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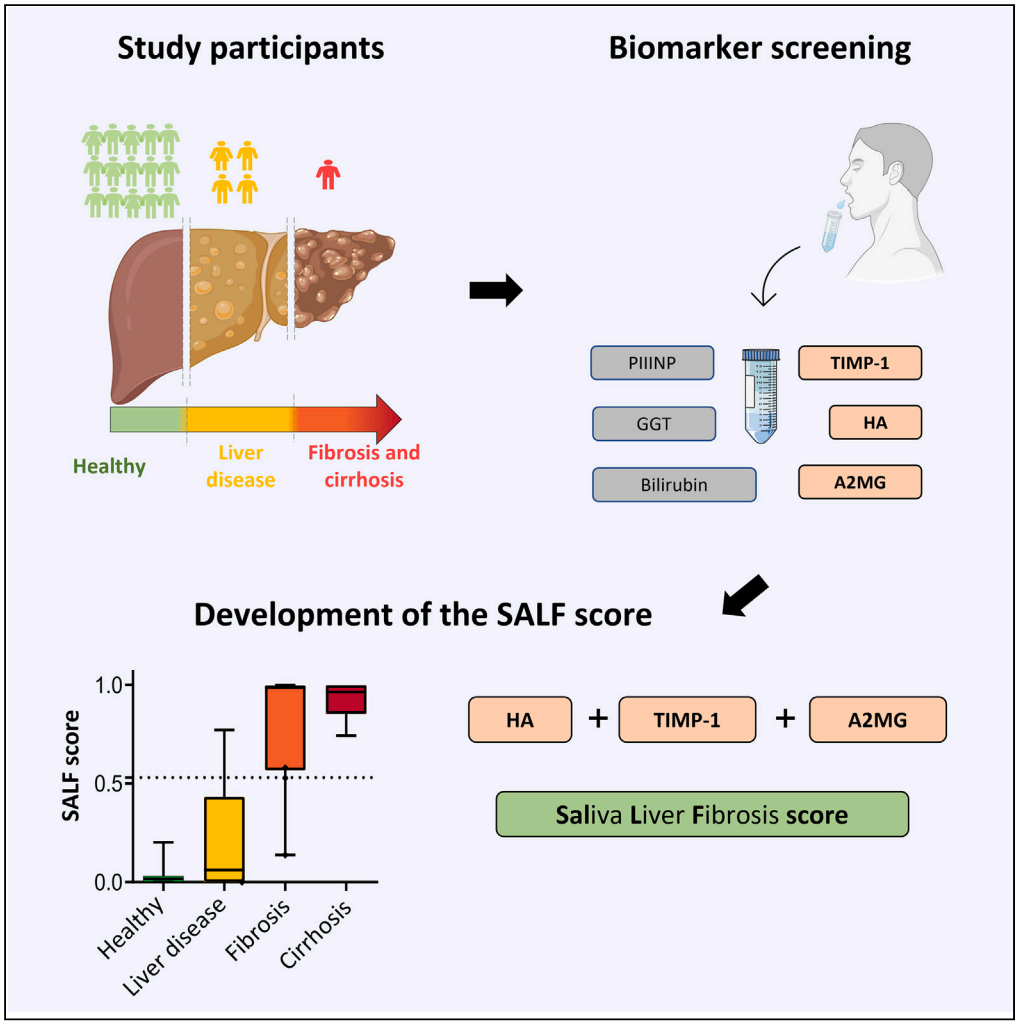

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Highlights

Currently used serum biomarkers for liver cirrhosis can be detected in saliva samples

The SALF score can accurately diagnose liver fibrosis and cirrhosis

The performance of the SALF score is similar to that of serum-based clinical tests

The biomarker panel correlates with the degree of liver fibrosis

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A salivary biomarker panel to detect liver cirrhosis

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SUMMARY

Limited access to diagnostic tests for liver fibrosis remains one of the main reasons for late diagnosis, especially in rural and remote communities. Saliva diagnostics is accessible with excellent patient compliance. The aim of this study was to develop a saliva-based diagnostic tool for liver fibrosis/cirrhosis. Salivary concentrations of hyaluronic acid (HA), tissue inhibitor of metalloproteinase-1 (TIMP-1), and α-2-macroglobulin (A2MG) were significantly increased ($p < 0.05$) in patients with liver fibrosis/cirrhosis. By combining these biomarkers, we developed the Saliva Liver Fibrosis (SALF) score, which identified patients with liver cirrhosis with an area under the receiver operating characteristic curve (AUROC) of 0.970 and 0.920 in a discovery and validation cohorts, respectively. The SALF score had a performance that was similar to that of the current Fibrosis-4 (AUROC:0.740) and Hepascore (AUROC:0.979). We demonstrated the clinical utility of saliva to diagnose liver fibrosis/cirrhosis with a potential to improve the screening for cirrhosis in asymptomatic populations.

INTRODUCTION

The accurate assessment of the degree of fibrosis in the liver is crucial for the clinical management of chronic liver disease patients. As liver-related morbidity and mortality are linked to the degree of liver fibrosis, a screening program to identify individuals with liver fibrosis becomes important not only to manage complications but also to monitor them for the progression to hepatocellular carcinoma (HCC). Liver/hepatic fibrosis is a common feature in the majority of chronic liver diseases and is characterized by the progressive substitution of the liver parenchyma by scar tissue as a response to sustained injury.[1] In its advanced stage, known as liver cirrhosis (LC), it can cause serious complications such as ascites, bleeding from esophageal varices, hepatic encephalopathy, HCC, and liver failure.[2] Indeed the vast majority of HCC cases develop in the context of a cirrhotic liver, and HCC is now the third leading cause of cancer-related death worldwide.[3] Thus, the reliable assessment of the degree of fibrosis is an important factor to guide therapeutic decisions and determine prognosis in patients with chronic liver disease.[4]

