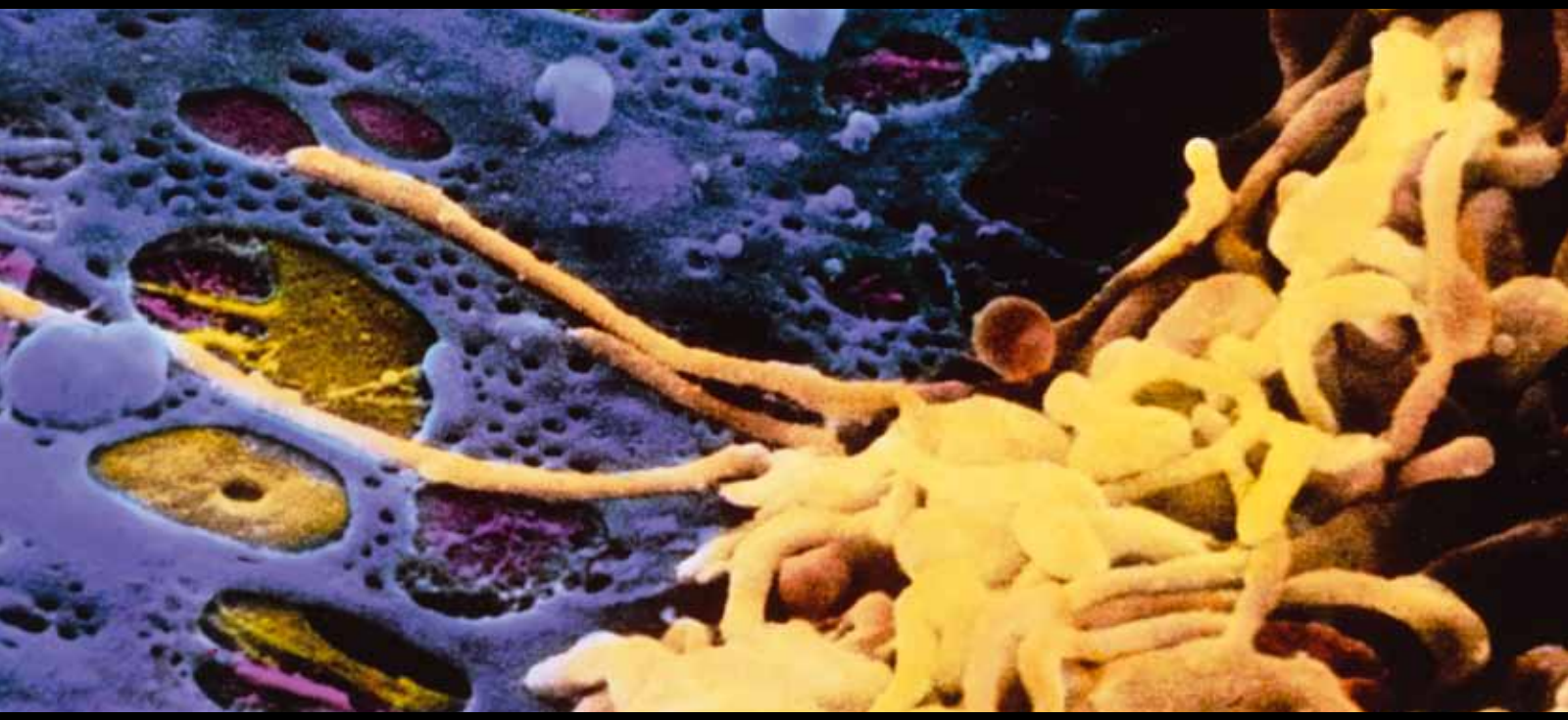
International Journal of Hepatology

Biomarkers in Liver Disease: Emerging Methods and Potential Applications

Guest Editors: Guruprasad P. Aithal, Neil Guha, Jonathan Fallowfield, and Laurent Castera



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Editorial

Biomarkers in Liver Disease: Emerging Methods and Potential Applications

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1. Introduction

Biomarker research represents an evolving area within hepatology. The growing burden of global liver disease, the absence of symptoms until late in the natural history of a disease which may take decades to manifest, the presence of an invasive reference test (liver biopsy) to assess disease severity, and the lack of robust tools to assess the efficacy of therapeutic interventions are some of the key drivers for this research.

The National Institute of Health defines a biomarker as "A characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [1]. Moreover, biomarkers can be classified into hierarchical systems based on their ability to assess natural history (type 0: prognosis), biological activity (type 1: response to therapy), and therapeutic efficacy (type 2: surrogate for clinical efficacy) [2].

The spectrum of pathological injury that occurs in liver disease including steatosis, necroinflammation, apoptosis, and fibrosis enhances the pool of potential biomarkers. Furthermore, advances in technology platforms have created an exponential rise in the discovery of putative mediators of pathophysiological injury. This has been countered by the growing need to align surrogate markers of injury with clinical consequences of injury in order to achieve diagnostic, prognostic, and therapeutic effectiveness. This timely special edition comprises original articles and reviews in the subject areas of biomarker discovery, biomarkers of liver injury, and biomarkers to assess the consequences of liver injury.

2. Methods of Biomarker Discovery

Advances in instrumentation design have driven biomarker discovery. The advent of modern biological mass spectroscopic techniques in the 1990s and the evolution of 2-dimensional polyacrylamide gel electrophoresis (2D SDS PAGE) from a highly specialist technique to one that could be carried out in most laboratories around the world drove the development of large-scale 'omics biomarker discovery projects. Advances in microlitre flow rate HPLC, that could be coupled directly to mass spectrometers (nano-LC/MS), and computing to analyse the data gave further impetus to this work. It became possible to quantify and identify many thousands of proteins from diseased and healthy tissue in a single experiment. Biomarker discovery projects ([3] metabonomics; [4] lipidomics; [5] proteomics; [6] SELDI and transcriptomics) demonstrate the ability to identify novel markers of liver disease. Proteomics, transcriptomics, lipidomics, and metabonomics offer the ability to discover completely novel markers of disease and its progression. This *de novo* approach to biomarker discovery leads to a great challenge of marker validation. There may be little or no obvious mechanistic connection between the putative marker and disease, demonstrating that a link can be very time and resource intensive.

Mechanism-focussed biomarker discovery has also benefited from advances in instrumental design and technology. These projects are based on prior disease knowledge and are much more limited in scope but, if successful, are more likely to identify a disease-relevant marker. Standard ELISA assays methodologies have been developed to use valuable patient samples more efficiently by allowing many analytes to be quantified simultaneously. In array or planar assays, a series of primary antibodies are bound to a surface in discrete spots, sample, and secondary antibody, and detection reagents are passed over the array and the location of the signal is determined using imaging technology. Bead-based technologies rely on a mixture of antibody-labelled beads which are then quantified using flow cytometers or dedicated analysers. From 30 to 50 proteins can be analysed per experiment using panels of antibodies that have been optimised to minimise cross-reactivity. Miniaturisation of liquid handling and high-density microplates, currently up to 1536 samples per plate, reduces reagent and patient sample usage when carrying out enzyme activity-based biomarker discovery. A typical 96-well microplate will require 100 μL reaction mix per well, the high density; 1536-well plates require only 5 μL per well, a reduction of 20-fold in sample consumption. Unfortunately, the additional costs that are incurred to ensure accurate reagent dispensing and reaction monitoring are not trivial. S. K. Hartwell, in this issue, describes an alternative approach using flow injection to minimise reagent consumption where sample numbers and volumes may be limited. The use of commonly available laboratory equipment aims to minimise costs and to open up the technology to laboratories with limited resources.

3. Biomarkers of Liver Injury

The pathological processes of steatosis, necroinflammation, oxidative stress, apoptosis, and fibrosis are common to a number of diverse liver diseases. The ability to define these individual entities is advantageous for determining the mechanistic evidence of efficacy, using biomarkers, for proposed treatment strategies. A difficulty remains that the pathological processes are often interdependent or cocorrelated, and thus, delineating biomarkers specific to one mode of injury can be challenging. This is illustrated by the article in this special edition by N. Mousa and coworkers describing the association of alpha fetoprotein (AFP) and liver steatosis in genotype 4 infection in chronic viral hepatitis. The authors postulate that the elevation in AFP is secondary to increased production from hepatic progenitor cells as a response to regeneration following injury. In this study, steatosis was also associated with the presence of necroinflammation and fibrosis, and thus, it is not clear whether it is the extent of liver injury or steatosis *per se* that leads to the elevation in AFP. There exists a wider debate in the literature on whether benign steatosis (in the absence of significant steatohepatitis or fibrosis) has clinical significance. In viral hepatitis, steatosis is most commonly seen in genotype 3 infection and improves following successful viral eradication [7]. In long-term studies based on pathological features at baseline biopsy, steatosis has not been shown to adversely affect outcome in nonalcoholic fatty liver disease [8, 9].

Natural history studies have shown that the presence and stage of fibrosis at the index liver biopsy provide prognostic information about the subsequent rate of fibrosis progression ([10–12] and the development of liver-related outcomes [9, 13]). It is therefore no surprise that over the last decade much of the focus has been to define novel biomarkers based on the pathological presence of fibrosis. The success and limitations of this strategy have been outlined elsewhere [14]. Defining surrogates of pathological entities other than liver fibrosis is both necessary and advantageous for a number of reasons. Liver fibrosis is essentially a generic wound-healing response and final common pathway resulting from a spectrum of hepatic insults. Moreover, particular characteristics of the hepatic scar including the composition and physical/biochemical attributes that limit remodelling and angioarchitectural changes have hitherto made the delivery of effective antifibrotic therapy challenging. The ability to intervene "upstream" in the injury process may yield a larger repertoire of therapies with the allure of enhanced targeting and superior drug profiles. Apoptosis in the liver may be one such example. Whilst the engulfment of apoptotic bodies by activated hepatic stellate cells (HSCs) may induce TGFβ and collagen-α1 synthesis and promote fibrosis, paradoxically, in preclinical models, resolution of fibrosis depends on the removal of activated HSCs via apoptosis. Thus, the detailed characterisation of apoptosis may provide critical insights into both fibrogenesis and fibrinolysis. J. B. Chakraborty and colleagues provide a comprehensive review in this special edition of the mechanisms of apoptosis in the liver, candidate apoptosis-related biomarkers, and the potential for clinical translation (e.g., assessing treatment response and/or monitoring the regression of fibrosis).

4. Biomarkers Assessing the Consequences of Liver Injury

Following long-term liver injury, the evolution of liver fibrosis to cirrhosis is associated with (1) architectural disturbance; (2) angiogenesis and haemodynamic changes (intra- and extrahepatic) resulting in portal hypertension; (3) a propensity for carcinogenesis. In the event of the injury not being removed, a proportion of affected individuals will have complications of liver failure, bleeding, hepatocellular carcinoma, and death. The ability of biomarkers (at baseline and/or changing over time) to predict these events directly has the potential to improve prognosis and provide a meaningful assessment of clinical effectiveness (as opposed to therapeutic efficacy indicators such as reduction in fibrosis). In hepatology, the limitations of liver biopsy and rather restrictive pathological scoring systems have encouraged the extrapolation of biomarkers (originally based upon pathological end points) to hard clinical end points. There are a number of studies demonstrating that noninvasive biomarkers (including serum analytes and transient elastography) measured at baseline predict liver-related outcomes between 5 and 8 years [15–17].

In this special edition, original research presented by N. Palaniyappan and colleagues has investigated the prognostic accuracy of validated scoring systems for detecting long-term outcomes in alcoholic hepatitis. These scoring systems showed a uniformly poor prognostic performance in detecting mortality at one year (AUC ranges from 0.5 to 0.66), in contrast to abstinence from alcohol within three to six months of initial diagnosis which was associated with an AUC of 0.83. This not only highlights the importance of abstinence but also that dynamic measurement, in this case of behaviour, can have a significant influence on prognosis in the context of liver disease.

Portal hypertension underpins the major complications of liver disease including variceal bleeding, ascites, and renal failure. Both existing and emerging therapeutic strategies in the context of established cirrhosis are directed towards lowering portal hypertension. The gold standard for its assessment remains the hepatic venous pressure gradient (HVPG). Whilst a wealth of evidence supports its prognostic value and utility in directing management [18, 19], it remains an invasive test that is only available in specialist centres. Thus, the search for robust biomarkers that offer a noninvasive alternative to HVPG is important if portal hypertension is to be assessed in routine clinical practice. The review by V. K. Snowdon and colleagues succinctly outlines the pathophysiological basis of portal hypertension and, in particular, uses examples of recent advances in endothelial cell biology/fibrosis and angiogenesis research to support the rationale for emerging biomarkers in this area.

Hepatocellular carcinoma (HCC) is the fifth leading cause of death from cancer in men, the seventh leading cause of death from cancer in women, and the fastest rising cause of cancer mortality worldwide. The majority of patients present at an advanced stage when treatment options are very limited and, consequently, HCC carries a dismal prognosis (overall median survival of 14 weeks, 1-year survival of 13%). Current screening strategies that rely on AFP and ultrasound are widely accepted but have only modest diagnostic accuracy with sensitivity rates between 25% and 65% [20]. There is an urgent need to discover and implement better diagnostic tools for this malignancy that may permit earlier and more accurate detection and the review by T. Behne and M. S. Copur outlines emerging biomarkers that have potential clinical utility.

To provide stratified care for patients with liver disease, we urgently need noninvasive tools that can effectively phenotype patients based on their degree of liver injury, natural history, and clinical outcomes. It is unthinkable that the choice of intervention in an individual patient still remains, in many circumstances, an empirical exercise involving "trial and error." Biomarker research and its dissemination should aim to overcome these barriers to individualising care.

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Research Article

The Utility of Scoring Systems in Predicting Early and Late Mortality in Alcoholic Hepatitis: Whose Score Is It Anyway?

