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ABOUT COVER

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AIMS AND SCOPE

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INDEXING/ABSTRACTING

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Retrospective Study

Usefulness of Mac-2 binding protein glycosylation isomer in non-invasive probing liver disease in the Vietnamese population

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Abstract

BACKGROUND
Early diagnosis is critical for successful intervention before liver disease progresses to cirrhosis and hepatocellular carcinoma.

AIM
To examine a novel biomarker for probing early liver disease quickly using an automated immunology system.

METHODS
This was a cross-sectional study. 140 patients at various stages of liver disease were randomly selected. The cohort consisted of patients who were treatment naïve and currently undergoing therapy. We included patients with diverse liver disease etiologies. Mac-2 binding protein glycosylation isomer (M2BPGi) levels in addition to different clinical parameters, co-morbidities and transient elastography results were collected and compared.

RESULTS
M2BPGi levels were significantly correlated with transient elastography for liver fibrosis staging across all disease etiologies. Statistically significant differences were observed in patients with F0-1; F2 and > F3 liver fibrosis. Further examination showed that M2BPGi levels were two-fold higher in F4 than F3 hepatitis C (HCV) patients. M2BPGi was observed to be etiology-specific and HCV patients had higher mean M2BPGi levels. We also observed significant correlations with aspartate aminotransferase to platelet ratio index and fibrosis-4 index as well as HBV DNA levels. Mean M2BPGi levels for HBV patients with a viral load lower than 2000 IU/mL was 1.75-fold lower than those with a viral load greater than 2000 IU/mL.

CONCLUSION
M2BPGi was observed to be a good indicator of early liver disease in patients with different etiologies. Our results provide reference cut-offs for different causes of liver disease and demonstrated the utility of this marker for early

disease monitoring. This is useful for remote regions in developing countries.

Key words: Hepatitis B; Hepatitis C; Noninvasive fibrosis markers; Mac-2 binding protein glycosylation isomer; Liver disease

Core tip: Mac-2 binding protein glycosylation isomer levels can be used for non-invasive liver fibrosis staging in the Vietnamese population with mixed etiologies. In early evaluations, significantly higher levels of this marker were observed in cirrhotic patients and showed good correlations with viral load testing in hepatitis B. This marker is convenient and useful, especially in resource limited countries for fast turnaround to assess liver disease.

INTRODUCTION

Chronic liver disease resulted in a high global burden of 1.5 billion people in 2017, which was mostly due to non-alcoholic fatty liver disease (NAFLD, 60%), hepatitis B (HBV, 29%), hepatitis C (HCV, 9%), and alcoholic liver disease (ALD, 2%)[1]. Chronic liver disease progressively leads to liver fibrosis, cirrhosis, and finally hepatocellular carcinoma (HCC). Globally, liver disease accounts for 2 million deaths per year, of which 1 million are due to cirrhosis and 1 million are due to hepatocellular carcinoma[2]. Vietnam has a high prevalence of hepatitis B and C, with a high HBV surface antigen (HBsAg) rate of 8.8%-19.0% and a high anti-HCV rate of 1.0%-3.3%[3]. It is estimated that HBV-related liver cancer and HCC will increase to 9400 and 25000, respectively, and HBV-related mortality will increase to 40000 in 2025 in Vietnam[4]. Given the disease impact in Vietnam, efforts to provide early detection of liver disease are critical.