Liver biopsy is the gold standard method for the assessment of liver fibrosis.[5] However, the inaccuracy of biopsy sampling can result in failure to recognize cirrhosis in up to 20% of patients.[6] As such, liver biopsy is not a recommended tool for screening individuals at-high risk of progressive liver fibrosis.[7] Recently, non-invasive methods to detect liver fibrosis have gained attention. These methods consist of "biomarker approach", mainly using the quantification of biomolecules present in serum, and a "physical approach", using ultrasound- or magnetic resonance-based technologies.[8] In the biomarker approach, a score is calculated based on the measurements of a combination of clinical and laboratory variables.[9] As an example, the Fibrosis-4 (FIB-4) score is composed of age, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and platelet count, with an area under the receiver operating characteristic curve (AUROC) of 0.800 for the detection of fibrosis (77% sensitivity and 79% specificity).[10] Other scores use extracellular matrix-related molecules such as the Enhanced Liver Fibrosis (ELF) score, based on the measurement of type III procollagen peptide (P3NP), hyaluronic acid (HA), and tissue inhibitor matrix metalloproteinase-1 (TIMP-1).[11] Several studies report the ELF score as the most accurate serum-based test to detect advanced fibrosis and cirrhosis (AUROC 0.78–0.84).[4,12]

Recently, human saliva has gained attention as a diagnostic medium because it mirrors the general health status of an individual.[13–17] Saliva contains molecules that are synthesized by the salivary glands as well as

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Table 1. Characteristics of participants in the discovery and validation cohorts

	Discovery cohort	Validation cohort
n	40	95
Male, %	23 (57)	55 (57)
Age, years	60 (9)	64 (7)
AST, IU/L	40 (22)	52 (20)
ALT, IU/L	50 (18)	58 (24)
AP, IU/L	88 (29)	78 (14)
Albumin, g/dL	36 (4)	39 (6)
Cause of liver disease		
MAFLD, %	23 (76.6)	67 (82.8)
Alcohol, %	1 (3.3)	3 (3.7)
Viral hepatitis, %	2 (6.7)	5 (6.1)
Other, %	4 (1.4)	6 (7.4)
Liver fibrosis stage		
% of patients without fibrosis	20 (50.0)	54 (56.8)
% of patients with intermediate fibrosis	10 (25.0)	10 (10.6)
% of patients with liver cirrhosis	10 (25.0)	31 (32.6)

Data are expressed as means (standard deviation) or number (proportion).
AST: aspartate aminotransferase, ALT: alanine aminotransferase, AP: alkaline phosphatase, MAFLD: metabolic-associated fatty liver disease.

biomarkers that are transported from the bloodstream.[18,19] Many studies have shown that saliva is useful for the detection of diseases of the oral cavity[20–22] and also for systemic conditions including heart disease,[19,23,24] type 2 diabetes mellitus,[25] viral infection,[26,27] and several types of cancer (breast, lung, ovarian, liver, gastric, and pancreatic).[27–33] The non-invasive nature of saliva collection leads to excellent patient compliance for testing, rendering saliva to be an ideal diagnostic medium when screening people from socioeconomically disadvantaged communities, patients from rural and isolated regions, and indigenous populations. There is some evidence suggesting that liver function parameters can be evaluated in saliva,[34] and metabolite signatures have been developed to discriminate between healthy individuals and patients with liver cirrhosis or HCC.[35] However, this is the first study to use saliva as a sample matrix to detect liver fibrosis.

We hypothesize that currently used serum biomarkers to evaluate liver fibrosis can also be detected in saliva samples. In this pilot study, we investigated the clinical utility of six salivary biomarkers that are routinely used in clinical practice to evaluate liver fibrosis, and these include: γ-glutamyl transferase (GGT), total bilirubin, α-2-macroglobulin (A2MG), P3NP, HA, and TIMP-1—in paired serum and saliva samples from patients with various degrees of liver fibrosis. We have developed the Saliva Liver Fibrosis (SALF) score, the first saliva-based algorithm to diagnose liver fibrosis/cirrhosis with a potential to reduce the disease burden in rural and remote communities.

RESULTS

Characteristics of participants

The clinical characteristics of the patients included in the discovery (n = 40) and validation (n = 95) cohorts were similar (Table 1). The average age of participants in the discovery cohort and validation cohort were 60 ± 9 years and 64 ± 7 years, respectively (p = ns), and the gender distribution was similar in the groups (57% male in the discovery vs. 57% male in the validation cohort). Three main causes of liver disease were identified in the populations: metabolic-associated fatty liver disease (MAFLD) (discovery: 76.6%, validation: 82.8%), alcoholic liver disease (ALD) (discovery: 3.3%, validation: 3.7%), and chronic viral hepatitis (discovery: 6.7%, validation: 6.1%). These etiologies were evenly distributed between the cohorts. Regarding the stage of fibrosis as measured using TE, the discovery cohort had a higher proportion of patients with intermediate degrees of liver fibrosis (25.0% in the discovery cohort vs. 10.6% in the validation). No