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Background. Alcoholic hepatitis (AH) is a distinct clinical entity in the spectrum of alcoholic liver disease with a high short-term mortality. Several scoring systems are being used to assess the severity of AH but the ability of these scores to predict long-term survival in these patients is largely unknown. *Aims*. We aim to assess the utility of five different scoring systems Child Pugh (CP), model for end-stage liver disease (MELD), Maddrey's discriminant function (mDF), Glasgow AH score (GAHS), and age-bilirubin-INR-creatinine (ABIC) score in predicting shot-term and long-term survival in patients with AH. *Methods*. Patients with histological evidence of AH were identified from our database. The clinical and biochemical parameters were used to calculate the 5 different scores. The prognostic utility of these scores was determined by generating an ROC curve for survival at 30 days, 90 days, 6 months, and 1 year. *Results and Conclusions*. All 5 scores with the exception of CP score have a similar accuracy in predicting the short-term prognosis. However, they are uniformly poor in predicting longer-term survival with AUROC not exceeding 0.74. CP score is a very poor predictor of survival in both short and long term. Abstinence from alcohol was significantly ($P < 0.05$) associated with survival at 1 year.

1. Introduction

Alcoholic hepatitis (AH) is one of the most recognised "acute on chronic" liver syndromes, wherein patient presents with symptoms and signs of acute decompensation with evidence of chronic liver disease, in the setting of ongoing or recent consumption of excess alcohol [1]. Patients present with progressive jaundice, tender hepatomegaly, and evidence of systemic inflammatory response (SIRS) with characteristic liver biopsy findings of ballooned hepatocytes and Mallory bodies (eosinophilic inclusion bodies) surrounded by neutrophils [2]. AH is a cause of considerable mortality and morbidity in the Western population. A Danish study reported a 28-day mortality rate of 15% among patients hospitalised for AH [3]. A pooled one-month mortality of patients with AH who were treated with placebo in randomised control trials (RCTs) was 22.44% in US and 18.45% in Europe [4]. Short-term prognosis of alcoholic hepatitis is worse than that of decompensated cirrhosis as defined by the system agreed at the Baveno IV consensus conference; 1-year probability of mortality is 20% in decompensated cirrhosis [5]. Hence, it is important to distinguish patients with AH, in particular those with much worse short-term prognosis, from those with decompensated cirrhosis so that the former group are targeted for specific potentially effective treatments [6–8].

Several scoring systems have been developed and used to assess the severity of AH and to predict survival in these patients. Maddrey's discriminant function (DF) has been used in clinical practice for more than 30 years [9]. A DF of 32 is used to stratify a patient's severity of AH, patients with a score of ≥ 32 having a high short-term mortality [10]. Model for end-stage liver disease (MELD) score was initially developed to predict survival in patients with cirrhosis and portal hypertension, but was found to detect short-term survival in patients with AH with good accuracy [11]. However, the cut-off value for MELD score in detecting severe AH is

Table 1: Histological features in alcoholic hepatitis.

(i) Steatosis
(ii) Ballooned hepatocytes
(iii) Lobular inflammation
(iv) Eosinophilic inclusion bodies—Mallory bodies
(v) Neutrophil infiltration
(vi) Megamitochondria

still controversial with various studies using different values to assess the accuracy of the score. The Glasgow alcoholic hepatitis (GAH) score identifies the subgroup of patients with a DF of >32 who will recover without steroids [12]. The ABIC score was developed to categorise patients with AH into high-, moderate-, and low-risk groups based on the risk of death at 90 days and 1 year [13]. The Lille score evaluates the response in serum bilirubin after a 7-day course of corticosteroid therapy and aids the decision in either stopping the corticosteroids or completing a 28-day course [14]. The prognostic value of portal pressure, measured by hepatic venous pressure gradient (HVPG), in chronic liver disease has been well recognised [15, 16]. However, the influence of portal pressure in AH as a distinct entity itself has not been established.

The ability of these various scores to predict long-term survival in patients with AH is largely unknown. Therefore, we attempt to assess the utility of the different published scoring systems in predicting short-term (30 and 90 days) and long-term (6 and 12 months) survival in patients with AH.

2. Methods

We identified patients with the diagnosis of AH from our histological database. The decision to refer patients for liver biopsy was made by the primary physician, which was a hepatologist in all cases. As it is our practice to perform liver biopsy through transjugular approach whenever alcoholic hepatitis is suspected, the histological findings, all the transjugular liver biopsies performed in Queens Medical Centre (QMC), Nottingham University Hospitals between 2004 and 2007, were reviewed from the online hospital database. Biopsies showing histological evidence of AH (Table 1) as determined by a single pathologist (PK) were included in the analysis. All the patients undergoing transjugular biopsies have their HVPG measured simultaneously and this was used to identify the degree of portal hypertension in these patients.

The clinical and biochemical parameters for these patients with biopsy-proven AH was collected from the patient notes and electronic database. These parameters were used to calculate the scoring systems that have been described to guide the management of patients with AH. The measurement units of the biochemical parameters were converted to the relevant units as dictated by the derivation formula of these scores.

Abstinence from alcohol in the short term (3–6 months from the histological diagnosis of AH) among these patients was determined during their follow-up clinic appointment or any hospital admissions in this period.

Short-term (30 and 60 days) and long-term (90 days and 1 year) survival was evaluated in these patients. The prognostic value of the scoring systems was determined by generating a receiver operating (ROC) curve and the area under the curve was calculated.

Table 2: Patient demographics and histological findings in the 44 biopsy proven alcoholic hepatitis.

Sex	
Male	25 (56.8%)
Female	19 (43.2%)
Mean age, in years	48
Liver biopsy findings	
Steatosis	42 (95.4%)
Necrosis	10 (22.7%)
Neutrophil infiltration	43 (97.7%)
Mallory bodies	26 (59.1%)
Ballooned hepatocytes	29 (65.9%)
Acidophilic bodies	2 (4.5%)
Giant mitochondria	1 (2.27%)
Underlying stage of liver disease	
Cirrhosis	18 (40.9%)

Table 3: Prognostic scores of 44 patients with histological evidence of AH at the time of biopsy.

Prognostic scores	*N*/Median
Child-Pugh score (±SD)	10.5 (±2.38)
A (%)	5 (11.4%)
B (%)	8 (18.2%)
C (%)	31 (70.6%)
Model of end-stage liver disease (MELD) (±SD)	18.5 (±6.51)
Maddrey's discriminant factor (MDF) (±SD)	31.6 (±19.7)
Glasgow alcoholic hepatitis (GAH) score (±SD)	7 (±1.47)
ABIC score	7.19 (±1.47)
HVPG, mmHg (±SD)	13 (±6.47)

3. Results

Over the study period, 140 transjugular liver biopsies were performed in QMC. 44 of these biopsies showed histological evidence of AH. The patient demographics and characteristics of their biopsies are summarised in Table 2. Almost all the biopsies demonstrated steatosis and neutrophil infiltration with over half of them having Mallory bodies and swollen hepatocytes. 40.9% (18/44) of the biopsies showed evidence of underlying cirrhosis.

The clinical and biochemical parameters of these 44 patients with biopsy-proven AH were used to calculate the various scoring systems that have been proposed (Table 3). The HVPG measurement was not available for a single patient due to malfunctioning instruments during the transjugular liver biopsy.

Table 4: The area under the receiver operating characteristic (AUROC) for prognostic scores for short- and long-term mortality in patients with AH.

Prognostic score	30-day mortality		90-day mortality		6-month mortality		1-year mortality	
	AUROC	95% CI	AUROC	95% CI	AUROC	95% CI	AUROC	95% CI
CP	0.53	0.25–0.8	0.47	0.21–0.73	0.55	0.35–0.76	0.5	0.31–0.69
mDF	0.79	0.64–0.94	0.81	0.67–0.95	0.72	0.54–0.91	0.63	0.43–0.82
GAHS	0.78	0.54–1	0.81	0.61–1	0.73	0.54–0.92	0.64	0.44–0.84
ABIC score	0.74	0.46–1	0.79	0.55–1	0.67	0.44–0.91	0.66	0.45–0.87
MELD	0.84	0.71–0.96	0.85	0.74–0.97	0.74	0.56–0.92	0.64	0.44–0.83

Follow-up data for one patient was not available as the patient's care was transferred to another hospital. The cumulative 30-day and 90-day mortality in this subgroup was 11.6% (5/43) and 14.0% (6/43). The long-term survival data was unavailable for another patient whose followup was lost. The respective 6-month and 1-year cumulative mortality in this cohort was 21.4% (9/42) and 26.2% (11/42).

In predicting the short-term (30 and 90 day) prognosis in this cohort of patients, GAHS, Maddrey's DF, MELD, and ABIC scores all have a similar accuracy as demonstrated by their AUROC. However, they are uniformly poor in predicting survival beyond 6 months with AUROC not exceeding 0.74. Childs-Pugh score has been shown to be a very poor predictor of survival in both short and long term (Table 4). Clinically significant portal hypertension (HVPG $\geq$ 10 mmgHg) is neither associated with short-term nor long-term prognosis (P = nonsignificant).

Abstinence from alcohol in 3 to 6 months from the diagnosis of AH was significantly associated with survival at the end of the year ($P < 0.05$) and predicted survival with an AUROC of 0.83 (95 CI: 0.71–0.95).

4. Discussion and Conclusion

Despite decades of debate and controversy, the role of histology in identifying the specific cohort described in these studies is still not unequivocally established. In a study that analysed 41 patients biopsied within a month of first presentation with decompensated alcoholic liver disease, none of the histological features were predictive of survival by Cox multivariate analysis [17]. In contrast, a recent study showed that the positive likelihood ratio of the presence of SIRS and clinical features in diagnosing AH is only 1.2, while histological criteria had the best area under the curve in the prediction of adverse outcome [18]. In addition to the lack of consensus, patients with AH have low platelet count and coagulopathy necessitating transjugular approach for the liver biopsy. As transjugular liver biopsy is available in limited number of centres, algorithms and scores using clinical and simple laboratory parameters are widely used in the clinical management of these patients.

We have described a comparison of 5 different prognostic scores in their value of predicting short- and long-term survival in patients with AH. All the scores with the exception of the CP score have a reasonable accuracy in predicting 30- and 90-day mortality. CP score was originally described to assess the operative risk is patients with established cirrhosis and was developed to predict their survival [19]. The relative ease by which it can be calculated at the bedside has meant that it has remained popular among clinicians. However, its use is limited in predicting prognosis in patients with "acute on chronic liver failure," in particular those with alcoholic hepatitis.

As with CP score, MELD is not a system developed specifically to evaluate AH. MELD score was described initially to predict survival following elective transjugular intrahepatic portosystemic shunts (TIPSS) for the prevention of variceal rebleeding or for the treatment of refractory ascites [20]. Utility of MELD score has since extended to assess the mortality risk in patients with end-stage liver disease and to aid organ allocation priorities in transplant centres [11]. Strength of MELD score is that it functions as a continuous variable and hence the clinical outcome can be accurately estimated based on a particular individual's MELD score. In our cohort, MELD performs well in predicting short-term outcome in AH. However, estimation of MELD score requires the use of a calculator and there is no consensus on the optimal cut-off value for this score as different studies have chosen different tradeoffs in setting the test threshold (sensitivity and specificity) [21–24].

Value of Maddrey's DF has been verified by more than 30 years experience [10]. Its main use has been in determining the group of patients with AH that might benefit from corticosteroid therapy. However, Maddrey's DF has often been used to assess the severity of biopsy proven AH and this has not yet been accepted as a standard practice. The GAH score depends entirely on simple clinical and laboratory parameters and has been shown to have a higher overall accuracy compared to the MELD and Maddrey's DF in predicting in-hospital death [12]. It also stratifies the group of patients with a high Maddrey's DF who will recover without being treated with steroids. The ABIC score was developed in an attempt to risk stratify the death in patients with AH at 90 days and 1 year [13]. It stratifies patients into high-, moderate-, and low-risk groups. In our study, the performance characteristics of both GAH and ABIC scores were comparable to that of Maddrey's DF. However, the GAH and ABIC scores have not been verified in countries out of which they were derived in.

The influence of portal pressure in the setting of alcoholic hepatitis is not well established. Rincon et al. attempted

to evaluate the prognostic value of HVPG in patients with acute alcoholic hepatitis and Maddrey's DF of greater than 32 [25]. HVPG of more than 22 mmHg was found to be an independent predictor of in-hospital mortality. Long-term survival was not evaluated in this cohort. In our study, we failed to demonstrate any association between clinically significant portal hypertension (HVPG $\geq$ 10 mmHg) with short- and long-term survival.

Our study has demonstrated that majority of the prognostic scores are comparable in their performance characteristics for predicting short-term mortality in patients with AH, and hence, are of similar utility in clinical practice. However, all the scores that we evaluated are uniformly poor in predicting longer-term survival beyond 6 months. We have shown that the abstinence in the first 3 months following the diagnosis of AH is associated with survival at the end of 1 year. It has been established that abstinence from alcohol is the most important intervention for patients with alcoholic liver disease (ALD). Abstinence has been shown to improve outcome and histological features in all stages of ALD, with improvement noticed in 3 months [26]. As alcohol abstinence is important in improving long-term survival, clinicians should remain focussed in ensuring that these patients have the opportunity to be abstinent from alcohol and continue to do so. The role of pharmacological agents in helping to sustain abstinence is still unclear and requires further investigation.