Early diagnosis and treatment are key to improve disease outcomes as it can halt further liver disease progression and save lives. At present, pan-genotypic drug combinations of direct acting antivirals such as sofosbuvir/velpatasvir and glecaprevir/pibrentasvir have demonstrated high efficacy against different HCV genotypes[5]. Long-term HBV antivirals have been effective in managing the disease[6]. Nonetheless, most patients are in a chronic state and different degrees of liver fibrosis have manifested. Management of these patients requires clear understanding of their liver fibrosis staging[7,8]. At present, liver biopsy is considered the gold standard for liver fibrosis staging. However, liver biopsy has its limitations such as sampling bias and inter-observer variations[9]. In addition, liver biopsy is invasive and it is not feasible to carry out repeated liver biopsies for regular monitoring. In advanced stage liver disease patients (> F3), a 3-6 monthly evaluation is required. In response to these limitations, non-invasive tests using imaging methods such as transient elastography (TE)[10], magnetic resonance elastography[11], ultrasound-based elastography[12], and acoustic radiation force impulse[13] have emerged. Serum biomarkers have also been explored. These included hyaluronic acid, type IV collagen, type III procollagen-N-peptide[14], soluble Axl[15], and lincRNA-p21[16] among others. Surrogate markers (aspartate aminotransferase to platelet ratio index (APRI)[17], and Fibrosis-4 index (FIB-4)[18] are also some of the commonly used tests to assess liver disease. A number of these tests require costly equipment, and patient results turnaround and waiting times may limit their usefulness. On the other hand, other tests lack sufficient clinical sensitivity and specificity.

In recent years, Mac-2 binding protein glycosylation isomer (M2BPGi) was identified in patients with liver fibrosis[19]. Serum M2BPGi levels were found to correlate with liver fibrosis stage[20], reflect impaired liver function or cirrhosis and hepatectomy-related complications after surgery[21], correlate with liver stiffness[22], and was negatively correlated with sustained virological response (SVR)[23]. In

addition to liver fibrosis in HCV and HBV, M2BPGi can be used to assess liver fibrosis in primary biliary cirrhosis[24], biliary atresia[25], primary sclerosing cholangitis[26], and NAFLD[27]. In this study, we examined M2BPGi as a biomarker for early monitoring of liver disease in a Vietnamese population. We compared M2BPGi levels with different clinical parameters, co-morbidities and TE results. M2BPGi was measured from blood samples using a sandwich immunoassay. The aim of the current study was to examine this novel diagnostic biomarker for probing early liver disease quickly and easily using an automated immunology system.

MATERIALS AND METHODS

Study design and patient demographics

A cross-sectional study design was employed in this study. Institutional review board approval was waived as residual blood samples were used in the evaluation. The results of this study did not alter the course of treatment in these patients. Other than patient history and current laboratory results, no other patient identifiers were used. We randomly selected patients with diverse liver disease etiologies who were treated in MEDIC Medical Center, Ho Chi Minh City, Vietnam from January to April 2019. Patients with confirmed liver cancer were excluded. Routine clinical investigations as part of standard of care included liver function tests, TE and other associated fibrosis measurements. A total of 140 patient samples were analyzed.

Liver disease probing and measurements

As part of standard of care, TE (Fibroscan, Echosens, France) was performed on all patients. Staging of liver fibrosis in patients was based on the METAVIR scoring system (F0 to F4, where F0: No fibrosis and F4: Cirrhosis) and cut-offs adopted from manufacturer's recommendations. Plasma and serum blood samples were taken from each patient for complete blood count and clinical biochemistry investigations. Liver function tests including alanine aminotransferase (ALT), aspartate aminotransferase (AST) alkaline phosphatase (ALP), albumin and bilirubin were measured using a routine laboratory chemistry analyzer (Roche, Switzerland). APRI was calculated using the following equation: AST (/upper limit of normal) × 100/platelet count (PLT) ($\times 10^9$/L)[17]. FIB-4 was calculated using the following equation: FIB-4 = age (years) × AST (U/L)/[PLT (10^9/L) × ALT (U/L)$^{1/2}$][18]. PLT was obtained using a hematology analyzer among other blood parameters (Sysmex, Japan). Serum M2BPGi levels were measured on the HISCL 5000 automated immunoassay analyzer (Sysmex, Japan). 10 µL of sample were used and M2BPGi levels were measured by a sandwich immunoassay. Each reaction took 17 min and was performed automatically alongside HBsAg and anti-HCV assays. M2BPGi was expressed as the cut-off index (COI) and calibrated using the manufacturer's calibrators.

Statistical analysis

All categorical variables were expressed as mean ± SD. Student *t* tests were used to compare two categorical variables and one-way analysis of variance (ANOVA) was used to compare multiple groups of liver fibrosis stages. A difference of $P < 0.05$ was considered statistically significant. Analysis was computed using Prism version 7.0 (Graphpad Inc., United States).

