravedad de la HA y la CA.*

Palabras clave: *Enfermedad hepática alcohólica. Histona H4. Gravedad.*

Introduction

Alcohol abuse is a public health care problem. The pathogenesis of alcoholic liver disease (ALD) involves a series of complex interactions, including toxic alcohol metabolites, cytokines, oxidative stress, endotoxins, as well as genetic and immunological factors[1-6].

The accumulation of neutrophils in the liver of ALD patients has been reported to be associated with poor prognosis[6-8]. Disruption of the intestinal barrier due to alcohol leads to bacterial translocation from the gut and increased lipopolysaccharide concentrations in the portal circulation, which in turn triggers the recruitment of neutrophils into the liver[4,6].

In response to bacterial products and chemical agents, neutrophils secrete extracellular traps (NET), including DNA fragments, histones, and bactericidal proteins, a process called NETosis[8]. Histones are basic proteins functioning in the assembly of DNA molecules, participate in the cellular innate immune response, and are responsible for immune-mediated immunothrombosis through toll-like receptor (TLR) activation[8-10]. NET, including histones, revealed diagnostic and prognostic significance in some clinical conditions, including sepsis, trauma, venous thromboembolism, and inflammatory bowel diseases[8]. Neutrophils and histones exert harmful and protective effects on inflammation, called double-edged swords[8].

Exaggerated NETosis can cause tissue damage, and excessive release of the histones from neutrophils into circulation was associated with liver injury in the experimental models of ALD[6,11-14]. In acute liver failure, hepatocyte necrosis triggers the release of damage-associated molecular patterns (DAMPs) that activate immune cells in the liver and circulation, and histones are members of DAMPs[9].

Regarding the associations between the extracellular release of histones in the liver and the pathogenesis of ALD, specifically, we aimed to evaluate whether the serum histone H4 (sHH4) levels of the patients with ALD could serve as diagnostic and prognostic markers correlating to the clinical and laboratory traits of the diseases.

Methods

Study population

Sixty-six patients with ALD and 47 healthy controls (HCs) admitted to our gastroenterology inpatient and outpatient services between October 2021 and July 2023 were enrolled in the study. The Local Ethics Committee approved the study (215/October 27, 2021). The study protocol complies with the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008), as reflected in a priori approval by the Institution's Human Research Committee. Written informed consent was obtained from all participants.

Participants with clinical conditions that can affect sHH4 levels, such as sepsis, inflammatory bowel diseases, or any malignancies, including hepatocellular carcinoma, venous thromboembolism, or trauma, were excluded from the study. Volunteers with severe organ failure, including acute liver failure, acute or chronic infections, autoimmune diseases, diabetes mellitus, and any cause of acute and chronic liver disease, as well as those with acute or chronic hepatitis B (including HBeAg-negative chronic hepatitis B infection) and C infection and illicit drug use, were also excluded from the study.

The patients using excessive alcohol of > 60 g/day for men and > 40 g/day for women for at least 5 years were enrolled in the study[1,15]. The diagnosis of alcohol use disorder was made by a psychiatrist after an interview, according to the Diagnostic and Statistical Manual of Mental Disorders[16]. The HCs were not using alcohol, and no hepatic steatosis or hepatomegaly was reported in ultrasonography (USG).

Data collection

The dose and the duration of alcohol use were noted for each patient with ALD. The body mass index (BMI), comorbidities, and medications were recorded for all participants. Based on the disease history, physical findings, laboratory results, and radiological results, patients with ALD were assigned into three

groups: alcohol-associated steatotic liver (ASL), alcoholic hepatitis (AH), and alcoholic cirrhosis (AC). USG was applied to all participants for the radiologic evaluation.

AH was defined according to clinical and laboratory criteria. In this group, the alcohol intake was continuous for 6 months or more with < 60 days of abstinence before the onset of jaundice, and serum aspartate transaminase (AST) values were higher than alanine aminotransferase (ALT) values (AST/ALT ratio > 1.5), serum total bilirubin > 3.0 mg/dL, and without any other cause[1,15]. Cirrhotic patients were not included in the AH group.

AC was diagnosed according to the stigmata of cirrhosis in the physical examination and to the results of the biochemical tests along with the USG reports. Patients with ALS were non-cirrhotic and had no clinical or laboratory criteria for AH. The ALD patients did not present with the clinical and laboratory findings of acute liver failure and hepatic encephalopathy.