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Review Article

Noninvasive Evaluation of Portal Hypertension: Emerging Tools and Techniques

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Portal hypertension is the main cause of complications in patients with cirrhosis. However, evaluating the development and progression of portal hypertension represents a challenge for clinicians. There has been considerable focus on the potential role of noninvasive markers of portal hypertension that could be used to stratify patients with respect to the stage of portal hypertension and to monitor disease progression or treatment response in a longitudinal manner without having to undertake repeated invasive assessment. The pathogenesis of portal hypertension is increasingly understood and emerging knowledge of the vascular processes that underpin portal hypertension has paved the way for exploring novel biomarkers of vascular injury, angiogenesis, and endothelial dysfunction. In this paper we focus on the pathogenesis of portal hypertension and potential noninvasive biomarkers with particular emphasis on serum analytes.

1. Clinical Importance of Portal Hypertension

Portal hypertension (PHT) is the most important consequence of cirrhosis and its presence is a hard endpoint for clinically relevant outcomes in terms of varices, ascites, hepatorenal syndrome, and encephalopathy [1]. The current gold standard for measuring PHT and its severity is measurement of the hepatic venous pressure gradient (HVPG). The prognostic value of PHT measurement at different stages in the natural history of chronic liver disease is well established, with cut-off values for the development of complications (HVPG > 10 mmHg) and variceal rupture (HVPG > 12 mmHg) [2, 3]. A reduction in HVPG (e.g., after drug therapy) below 12 mm Hg or by >20% from baseline is associated with a significant reduction in complications and death. In addition, HVPG is also emerging as a reliable endpoint to assess disease progression and therapeutic response in chronic liver disease. The importance of PHT is summarised in Figure 1 showing how changes in the HVPG affect clinical outcomes. Although HVPG measurement is safe and relatively simple to perform, it is invasive, costly, and only performed in specialist centres [4]. A recommendation from the Baverno V Consensus Workshop on Methodology of Diagnosis and Therapy in PHT was to identify noninvasive tools for detecting PHT [5], which could have clinical utility for monitoring changes in PHT over time.

2. Pathophysiology of Portal Hypertension

In cirrhosis, PHT is initiated by an increase in intrahepatic vascular resistance (IHVR) and then exacerbated by changes in the systemic and splanchnic circulation that increase the portal inflow. Increased IHVR is caused not only by mechanical factors (e.g., fibrotic scars and regenerative nodules that distort the hepatic vascular architecture), but also by a reversible dynamic component mediated by an increase in vascular tone due to the active contraction of myofibroblasts around the hepatic sinusoids and in fibrous septa (Figure 2). This dynamic component (which accounts for ~30% of increased IHVR) reflects a functional disturbance of the liver circulation, secondary to increased production of vasoconstrictors (e.g., endothelin-1) and reduced release of endogenous vasodilators (mainly nitric oxide, NO) [6–9].

HVPG	Prognosis	Reference
<10 mm Hg	Predicts patients with cirrhosis that do not decompensate (median 4-year followup)	Ripoll et al. (2007) [3]
>10 mm Hg	Predicts the development of varices	Groszmann et al. 2005 [2]
Reducing <12 mm Hg or >20%	Prevents rebleeding (secondary prophylaxis)	Villanueva et al. 1996 [6]
>20 mm Hg	Predicts ITU stay, hospital stay, short-term and long-term mortality in acute variceal bleed	Moitinho et al. 1999 [7]

Figure 1: Clinical importance of portal hypertension.

Decreased expression of endothelial NO synthase (eNOS) protein, decreased phosphorylation of eNOS by the serine-threonine kinase AKT, the presence of inhibitory substances (e.g., asymmetric dimethylarginine, ADMA), and hyporesponsiveness to NO underlie this endothelial dysfunction [10–12]. In contrast, extrahepatic endothelial cells have the opposite phenotype producing excessive NO which contributes to increased portal blood flow and an increase in PHT.

Angiogenesis has also been shown to influence PHT, with studies demonstrating that the maintenance of increased portal pressure, hyperkinetic circulation, splanchnic neovascularization, and portosystemic collateralization is regulated by vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) [13].

There has been considerable interest in the identification of reliable noninvasive biomarkers for PHT including imaging techniques, routine laboratory tests, serum markers of inflammation and fibrosis, and quantitative assays of liver function which have all shown varying levels of diagnostic accuracy for PHT. The role of imaging markers has been comprehensively addressed in recent reviews [14–16]. The aim of this paper is to review the potential role of noninvasive techniques in evaluating PHT. We have focussed on serum biomarkers with particular emphasis on those that have been identified as being involved in the pathogenesis of PHT. These include novel serum markers associated with vascular injury, angiogenesis, and altered endothelial phenotypes.

3. Assessment of Simple Clinical Parameters

3.1. Clinical Manifestations of PHT. There are several clinical features that indicate the presence of PHT including ascites, splenomegaly, and caput medusa. Hypotension and tachycardia may reflect a hyperkinetic circulation. However, these signs often develop late in the natural history of PHT, can be caused by other diseases, (e.g., portal vein thrombosis or malignancy), and their presence varies between patients. A systematic review of the diagnostic accuracy of physical findings established that they had low sensitivity in compensated disease [17].

3.2. Platelet : Spleen Ratio (Giannini Index). Thrombocytopaenia (platelet count <150,000/uL) is a common complication in patients with chronic liver disease. Moderate thrombocytopaenia (platelet count 50,000–75,000) occurs in ~13% of patients with cirrhosis. Multiple factors can contribute to the development of thrombocytopaenia, including splenic platelet sequestration, bone marrow suppression by chronic hepatitis C infection, and antiviral treatment with interferon-based therapy. Reductions in the level or activity of the haematopoietic growth factor thrombopoietin (TPO) may also play a role. Thrombocytopaenia has been shown to be an independent predictor of significant PHT and the presence of varices, with HVPG and platelet count showing significant negative correlation [18]. However, no specific platelet value has been found to accurately predict the presence of varices and although there is a statistical correlation, a change in the platelet count is not a reliable surrogate of reciprocal changes in portal pressure/HVPG [19]. When combined as the platelet : spleen ratio by Giannini, a 100% negative predictive value for presence of varices with a ratio of over 909 was shown [20]. This ratio has been validated and is simple and cheap [21]. However, criticisms of this simple test are that thrombocytopaenia is often a late sign of PHT, it can occur due to other conditions such as bone marrow

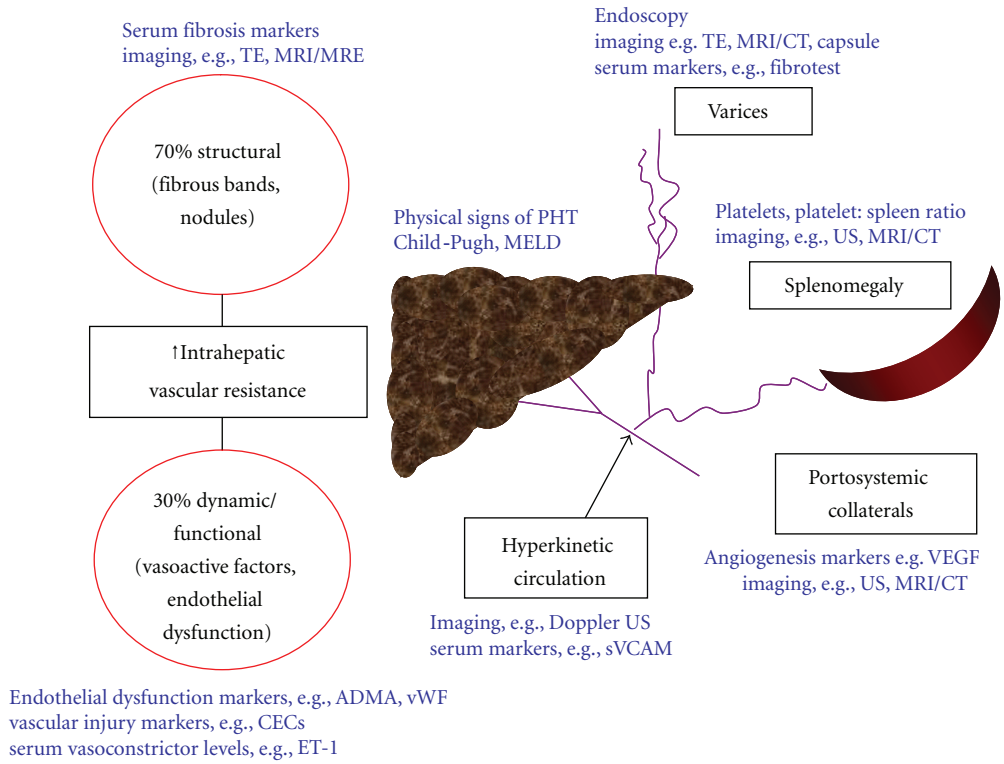

Figure 2: Schematic representation of the pathophysiology of portal hypertension with corresponding potential noninvasive markers. TE: transient elastography; MRI(E): magnetic resonance imaging (elastography); US: ultrasound; CECs: circulating endothelial cells; ADMA: asymmetric dimethylarginine; vWF: von Willebrand factor; ET-1: endothelin-1; MELD: Model for End-Stage Liver Disease.

suppression, and there is a degree of interobserver variability when measuring the spleen size.

It is worth noting that splenomegaly in cirrhosis is not simply caused by portal congestion, but is mainly due to tissue hyperplasia and fibrosis. Although a slight reduction in spleen size has been reported after liver transplantation for cirrhosis [22], evidence of regression of splenomegaly in parallel with a reduction in portal pressure is lacking. Indeed, complete resolution of splenomegaly has never been described, presumably because the architectural changes are at least in part irreversible. This calls into question the utility of splenomegaly as a dynamic marker of PHT.

3.3. Serum Markers of Hepatic Failure. The degree of hepatic failure as indicated by low albumin, prolonged prothrombin time, raised bilirubin, or stratification by Child-Pugh score has been shown in various studies to correlate with severe PHT and the prevalence/grade of varices. However none have been shown to correlate with the degree of PHT and are therefore not accurate enough to determine the severity of PHT in clinical practice [18, 19, 23].

4. Assessing the Structural Component of PHT

4.1. Serum Markers of Hepatic Fibrosis. The extent of hepatic fibrosis influences IHVR and therefore portal pressure, which would suggest that markers of fibrosis may also act as markers of PHT [24]. However, there have been relatively few studies exploring the use of serum fibrosis markers in PHT. Examples of potential analytes include constituents of the basal lamina (e.g., laminin) or major constituents of loose connective tissue (e.g., hyaluronic acid). These markers are found in the blood and have been correlated with hepatic fibrosis [25]. Several studies have shown that serum laminin levels correlate with HVPG in patients with fibrosis and compensated cirrhosis [26, 27]. For the prediction of severe PHT (HVPG > 12 mmHg), serum laminin had a positive predictive value (PPV) of 85% and negative predictive value (NPV) of 43% [28]. Correlation has also been shown between the serum hyaluronic acid concentration and HVPG [29]. To date, studies have only involved small numbers of patients and larger-scale studies are needed to determine the clinical utility of serum fibrosis markers for the evaluation of PHT.

FibroTest (FT) is a panel of biochemical markers that has been extensively validated for the diagnosis of advanced fibrosis and cirrhosis [14]. Thabut and coworkers conducted a prospective study in 130 patients (with or without cirrhosis) undergoing transjugular liver biopsy. The HVPG was also measured along with serum collection for FT. There was significant correlation between FT and HVPG, but this correlation was weaker in patients with established cirrhosis. The FT result was significantly higher in those with PHT, the area under the receiver operator curve (AUROC) for the diagnosis of severe PHT (HVPG > 12) was 0.79, indicating

that this test was not superior to platelet count or Child-Pugh score (0.79 and 0.78, resp.) in diagnosing PHT [30]. Another study performed in 268 patients with chronic hepatitis C compared FT to other potential markers of PHT. For FT, the AUROC for the diagnosis of all varices was 0.72 and 0.76 for large varices, with a sensitivity of only 70% [16]. Despite showing promise, FT has not yet been shown to be a reliable test for clinically significant PHT.

4.2. Measurement of Liver Stiffness. The role of transient elastography (TE) has been explored in several reviews [14, 15]. The degree of liver stiffness has been shown to strongly predict the presence of advanced fibrosis or cirrhosis [31] and also correlates with HVPG. A very recent study by Robic and colleagues showed that the liver stiffness measurement (LSM) can be as useful as HVPG in predicting clinical decompensation and PHT-related complications [32]. In this study an LSM of 21.1 kPa or greater gave an AUROC of 0.845 for predicting portal hypertensive complications, with HVPG giving an AUROC of 0.837. No patients with an LSM <21.1 kPa developed any portal hypertensive-related complications. TE is therefore emerging as a leading diagnostic marker for PHT, although a major disadvantage of this technique is the inability to interpret scans in nearly 1 in 5 cases mostly due to obesity and limited operator experience [33]. Additionally, outside of specialist centres, many hospitals may not have access to this resource. Magnetic resonance elastography (MRE) is a promising modality for the noninvasive assessment of liver fibrosis. MRE of the spleen is also feasible and has shown promise as a quantitative method for predicting the presence of oesophageal varices in patients with advanced liver fibrosis [34]. However, MRE is currently too expensive and time consuming for widespread implementation in clinical practice.

4.3. Serum Markers of Angiogenesis. Both VEGF and PDGF are critical to angiogenesis, a process that contributes significantly to PHT by expanding the splanchnic vascular bed and thereby increasing portal blood flow. In addition, VEGF-dependent angiogenesis is important in portosystemic collateral vessel formation including varices. VEGF plays the predominant role in stimulating proliferation of endothelial cells and endothelial tube formation, whereas PDGF regulates vessel stability via the attachment of mural and pericyte cell populations to the endothelium. Increased VEGF expression has been shown by immunohistochemistry and western blot in the mesenteric vessels of animals with PHT, with levels correlating with increasing PHT [35]. Combined blockade of VEGF and PDGF after the development of PHT significantly decreased portal pressure and mesenteric blood flow with reduced expression of VEGF and PDGF [13]. Interestingly, this effect was not observed in models where PHT was just developing. In a model of carbon-tetrachloride- (CCl_4-) induced cirrhosis, animals with PHT had significantly increased levels of intestinal and plasma VEGF but there was no correlation between plasma VEGF levels and portal pressure [36]. This contrasts with a small human study investigating the role of Octreotide in PHT which showed a significant correlation between HVPG and the serum VEGF level [37]. It appears that VEGF and PDGF have a synergistic interaction in the pathogenesis of PHT through regulation of splanchnic neovascularisation and portosystemic collateral formation. However, data to support a diagnostic role for these markers in PHT is currently lacking.