RESULTS

Patient characteristics and experimental design

Patient demographics are shown in Table 1. A total of 140 patients were enrolled. This was a cross-sectional study involving randomly selected patients with diverse liver disease etiologies to assess the versatility of the marker M2BPGi. All patients were treated in the MEDIC Medical Center and assessed by their attending physicians for liver disease stage. The median age of the study cohort was 52 years and all suffered from chronic hepatitis. Among the patient group, the number of male and female patients was almost equal (52.9% *vs* 47.1%, respectively). Most of the selected patients were HBV or HCV patients, of which around 60% were HBV patients, and 2 were patients co-infected with HBV and HCV. The HBV and HCV cohorts, 35% and 13%, respectively, had immediate family members with the same condition or had progressed to HCC. In Vietnam, HBV is the predominant cause of HCC and was critical in this study. The range of TE, APRI, FIB-4 and M2BPGi measurements were 3–46.4 kPa, 0.07–7.02, 0.23–9.97 and 0.07- > 20 COI, respectively. Among the 140 patients, 35% of patients had higher than normal AST, ALT or Gamma glutamyl

transferase. Alpha fetoprotein (AFP) measurements were obtained in these patients as part of an early indication of HCC. Only 1 patient (112 ng/mL) in this cohort had higher than normal AFP level.

M2BPGi levels were correlated with other fibrosis markers in HCV patients

As shown in Figure 1A, M2BPGi levels increased with increasing fibrosis stages (as measured by TE) in HCV patients. The results were statistically significant when comparing the F2 cohort with F0-1 subjects. Patients with significant fibrosis require regular monitoring and this provides a quick method to stratify patient groups for clinicians. We also observed significantly more patients with severe fibrosis or cirrhosis than patients with normal liver in HCV positive individuals. In an attempt to further evaluate the discriminatory role of M2BPGi, we divided patients with > F3 fibrosis into the F3 and F4 groups (Figure 1B). The results showed a statistically significant difference in M2BPGi levels between F3 and F4 patients. The cirrhotic group (F4) had two-fold higher M2BPGi levels. These data supported the accurate staging of liver fibrosis using M2BPGi levels. More data are required to ensure statistical power for medical decisions; however, preliminary data demonstrated strong evidence of a clear discriminatory role. Within this cirrhotic patient group, some patients were currently undergoing treatment and some were treatment naïve. M2BPGi levels were observed to be insignificant between these two groups (Figure 1C). Previous reports have shown that M2BPGi may be affected by the treatment for viral hepatitis. In a comparison of fibrosis staging methods, we selected only treatment-naïve patients (Figure 2) to ensure an unbiased correlation. As shown in Figure 2, we found that M2BPGi levels showed good correlation with TE (A), FIB-4 (B), and APRI (C) in untreated patients ($P < 0.001$).

M2BPGi levels were correlated with other fibrosis markers in HBV patients

Similar to the results in the HCV group, we observed significant correlations in HBV patients. The limitation of the current study was that the distribution among this cohort leaned towards the early liver disease group with fewer severe fibrosis and cirrhosis patients. Nonetheless, from Figure 3A, it can be seen that M2BPGi levels increased with increasing fibrosis stage. The maximum detected M2BPGi level in patients with > F3 fibrosis staging was 4.0 COI with a mean value of 2.0 COI. This was more than 2-fold higher than early stage patients (F0-1) with a mean M2BPGi level of - 0.8 COI. Compared with a similar early disease group in HCV patients, the mean levels were significantly lower in the HBV cohort indicating the need for etiology-specific cut-offs using M2BPGi. Further division of > F3 patients into F3 and F4 groups showed the same trend as previously seen in the HCV cohort (Figure 3B). More datapoints are required for better statistical comparison in advanced stage patients. In the same cohort, we attempted to understand if M2BPGi correlated with HBV DNA levels. HBV viral load is a required parameter during treatment monitoring especially in addressing treatment discontinuation and efficacy. From Figure 4, it can be seen that the mean M2BPGi level for hepatitis B patients with a viral load of < 2000 IU/mL was 1.75-fold lower than the group with a viral load greater or equal to 2000 IU/mL.