Assessment of the severity of disease in patients with ALD

All participants had blood chemistry tests, including the complete blood count, C-reactive protein (CRP), and D-dimer values, before USG. According to USG, the presence of hepatic steatosis was recorded in all participants and in patients with AH and AC, the presence of ascites was noted[17]. The patients with AH and AC underwent upper gastrointestinal endoscopy, and the presence of esophageal and/or gastric varices was recorded.

The fibrosis-4 (FIB-4) index was used to predict liver fibrosis for patients with ALD[18]. The Maddreys-discriminant function (MDF) tests and the Model for End-stage Liver Disease Sodium (MELD-Na) scores were calculated in patients with AH[19,20]. The patients with AC were classified according to the Child-Pugh classification (CPC A, B, C). The MELD-Na scores were also noted in the cirrhotic patients[21,22].

Measurement of sHH4

The serum for histone H4 (HH4) was separated from venous blood samples. After centrifugation at 5000r *g* for 10 min at 30nut the supernatant serum was stored in Eppendorf tubes at (–) 80be for 12-24 months. For the enzyme-linked immunosorbent assay (ELISA) measurement of sHH4, the Human HH4 (HIST1H4A) Bioassay Technology Laboratory Kit (Cat. No. E5420 Hu, Lot:202305012) was used (Intra-Assay: CV < 8%, Inter-Assay: CV < 10%) with a microplate reader (Biotech Epoch 2 Microplate ELISA Reader, USA).

Statistical analysis

Statistical analyses were performed using jamovi version 2.3.28.0 and easyROC version 1.3.1[23,24]. Descriptive statistics were presented as median and mean with quartiles and standard deviations, respectively, for continuous variables and as frequencies with percentages for categorical ones. Mann-Whitney U and Kruskal-Wallis compared non-parametric continuous variables and analysis of variance for those with parametric features. Furthermore, the χ^2 test was used to test equity with categorical variables. Diagnostic accuracy was studied by receiver operating characteristics (ROCs) curved analysis. The confidence level for statistical significance was defined as 95%.

Results

The study evaluated 66 patients with ALD (62 males, 4 females) and 47 HCs (42 males, 5 females). The demographic, clinical, and histopathological characteristics of the study population are presented in table 1. For gender, males dominated the study population, and there was no statistically significant difference among the groups. The mean age of the patients was the highest in the AC patients (p = 0.001). The mean BMI of the HC group was the lowest, but it was similar among the ALD groups (p < 0.001) (Table 1).

The neutrophil counts were similar in all groups, whereas the median CRP value was the lowest in the HC group (Table 1). While the median Fib-4 index was the highest in the AH group, no difference existed between the patients with AH and AC (p < 0.001) (Table 1). The median D-dimer values were similar between the AH and AC and between the ALS and HC groups (p < 0.001).

In total, sHH4 concentrations were higher in the ALD patients compared to the HCs (878.91 [391.54-1985.92] vs. 451 [359.91-922] ng/L, respectively, p = 0.034). Regarding sHH4 concentrations, there was a statistically significant difference between the subgroups of ALD patients and the HCs (p = 0.002) (Table 1). The median sHH4 value was the highest in the AH patients and the lowest in the HCs (3572.32 ng/L vs. 451 ng/L, respectively, p = 0.002). The median

Table 1. Clinical, demographic, laboratory characteristics, and serum histone H4 values in patients with alcoholic liver diseases and healthy controls