In patients with cirrhosis, serum levels of soluble vascular adhesion molecule (sVCAM-1) have been associated with increasing liver fibrosis and are related to angiogenesis. Although serum sVCAM-1 levels did not correlate with HVPG, it could represent a marker of the hyperkinetic circulation and levels were closely related to clinical stage (Child-Pugh, MELD scores) [38].

5. Dynamic Functional Component of Portal Hypertension

5.1. Markers of Increased Vasoconstriction. As PHT is associated with hyperproduction of endogenous vasoconstrictors, measurement of these factors in the serum could be used to evaluate PHT noninvasively. Serum endothelin-1 (ET-1) levels are elevated in portopulmonary hypertension and associated with a poor outcome [39] and have also been shown to correlate with HVPG values in patients with cirrhosis [40]. Thus, serum endothelin levels could be used to evaluate the degree of PHT, although further studies are needed to determine the clinically relevant levels.

Urotensin II (U-II), a somatostatin-like cyclic peptide, was recently identified as the most potent human vasoconstrictor peptide. One study suggested that U-II was an important marker of the severity of PHT in children with chronic liver disease and correlated with Child-Pugh score, paediatric end-stage liver disease score, and long-term clinical outcome [41]. In another study, in adults with cirrhosis and hyperkinetic circulation but with normal serum creatinine, U-II levels were notably higher than in healthy subjects; however there was no correlation with cardiac index or other haemodynamic parameters observed [42].

5.2. Markers of Endothelial Dysfunction. Endothelial dysfunction is a major determinant of the increased intrahepatic vascular tone observed in cirrhosis and a number of markers reflecting this dysfunction have been identified.

NO synthesis can be inhibited by the endogenous circulating amino acid asymmetric dimethylarginine (ADMA). ADMA is synthesized via enzymatic methylation of L-arginine residues in proteins and is released during proteolysis and metabolized to citrulline and dimethylamine in the liver, with impaired liver function associated with increased plasma levels of ADMA. There have been several studies linking ADMA to endothelial dysfunction in cardiovascular disease and multiorgan failure [43, 44]. Laleman and colleagues examined different animal models of cirrhosis and PHT and showed that bile-duct-ligated (BDL) animals exhibited normal eNOS levels in contrast

to thioacetamide and carbon-tetrachloride-induced models of cirrhosis, suggesting that posttranslational regulatory mechanisms are involved in the defective production of NO in some causes of cirrhosis [12]. In BDL-treated animals ADMA levels were significantly elevated suggesting a possible role for ADMA in inhibiting eNOS. Lluch and coworkers showed that peripheral blood levels of ADMA correlated with the degree of liver failure and decompensation in patients with alcohol-related cirrhosis [45]. In a further study involving patients with compensated chronic hepatitis C cirrhosis, a positive statistically significant correlation was found between HVPG and ADMA [46]. This was the first study to observe a correlation between the degree of PHT and ADMA levels. Further mechanistic studies are needed to define ADMA metabolism and function in PHT.

Von Willebrand factor (vWF), P-selectin, and 8-iso-PGF2a have also been identified as surrogate markers of endothelial dysfunction and levels of these factors are increased in patients with cirrhosis compared with controls. In patients with PHT, vWF levels significantly correlated with HVPG, Child-Pugh, and MELD scores. In addition, peripheral vWF levels with a cut-off value of 216 U/dL (Youden index) were also predictive of clinical outcomes (PHT-related events and liver transplantation) [47].

5.3. Markers of Vascular Injury: Circulating Endothelial Cells (CECs). CECs are a specific population of endothelial cells in peripheral blood. They exceed 10 μm in size and are characterized by the expression of at least two different endothelial markers and absence of expression of leukocyte markers [48]. They are present in very low levels in healthy individuals. Elevated levels of CECs have been observed in a variety of diseases associated with vascular damage and are considered to reflect the severity of vascular injury [11]. Abdelmoneim and colleagues [49] performed a small study on patients with cirrhosis, with or without PHT, the latter being defined by the presence of varices, splenomegaly, ascites, encephalopathy, and/or HCC versus age- and sex-matched controls. The number of CECs was significantly elevated in patients with cirrhosis compared to controls. However, HVPG was not measured in these subjects such that conclusions regarding the clinical potential of CECs as a biomarker for PHT are limited. When combined with the platelet count (PC) as CEC/PC with a cut-off value of 0.21, the sensitivity for diagnosing cirrhosis was 100% with a specificity of 73% and AUROC of 0.8. Additionally correlation was seen with a rising CEC/PC and presence of decompensation. A further larger study is needed in patients where CECs levels and CEC/PC are correlated with the HVPG.

6. Markers of Modifications in Splanchnic Circulation and Hyperkinetic Syndrome

The extrahepatic endothelial phenotype is that of excess NO production causing peripheral vasodilatation and increased blood flow through the mesenteric vessels and portal vein. This exacerbates the portal pressure. Imaging of the portal and systemic circulation has been performed using duplex Doppler ultrasound, CT, and MRI. Detailed discussion of these modalities is beyond the scope of this paper but noninvasive imaging has shown promise in detecting portosystemic collaterals and changes in portal vein expiration diameter, hepatic vein waveforms, and splenic pulsatility which all have varying discriminatory ability in detecting changes in PHT [14].

7. Video Capsule Endoscopy (VCE)

The presence of varices is objective evidence of the presence of severe PHT. Rather than pure search for surrogate markers of PHT, there has been much interest in the use of capsule endoscopy in diagnosing varices. Promising results in pilot studies led to two larger studies. De Franchis et al. [50] showed, in a study of 288 cirrhotic patients undergoing endoscopy for either screening or surveillance, that VCE had 84% sensitivity and 92% PPV for detecting all oesophageal varices. For determining the size of the varices and need for surveillance versus treatment, it was shown that VCE had an 87% PPV and 92% NPV suggesting that as a noninvasive tool it is promising [50]. Lapalus et al. [51] showed similar encouraging results in a study of 120 patients with PHT undergoing VCE followed by endoscopy. They found VCE had 77% sensitivity and 90% PPV for diagnosing oesophageal varices [51]. Concordance between the two blinded endoscopists was good, particularly with regard to who required prophylaxis. However, recent evidence from a clinical study by Chavalitdhamrong et al. [52] has shown overall accuracy for detection of oesophageal varices at only 63.2% with 51.5% sensitivity for other significant upper GI lesions such as portal hypertensive gastropathy or gastric varices, suggesting that there are fairly major discrepancies in the sensitivity and specificity between operators [52]. It is clear that standard endoscopy is superior to VCE. Although it does show promise as a noninvasive tool, its role may be in patients who require screening whilst on treatment, but do not tolerate standard endoscopy well.

8. Conclusion

PHT is a robust outcome measure which has proven prognostic significance in chronic liver disease and the potential for use in monitoring disease progression and treatment efficacy. In this paper we have outlined the pathogenesis of PHT and discussed a range of candidate serum biomarkers that have been identified. At present, transient elastography appears to represent the most promising noninvasive technique that could potentially replace HVPG measurement for PHT or endoscopy for variceal detection. The potential role of serum markers for the evaluation of PHT remains unproven, but will increasingly be assessed in prospective clinical studies. Further advances in our understanding of the underlying mechanisms responsible for the development and progression of PHT will continue to reveal additional biomarker targets.

9. Methods

Referred papers were identified by MEDLINE search through the PubMed database by combining the keyword "portal hypertension" with the keywords "biomarkers, serum, fibrosis, endothelial cell and angiogenesis." Additional papers were identified by searching of references through retrieved papers.

Conflict of Interests

There is no conflict of interests.

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Research Article

Increased α-Fetoprotein Predicts Steatosis among Patients with Chronic Hepatitis C Genotype 4

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Background. The prognostic importance of α-fetoprotein (AFP) level elevation in patients with chronic hepatitis C and its clinical significance in steatosis associated with HCV infection remain to be determined. The present paper assessed clinical significance of elevated AFP in patients with CHC with and without steatosis. *Methods*. One hundred patients with CHC were divided into 50 patients with CHC and steatosis and 50 patients with CHC and no steatosis based on liver biopsy. *Results*. AFP was significantly increased in CHC with steatosis than patients without steatosis ($P < 0.001$). Highly significant positive correlation was found between serum AFP and necroinflammation as well as the severity of fibrosis/cirrhosis and negative significant correlation with albumin level in chronic HCV with steatosis ($P < 0.001$) but negative nonsignificant correlation with ALT and AST level ($P \leq 0.778$ and 0.398), respectively. Highly significant increase was found in chronic hepatitis patients with steatosis than CHC without steatosis regarding necroinflammation as well as the severity of fibrosis/cirrhosis and AFP ($P < 0.001$). *Conclusion*. Patients with chronic HCV and steatosis have a higher AFP levels than those without steatosis. In chronic HCV with steatosis, elevated AFP levels correlated positively with HAI and negative significant correlation with albumin level.

1. Introduction

Chronic hepatitis C (CHC) is thought to affect more than 170 million people worldwide, and it has been shown that steatosis occurs in approximately 50% of patients with CHC [1]. Steatosis also occurs more than twice as frequently in patients with CHC than in the general population [2]. Both viral and host metabolic factors have been reported to contribute to the genesis of hepatic steatosis in patients with CHC. Most steatosis is mild, with the more severe cases usually occurring in genotype 3 virus infection [3].

Serum alpha-fetoprotein (AFP) is a fetal glycoprotein produced by the yolk sac and fetal liver [4]. Following birth, AFP levels decrease rapidly to less than 20 ng/mL and increase significantly in certain pathologic conditions. Serum AFP is a debated but routinely used marker for hepatocellular carcinoma (HCC) in patients with chronic liver disease [5]. Benign circumstances that may produce elevations of AFP include cirrhosis, hepatic necrosis, acute hepatitis, chronic active hepatitis, ataxia-telangiectasia, and pregnancy [6, 7]. Elevated serum AFP levels may also be due to altered hepatocyte-hepatocyte interaction and the loss of normal architectural arrangements [8]. Based on the results of Ray et al. about 91% of the Egyptian patients with chronic HCV were infected with HCV genotype 4 [9]. In this study, we evaluated serum AFP and its clinical significance in Egyptian patients with chronic hepatitis C mostly genotype 4 (based on the results of Ray et al.) with and without steatosis.

2. Subjects and Methods

This prospective study included 100 patients with CHC divided into two groups (50 patients with steatosis and 50 patients without steatosis) based on liver biopsy. Both groups were adjusting as regarding age, sex, and for risk factors for steatosis (BMI, DM, and hyperlipidemia). They

were referred to Mansoura university Hospital, from October 2010 to December 2011, for liver biopsy and searching for chronic HCV management. The study protocol confirmed to the ethical guidelines of 1975 Declaration of Helsinki. Informed consent was obtained from all patients. CHC was defined according to positive serum HCV-Ab and HCV-RNA for at least 6 months and elevated levels of serum aminotransferases (AST, ALT). The patient's medical histories were taken including, age, gender, weight, height, and body mass index (BMI). AFP was studied with ELISA method using Abbott laboratory reagents, USA (normal level of AFP was defined as <8.1 ng/mL); serum fasting triglyceride, fasting blood sugar, albumin, AST, ALT, total bilirubin, and HCV-RNA viral load were determined. Biochemical tests and HCV-RNA (viral load IU/mL) were performed by autoanalyzer (Selecta, Germany) and Cobas Amplicore monitor version 2 (Roche Molecular Systems, Branchburgh, NJ, USA), respectively. BMI was calculated by the following formula: weight (kg)/height^2 (m^2).

Exclusion criteria were patients with diabetes mellitus, hypertension, and patients who had any serological evidence of infection with other viruses (HBV and HIV); all other known causes of liver diseases were excluded on the basis of analytical, clinical, and epidemiological data: autoimmunity, metabolic and genetic disorders, NASH, alcohol intake, drug toxicity, and patients with decompensated cirrhosis. Hepatocellular carcinoma and other causes of high levels of AFP like cancer of the testes or ovaries and metastatic liver cancer were excluded using ultrasound as the predominant screening method.

Percutaneous liver biopsy (≥15 mm in length) was performed for all the patients. Liver biopsy specimens were reviewed by a single pathologist. For each liver biopsy specimen, hematoxylin and eosin and Masson's trichrome stains were available. The extent of hepatic steatosis was assessed and graded as none (steatosis < 5%), mild steatosis (steatosis 5–33% of hepatocytes), moderate steatosis (steatosis 34–66% of hepatocytes), and sever (steatosis > 66% of hepatocytes) according to histological scoring system of Kleiner et al. [10]. The histological activity (grade) and degree of fibrosis (stage) of the liver biopsy were assessed according to the modified histological activity index (HAI) of Ishak et al. [11]. Histological activity was considered as minimal (score 1–3), mild (4–8), moderate (9–12), and severe (13–18). Fibrosis was staged separately on a scale 0–6, corresponding to no fibrosis (0), mild (1-2), moderate (3-4), and severe or cirrhosis (5-6).