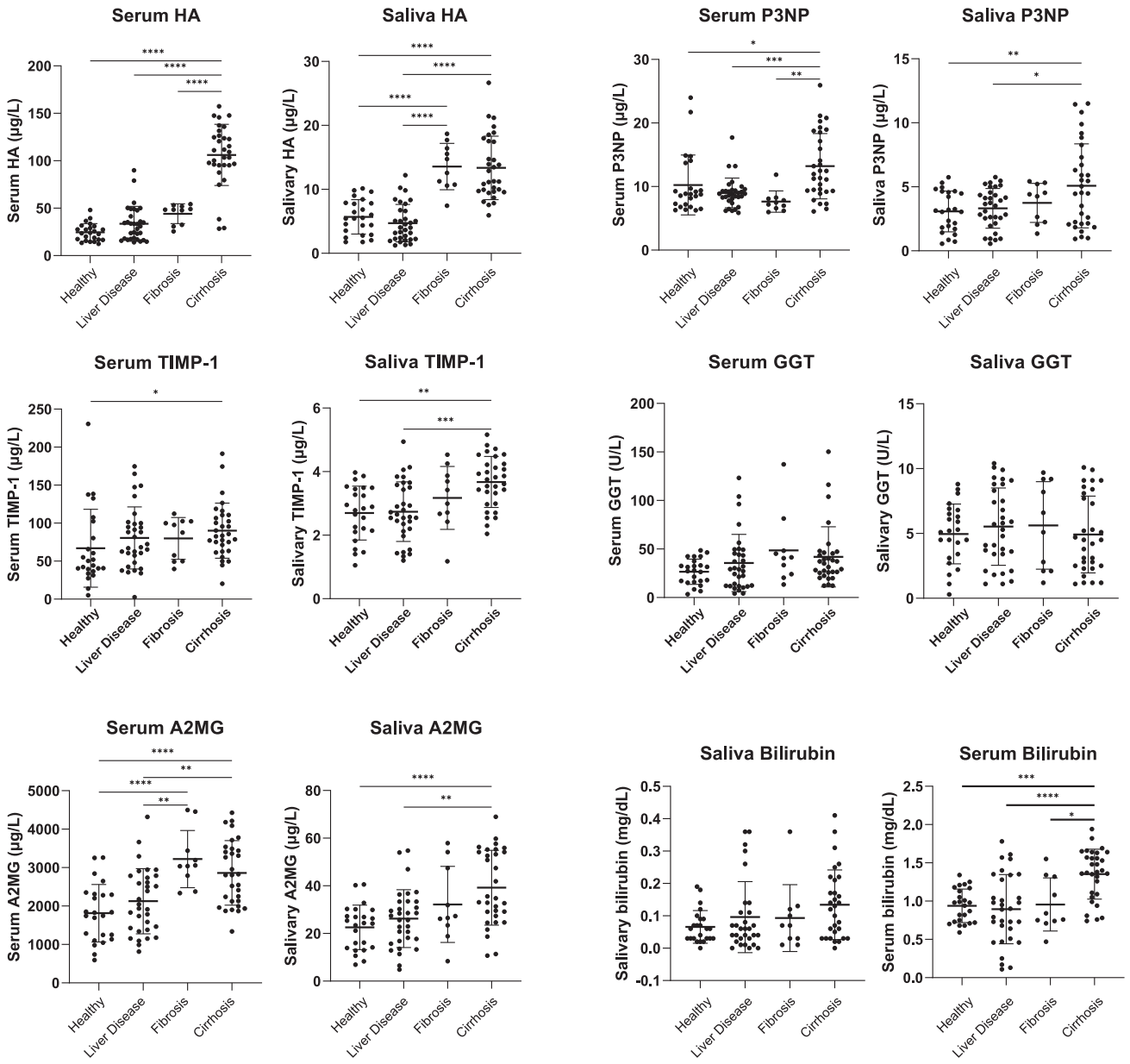


Figure 1. Concentrations of HA, P3NP, TIMP-1, A2MG, total bilirubin, and GGT in paired serum (left) and saliva (right) samples from healthy controls, patients with liver disease without fibrosis, liver fibrosis, and liver cirrhosis patients

Significant differences are indicated by *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), and ****($p < 0.0001$).

differences were observed between the discovery and validation cohorts regarding the clinical parameters and serum concentration of liver enzymes.

Biomarker quantification in serum and saliva samples

Six currently used serum biomarkers for the detection of liver cirrhosis (LC) were measured in paired serum and saliva samples. For HA, TIMP-1, P3NP, and A2MG, the concentrations were measured using ELISA. Spike and recovery tests were performed to validate the commercially available kits with saliva samples. Significantly higher mean serum concentrations ($p < 0.05$) of HA, A2MG, P3NP, and total bilirubin were detected in the serum of LC patients compared to controls (Figure 1, Table 2). Patients in the intermediate fibrosis (IF) cohort also showed significantly higher mean concentrations of A2MG ($p < 0.05$) compared

Table 2. The quantification of liver fibrosis biomarkers in serum and saliva of patients with liver cirrhosis, intermediate degrees of fibrosis, non-fibrotic liver conditions and healthy controls in the discovery set

	HC	LD	IF	LC	p value
Age, years	65 (8)	59 (10)	64 (9)	61 (10)	0.3054
Male, %	8 (57)	23 (57)	6 (60)	18 (58)	0.3648
LSM, kPa	5.3 (1.4)	5 (1.2)	9.8 (1.4)	22.6 (4)	<0.0001
Serum HA, μg/L	26.2 (9.3)	41.4 (25.8)	44.2 (10.4)	103.5 (33)	<0.0001
Saliva HA, μg/L	5.6 (2.4)	5.8 (3.7)	13.6 (3.6)	13.2 (3.8)	<0.0001
Serum TIMP-1, μg/L	71.5 (64.1)	88.2 (40.1)	79.9 (27.5)	101.7 (27.3)	0.4414
Saliva TIMP-1, μg/L	2.8 (0.9)	3 (1)	3.2 (1)	3.8 (0.7)	0.0847
Serum A2MG, mg/L	2.1 (0.8)	2.2 (0.7)	2.9 (0.8)	2.9 (1)	0.0488
Saliva A2MG, μg/L	20.4 (7)	24.5 (10.5)	32.2 (15.9)	34.2 (13.6)	0.0475
Serum P3NP, μg/L	9.3 (2.9)	9 (1.7)	7.6 (1.7)	12.2 (4.7)	0.0144
Saliva P3NP, μg/L	3.4 (1.6)	2.9 (1.4)	3.7 (1.5)	4.5 (2.8)	0.2803
Serum bilirubin, mg/L	0.85 (0.32)	0.75 (0.38)	0.96 (0.35)	1.38 (0.36)	0.0016
Saliva bilirubin, mg/dL	0.07 (0.06)	0.07 (0.07)	0.09 (0.1)	0.14 (0.12)	0.4087
Serum GGT, IU/L	23.4 (15.4)	22.1 (12)	48.5 (36.4)	29.3 (11.7)	0.0331
Saliva GGT, IU/L	4.3 (2.1)	5.6 (3)	5.6 (3.4)	5.2 (2.9)	0.6953
ELF score	6.2 (0.4)	7.7 (0.6)	8.7 (0.4)	9.8 (0.5)	<0.0001
Hepascore	0.1 (0.1)	0.2 (0.2)	0.4 (0.2)	0.7 (0.3)	<0.0001
FIB-4	N/A	1.1 (0.4)	1.8 (0.3)	3.9 (1.6)	<0.0001
APRI	N/A	0.5 (0.2)	0.8 (0.3)	1.9 (0.5)	<0.0001

Data are expressed as means (standard deviation).
LSM: liver stiffness measurement, ELF: Enhanced Liver Fibrosis score, FIB-4: Fibrosis-4, APRI: AST to Platelet ratio index. Statistical significance determined using ANOVA.

to those in the liver disease (LD) cohort and controls. For TIMP-1, the mean serum concentration was significantly increased ($p < 0.05$) in LC patients compared to the controls, but no differences were observed between the LC and IF cohorts.