Parameters	Alcohol-associated steatotic liver (n = 34)	Alcoholic hepatitis (n = 6)	Alcoholic cirrhosis (n = 26)	Healthy control group (n = 47)	p
Gender, n (%) Male	33 (97.1)	5 (83.3)	24 (92.3)	42 (89.4)	0.522
Age, median (IQR)	47 (43-53) [c]	45 (41-47) [c]	60 (53-66)[a,b,d]	45 (32-61)[c]	0.001†
Duration of alcohol use (years), median (IQR)	20 (15-30) [c]	21 (15-28)	35 (25-47)[a]	-	< 0.001*
BMI (kg/m^2), mean ± SD	28.29 ± 2.8[d]	29.51 ± 3.75[d]	29.72 ± 3.85[d]	25.22 ± 3.62[a,b,c]	< 0.001*
Presence of hepatic steatosis, n (%)	34 (100)	5 (83.3) [c]	8 (30.7) [b]	-	< 0.001*
Presence of ascites, n (%)	-	3 (50)	11 (42.3)	-	0.732
Esophageal/gastric varices, n (%)	-	3 (50)	16 (61.5)	-	0.604
Fibrosis-4 index, median (IQR)	0.95 (0.74-1.98) [b, c]	4.47 (2.58-7.64) [a]	3.45 (2.52-6.08) [a]	-	< 0.001*
MELD-Na score, median (IQR)	-	20 (9-39)	14 (9-21)	-	0.358
MDF, median (IQR)	-	50.5 (17.5-164)	-	-	-
Child-Pugh class, n (%) A B C	-	-	 10 (38.5) 7 (26.9) 9 (34.6)	-	-
Child-Pugh score, median (IQR)	-	-	7 (5-10)	-	-
Neutrophils (×10^3/μL), median (IQR)	5.26 (3.99-6.55)	4.62 (4-7.2)	3.53 (2.66-6.14)	4.63 (3.79-5.69)	0.056
D-Dimer (ug/mL), median (IQR)	0.45 (0.22-0.82)[c]	0.99 (0.56-4)[d]	1.58 (0.46-4)[a,d]	0.26 (0.1-0.46)[b,c]	< 0.001*
CRP (mg/L), median (IQR)	5.89 (1.63-14.86)[d]	15.6 (8.02-27.5)[d]	7.08 (3-21.1)[d]	2 (0.91-3.36)[a,b,c]	< 0.001*
Serum histone H4 (ng/L), median (IQR)	706.18 (407.04-1376.89)[b]	3572.32 (2387.4-4856)[a,c,d]	789.57 (333.8-2233.8)[b]	451 (359.91-922)[b]	0.002†

*Statistically significant level was lower than 0.001. According to the pairwise comparison, it was different from alcohol-associated steatotic liver.
†Statistically significant level was lower than 0.01.
[a]alcohol-associated steatotic liver.
[b]alcoholic hepatitis.
[c]alcoholic cirrhosis.
[d]healthy control group.
IQR: inter quartile range; SD: standard deviation; BMI: body mass index; MELD-Na: model for end-stage liver disease sodium; MDF: Maddrey's discriminant function; CRP: c-reactive protein.

sHH4 values were similar in the ALS, AC, and HC groups (Table 1).

In the AH and AC groups, the median sHH4 values were higher in the patients with steatosis, but the differences were not statistically significant. Although not significant, the patients with ascites, esophageal, and/or gastric varices also had higher median sHH4 values. According to the CPC in the AC patients, the median sHH4 value was higher in the CPC-B group than in the CPC-A group, but there was no difference between the CPC-B and CPC-C groups (p = 0.026) (Table 2).

There were positive correlations between the sHH4 value and the duration of alcohol use and MDF scores in patients with AH (rho = 0.886, p = 0.019 for both). In the AC group, there was a positive correlation between the sHH4 values and MELD-Na scores (rho = 0.527, p = 0.006). No correlation was reported between the sHH4 values and the Child-Pugh scores, neutrophil counts, and FIB-4 scores in the ALD patients (Table 3).

ROCs curve analyses revealed that the sHH4 concentrations had a predictive value to differentiate AH from the ASL (Area under the curve: 0.931 [95% CI: 0.842-1.00, p < 0.001]; Fig. 1). Serum HH4 levels higher than 0.765 ng/L had a diagnostic accuracy for AH with a sensitivity of 100% (0.541–n/a) and a specificity of 76.5% (95% CI: 0.588-0.893).

Discussion

Harmful alcohol use is a rising global burden and causes morbidity and mortality in the younger ages of life, with over 3 million deaths worldwide every year[2].

Table 2. Serum histone H4 concentrations and clinical and laboratory variables in patients with alcoholic hepatitis and cirrhosis

Group	n	Serum histone H4 (ng/L) Median IQR		p
Alcoholic hepatitis (n = 6)				
Hepatic steatosis				
Absent	2	3572.3	(2050.4-4710)	1
Present	4	3621.7	(2387.4-4856)	
Ascites				
Absent	3	2721.6	(2387.4-4997)	1
Present	3	4423	(1379.2-4856)	
Esophagus and/or gastric varices				
Absent	3	2721.6	(2387.4-4997)	1
Present	3	4423	(1379.2-4856)	
Alcoholic cirrhosis (n = 26)				
Hepatic steatosis				
Absent	24	789.5	(329.8-2109.8)	0.677
Present	2	1409.5	(350.8-2468.2)	
Ascites				
Absent	15	391.5	(325.8-1985.9)	0.443
Present	11	962	(385.3-2377.2)	
Esophagus and/or gastric varices				
Absent	10	371.2	(305.3-1334.8)	0.182
Present	16	1233	(371.2-2287.8)	
Child-Pugh Classification				
A	10	345.4	(305.3-391.5)[b]	0.026*
B	7	2233.8	(447.9-2468)[a]	
C	9	1406	(617.1-2341.8)	

*Statistically significance level was lower than 0.05. According to the pairwise comparison, it was different from Child-Pugh Classification A[a], B[b].