M2BPGi levels are etiology-specific for early liver disease

During early analysis, it was observed that M2BPGi levels were significantly different among the HBV and HCV cohorts within the same fibrosis stages. To address these differences further, the patient cohort was divided into HCV, HCV with NAFLD, HBV, HBV with NAFLD, NAFLD, and ALD as shown in Figure 5 for early disease patients (F0-1). ANOVA showed significant differences among these major groups. Viral hepatitis related liver disease alone resulted in higher M2BPGi levels compared with alcoholic liver disease ($P < 0.01$) and NAFLD ($P < 0.01$). Interestingly we did not observe any statistical significance in viral hepatitis patients with NAFLD comorbidity (HCV *vs* HCV and NAFLD; HBV *vs* HBV and NAFLD). These findings show that M2BPGi levels may be dominated by the presence of viral hepatitis in patients. Overall, we found that in early disease patients, M2BPGi levels were highest in HCV groups, followed by HBV and subsequently alcoholic liver disease and finally NAFLD cases.

DISCUSSION

Non-invasive tests in probing liver disease are gaining attention as liver biopsy is insufficient in addressing critical disease management roles. These methods are also more patient friendly and allow repeated sampling. However, equipment investment is not easy for medical facilities in remote regions of Vietnam in relation to advanced

Table 1 Evaluated subjects' demographics and corresponding liver fibrosis marker results		
Parameters		**Parameter value**
Sample size (*n*)		140
Age (yr)	Range (mean ± SD)	26-81 (52 ± 16)
Gender, *n* (%)	Male	74 (52.9%)
	Female	66 (47.1%)
Etiology, *n* (%)	HBV	54
	HBV + NAFLD	31
	HCV	37
	HCV + NAFLD	13
	HCV + HBV	2
	NAFLD	1
	Alcoholic	2
M2BPGi (COI)	Minimum	0.065
	Median	0.863
	Maximum	> 20
TE (kPa)	Minimum	3
	Median	6.4
	Maximum	46.4
APRI	Minimum	0.0737
	Median	0.2997
	Maximum	7.0192
FIB-4	Minimum	0.2328
	Median	1.119
	Maximum	9.9664

HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Non-alcoholic fatty liver disease; M2BPGi: Mac-2 binding protein glycosylation isomer; TE: Transient elastography; COI: Cut-off index; APRI: Aspartate aminotransferase to platelet ratio index; FIB-4: Fibrosis-4 index.

elastography methods. Hence, it is crucial to have a quick and straightforward method to detect and stratify liver disease. Therefore, serum biomarkers derived from automated analyzers which are easy to interpret without skilled operators are desirable.

The results of the present study showed that M2BPGi levels were correlated with fibrosis stage derived from TE measurements and positively correlated with other surrogate markers (FIB-4 and APRI). This concurs with other studies[28,29]. However, no correlation was found between M2BPGi levels in treatment naïve and treated cirrhotic HCV patients. Treatment failure is typically associated with cirrhotic cases and the current results may be representative of this phenomenon. Ura *et al*[30] found that SVR is negatively correlated with M2BPGi levels, highlighting that treatment response may be reflected in serial M2BPGi measurements. Cirrhotic patients remain a key target group for treatment and surveillance, of which M2BPGi may be useful. One limitation of our study is the small sample size of cirrhotic patients (*n* = 16), which may be insufficient to demonstrate the effects of anti-viral treatment on M2BPGi levels in cirrhotic patients. Future studies will examine the effects of cirrhosis regression on M2BPGi levels in hepatitis patients, and patients can be further stratified based on time of onset of cirrhosis[31], which affects regression.

In this study other than METAVIR-based staging, we found that HBV viral load was correlated with M2BPGi levels, showing that M2BPGi may be used as a surrogate marker for HBV viral load and disease severity. In fact, some studies have shown that M2BPGi level is a predictor of HCC and death[32], HBV-related HCC recurrence[33], and liver function and prognosis[34]. Hence, M2BPGi may complement HBV viral load testing to better understand disease severity and prediction of disease outcomes. Several studies have shown that other than liver fibrosis staging, M2BPGi level is a predictor of HCC after SVR[35], HCC survival[36], HCC recurrence[37], and cirrhosis survival[38].