3. Statistical Analysis

The statistical analysis of data was done by using *Excel* program and *SPSS* program (statistical package for social science) version 10. Data are expressed as the mean ± SD. Mean values were compared with the Student's *t*-test (variables with normal distribution) or Mann-Whitney *U*-test (variables with nonnormal distribution). Categorical variables were compared using the chi-square test. Correlations were done using Pearson's correlation. All the tests performed were two sided and a P value < 0.05 was considered to be statistically significant.

4. Results

This prospective study included 100 patients with CHC (50 patients with steatosis and 50 patients without steatosis) as evidenced by liver biopsy. All patients were positive for anti-HCV antibodies and positive HCV RNA. Table 1 shows the comparison of clinical, biochemical, and histopathological characteristics of patients with and without steatosis. Patients with steatosis had a significantly higher AFP ($P < 0.001$) and necroinflammation and fibrosis/cirrhosis ($P < 0.001$). Also AST ($P < 0.001$), ALT ($P = 0.002$), total Bilirubin ($P = 0.023$), and prothrombin time ($P = 0.003$) were significant high in chronic HCV with steatosis than without steatosis. However, age, triglyceride, fasting blood glucose, albumin, BMI, and HCV RNA were not significantly different between the two groups.

Table 2 showed a significant positive correlation between higher serum AFP levels, with the severity of periportal necroinflammation, as well as the severity of fibrosis/cirrhosis ($P < 0.001$). A significant negative correlation was found between serum AFP and serum albumin ($P < 0.001$). Also a negative correlation but not significant was found between serum AFP, AST ($P = 0.398$), and ALT ($P = 0.778$).

5. Discussion

Hepatitis C virus (HCV) is a member of the Flaviviridae family responsible for acute and chronic liver disease [12]. Infection with HCV is common, with an average worldwide prevalence of 3% [13]. Acute HCV infection becomes persistent in about 85% of cases [14] and may cause chronic hepatitis leading to cirrhosis and, eventually, hepatocellular carcinoma [15].

The reported prevalence of steatosis in patients with chronic hepatitis C varies between 40% and 80%, depending on the features of the population studied in terms of alcohol consumption, prevalence of obesity, diabetes, and other risk factors [16]. The prevalence of steatosis in HCV is approximately 2-fold higher than in another common chronic liver disease like hepatitis B [17], suggesting that HCV may directly cause steatosis, at least in some patients. All genotypes are steatogenic, but numerous reports showed that steatosis was more frequent and more severe in patients infected with genotype 3 [18–20].

AFP is a glycoprotein that is normally generated during conception by the fetal liver and yolk sac. In clinical practice, AFP levels are elevated in various clinical situations, which include hepatocellular carcinoma, acute or chronic viral hepatitis, chronic liver disease, and gonadal tumors [21].

In this study we tried to determine the level of AFP among patients with chronic HCV, and evaluate its relation to the presence of steatosis among 100 patients with chronic HCV (50 with steatosis and 50 without steatosis) enrolled in our study.

Table 1: Clinical, biochemical and histopathological characteristics of patients with and without steatosis.

	Steatosis + VE	Steatosis − VE	*P* value
Age (years)	41.62 ± 6.37	43.68 ± 6.94	0.126
Grade (Ishak grading)	5.90 ± 2.21	2.48 ± .1.50	0.001*
Stage of fibrosis (Ishak staging)	2.72 ± 1.93	.50 ± .50	0.001*
AFP (ng/mL)	8.64 ± 3.18	3.56 ± .81	0.001*
Triglyceride (mg/dL)	108.95 ± 26.39	102.68 ± 31.65	0.323
FB glucose (mg/dL)	106.76 ± 75.84	101.31 ± 14.35	0.44
Alumin (g/dL)	4.40 ± .30	4.50 ± .28	0.115
AST (u/L)	53.62 ± 20.83	34.42 ± 15.26	0.001*
ALT (u/L)	57.72 ± 20.43	45.22 ± 18.47	0.002*
Total Bilirubin (mg/dL)	0.82 ± .35	0.62 ± .34	0.023*
Prothrombin time concentration %	88.09 ± 7.39	84.50 ± 3.64	0.003*
BMI	29.3 ± 5.2	28.9 ± 3.2	0.695
HCV RT-RNA (IU/mL)	224529.1 ± 308275.96	298415.9 ± 320004.30	0.302

AFP, Alpha-fetoprotein; FB glucose, Fasting blood glucose; AST, aspartate aminotransferase; ALT, alanine aminotransferase; BMI, body mass index; RT-PCR, reverse transcriptase-polymerase chain reaction.

Table 2: The Pearson's Correlation between pathological and biochemical parameters with AFP in CHC patients with steatosis.

	AFP	
	R	*P*
Necroinflammation	*0.759	0.001
Fibrosis	*0.759	0.001
Albumin (g/dL)	−0.710	0.001
ALT (u/L)	−0.041	0.778
AST (u/L)	−0.122	0.398

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Similar to previous studies [6, 7] we found that AFP was increased in patients with chronic HCV in the current study. Although both groups (with or without steatosis) are similar regarding underlying etiology (HCV) and were adjusting for risk factors of high levels of AFP and steatosis, AFP was highly significant among patients with chronic HCV and steatosis than patients without steatosis. Regarding necroinflammmation and fibrosis/cirrhosis, a significant increase in patients with HCV and steatosis than without steatosis was found. Our results were consistent with the previous reports [22, 23] which revealed that HCV is associated with steatosis in a large portion of cases and that steatosis is associated with worsening fibrosis. In addition, steatosis induces chronic hepatic inflammation, reactive oxygen species, and DNA damage in animal models [24–26]. So steatosis is associated with more degree of inflammation and fibrosis. Hepatic progenitor cells (HPCs) arise in the periportal region of the liver and may be responsible for liver regeneration. They express high levels of AFP, certain keratin markers, and GGT [27–29]. Their presence is related to the severity of fibrosis [30], and their activation has been documented in parallel with cells associated with the development of fibrosis (stellate cells) [31]. Since steatosis among patients with chronic HCV infection was associated with an increase in both the number of HPC and the extent of the ductular reaction as provided by Clouston et al., so these provide a potential mechanism whereby steatosis contributes to the increase in AFP [32].

Another explanation of increased AFP was provided by the results of others [27–31]; they concluded that the presence of more fibrosis is associated with increasing number and more activation of hepatic progenitor cells. Since the group with steatosis has more fibrosis/cirrhosis than group without steatosis, hence, the joint association observed in this study among increased AFP in chronic HCV with steatosis than none steatotic group.

The aminotransferases are also important biological markers that are widely used for liver diseases. Elevation of the activity of these enzymes in serum is believed to result from their leakage from damaged cells, and so this reflects hepatocyte injury. These enzymes are elevated in many forms of liver diseases and especially those diseases that are associated with significant hepatocyte necrosis such as acute viral hepatitis, which is the most common cause of massive aminotransferases elevation [33]. In this study the levels of aminotransferases AST and ALT are significantly increased in chronic HCV with steatosis than patients without steatosis, indicating more hepatocytes necrosis in patients with steatosis. Our result was in agreement with Hepburn et al., who found significant increase in ALT and AFP level among patients with steatosis versus without steatosis [34].

Our results showed that serum AFP levels were correlated with the severity of periportal necroinflammation as well as the severity of fibrosis/cirrhosis among chronic HCV with steatosis. These results were consistent with the report of Chu et al., which revealed that higher serum AFP levels were correlated with the severity of periportal necroinflammation as well as the severity of fibrosis/cirrhosis. AFP production is enhanced in the presence of injury, possibly resulting

from increased hepatocyte turnover [35]. Hu et al. found a similar correlation between AFP and measures of liver disease activity and severity [36]. The obvious increase in AFP and biochemical values suggests that inflammation, necrosis, and hepatocellular injury are the most common cause of elevated AFP in the studied groups.

In our study a negative correlation was found between lower serum albumin and AFP among patients with steatosis. According to previous reports, the AFP and albumin genes are characteristically arranged in tandem by a similar structure and are believed to be derived from a common ancestral gene [37]. Previous reports have described that reciprocal changes in albumin and AFP gene transcription existed during liver regeneration [38, 39]. The possible mechanism may attribute to the switching action of the AFP enhancer from the AFP promoter to the albumin promoter, which leads to a decrease in AFP expression and an increase in albumin expression [37]. Thus, in patients with chronic Hepatitis C, a reactive expression of the AFP gene, as shown in hepatic necroinflammation and hepatocellular proliferation, may be associated with a decrease in albumin gene transcription and may lead to a lower serum albumin level.

The relationship between the level of aminotransferases and AFP is not definite as a rise in the level of aminotransferases enzymes can be an attribute to damage of hepatocytes, while the level of AFP, and especially a markedly increased level (>400 ng/mL), is rather due to a neoplasm, hepatocellular carcinoma [33]. In this study a negative correlation between the ALT, AST, and AFP (Table 2) was found but not statistically significant. Chu et al. found no significant different in serum transaminase levels in patients with or without elevated serum AFP [35], but against this result is that of Goldstein et al., who found that increasing serum AFP values were significantly correlated with increasing ALT values [8].

There seems to be important correlations among the necroinflammation as well as the severity of fibrosis/cirrhosis and albumin with serum AFP. The necroinflammation as well as the severity of fibrosis/cirrhosis and a lower serum albumin was shown to be predictor for elevation of serum AFP among chronic HCV with steatosis.

Our results were consistent with the report from Bayati et al., which revealed that an elevated serum AFP level was highly specific for the diagnosis of cirrhosis among patients with chronic hepatitis C. These findings indicated that hepatic fibrosis/cirrhosis is more important than necroinflammation in causing an elevation of serum AFP in patients with chronic hepatitis C [40]. Also chu et al. found that a lower serum albumin level was an independent factor in predicting elevated serum AFP [35].

In conclusion, this study has demonstrated that patients with chronic HCV and steatosis have higher AFP levels than those without steatosis. AFP correlates with necroinflammation as well as the severity of fibrosis/cirrhosis in chronic HCV with steatosis. Higher levels of serum AFP may correspond to the presence of steatosis among chronic HCV. They increased both the number of HPC and the extent of the ductular reaction in addition to increased fibrosis/cirrhosis levels providing a potential mechanism whereby steatosis contributes to the increase in AFP. So in the absence of traditional causes of elevated serum AFP, steatosis should be among the differential diagnoses of elevated serum AFP levels in patients with chronic HCV infection.

Abbreviations

AFP: Alpha-fetoprotein
CHC: Chronic hepatitis C
AST: Aspartate aminotransferase
ALT: Alanine aminotransferase
HCV: Hepatitis C virus
RT-PCR: Reverse transcriptase-polymerase chain reaction
BMI: Body mass index
HAI: Histological activity index.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Biomarkers for Hepatocellular Carcinoma

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The hepatocellular carcinoma (HCC) is one of the most common malignant tumors and carries a poor survival rate. The management of patients at risk for developing HCC remains challenging. Increased understanding of cancer biology and technological advances have enabled identification of a multitude of pathological, genetic, and molecular events that drive hepatocarcinogenesis leading to discovery of numerous potential biomarkers in this disease. They are currently being aggressively evaluated to establish their value in early diagnosis, optimization of therapy, reducing the emergence of new tumors, and preventing the recurrence after surgical resection or liver transplantation. These markers not only help in prediction of prognosis or recurrence but may also assist in deciding appropriate modality of therapy and may represent novel potential targets for therapeutic interventions. In this paper, a summary of most relevant available data from published papers reporting various tissue and serum biomarkers involved in hepatocellular carcinoma was presented.

1. Introduction

As molecular indicators of biological status, biomarkers, detectable in blood, urine, or tissue, can be useful for the clinical management of various disease states. Threshold concentrations can be utilized to identify the presence of various diseases. Concentration fluctuations have the potential to guide therapy in disease progression. Numerous biomarkers have been identified for various disease states. Research is ongoing to fully understand and evaluate the clinical significance of utilizing biomarkers. Time and money can be saved by avoiding empiric or broad treatment approaches to diseases of particular organs or systems, and ideally, biomarkers could serve as a measurement tool to detect disease presence and progression and to guide more targeted therapy. Many disease states, especially various types of cancer, can be better understood by the utilization of tumor biomarkers. Hepatocellular carcinoma (HCC) is one such cancer that can benefit from tumor biomarkers' diagnostic, therapeutic, and prognostic capabilities.

HCC is the fifth most common malignant tumor and the third leading cause of cancer-related deaths. Worldwide, there are about 626,000 new HCC cases and nearly 600,000 HCC-related deaths each year with an incidence equal to the death rate [1, 2]. Although the molecular mechanisms by which HCC develops remain largely unclear, a multitude of pathological, genetic, and molecular events that drive hepatocellular carcinogenesis has been identified.

Current gold standard and most commonly used biomarkers for patients at risk for HCC, alpha-fetoprotein (AFP) along with ultrasound every 6 to 12 months, is far from perfect. Serum AFP levels of more than 400 ng/mL are considered diagnostic; however, such high values are observed only in a small percentage of patients with HCC. Ultrasound surveillance even performed at every three monthly intervals cannot improve detection of small HCC because of limitations in recall procedures [3, 4].

With advances in understanding of tumor biology, along with the development of cellular and molecular techniques, the role of biomarkers related to early detection, invasiveness,

metastasis, and recurrence has attracted great deal of research interest resulting in discovery and utilization of several novel markers in this disease. In this paper we try to give an overview of available data on this burgeoning area of research.