All six biomarkers were successfully detected in saliva samples but at lower concentrations compared to serum. Mean concentrations of salivary HA, TIMP-1, and A2MG were higher in patients with LC ($p < 0.05$) compared to healthy control (HC). Furthermore, a significant increase ($p < 0.0001$) in the mean concentration of HA in saliva was also observed in patients in the IF cohort compared to HC and LD (Table 2). No significant differences were observed between the groups in either serum or salivary GGT levels, and the increase in total bilirubin observed in the serum samples was not observed in saliva. The Spearman's Rho correlation showed a significant positive correlation between the serum and salivary concentrations of HA ($r = 0.546$, $p < 0.01$), A2MG ($r = 0.326$, $p < 0.05$), and total bilirubin ($r = 0.482$, $p < 0.05$) (Figure S1).

Development of the SALF score

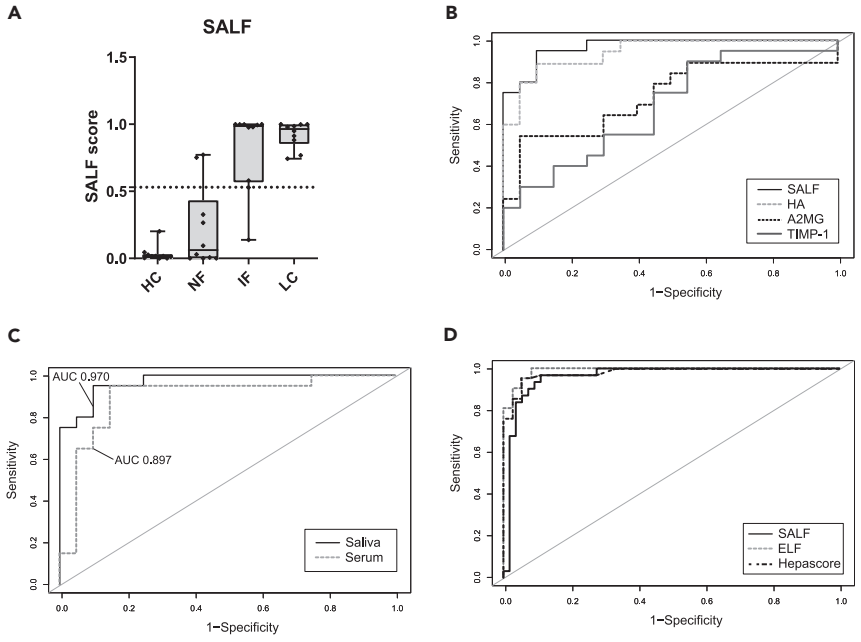
Overall, the highest AUROC was obtained when LC patients were compared to HCs. In serum, the biomarkers with the highest AUROC for the identification of cirrhosis vs. HCs were HA, total bilirubin, and TIMP-1 with AUROC values of 0.980, 0.840, and 0.750, respectively (Table 3). HA, A2MG, and TIMP-1 showed the best performance in saliva samples with AUROCs of 0.971, 0.850, and 0.830, respectively. When compared to the measurements in serum, the salivary biomarkers had slightly lower AUROCs, with an exception of TIMP-1, in which the performance in saliva was superior (salivary AUROC: 0.830 vs. serum AUROC: 0.750) (Table 3). Furthermore, the concentrations of these three biomarkers in saliva were independently associated with the degree of liver fibrosis as observed by a significant positive correlation between liver stiffness measurement (LSM) and HA ($r = 0.474$, $p < 0.001$), TIMP-1 ($r = 0.202$, $p = 0.046$), and A2MG ($r = 0.389$, $p < 0.001$) levels.

The salivary biomarkers assessed in the discovery set were then used in a logistic regression analysis to create a diagnostic algorithm for LC. The optimal model with the highest AUROC was obtained by

Table 3. Area Under the Curve (AUROC) for serum and saliva biomarkers in liver cirrhosis patients compared to patients with non-fibrotic liver disease and healthy individuals

	Cirrhosis vs. Healthy					Cirrhosis vs. LD					Cirrhosis vs. Healthy+LD					Cirrhosis+Fibrosis vs. Healthy+LD				
Variable	AUROC	Sens	Spec	PPV	NPV	AUROC	Sens	Spec	PPV	NPV	AUROC	Sens	Spec	PPV	NPV	AUROC	Sens	Spec	PPV	NPV
Serum HA	0.98	90	100	90.9	100	0.92	80	100	83.3	100	0.95	90	90	94.7	81.8	0.86	75	85	77.3	83.3
Saliva HA	0.97	90	100	90.9	100	0.91	90	80	88.9	81.8	0.95	90	90	94.7	81.8	0.95	90	90	90	90
Serum TIMP1	0.75	100	6	100	71.4	0.48	100	30	100	49.9	0.64	100	35	100	43.5	0.60	55	75	62.5	68.8
Saliva TIMP-1	0.83	100	60	100	71.4	0.72	90	50	83.3	64.3	0.78	90	55	91.7	50	0.68	90	45	81.8	62.1
Serum A2MG	0.73	90	60	85.7	69.2	0.59	40	90	60	80	0.66	90	45	90	45	0.76	60	85	68	80
Saliva A2MG	0.85	80	90	81.8	88.9	0.75	70	90	75	87.5	0.80	70	95	86.4	87.5	0.74	55	95	67.9	91.8
Serum GGT	0.59	90	50	83.3	64.3	0.66	100	30	100	48.8	0.63	90	45	90	45	0.70	90	45	81.8	62.5
Saliva GGT	0.64	40	90	60	80	0.51	100	20	100	45.6	0.56	60	65	76.5	46.2	0.56	45	80	59.3	69.2
Serum bilirubin	0.84	80	90	81.8	88.9	0.89	80	90	81.8	88.9	0.86	80	90	90	80	0.73	55	90	66.7	84.6
Saliva bilirubin	0.65	80	50	71.4	61.5	0.675	60	80	64.7	75	0.66	60	75	78.9	54.5	0.61	50	75	60	66.7
Serum P3NP	0.70	80	70	77.8	73.7	0.75	80	80	80	80	0.73	80	75	88.9	61.5	0.48	40	85	58.6	72.7
Saliva P3NP	0.64	40	100	62.5	100	0.67	50	90	64.3	83.3	0.66	40	100	76.9	100	0.64	55	75	62.5	68.5

LD: Liver Disease; Sens: Sensitivity (%); Spec: Specificity (%); PPV: Positive Predictive Value (%); NPV: Negative Predictive Value (%).