M2BPGi is etiology-specific and our results showed that M2BPGi levels are higher in HCV than HBV patients, which is consistent with Nishikawa *et al*[39] and patients with viral hepatitis have higher M2BPGi levels than those with NAFLD and ALD.
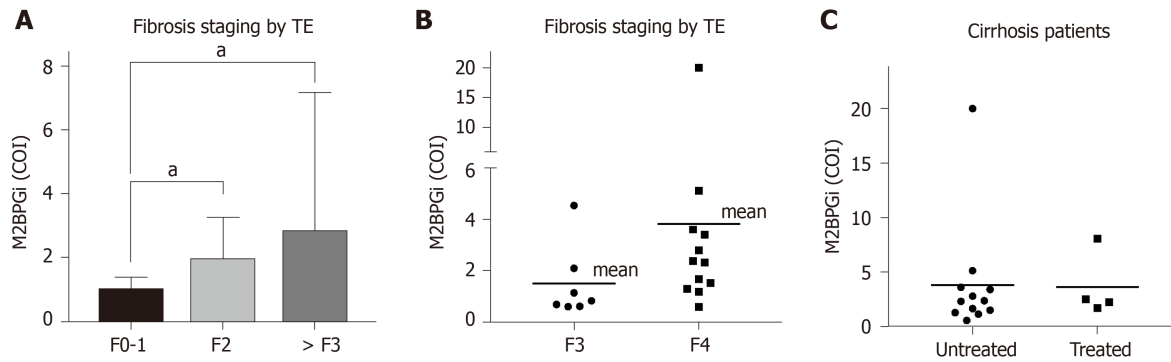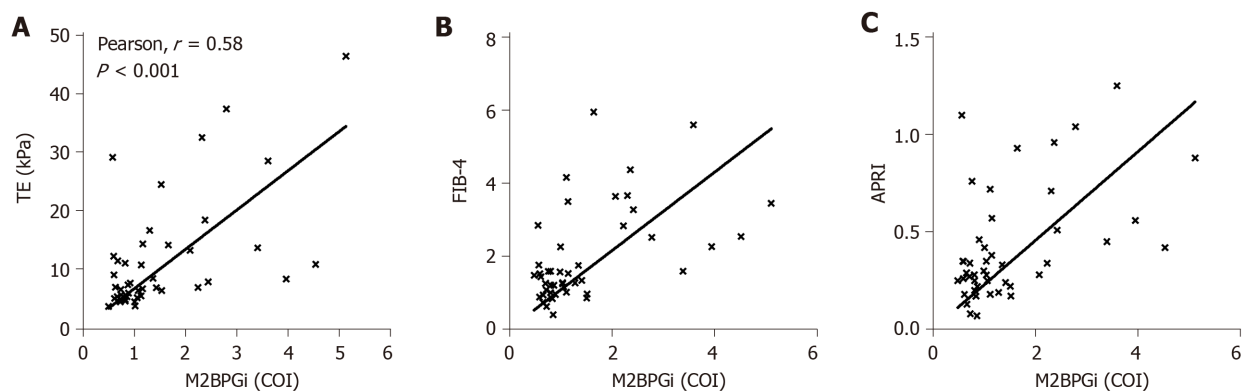
Figure 1 Comparison of Mac-2 binding protein glycosylation isomer levels against fibrosis staging by transient elastography in hepatitis C patients.
M2BPGi levels were plotted against fibrosis stages based on transient elastography. A: Mac-2 binding protein glycosylation isomer (M2BPGi) levels against fibrosis stages (F0-1, F2, and >F3); B: M2BPGi levels against fibrosis stages (F3 and F4); C: M2BPGi levels in treated and untreated cirrhosis patients. $^aP < 0.05$. M2BPGi: Mac-2 binding protein glycosylation isomer; COI: Cut-off index; TE: Transient elastography.