2. Biomarkers for Liver Cancer

2.1. Oncofetal and Glycoprotein Antigens

2.1.1. Alpha-Fetoprotein (AFP). The first serologic assay for detection and clinical followup of patients with hepatocellular carcinoma was alpha-fetoprotein (AFP) which has been the standard tumor biomarker for HCC for many years. It is a glycoprotein produced by the fetal liver and yolk sac during pregnancy. Serum AFP levels are often elevated in HCC, but this is not always the case. AFP levels may be elevated initially in the early stages of HCC and then drop or even normalize before rising again as disease progression occurs [5]. Additionally, AFP elevation has also been recognized in the presence of acute and chronic viral hepatitis as well as in patients with cirrhosis caused by hepatitis C. Given the multiple indications that present with elevated AFP levels, it is necessary to evaluate the significance of serum concentrations. In general, consistently elevated serum AFP levels greater than 500 ng/mL are indicative of HCC. Lower serum concentrations which are only transient in nature are more often present in benign liver disease [6]. If a patient has known risk factors for HCC, such as the presence of cirrhosis, increasing levels of AFP have been shown to correlate with the development of HCC [6]. Unfortunately, AFP serum concentrations do not correlate well with the prognostic values of HCC such as tumor size, stage, or disease progression, and ethnic variability may also exist. Furthermore, in some cases of HCC, AFP elevations are not apparent at all [7]. Total AFP can be divided into three different glycoforms, AFP-L1, AFP-L2, and AFP-L3-based on their binding capability to lectin *Lens culinaris* agglutinin (LCA). High percentage of AFP-L3 has been shown to be associated with poor differentiation and biologically malignant characteristics, worse liver function, and larger tumor mass [8].

2.1.2. Glypican-3. Glypican-3 (GPC3), a membrane-anchored heparin sulfate proteoglycan, has been demonstrated to interact with growth factors and modulate their activities. It binds to the cell membrane through the glycosylphosphatidylinositol anchors. GPC3 mRNA was upregulated significantly in tumor tissues of HCC compared to paraneoplastic liver tissue, liver tissues of healthy adults, and liver tissues of patients with nonmalignant hepatopathy. The expression of GPC3 (at both mRNA and protein levels) in the serum of HCC patients was significantly higher than that in the serum of healthy adults or patients with nonmalignant disease. It can be detected in 40–53% of HCC patients and 33% of HCC patients seronegative for both AFP and Des-gamma-carboxyprothrombin (DCP) [9, 10]. It has been shown that soluble GPC3 (sGPC3), the NH_2-terminal portion of GPC3, is superior to AFP in the sensitivity of detecting well or moderately differentiated HCC, and the simultaneous determination of both markers improves overall sensitivity from 50% to 72%. Recently, a study compared the survival rate between the GPC3-positive and GPC3-negative HCC patients. GPC3 positivity correlated with poor prognosis and identified as an independent prognostic factor for the overall survival on multivariate analysis [11].

Table 1: Diagnostic values of HCC serum markers [12–14].

Type of test	Sensitivity (%)	Specificity (%)
AFP-L3	61.6	92.0
DCP	72.7	90.0
AFP	67.7	71.0
AFP-L3 + DCP	84.8	97.8
AFP-L3 + AFP	73.7	86.6
DCP + AFP	84.8	90.2
AFP-L3 + DCP + AFP	85.9	59.0

2.2. Enzymes and Isoenzymes

2.2.1. Des-Gamma-Carboxy (Abnormal) Prothrombin (DCP). DCP is produced by the malignant hepatocyte and appears to result from an acquired posttranslational defect in the vitamin-K-dependent carboxylase system. DCP production is independent of vitamin K deficiency, although pharmacological doses of vitamin K can transiently suppress DCP production in some tumors. DCP levels greater than 0.1 AU/mL (100 ng/mL) on ELISA are highly suggestive of HCC or tumor recurrence. Normalization of DCP levels correlates well with successful tumor resection and appears to be an excellent marker of tumor activity. It is thought that the combination of AFP and DCP assays will increase the sensitivity of testing. The correlation between tumor size and DCP levels is not yet clearly defined. It appears that there is a correlation in DCP levels and large tumors; however, the same is not the case in small tumors (<3 cm) [15]. A cross-sectional case control study involving 207 patients determined that DCP is more sensitive and specific than AFP for differentiating HCC from nonmalignant liver disease. In this study there were 4 groups studied: normal healthy subjects; patients with noncirrhotic chronic hepatitis, patients with compensated cirrhosis, and patients with histologically proven HCC. Both DCP and AFP levels increased among the groups as disease severity increased (from normal to HCC), but DCP values had less overlap among the groups than AFP. Study results concluded that a DCP value of 125 mAU/mL yielded the best sensitivity and specificity for differentiating patients with HCC from those with cirrhosis and chronic hepatitis [16]. Sensitivity and specifity of total AFP, AFP glycoforms, DCP, and combinations of both markers have been summarized in Table 1.

2.2.2. Gamma-Glutamyl Transferase. Serum gamma-glutamyl transferase (GGT) in healthy adults is mainly secreted by hepatic Kupffer cell and endothelial cell of bile duct,

and its activity increases in HCC tissues. Total GGT can be divided into 13 isoenzymes by using polymer acrylamide gradient gel electrophoresis, and some of them can only be detected in the serum of HCC patients. Sensitivities of GGTII have been reported to be 74.0% in detecting large HCC and 43.8% in detecting small HCC. Sensitivity can be significantly improved with the simultaneous determination of GGTII, DCP, and AFP [17].

2.2.3. Serum Alpha-1-Fucosidase. Alpha-l-fucosidase (AFU) is a lysosomal enzyme found in all mammalian cells with a function to hydrolyze fucose glycosidic linkages of glycoprotein and glycolipids. Its activity increases in the serum of HCC patients (1418.62 ± 575.76 nmol/mL/h) compared with that in the serum of healthy adults (504.18 ± 121.88 nmol/mL/h, $P < 0.05$), patients with cirrhosis (831.25 ± 261.13 nmol/mL/h), and patients with chronic hepatitis (717.71 ± 205.86 nmol/mL/h). It has been reported that the sensitivity and specificity of AFU at the cut-off value of 870 nmol/mL/h were 81.7% and 70.7%, respectively [18]. AFU measurement is useful in association with AFP in early diagnosis of HCC and could serve as a valuable supplementary to AFP. It has been indicated that HCC will develop within few years in 82% of patients with liver cirrhosis, if their serum AFU activity exceeds 700 nmol/mL/h. The activity of AFU was reported to be elevated in 85% of patients at least 6 months before the detection of HCC by ultrasonography [19].

2.2.4. Human Carbonyl Reductase 2. This enzyme expressed in the human liver and kidney is important in detoxification of the reactive alpha-dicarbonyl compounds and reactive oxygen species deriving from oxidative stress in HCC. The human carbonyl reductase 2 levels have been shown to be inversely correlated to the pathological grading of HCC [20].

2.2.5. Golgi Phosphoprotein 2. Golgi phosphoprotein 2 (GOLPH2), a Golgi-apparatus-associated protein, has been shown to have a higher sensitivity than AFP in the detection of HCC [21]. A recent study found that GOLPH2 protein was highly expressed in tissues of HCC (71%) and bile duct carcinoma (85%) patients. GOLPH2 protein levels were detectable and quantifiable in sera by ELISA. In patients with hepatitis C, serial ELISA measurements in the course of the disease appear to be a promising complimentary serum marker in the surveillance of HCC [22].

2.3. Growth Factors and Their Receptors

2.3.1. Transforming Growth Factor-Beta (TGF-Beta). Belonging to a superfamily of polypeptide signaling molecules involved in regulating cell growth, differentiation, angiogenesis, invasion, and immune function, TGF-beta is a predominant form of growth factor family in humans. Its mRNA and protein are overexpressed in HCC compared with surrounding liver tissues, especially in small and well-differentiated HCCs [23]. However, no relationship has been shown between TGF-beta expression and posthepatectomy survival [24]. Serum TGF-beta level has been found to be elevated in HCC patients compared to healthy adults or patients with nonmalignant liver disease [25–27].

2.3.2. Tumor-Specific Growth Factor (TSGF). Malignant tumors release tumor-specific growth factor (TSGF) into peripheral blood during their growing period. Serum levels of TSGF may reflect the existence of tumor. TSGF can be used as a diagnostic marker in detecting HCC, and its sensitivity can reach 82% at the cut-off value of 62 U/mL and may have a higher accuracy with the simultaneous determination of other tumor markers. The simultaneous determination of TSGF (at the cut-off value of 65 U/mL), AFP (at the cut-off value of 25 ng/mL), and serum ferritin (at the cut-off value of 240 ng/mL) can reach a sensitivity and specificity of 98.4% and 99%, respectively [26].

2.3.3. Epidermal Growth Factor Receptor Family. The epidermal growth factor receptor (EGFR) family consists of four closely related transmembrane tyrosine kinase receptors: EGFR (erbB-1), c-erb-2 (Her-2/neu), c-erb-3 (HER-3), and c-erb-4 (HER-4). These bind with ligands of the EGF family, including EGF, TGF-alpha, and heparin-binding EGF. High levels of EGFR expression have been associated with early recurrence and reduced disease-free survival following resection of hepatocellular carcinoma [27].

2.3.4. Hepatocyte Growth Factor/Scatter Factor. Hepatocyte growth factor/scatter factor (HGF/SF) is a cytokine with a wide range of effects from embryonic development and liver regeneration. It is associated with molecular mechanisms of hepatocarcinogenesis via paracrine system involving its cellular receptor, c-met. High c-met expression has been shown in invasive-type HCC and has been associated with metastasis and reduced overall survival [28, 29].

2.3.5. Basic Fibroblast Growth Factor. This is a soluble heparin-binding polypeptide with a potent mitogenic effect on endothelial cells. Elevated levels above the median of >10.8 pg/mL have been shown to predict decreased disease-free survival [30]. Recent preliminary data with targeted therapy lenalidomide which inhibits fibroblast growth factor (FGF) showed promising and in some patients dramatic activity in HCC patients [31].

2.4. Molecular Markers

2.4.1. Circulating Nucleic Acids: mRNAs. The analysis of circulating nucleic acids in plasma offers another avenue for noninvasive monitoring of a variety of physiological and pathologic conditions [30, 31]. Numerous applications based on the detection of circulating cell-free nucleic acids in human plasma have been reported for the management of malignancies. The fundamental principle underlying these applications relates to the detection in plasma of extracellular nucleic acid molecules derived from diseased organs. Analysis of cell-free plasma RNA offers an opportunity for the development of pathology-related markers [32–34].

Alpha-Fetoprotein mRNA (AFP mRNA). Matsumura et al. first reported that single HCC cell could be detected in circulation by means of reverse-transcription polymerase chain reaction (RT-PCR), targeting AFP mRNA [35]. This led to further reports of the value of AFP mRNA as a predictor for HCC recurrence. Rather controversial results were attributed to the blood borne dispersion of both tumor cells and normal liver cells and the mistranscription of mRNA encoding AFP by peripheral mononuclear cells. The recurrence-free interval of HCC patients with postoperative serum AFP mRNA positivity has been reported to be significantly shorter than that of HCC patients with postoperative negativity (53% versus 88% at 1 year, 37% versus 60% at 2 years, $P = 0.014$) [34] and (52.6% versus 81.8% at 1 year, 15.6% versus 54.5% at 2 years, and 0% versus 29.2% at 3 years, $P < 0.001$) [36]. A meta-analysis showed that the expression of AFP mRNA one week after surgery was correlated with the recurrence of HCC [37].

Gamma-Glutamyl Transferase mRNA (GGT mRNA). Similar to AFP, GGT mRNA can be detected in the serum and liver tissues of healthy adults, patients with liver disease, benign liver tumor, HCC, and secondary tumors of the liver [38]. The two types of GGT mRNA, type A and type B, have been identified. Type B is the predominant one in cancerous tissue suggesting that changes in the expression of hepatic GGT mRNA may be related to the development of HCC [39]. Patients with HCC harboring type B GGT mRNA both in cancer and in noncancerous tissue had a worse outcome, earlier recurrence, and more recurrence-related mortality. The presence of type B GGT mRNA in cancerous tissue was statistically correlated with high serum level of AFP, daughter nodules, higher postresection recurrence rate than those without it (63.6% versus 14.3%), and lower postrecurrence survival. The presence of type B GGT mRNA in noncancerous liver tissue was significantly correlated with hepatitis C infection, high serum level of AFP, absence of infiltration of capsule, vascular permeation, daughter nodules, postresection recurrence, and postrecurrence survival [40].

Insulin-Like Growth Factor II (IGF-II) mRNA. Abnormal expression of IGF-II mRNA can be a useful tumor marker for diagnosis, differentiation, extrahepatic metastasis, and monitoring of postoperative recurrence in HCC. The determination of serum insulin-like growth factor-II (IGF-II) (at the cut-off value of 4.1 mg/g, prealbumin) has a sensitivity of 63%, specificity of 90%, and accuracy of 70% in the diagnosis of small HCC [41]. It can be a complementary tumor marker to AFP for diagnosis of small HCC. The simultaneous determination of IGF-II and AFP (at the cut-off value of 50 ng/mL) can improve the sensitivity to 80% and accuracy to 88% [42].

Albumin mRNA. Albumin is the most abundant protein in the body synthesized by the liver. mRNA of albumin is detectable in human plasma and could be a diagnostically sensitive marker for liver pathologies. Extracellular-based assays (circulating DNA/RNA) have been found to be better than cell-based assays (circulating tumor cells) in detection of preneoplastic lesions and micrometastases as plasma levels of circulating cancer-derived nucleic acid are higher than the levels of circulating cancer cells and are less prone to sampling errors. Cheung and colleagues studied the preoperative plasma samples obtained from 72 HCC patients who had undergone liver transplantation and found that patients with plasma albumin mRNA level (>14.6) had a significantly higher recurrence rate on multivariate analysis. High plasma albumin mRNA level predicted the 2-year recurrence rate with sensitivity and specificity of 73% and 70%, respectively [43].