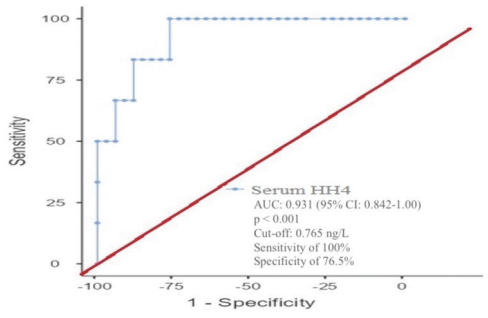

Figure 1. *Receiver operating characteristics curve analyses of the serum histone H4 concentrations, which differentiate alcoholic hepatitis from alcohol-associated steatotic liver (Area under the curve: 0.931 [95% CI: 0.842-1.00, p < 0.001]).*

Alcoholic fatty liver disease, alcohol-related hepatitis, and cirrhosis are consecutive disorders. Liver disease in alcohol use disorder is still a challenging enigma for practitioners[2,6].

Approximately 10-20% of alcoholic patients may subsequently develop hepatitis, and overall, 8-20% of patients with alcoholic steatosis can progress to cirrhosis[25]. Despite the excessive amount of alcohol ingestion, it is not known why some patients are more vulnerable to ethanol, which progresses to advanced liver disease and cirrhosis, while it may be steady in others. Unknown mechanisms may exist in the pathogenesis of ALD[5,6,25].

Neutrophils enhance lipid accumulation in the liver by inducing pro-inflammatory cytokine secretion in ALD[6,26,27]. Histones, classified as H1, H2A, H2B, H3, and H4, are the main members of the NET family, and HH4 is the most investigated molecule[28]. Neutrophils and histones exert harmful and protective effects on inflammation, called double-edged swords[8].

In a model of ethanol-related liver injury, the ethanol-derived acetate was converted to acetyl-CoA, which bound to specific sites on HH3 and HH4 proteins and disrupted the biological function of the cell by altering post-transcriptional protein modification through histone acetylation[11].

In a previous study, mice were treated with alcohol binges and LPS, and endotoxin significantly increased NET formation. In the efferocytosis process, in which apoptotic cells are removed by phagocytic cells, indicators of NET formation, including citrullinated histone-H3, increased in mouse hepatocytes, and citrullinated histone-H3 formation was attributed to the decreased clearance of NET[13].

Li et al. reported that histones are the critical mediators of liver injury in a heat shock (HS) setting through hepatocyte pyroptosis, a novel type of programmed cell death that causes pro-inflammatory cytokine secretion in murine hepatocytes[14]. Serum HH3 (sHH3) levels, which ELISA measured in HS mice, increased in a time- and dose-dependent manner compared to the control group, which was not exposed to HS injury. There were positive correlations between the sHH3 values, serum transaminase levels, and the histological activity scores of mice hepatocytes. Although HH3 was declared a pivotal mediator of hepatic injury, the other histone monomers were not evaluated in that study[14].

The aforementioned body of evidence demonstrates the pathogenic significance of histones in the experimental models of liver disease, and, as noted earlier, over-expressed histones in the tissue might diffuse into circulation[8,11-14]. Thus, we hypothesized that sHH4 concentrations might relate to the clinical traits of ALD patients.

Our results revealed higher sHH4 concentrations in the ALD patients compared to the HCs. Among the ALD groups, the mean sHH4 value was the highest in AH patients and the lowest in the ALS group. In the present study, increased extracellular HH4 release

Table 3. Correlations between the serum histone H4 values and the clinical and laboratory variables of the patients with alcoholic liver diseases

Parameters	Serum histone H4 (ng/L)					
	Alcohol-associated steatotic liver (n = 34)		Alcoholic hepatitis (n = 6)		Alcoholic cirrhosis (n = 26)	
	rho	p	rho	p	rho	p
Duration of alcohol use (years)	–0.255	0.146	0.886	0.019*	–0.005	0.981
Child-Pugh score	-	-	-	-	0.368	0.065
MELD-Na	-	-	0.771	0.072	0.527	0.006**
MDF	-	-	0.886	0.019*	-	-
Neutrophils (x10^3/μL)	–0.157	0.760	–0.371	0.468	0.302	0.134
Fibrozis-4 score	0.071	0.690	–0.143	0.787	0.278	0.169

*Correlation is significant at the 0.05 level. **Correlation is significant at the 0.01 level.
MELD-Na: model for end-stage liver disease sodium; MDF: Maddrey's discriminant function.

may be due to higher inflammatory activity in AH patients, as noted with high CRP values in that group.