Therefore, different cut-off values should be assigned to different types of patients. The mechanism of this difference in M2BPGi level between HBV and HCV patients remains unclear, but our observational study showed that M2BPGi is dominated by the presence of viral hepatitis. With larger sample sizes in future investigations, we will be able to establish a clear disease-based cut-off to implement M2BPGi in routine clinical use.

The present study demonstrated the feasibility of using M2BPGi level as a surrogate marker for liver fibrosis in a Vietnamese population. This method is convenient, easy to use, and can guide prevention and treatment efforts for viral hepatitis in remote regions. At present, there is no national program against HBV and HCV in Vietnam[3] and mortality due to liver cancer is high in Vietnam[40]. In contrast, M2BPGi tests are reimbursable in South Korea and Japan. In future, national programs for treating HCV and HBV could be rolled out along with such tests to reduce the prevalence of liver fibrosis, cirrhosis, and HCC in Vietnam and thereby reduce mortality.

In conclusion, this study is the first to investigate mixed etiology testing of this marker and demonstrated a significant correlation with existing routine assays and treatment monitoring. Our results provide reference cut-offs for different causes of liver disease and demonstrated the utility of this marker for early disease monitoring. This is useful for remote regions in developing countries.

Figure 2 Comparison of Mac-2 binding protein glycosylation isomer levels against other fibrosis markers in treatment naïve patients. A: Mac-2 binding protein glycosylation isomer (M2BPGi) levels against transient elastography; B: M2BPGi levels against FIB-4; C: M2BPGi levels against aminotransferase to platelet ratio index. M2BPGi: Mac-2 binding protein glycosylation isomer; COI: Cut-off index; TE: Transient elastography; FIB-4: Fibrosis-4 index; APRI: Aspartate aminotransferase to platelet ratio index.
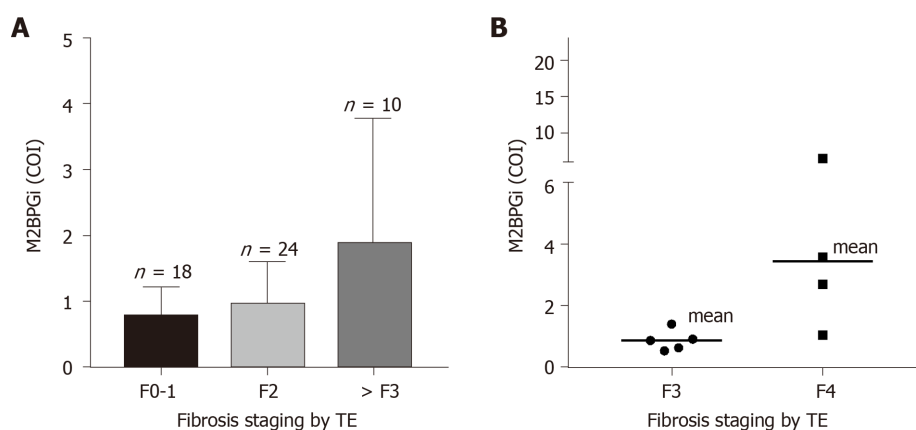
Figure 3 Comparison of Mac-2 binding protein glycosylation isomer levels against fibrosis staging by transient elastography in hepatitis B patients. A: Mac-2 binding protein glycosylation isomer (M2BPGi) levels against fibrosis stages (F0-1, F2, and > F3); B: M2BPGi levels against fibrosis stages (F3 and F4). M2BPGi: Mac-2 binding protein glycosylation isomer; COI: Cut-off index; TE: Transient elastography.
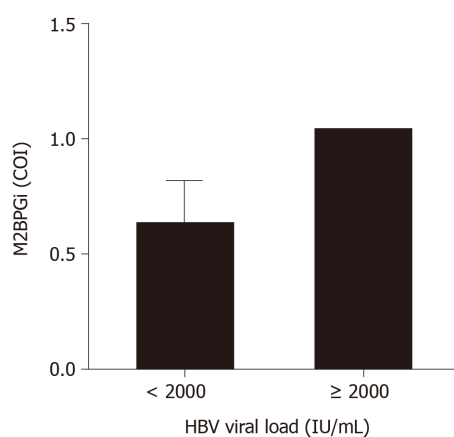
Figure 4 Comparison of Mac-2 binding protein glycosylation isomer levels against hepatitis B viral load. M2BPGi: Mac-2 binding protein glycosylation isomer; COI: Cut-off index; HBV: Hepatitis B virus.
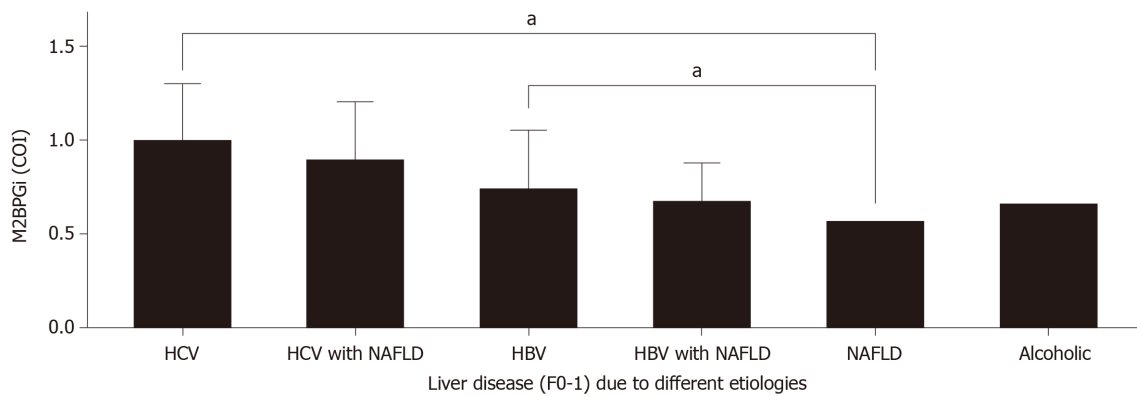
Figure 5 Mac-2 binding protein glycosylation isomer levels in early liver disease (F0-1) due to different etiologies.[a]$P < 0.01$. M2BPGi: Mac-2 binding protein glycosylation isomer; COI: Cut-off index; HBV: Hepatitis B virus; HCV: Hepatitis C virus; NAFLD: Non-alcoholic fatty liver disease.