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Algorithm	AUROC	Cut-off	Sens, %	Spec, %	PPV, %	NPV, %
SALF	0.970	0.51	95.0	90.0	94.7	90.5
Hepascore	0.979	0.50	69.8	91.1	98.9	85.7
ELF	0.991	9.00	100.0	91.1	93.0	90.0
FIB-4	0.740	1.28	82.6	71.0	67.3	69.0
APRI	0.820	0.50	78.8	81.4	66.7	71.9

Figure 2. The receiver operating characteristics (ROC) curve analysis of the Saliva Liver Fibrosis (SALF) score

(A) The SALF score for each individual was calculated using a logistic regression model combining the measurement of HA, TIMP-1, and A2MG (cutoff of 0.51 indicated as a dashed diagonal line).

(B–E) The performance of the SALF score was compared with its individual components, (C) between serum and saliva samples, and (D and E) with other serum-based diagnostic models used for the detection of liver cirrhosis. SALF: Saliva Liver Fibrosis score; ELF: Enhanced Liver Fibrosis score; FIB-4: fibrosis-4 score; APRI: AST-to-platelet ratio index; PPV: Positive Predictive Value; NPV: Negative Predictive Value.

combining salivary HA, TIMP-1, and A2MG, which was named Saliva Liver Fibrosis (SALF) score. The SALF score was calculated using the following algorithm SALF = (Y/Y+1), in which Y = EXP[-15.8454816 + (0.7944629*HA) + (1.3469354*TIMP-1) + (0.1541859*A2MG)]. The SALF scores of LC (0.921 $\pm$ 0.09) and fibrosis patients (0.819 $\pm$ 0.285) were significantly higher ($p < 0.0001$) than the score of the HCs (0.034 $\pm$ 0.05) and patients with non-fibrotic liver disease (0.061 $\pm$ 0.29) (Figure 2A). No significant differences in the SALF scores were observed between the cirrhosis and fibrosis cohorts. The SALF score showed a diagnostic performance which was significantly higher than its individual parameters for all the conditions tested (Figure 2B) and also superior to the combination of the same biomarkers in serum samples (Figure 2C). The SALF score was compared to other clinically validated serum algorithms to diagnose LC, in which the performance of the saliva score was superior to that of the FIB-4 (AUROC: 0.740) and AST-to-platelet ratio index (APRI) (AUROC: 0.820) and similar to that of the Hepascore (AUROC: 0.979). The ELF score showed the best performance, with an AUROC of 0.991, 100.0% sensitivity, and 91.7% specificity for the detection of fibrosis (Figures 2D and 2E).

Validation of the SALF score

To further investigate the clinical utility of the developed SALF score, the performance of the algorithm was validated using an independent cohort of patients with different degrees of fibrosis: 14 HCs, 40 patients with non-fibrotic liver conditions (LD), 10 patients with intermediate degrees of hepatic fibrosis (IF), and 31 LC patients. In the validation cohort, the concentrations of HA, TIMP-1, and A2MG were significantly increased in the saliva of cirrhosis patients when compared to patients in the control and liver disease cohorts ($p < 0.05$, Figure 3A). Furthermore, the mean concentration of salivary HA was increased in the

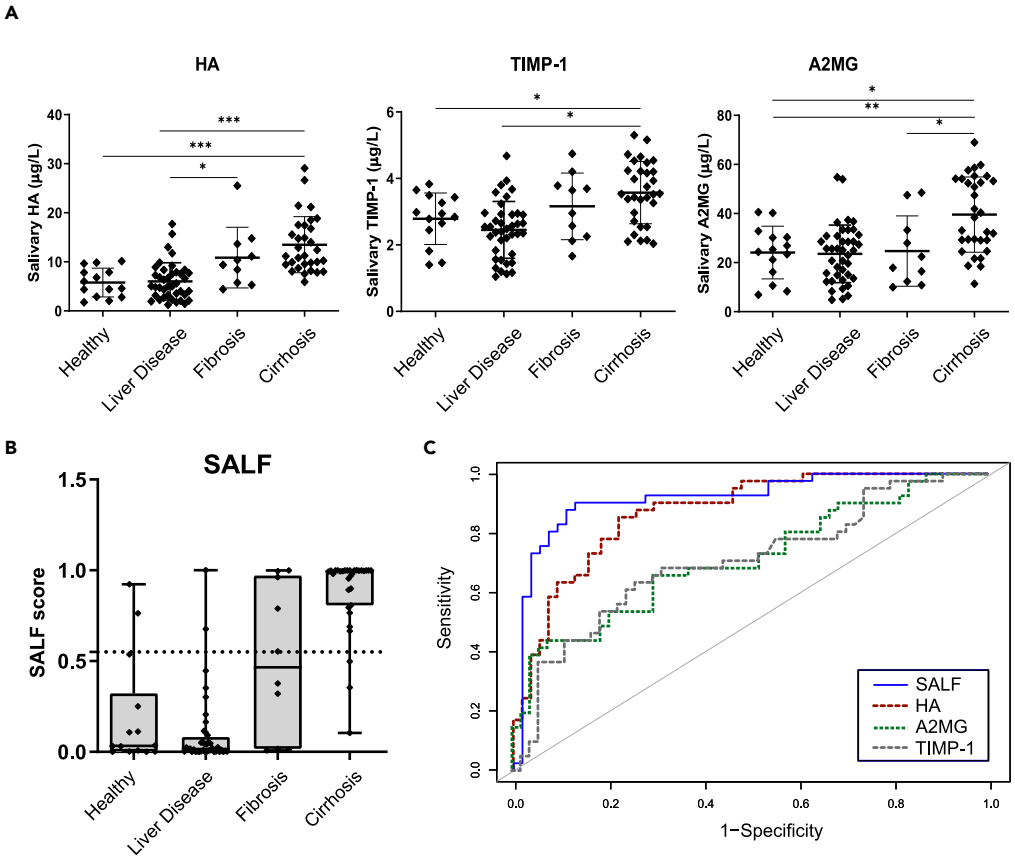

Figure 3. Validation of the SALF score in an independent cohort

(A) The concentrations of HA, TIMP-1, and A2MG were measured in the saliva of healthy controls and liver disease, fibrosis, and cirrhosis patients.