MicroRNAs (miRNAs). MicroRNAs (miRNAs) are a family of endogenous, small (21–23 nucleotides), noncoding but functional RNAs, which have been found in worms, flies, and mammals including human beings [44]. It is estimated that there are about 1,000 miRNA genes in the human genome with approximately 500 miRNA genes being already identified [45]. Similar to mRNA, HCC-associated miRNAs could be used as diagnostic and prognostic biomarkers of HCC with a potential for even greater accuracy. MiRNAs can accurately predict whether liver cancer will spread and whether liver cancer patients will have shorter or longer survival. MicroRNAs regulate gene expression by binding to specific messenger RNAs and prevent their translation into protein. Because each type of miRNA is able to downregulate hundreds of genes at a time, they can control entire transcriptional programs that determine fundamental cellular properties and behavior. Accordingly, miRNA profiling has emerged as an extremely valuable method for phenotyping and subclassifying tumors [44]. Compared to conventional gene expression profiling (in which protein-coding, messenger RNAs are examined), miRNA analysis has several advantages. Due to the stability of miRNAs, formalin-fixed samples (rather than frozen tissue) can be used. Furthermore, the interrogation of hundreds of miRNAs (and often significantly fewer) yields as much information as might be gleaned from examining thousands of messenger RNAs.

Many independent groups have conducted comprehensive analyses of miRNAs in HCC, and a plethora of information on miRNA markers has been identified. Many of these miRNA signatures correlate with important biological parameters, such as metastasis [46–48], differentiation [49–51], HBV or HCV infection [52, 53], tumor recurrence [54], and patient survival [55, 56]. Some miRNAs are involved in HCC carcinogenesis by promoting cancer stem cell and by controlling cell proliferation and apoptosis; others are associated with HCC progression by controlling cell migration and invasion. These HCC-associated miRNAs not only provide new insights into the molecular basis of HCC but also serve as new tools for HCC diagnosis and prognosis. Currently a few miRNA signatures, however, could potentially be used in this area. Some miRNAs have been validated in an independent cohort, paving the way for clinically useful platforms to assess HCC risk and outcome. This promising area of research awaits further validation in prospective studies [57].

Table 2: Various HCC biomarkers and their clinical use.

HCC marker	Clinical use
Alpha-fetoprotein	Early diagnosis, monitoring, and recurrence
Lens culinaris agglutinin reactive AFP (AFP-L3%)	Early diagnosis and prognosis, vascular invasion
Des-gamma-carboxy prothrombin (DCP)	Early diagnosis and prognosis, portal vein invasion and metastasis
Gamma-glutamyl transferase	Early diagnosis complementary to other markers
Alpha-l-fucosidase	Early diagnosis
Glypican-3	Early diagnosis
Human carbonyl reductase 2	Prognosis
Golgi phosphoprotein 2	Tumor aggressiveness
Transforming growth factor beta	Tumor invasiveness
Hepatocyte growth factor (HGF)	Prognosis and disease recurrence
Transforming growth factor-b (TGF-b)	Prognosis invasiveness
Tumor specific growth factor	Diagnosis complementary to other markers
Epidermal growth factor receptor family	Early recurrence
Hepatocyte growth factor	Metastasis reduced survival
Micro RNAs	Tumor spread and survival

2.5. Pathological Biomarkers. Finally there have been reports of pathological biomarkers of HCC for diagnosis and prognosis. Some of these diagnostic biomarkers focus on immunochemical staining patterns to distinguish high-grade dysplastic nodules and well-differentiated HCC. The best type of immunostaining for this difficult condition has been reported to be the combination of heat-shock protein 70 (HSP70), glypican-3 (GPC3), and glutamine synthetase (GS). For prognostic use a number of histological and immuno-histochemical markers such as markers of cell proliferation (Ki67), apoptosis or cell survival (survivin), cell adhesion molecules (E-cadherin), neoangiogeneis (VEGF), and more have been looked in small studies showing promise; however, most of these markers have not been validated in large studies [57]. Various HCC biomarkers and their clinical use have been summarized in Table 2.

3. Discussion

Hepatocarcinogenesis is a complex multistate process usually occurring after many years of chronic exposure to several mitogenic and mutagenic environments precipitating random genetic alterations. Recent evidence suggest that intrinsic biologic characteristics of the tumor in terms of proliferation and invasiveness are probably related to different composition and activity of the microenvironment, leading to very different clinical outcomes. HCC is rather unique with its ability to synthesize various tumor-related proteins rendering itself more suitable to biomarker-related research than other tumors. Because of the large multitude of biomarkers reported in this disease, selecting the biomarkers which would be most useful in clinical practice has been more than challenging. In this rather brief overview, we tried to focus on most widely used and accepted biomarkers.

Despite its limitations, serum AFP still remains the most widely used tumor marker in clinical practice. Recent research favors the circulating hepatoma-specific AFP subfraction AFP-L3 and DCP over AFP alone in differentiating HCC from nonmalignant hepatopathy and detecting small HCC. Furthermore, some other tumor markers, such as GPC3, GGT II, AFU, have been shown to be supplementary to AFP and DCP in the detection of HCC. Some of them even can be detected in HCC patients seronegative for both AFP and DCP, thus indicating that the simultaneous determination of these markers may improve the accuracy.

However, most exciting and promising area of research in this disease has been the identification of a new group of molecules called miRNAs. MiRNAs have been discovered to be aberrantly expressed in HCC, and some of them are functionally involved in HCC carcinogenesis and progression. Furthermore, certain microRNAs are associated with HCC or related to HCC subtypes, implicating the potential use of microRNAs in HCC patient stratification of diagnosis and prognosis. Some of these HCC-associated miRNAs have been validated in independent cohorts. This brings the possibility of developing clinically useful platforms to develop HCC diagnosis, risk assessment, and patient risk stratification with the ultimate goal of personalized therapy.

4. Conclusion

Research into the molecular biology of hepatocarcinogenesis has identified numerous biomarkers which could provide additional information for HCC biologic behavior metastasis and recurrence to that gained from traditional histopathological features. A large number of biomarkers have been shown to have potential predictive significance. However, most of them have been studied retrospectively. Efforts should be directed towards prospective clinical trials in evaluating the prognostic significance of these markers. These molecules not only help in prediction of prognosis for patients with HCC but may also assist in deciding appropriate

modality of therapy and represent novel targets for therapeutic interventions.

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Review Article

Mechanisms and Biomarkers of Apoptosis in Liver Disease and Fibrosis

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Liver fibrosis and cirrhosis are a major cause of morbidity and mortality worldwide. Development of the fibrotic scar is an outcome of chronic liver diseases of varying aetiologies including alcoholic liver disease (ALD) nonalcoholic liver disease (NAFLD) including non-alcoholic steatohepatitis (NASH) viral hepatitis B and C (HBV, HCV). The critical step in the development of scar is activation of hepatic stellate cells (HSCs), which become the primary source of extracellular matrix. Aberrant apoptosis is a feature of chronic liver diseases and is associated with worsening stages of fibrosis. However, apoptosis is also the main mechanism promoting the resolution of fibrosis, and spontaneous or targeted apoptosis of HSC is associated with regression of fibrosis in animal models and patients with chronic liver disease. Given the importance of apoptosis in disease progression and resolution, there is much interest in precisely delineating the mechanisms involved and also developing biomarkers that accurately reflect the underlying pathogenesis. Here, we review the mechanisms driving apoptosis in development of liver disease and use of apoptosis -related biomarkers to aid in clinical diagnosis. Finally, we will also examine the recent literature regarding new insights into mechanisms involved in apoptosis of activated HSCs as possible method of fibrosis regression.

1. Introduction

Liver injury leading to fibrosis occurs in response to a variety of insults including alcohol, viral hepatitis, steatosis and insulin resistance, autoimmune disease, excessive deposition of iron or copper, and congenital abnormalities. Fibrosis is the consequence of an overactive wound healing process in response to the injury [1]. A key step in this process is activation and proliferation of HSC from periportal and perisinusoidal areas [2]. Under normal conditions, the HSC resides in the space of Disse in a quiescent phenotype storing retinoids including vitamin A [3]. Upon liver injury HSCs transform to an active phenotype, positive for alpha smooth muscle actin (α-SMA) and producing excessive fibrillar collagens, proinflammatory cytokines including IL-6, IL-8, MCP1, and inhibitors of matrix proteases. Initially, this process is driven by an inflammatory response and results in a controlled deposition of extracellular matrix; however, if the underlying insult persists, there is an excessive deposition of extracellular matrix including cross-linking of collagen and impairment of hepatocyte regeneration [4].

In chronic liver diseases, liver cell death is a prominent feature and correlates with worsening fibrosis [5, 6]. The cell death can occur by one of two mechanisms: necrosis or apoptosis. Apoptosis is a highly synchronised procedure requiring cellular ATP; conversely death by necrosis is ATP independent. In recent years, it has been suggested that both apoptosis and necrosis can occur in response to a single initiating factor; however, the ultimate fate of the cell is thought to depend largely on the severity of the initial damage signal. It is likely that other forms of cell death such as autophagy (self digestion) [7], paraptosis [8], necroptosis [9], and oncosis [10] also play an important role in fibrogenesis; however, in this paper, we will focus specifically on apoptosis.

2. Apoptosis or Programmed Cell Death (PCD)

Apoptosis is a normal physiological process and is characterised by a well-synchronised sequence of morphological events. The dying cell undergoes nuclear and cytoplasmic

condensation, blebbing of the plasma membrane, and eventually breaking apart into membrane-enclosed particles termed apoptotic bodies containing intact organelles, as well as portions of the nucleus [11, 12]. These apoptotic bodies are recognised, engulfed, and degraded by professional phagocytes, innate immune cells, and HSC [13]. Within the liver, the major cell type to eliminate apoptotic bodies is the resident liver macrophage, the Kupffer cell. In comparison, necrosis, is a pathological or accidental mode of cell death, characterised by irreversible swelling of the cytoplasm and distortion of organelles, including mitochondria [14]. Eventually there is loss of membrane integrity resulting in cell rupture and release of cellular contents. Necrosis occurs when cells are subjected to toxic stimuli such as hyperthermia, metabolic poisons, and direct cell trauma. Several important biochemical markers of apoptosis have been identified, including nuclear DNA fragmentation, activation of aspartate-specific proteases known as caspases and cell surface externalization of phosphatidylserine (PS) residues, expression of several death ligand TNFs, FasL, or overexpression of death receptors including TRADD, Fas, and DR5 [15]. Although cell death may transpire in the absence of caspases [16], the characteristic morphological features that define apoptosis are dependency of caspase activation and cleavage of specific cellular proteins or "death" substrates within the cell. Apoptosis may therefore be viewed, in biochemical terms, as a caspase-mediated form of cell death. At present, two major pathways that link apoptosis have been identified: (a) intrinsic or mitochondrial and (b) extrinsic or death receptor related.

2.1. Mitochondrial or Intrinsic Pathway of Apoptosis. The intrinsic pathway involves the regulation of apoptosis by mitochondria and is characterized by the release of mitochondrial intermembrane space proteins including cytochrome c, apoptosis-inducing factor (AIF), second mitochondrial activator of caspases (Smac), direct IAP binding protein with low pI (DIABLO), endonuclease G, and Omi/HtrA2 into the cytosol [17]. Cytosolic cytochrome c subsequently activates a multiprotein complex referred to as the apoptosome, which in turn leads to cleavage of procaspase-9 and downstream effector caspases (e.g., caspase-3), resulting in cell death. As such, the mitochondria have emerged as a novel target for anticancer chemotherapy. This target is based on the observation that several conventional and experimental chemotherapeutic agents promote the permeabilization of mitochondrial membranes in cancerous cells to initiate the release of apoptogenic mitochondrial proteins. This ability to engage mitochondrial-mediated apoptosis directly using chemotherapy may be responsible for overcoming aberrant apoptosis regulatory mechanisms commonly encountered in cancerous cells. Interestingly, several putative cancer chemopreventive agents also possess the ability to trigger apoptosis in transformed, premalignant, or malignant cells in vitro via mitochondrial membrane permeabilization [18]. This process may occur through the regulation of Bcl-2 family members or by the induction of the mitochondrial permeability transition. Thus, by exploiting endogenous mitochondrial-mediated apoptosis inducing mechanisms, certain chemopreventive agents may be able to block the progression of premalignant cells to malignant cells or the dissemination of malignant cells to distant organ sites as means of modulating carcinogenesis *in vivo*.

2.2. Death Receptor or Extrinsic Pathway of Apoptosis. The extrinsic pathway is triggered by ligation of death receptors, such as CD95 or the agonistic TRAIL receptors TRAIL-R1 and TRAIL-R2, by their cognate ligands or agonistic antibodies, which results in receptor trimerization, clustering of the receptors death domains, and recruitment of adaptor molecules (e.g., Fas-associated death domain FADD) through homophilic interaction mediated by the death domain [19]. FADD in turn recruits caspase-8 to the activated CD95 receptor to form the CD95 death-inducing signaling complex (DISC). Oligomerization of caspase-8 upon DISC formation drives its activation through self-cleavage. Caspase-8 then activates downstream effector caspases such as caspase-3 [20]. Links between the receptor and the mitochondrial pathway exist at different levels. Upon death receptor triggering, activation of caspase-8 results in cleavage of proapoptotic Bid protein, a Bcl-2 family protein with a BH3 domain only, whose truncated form is inserted into the mitochondrial outer membrane and promotes cytochrome c release and consequent activation of the apoptosome, thereby initiating a mitochondrial amplification loop [17]. In addition, cleavage of caspase-6 downstream of mitochondria can feed back to the receptor pathway by cleaving caspase-8 [21]. The idea to specifically target death receptors to trigger apoptosis in tumour cells is attractive for cancer therapy, as death receptors have a direct link to the cell death machinery. Also, apoptosis upon death receptor triggering is considered to occur independently of the p53 tumour suppressor gene, which is impaired in the majority of human tumours [22].