ARTICLE HIGHLIGHTS

Research background
Non-invasive and rapid testing of liver disease for chronic hepatitis patients is crucial given its high prevalence in Vietnam.

Research motivation
Liver disease can be managed properly with timely treatment and control. Mac-2 binding protein glycosylation isomer (M2BPGi) offers the capability to stage fibrosis severity quickly and assess treatment response with longitudinal measurements.

Research objectives
This study aims to compare M2BPGi, a blood-based biomarker with existing methods of non-invasive testing and elastography in chronic hepatitis. In a preliminary assessment of treatment response, hepatitis B DNA concentrations were correlated with M2BPGi levels in respective patients.

Research methods
In patients with liver disease of different etiologies, M2BPGi levels in residual blood samples were measured. Comparisons with transient elastography (TE) were made to establish preliminary clinical cut-offs. Pearson correlations were tested using different liver disease markers to establish any significant trends. M2BPGi levels in early disease patients were compared to show etiology specificity.

Research results
We established clear correlations between M2BPGi with TE and other non-invasive biomarkers. For fibrosis staging of both hepatitis B and C patients, we observed statistically significant correlations with M2BPGi. M2BPGi levels in early disease were higher in viral hepatitis patients indicating the need to establish different cut-offs. The results were also significantly correlated with hepatitis B viral load, which established the possibility of treatment assessment.

Research conclusions
M2BPGi level is a useful addition to the current routine assessment of chronic hepatitis patients. This is performed by a routine immunoassay that enables fast turnaround time and quicker reporting for patients.

Research perspectives
We envision this research to have clinical potential to improve treatment monitoring procedures and rapid assessment of liver disease. In a resource limited setting, this marker presents useful results to ensure patients are linked to care promptly.

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