(B and C) The SALF score was calculated and (C) ROC analysis was performed to assess the performance of the SALF score for the diagnosis of liver fibrosis (LC + IF vs. HC + LD) compared to the individual components.

patients with liver fibrosis compared to those with liver disease ($p < 0.05$). The SALF score for each patient was calculated according to the previous algorithm. The median SALF scores of cirrhosis patients (0.88 ± 0.21) were significantly higher ($p < 0.01$) than those in the HC (0.20 ± 0.31) and liver disease cohorts (0.09 ± 0.20). The fibrosis cohort showed SALF scores (0.50 ± 0.41) that were significantly different from scores in patients with LC ($p < 0.01$), liver disease ($p < 0.01$), and controls ($p < 0.05$). No statistically significant differences were observed between controls and liver disease patients ($p = 0.563$) (Figure 3B). Using the optimal cutoff of 0.51, the SALF score showed an AUROC of 0.962, with 87.1% sensitivity, 94.4% specificity, 92.7% positive predictive value (PPV), and 90.0% negative predictive value (NPV) to detect cirrhosis against those without fibrosis (healthy + liver disease). Importantly, for the detection of significant/advanced liver fibrosis (LC + IF), the AUROC, sensitivity, specificity, PPV, and NPV were 0.920, 90.2%, 87.0%, 92.2%, and 84.1%, respectively. Similar to the discovery cohort, the performance of the combinatorial algorithm was superior to the performance of its components individually (Table 4, Figure 3C). Considering the discovery and validation cohorts, the SALF score also showed a strong correlation with LSM measurements ($r = 0.616$, $p < 0.001$, Figure S2).

DISCUSSION

Approximately two-thirds of patients with liver disease are diagnosed with advanced stages of disease, despite the innovations in non-invasive diagnosis of liver fibrosis. The importance of early diagnosis and management of chronic liver disease is also recognized by the international liver associations.[36,37] In Australia, 60% of HCC cases have undiagnosed cirrhosis, and up to 66% of MAFLD-associated cirrhosis cases are not initially diagnosed during routine care.[38,39] Several non-invasive approaches, such as blood

Table 4. Accuracy of the detection of significant fibrosis (LC + IF vs. HC + LD) in the discovery, validation, and total cohort using the SALF score, in comparison to its constitutes

	AUROC	Sens, %	Spec, %	PPV, %	NPV, %
Discovery cohort					
SALF	0.970	95	90	94.7	90.5
HA, μg/L	0.948	90	90	90	90
TIMP-1, μg/L	0.682	90	45	81.8	62.1
A2MG, μg/L	0.738	55	95	67.9	91.8
Validation cohort					
SALF	0.920	90.2	87	92.2	84.1
HA, μg/L	0.873	85.4	77.8	87.5	74.5
TIMP-1, μg/L	0.703	63.4	74.1	72.7	65
A2MG, μg/L	0.711	43.9	92.6	68.5	81.8
Total cohort					
SALF	0.936	90.2	89.2	91.7	87.3
HA, μg/L	0.894	78.7	86.5	83.1	82.8
TIMP-1, μg/L	0.706	70.5	664.9	72.7	62.3
A2MG, μg/L	0.716	54.1	83.8	68.9	73.3

fibrosis tests and liver elastography to detect liver fibrosis have been reported. These tests facilitate early diagnosis and may avoid the need for liver biopsy. Some of the drawbacks relating to current diagnostic workflow are limited awareness and use by clinicians in the primary care setting, difficulty in accessing these investigations which are often restricted to major centers, expense, and the need for venepuncture. Improving access and uptake of tests for fibrosis and the screening of populations at risk are two major unmet clinical needs to achieve earlier diagnosis of liver fibrosis and to tailor earlier targeted interventions to prevent complications of the disease. There are two contemporary matters to consider in this context. Firstly, the increasing global burden of MAFLD will compound the problem of patients presenting with advanced disease and related complications. Secondly, access to diagnostic tests in disadvantaged, rural and indigenous communities is limited. Recent studies have highlighted the increasing burden of chronic liver disease in indigenous, rural, and regional communities especially linked to lower income and levels of education, restricted access to care, and older ages of the population.[40,41] In this context, the development of a readily accessible, cost-effective screening test to identify patients who require close monitoring or further intervention would significantly improve patient outcomes.

The present pilot study was aimed to develop a simple saliva-based score for the detection of liver fibrosis, named Saliva Liver Fibrosis (SALF) score. We analyzed six of the main serum markers for fibrosis—HA, TIMP-1, A2MG, GGT, total bilirubin, and P3NP—in serum and saliva samples from liver disease patients with different degrees of fibrosis and healthy volunteers. The SALF score accurately detected patients with LC within a population of healthy individuals and patients with underlying liver disease. In addition, the score can be further developed to be used for the detection of earlier stages of liver fibrosis, as demonstrated by the performance of the novel algorithm in patients with intermediate degrees of fibrosis (F2 and F3).

These findings were subsequently validated in an independent cohort of patients. We found that a SALF score $\geq$0.51 provided an AUROC of 0.970 and 0.920 to detect LC in the discovery and validation sets, respectively, with high sensitivity (95.0% and 90.2%) and specificity (90.0% and 87.0%). This model showed a diagnostic performance which was similar to that of the ELF score (AUROC 0.690–0.990)[42] and the Hepascore (0.730–0.850).[43] Furthermore, the SALF score showed a better performance in saliva than the combination of the same biomarkers in serum.

Serum levels of HA, A2MG, and TIMP-1 have been previously linked to excessive fibrogenesis in the liver. HA is a polysaccharide that provides structural and functional support within the extracellular matrix.[44] It has been used as a non-invasive marker for LC caused by viral, metabolic, and environmental factors.[45,46] TIMP-1 modulates the

extracellular matrix degradation by regulating the activity of matrix metalloproteinases (MMPs). The circulating concentrations of TIMP-1 were reported to increase with the progression of chronic liver disease.[47] A2MG is a proteinase inhibitor of the catabolism of matrix proteins, therefore enhancing the fibrotic process in the liver.[48,49] Although these molecules have been previously detected in saliva, there are no reports of clinical studies investigating their application for the detection of liver fibrosis.[44,50–52]

Saliva-based diagnosis of liver fibrosis provides a remarkable opportunity for screening of populations at risk of liver fibrosis. Our results show that SALF score can detect patients with significant to advanced liver fibrosis. Based on our observations, we propose that the best application of a simple saliva test could be to identify those patients with at least intermediate grades of fibrosis, such as those who have a higher SALF score and may require further more sophisticated evaluation with serum markers, Fibroscan, or liver biopsy. A simple screening test for liver fibrosis will also reduce the burden of unnecessary testing. It is understood that only 20% of the population with MAFLD develop significant hepatic fibrosis. Many of the remaining 80% undergo further unnecessary and expensive testing since clinical evaluation and standard biochemical tests do not allow clinicians to identify the group with significant fibrosis. If confirmed in larger studies, and given the lower costs and almost universal compliance of collection, saliva testing and application of the SALF score may become a simple and inexpensive way to identify the patients at higher risk who warrant further investigation.