In the AC patients, the mean sHH4 value was the lowest in CPC-A patients, whereas it was the highest in the CPC-B group. In the CPC-A group, as expected, the patients had compensated disease. Although not statistically significant, patients presenting with ascites and varices had higher median sHH4 concentrations. Necrotic hepatocytes that are exposed to ethanol toxicity can act as DAMPs and induce the activation of innate immunity, further aggravating liver injury and subsequent fibrosis, a process that is partly due to histone activation[4,6,9,14]. In our study, increased sHH4 values might indicate susceptibility to hepatic decompensation in AH and AC patients. Nevertheless, the number of patients was low in the CPC groups, and it can be a focus for future research.

Serum D-dimer values are representative of thrombosis, and histones promote thrombosis through TLR activation[8,10,14]. In the present study, the mean D-dimer values of the ALD patients were higher in the AC and AH patients and this result might be a clue for the procoagulant feature of severe ALD.

The Fib-4 index was reported to have a predictive value for hepatocellular carcinoma in alcoholic patients[29]. There was no correlation between the FIB-4 index and sHH4 values in the ALD patients, even in the AC group, who are more prone to developing carcinoma. As a predictive method, it is possible that the Fib-4 index may not exhibit the disease severity in ALD. On the other hand, performing a liver biopsy could be more precise for scoring the fibrosis. However, it is not recommended for ALD patients with significant alcohol use and with no other cause of chronic liver disease because of its complications and ethical considerations[29].

No correlation was reported between the sHH4 values and the MELD-Na values in patients with AH. However, MDF scores, which are a good predictor of mortality, were strongly correlated to sHH4 values[19]. Despite the small sample size of the AH patients, sHH4 values had a predictive value to differentiate AH from ALS. Larger sample-sized cohorts might confirm the diagnostic value of sHH4. In patients with AH, in which time- and dose-dependent continuous ethanol ingestion is the cause, it was noteworthy that the duration of alcohol use was strongly correlated with sHH4 concentrations. The CPC scores did not correlate to sHH4 values in the AC patients, but there were positive correlations between the sHH4 concentrations and MELD-Na values. This may partly be due to the subjective components of the CPC[21]. sHH4 values may be a prognostic marker in patients with AH and AC.

Although the median sHH4 was different among the groups, neutrophil values were similar in the present study. The dichotomous choice of neutrophils is phagocytosis or NETosis, which relates to pro-inflammatory cytokines, chemical stimuli, and antigen size[28]. As noted earlier, histones are known as DAMPs, and cell death in hepatocytes promotes more histone release into circulation from neutrophils[9]. Thus, rather than the counts of neutrophils, the pathogenic significance of neutrophils might exert an influence on liver injury.

Inhibition of NET formation through an anti-citrullinated protein antibody that specifically binds to

citrulline in histones 2A and 4 has been declared to have therapeutic potential in the experimental arthritis model[30]. Li et al. noted that hepatotoxicity and inflammation due to HH3 are reduced after anti-H3 antibody treatment in the HS-induced liver injury model[14]. Abstinence from alcohol is the primary goal in treating alcohol use disorder, and anti-histone treatment might be a candidate modality to abolish liver injury in ALD.

This study is the first clinical trial of the serum HH4 values in patients with ALD and should be considered a preliminary step for future research. The major limitation is the relatively small size of the population, especially in the AH group, as it was a single-centered trial.

Conclusion

In patients with AH and AC, sHH4 concentrations might delineate disease severity. If proven further, larger cohorts may confirm the relationship between the clinical and laboratory traits of disease and sHH4 values in patients with ALD. Diagnostic strategies with the possibility of therapeutic interventions can be developed by identifying new practical and objective biomarkers in ALD.

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Conflicts of interest

The authors declare no conflicts of interest.

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Ethical considerations

Protection of humans and animals. The authors declare that the procedures followed complied with the ethical standards of the responsible human experimentation committee and adhered to the World Medical Association and the Declaration of Helsinki. The procedures were approved by the institutional Ethics Committee.

Confidentiality, informed consent, and ethical approval. The authors have followed their institution's confidentiality protocols, obtained informed consent from patients, and received approval from the Ethics Committee. The SAGER guidelines were followed according to the nature of the study.

Declaration on the use of artificial intelligence. The authors declare that no generative artificial intelligence was used in the writing of this manuscript.

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