In comparison to other non-invasive sampling methods such as blood, feces, and urine, saliva presents the advantage of better patient compliance.[53] Furthermore, specific discovery is not necessary for collection, which can be performed at home by the patient, and further facilitates sequential sampling.[54] Finally, the introduction of saliva diagnosis for liver disease would significantly improve health care in rural and geographically isolated regions. Roberts et al. showed that the prevalence of MAFLD in rural regions of Australia is considerably higher (36%) than the average prevalence in white populations (25%).[41] A similar disparity was observed in the United States, where patients with end-stage liver disease admitted to hospitals in rural areas had over twice the odds of experiencing in-hospital mortality compared to urban hospitals.[55] In this context, saliva presents the advantage of being stored without the need for special laboratory equipment,[53] collected into stabilizing buffers for short-term transport[53,56] and, more recently, applied to point-of-care devices.[57]

In this pilot study, we have demonstrated the potential clinical utility of saliva as a sample matrix to diagnosis of liver fibrosis/cirrhosis. We have shown that serum biomarkers can be detected in saliva samples and are significantly increased in patients with LC compared to healthy individuals and patients with underlying liver disease. We developed the first saliva-based score for the diagnosis or screening for liver fibrosis. The SALF score has the potential to improve the screening for cirrhosis in high-risk asymptomatic populations, potentially decreasing the proportion of patients who progress to liver failure and/or cancer.

Limitations of the study

The major limitation of the current study is that the great majority of LC patients were not submitted to a liver biopsy. Although considered the gold standard to diagnose LC, biopsies are not suited for screening purposes and are usually reserved for long-term follow-ups in patients with a high risk of advanced liver disease.[58] Therefore, transient elastography was used to stratify liver disease patients according to their proposed degree of fibrosis. Although transient elastography has shown a good diagnostic accuracy, there are technical limitations regarding its reliability for obese patients and/or people with large amounts of chest wall fat.[59] Furthermore, the biomarkers in serum were measured using commercially available ELISA kits, which may present results that are different from results of the tests conducted in a clinical pathology laboratory. A second limitation of the study is the sample size, which is relatively small and does not allow for the classification of patients according to etiology. Thus, future multi-center studies involving a large number of patients with histology-proven liver fibrosis are necessary.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.107015.

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AUTHOR CONTRIBUTIONS

Conception and design: DC, CP; Development of methodology: LTFL, DC, XZ, CP; Acquisition of data: LTFL; Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): LTFL, XZ, DC, CP, DB; Drafting manuscript: LTFL, DC; Revision of the manuscript: CP, DC, DB, XZ, KB; Administrative, technical, or material support: DC, CP, KB; Study supervision: CP, DC. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

Some of the authors are inventors on a provisional patent application that has been filed to the Australian Patent Office (reference 2022901434). The authors have no relevant financial or non-financial interests to disclose.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Saliva Samples	Saliva and Liquid Biopsy Translational Laboratory biobank, Griffith University, Australia	N/A
Chemicals, peptides, and recombinant proteins		
TMB Substrate Solution	Thermo Fisher Scientific	cat#:N301
TWEEN 20	Merck	cat#: P9416
Critical commercial assays		
Human TIMP-1 DuoSet	R&D Systems	cat#:DY970
Hyaluronan DuoSet	R&D Systems	Cat#:DHYAL0
Human alpha-2-macroglobulin DuoSet	R&D Systems	Cat#:DY1938
Human Procollagen Type III N-Terminal Propeptide ELISA kit	MyBioSource	cat#:MBS045955
Bilirubin Assay Kit	Abcam	cat#:ab235627
Gamma Glutamyl Transferase (GGT) Assay Kit	Abcam	cat#ab241029
Human TIMP-1 DuoSet	R&D Systems	cat#:DY970
Software and algorithms		
R Project for Statistical Computing, R Bioconductor	The Comprehensive R Archive Network	https://www.r-project.org/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Professor Chamindie Punyadeera (c.punyadeera@griffith.edu.au).

Materials availability

All materials are ' in the STAR Methods key resources table, and there is no new unique reagent in this study.

All the information and the requests of materials in this paper should be directed to the lead contact: c.punyadeera@griffith.edu.au.

Data and code availability

All data has been included in main figures and supplementary information. All data reported in the paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyse the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Ethics statement

The study complies with the 2013 Declaration of Helsinki(36). Human research ethics approval was obtained from the Greenslopes Research and Ethics Committee (approval number: 18/06), Queensland University of Technology (approval number: 2000000690) and The University of Queensland (approval

number: 2000000690). Patients were recruited from the Greenslopes Private Hospital, Brisbane, Australia. All patients provided written consent prior to inclusion in the study.

Participant cohorts

Patient samples were collected from patients attending the Queensland Gastroenterology clinic, Greenslopes Hospital, Brisbane, Australia. The patients were diagnosed with liver disease, liver fibrosis or liver cirrhosis on the basis of a transient elastography examination. In this study, a total of 135 participants aged between 50- and 87-years old were recruited (78 male and 57 female). The influence of sex and age was analysed and results are shown in Table 2.

For the discovery cohort, 10 liver cirrhosis patients (LC), 10 chronic liver disease patients without fibrosis (LD), 10 patients with intermediate degrees of fibrosis (IF), and 10 healthy controls (HC) were recruited. Liver fibrosis and/or cirrhosis were assessed using transient elastography (TE, FibroScan 502®, Echosens, France) by a trained operator. To obtain a liver stiffness measurement (LSM), a probe was placed in the intercostal space over the right hepatic lobe of the patient. Patients were fasting for three hours before the examination. LSM results with at least 10 valid readings and an interquartile range (IQR) of less than 30% of the median LSM value were required for inclusion in the study.[60] The absence of fibrosis in the healthy and LD cohorts was designated by a LSM $\leq$ 7.0 kPa, and liver cirrhosis was designated by an LSM $\geq$ 14.0 kPa.[61] Patients with LSM values between 7.0 kPa and 13.0 kPa were classified as having an intermediate degree of hepatic fibrosis.

The validation cohort was composed of 95 individuals, classified as: 14 healthy controls (HC), 40 patients with non-fibrotic liver conditions (LD), 10 patients with intermediate fibrosis (IF) and 31 patients with liver cirrhosis (LC).

METHOD DETAILS

Reagents

The following commercially available ELISA kits were used for biomarker quantification: Human TIMP-1 DuoSet (R&D Systems, Minneapolis, MN, USA, cat#:DY970); Hyaluronan DuoSet (R&D Systems, Minneapolis, MN, USA Cat#:DHYAL0); Human alpha-2-macroglobulin (R&D Systems, Minneapolis, MN, USA Cat#:DY1938); Human Procollagen Type III N-Terminal Propeptide (MyBioSource, San Diego, CA, USA, cat#:MBS045955). Total bilirubin and GGT were quantified using colorimetric assays (Bilirubin Assay Kit, Abcam, Cambridge, UK, cat#:ab235627; Gamma Glutamyl Transferase (GGT) Assay Kit, Abcam, Cambridge, UK, cat#ab241029).

Sample collection

Blood samples were collected using SST tubes (Greiner VACUETTE®), allowed to sit for 30 minutes at room temperature allowing it to clot, and centrifuged at 500 x g for 15 minutes at 4°C. Serum was immediately separated and aliquots were kept at -80°C until analysis. For the collection of saliva, participants were asked to refrain from eating and drinking (except water) for two hours. Unstimulated saliva samples were collected using the drool method.[62] In brief, patients were asked to sit in an upright position, lean forward to pool the saliva in the front of their mouth, and expectorate into a 50 mL tube kept on ice. To minimize sample contamination from food, volunteers were asked to rinse their mouths with water prior to collection. Samples were placed on ice, transported to the laboratory, and stored at -80°C.

Biomarker quantification

Using paired serum and saliva samples, six biomarkers indicate of liver fibrosis/cirrhosis were measured: hyaluronic acid (HA), tissue inhibitor of metalloproteinase 1 (TIMP-1), procollagen III amino-terminal propeptide (P3NP), γ-glutamyl transferase (GGT), total bilirubin, and α-2-macroglobulin (A2MG). These biomarkers were selected because they form part of currently in use two liver cirrhosis scoring systems, the Enhanced Liver Fibrosis (ELF) score and Hepascore.[63,64]

The following commercially available ELISA kits were used to quantify the biomarkers concentration in blood and saliva samples: Human TIMP-1 DuoSet (R&D Systems, Minneapolis, MN, USA, cat#:DY970); Hyaluronan DuoSet (R&D Systems, Minneapolis, MN, USA Cat#:DHYAL0); Human alpha-2-macroglobulin (R&D Systems, Minneapolis, MN, USA Cat#:DY1938); Human Procollagen Type III N-Terminal Propeptide

(MyBioSource, San Diego, CA, USA, cat#:MBS045955). Total bilirubin and GGT were quantified using colorimetric assays (Bilirubin Assay Kit, Abcam, Cambridge, UK, cat#:ab235627; Gamma Glutamyl Transferase (GGT) Assay Kit, Abcam, Cambridge, UK, cat#ab241029).

ELISA was conducted according to the manual instructions. Briefly, 100 μL of capture antibody were added to a 96-well microplate and incubated at 4°C for 16 hours. Blocking was performed with an incubation with 5% BSA for 1 hour at room temperature, followed by five washes with 350 μL of a 0.1% Tween 20 solution. A serial dilution of standards, samples and blank were added to the pre-coated microplates, incubated for 2 hours at room and washed five times. Biotinylated detection antibody was added to the wells (100 μL) and the plate was sealed and incubated for 2 hours at room temperature. HRP-streptavidin beads were 40-fold diluted, added to the wells and incubated for 20 minutes. HRP reaction was developed using 100 μL of TMB substrate (Thermo Fisher Scientific, cat#:N301) for 15 minutes, followed by stop solution.

Blood fibrosis test

The Enhanced Liver Fibrosis (ELF) score was calculated based on the algorithm proposed by Parkes et al.[64] The Hepascore values were obtained using the logistic regression model proposed by Adams et al.[63] The fibrosis-4 (FIB-4) and the AST-to-platelet ratio index (APRI) were determined using the patients laboratory measurements. Briefly, FIB-4 was calculated using the formula: age ([yr] x AST [U/L]) / ((PLT [10(9)/L]) x (ALT [U/L])(1/2)) and the APRI was generated using [(AST/upper limit of the normal AST range) X 100]/Platelet Count.[65,66]

Development and validation of the salivary biomarker score

The diagnostic accuracy of the biomarkers was evaluated using receiver operating characteristic (ROC) curve analysis. A logistic regression predictive model was applied to the three biomarkers with the highest area under the curve (AUROC) to calculate an individual score for each patient. This model, referred to as Saliva Liver Fibrosis (SALF) score, was then validated using an independent cohort of patients with different degrees of liver fibrosis (n=95). The optimal cut-off values were determined based on the highest Youden's index.[67]

QUANTIFICATION AND STATISTICAL ANALYSIS

The software GraphPad Prism 9 version (GraphPad Software Inc., La Jolla, CA, USA) and R (R Development Core Team, Vienna, Austria) were used for statistical analysis. The diagnostic performance of the biomarkers to predict liver cirrhosis was assessed using receiver-operating characteristic (ROC) curve analysis. Continuous variables were tested for normality using the Shapiro–Wilk normality test. Kruskal–Wallis test was performed on data with non-normal distribution to compare values between multiple groups. One-way ANOVA was performed for group comparison in data with a normal distribution. GraphPad was used to generate standard curves for the ELISA assays by plotting the absorbance in the y-axis and concentration of the analyte in the x-axis. The concentration of the analyte in the samples was deduced from the standard curve using a nonlinear regression equation. Biomarker concentration is expressed as mean ± SD. Correlation studies were executed using Spearman's correlation test.