3. Apoptosis in Liver Disease

Apoptosis can occur in response to viral infection, and exposure to any kind of hepatocarcinogen, excessive alcohol consumption or due to genetic mutations. The liver resident cells express high levels of cell-death-associated receptors, for instance hepatocytes, cholangiocytes, activated stellate cells, and Kupffer cells all express Fas. High expression of the Fas receptor not only helps to maintain liver homeostasis but also helps to eliminate virally infected cells of liver by the immunocytes [23]. Fas/FasL signalling has been largely implicated in liver pathophysiology but the mitochondrial intrinsic pathways are also involved in liver homeostasis. Bid, one of the BH3 subfamily proteins, is cleaved by caspase-8, and the truncated Bid then translocates to the mitochondria where it activates the intrinsic apoptosis pathway. It has been shown that bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis [24]. These studies implicate both extrinsic and intrinsic apoptotic pathways in maintaining normal liver physiology.

3.1. Viral Hepatitis. There are seven different types of viral hepatitis, among them hepatitis B (HBV) and hepatitis C (HCV) are the major cause of hepatic cell destruction leading to chronic hepatitis, fibrosis and increased risk of formation of hepatocellular carcinoma [25]. It is now well established that cytotoxic T cells induce apoptosis of virally infected hepatocytes via Fas/FasL and perforin-mediated pathways [26]. FasL-expressing infiltrated mononuclear cells are found in the hepatitis-C-infected patients [27]. Conversely, failure of immune cells to eliminate virally infected hepatocytes leads to viral persistence and immune surveillance causing chronic hepatitis and initiating fibrogenesis. In vivo silencing by small interfering RNA (siRNA) duplexes targeting the gene Fas protects mice from liver failure and fibrosis in two models of autoimmune hepatitis [16]. In addition to viral-induced damage, host factors such as the presence of steatosis are associated with increased hepatocyte apoptosis in patients with HCV infection. Additionally, hepatocyte apoptosis in patients with steatosis correlates with grade of fibrosis, implicating a direct link between apoptosis and fibrosis [28].

3.2. Alcoholic Steatohepatitis. Excessive alcohol consumption may lead to changes in fat metabolism, cause chronic inflammation, and over time promote the development of a chronic hepatitis and fibrosis called alcoholic steatohepatitis (ASH) [29]. Hepatocyte ballooning and apoptotic hepatocytes are the common features in the liver of patients with ASH [30]. Excessive hepatocyte apoptosis stimulates inflammation and results in the production of proinflammatory cytokines and reactive oxygen species by innate immune cells [31]. Additionally, oxidative stress-induced hepatocyte apoptosis is one of the consequences of acute alcohol injury [32]. Defects in endocytosis leading to accumulation of apoptotic bodies followed by inflammation may also play an important role in alcoholic liver injury. It has been found that in ethanol induced impairment receptor mediated endocytosis model defects in asialoglycoprotein receptor, which lead to defective uptake of apoptotic bodies causing severe liver injury [33]. CYP2E1 is one of the key markers upregulated in alcoholic liver disease [34]. CYP2E1 is an isoform of cytochrome p450 related to free radical generation. Increased free radical production causes DNA damage and lipid peroxidation increasing the severity of the disease by inducing oxidative stress induced hepatocytic damage [35].

3.3. NAFLD and NASH. Apoptotic death of hepatocytes is a common feature of nonalcoholic steatohepatitis and is associated with fibrosis [5]. Increased expression of death receptors like Fas and TNF-R has been found in most of the NASH patients. Both extrinsic and intrinsic apoptotic pathways are involved in NASH-induced hepatocyte death [36, 37]. Caspase-3 and caspase-7 are activated with disease progression and subsequently cleave a major filamentous protein known as cytokeratin-18 (CK-18) [38]. Endoplasmic reticulum stress-induced apoptosis is a feature of multiple diseases including cystic fibrosis, diabetes, and Parkinson's and prion-related diseases [39] and may be an important mechanism in NAFLD and NASH. The presence of hepatic steatosis is associated with an increase in ER stress and a subsequent increase in hepatocyte apoptosis and liver injury [40].

3.4. Liver Fibrosis. Liver fibrosis is the consequence of chronic liver injury. After toxic exposure hepatocytes undergo apoptosis and hepatic stellate cells migrate to the site of injury to engulf the apoptotic bodies. This engulfment promotes activation of the hepatic stellate cells to hepatic myofibroblasts, and in their activated state these cells promote deposition of extracellular matrix and scar formation in the liver. Recently it has been demonstrated that hepatocyte-specific disruption of Bcl-xL induces continuous hepatocyte apoptosis and fibrogenesis [41]. Several proinflammatory cytokines including IL-6, TNF-α induce unresolved hepatocytic inflammation. The profibrogenic cytokine TGF-β is secreted by the immune cells gathered at site of injury to phagocytose the apoptotic bodies, further fuelling the inflammatory and fibrogenic reaction [42].

3.5. Hepatocellular Carcinoma. Hepatocellular carcinoma (HCC) is the consequence of exposure to carcinogen or environmental pollutants, chronic viral infection, and obesity and is the 3rd major cause of cancer death worldwide. HCC is a slow progressing disease. During the initiation phase of this disease the balance between apoptosis and cell proliferation of hepatic cells is disrupted and favours proliferation, whereas hepatocytes undergo high levels of hepatocytic cell death. In response to this injury, innate immune cells migrate to the site of damage and release a plethora of proinflammatory cytokines and free radicals generating an inflammatory microenvironment, which promotes cancer progression. After chronic exposure to rounds of liver injury and inflammation hepatocytes develop mechanisms to evade apoptotic death; this results in the accumulation of damaged hepatocytes that eventually become HCC. These mechanisms include the persistent downregulation of proapoptotic molecules and upregulation of antiapoptotic proteins. Fas receptor and Fas ligand are highly expressed on hepatocytes; however, levels of these proteins are diminished during the disease progression [43]. Concurrently, decreased expression of other downstream molecules from the Fas family including FADD (Fas Associated death domain) and FLICE have been observed during HCC development [44]. Loss of other death receptors including TRAIL-R has also been linked to neoplastic growth and reduced apoptosis in HCC [45]. Anti-apoptotic factor, brain and reproductive organ-expressed protein (BRE), is a death-receptor-associated protein and is upregulated in HCC. BRE binds to tumor necrosis factor receptor-1 and Fas, and in cell lines it has been shown to attenuate apoptosis by inhibiting t-Bid-induced activation of the mitochondrial pathway [46]. Normal liver homeostasis is maintained by a balance of proapoptotic and antiapoptotic genes but in the majority of cases of HCC it has been shown that there is an overexpression of antiapoptotic genes. This imbalance can be caused by different mechanisms. For example, Otsuka et al. reported that the hepatitis C

virus inhibits apoptosis by overexpressing Bcl-xL [47]. One of the important antiapoptotic proteins XIAP (inhibitor of caspases) helps to bypass apoptotic pathway in HCC progression [48]. Growth arrest DNA damage-inducible gene 45β (GADD45beta) regulates apoptotic cell death in response to DNA damage. Downregulation of GADD45beta has been observed in HCC [49]. Another important regulator of liver cancer progression is the tumour suppressor gene p53. This gene is activated when there is DNA damage, but, in most cases of HCC, the p53 gene is mutated. Kraus and colleagues postulated that this was a result of oxidative stress and that this provided a link between chronic inflammation and genomic changes observed in precancerous cells [50].

4. Biomarkers of Apoptosis in Liver Disease

One of the most promising biomarkers of apoptosis is CK-18. Serum levels of CK-18 are markedly increased in patients with NASH compared with patients with steatosis or normal biopsies [51, 52]. Additionally, serum levels of uncleaved CK-18 are able to distinguish between simple steatosis and NASH. More recently, in a prospective study examining the utility of apoptosis biomarkers to predict fibrosis in patients with NASH, both full length and caspase-cleaved CK-18 were able to discriminate different stages of fibrosis with healthy controls [53]. Patients with HCV infections also have higher levels of serum CK-18 correlating with serum transferase activity; however, some patients had elevated serum CK-18 with normal transaminases, suggesting that CK-18 may be an earlier maker of liver damage. The utility of serum CK-18 levels in discriminating different stages of HCV and ALD, for example, needs to be studied further but it may provide a minimally invasive method of assessing the underlying liver injury.

5. Increased Apoptosis of Hepatocytes May Directly Contribute to Fibrogenesis

There is mounting evidence that suggests phagocytosis of apoptotic bodies by hepatic stellate cells may directly stimulate fibrogenesis [54]. Although liver macrophages are thought to be the main cell involved in phagocytosis, endothelial cells and fibroblasts have been demonstrated to clear apoptotic bodies [42, 54]. Both HSCs and Kupffer cells (KC) express the phosphatidyl serine receptor suggesting that both cell types are able to internalize apoptotic bodies. Engulfment of apoptotic bodies by KC is associated with a marked increase in profibrogenic factors including TRAIL, TNF-α, FasL, and TGFβ1 mRNA expression 24 hours following exposure to apoptotic bodies derived from hepatocytes. Kupffer cells isolated from bile duct ligated (BDL) mice also show an increase in the expression of TRAIL-associated ligands compared with sham-operated animals. Additionally, depletion of KC from BDL mice was associated with a significant reduction in hepatocyte apoptosis and liver injury and a concurrent reduction in α-SMA and col1A1 mRNA expression [42]. Increase in the expression of TRAIL markers on KC may be suitable biomarkers for pathogenic hepatocyte apoptosis. Subsequent perpetuation of hepatocyte apoptosis is associated with an increase in inflammation and liver fibrosis. This suggests that engulfment of apoptotic bodies may prolong the cycle of liver injury and that targeting of KCs maybe a viable therapeutic option in cholestasis to reduce liver fibrosis.

Phagocytosis of hepatocyte-derived apoptotic bodies by stellate cells has also been implicated in fibrogenesis. Apoptotic bodies express phosphatidyl serine, which acts as an engulfment signal. Canbay and colleagues found that Lx-1 cells (human hepatic stellate cell line) express the phosphatidyl serine (PS) receptor and that these cells show a significant increase in TGF-β1 and Col1a1 mRNA expression after 48-hour incubation with apoptotic bodies. Blocking of engulfment of apoptotic bodies by Nocodazole abrogates the further increase in α-SMA and TGF-β1 suggesting that the increase in fibrogenic nature of the HSC was specifically as a consequence of apoptotic body engulfment [54].

6. Apoptosis and Reversal of Liver Fibrosis

6.1. Clinical Evidence for Reversal of Fibrosis Apoptosis. Until recently, transplantation was considered the only viable treatment option for cirrhosis and severe forms of liver disease [55]. However, there is a growing body of clinical evidence that suggests fibrosis is somewhat reversible. Successful treatment of underlying viral infection in patients with HCV and HBV is associated with regression of liver fibrosis and, in some instances, reversal of cirrhosis upon liver biopsy [56–58]. Similarly in cholestatic-type liver diseases, regression of liver fibrosis was reported after biliary drainage in patients with stenosis of the common bile duct. Additionally, abstinence from alcohol [59] and weight loss have also been associated with an improvement in histological analysis of fibrosis.

6.2. Experimental Evidence for Spontaneous Apoptosis-Mediated Regression of Apoptosis. Summarised in Figure 1 there is a considerable experimental evidence suggesting that apoptosis of activated HSCs is the main mechanism associated with regression of fibrosis. There are two seminal papers that show spontaneous regression of fibrosis mediated by apoptosis. Firstly, Iredale and colleagues discovered that in rats treated with carbon tetrachloride to induce significant liver fibrosis, α-SMA-positive cells, fibrosis and hydroxy proline content returned to an almost histologically normal state 28 days following cessation of liver injury. Importantly, dual staining for TUNEL and α-SMA showed spontaneous resolution of fibrosis was associated with an increase in apoptosis of nonparenchymal α-SMA-positive cells [60]. This hypothesis has been subsequently confirmed in an additional *in vivo* model of fibrosis. Issa and colleagues ligated the bile duct of rats for 21 days to induce fibrosis, then biliodigestive anastomosis was performed and rats were allowed to recover for 45 days. There was a 5-fold decrease in α-SMA-positive myofibroblasts with rapid apoptosis indicated by TUNEL-positive cells two days after anastomosis [61].

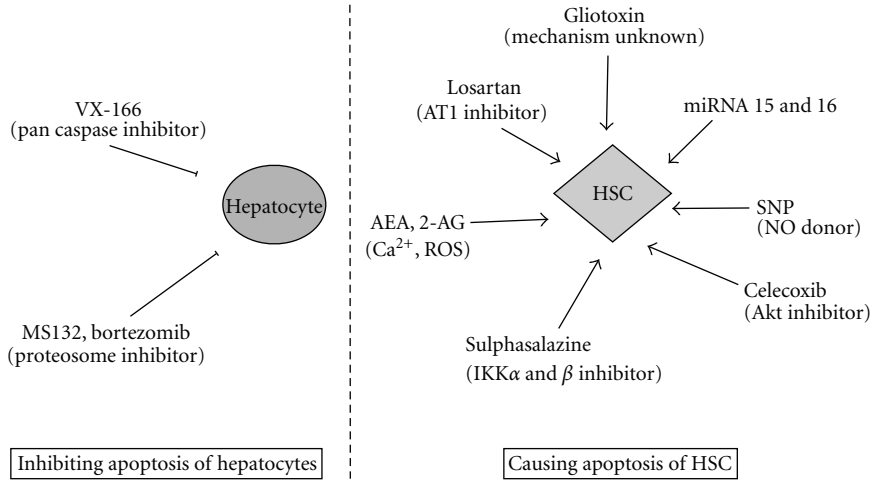

Figure 1: Mechanisms for targeting apoptosis to treat liver disease and cause fibrosis regression.

This method of deleting collagen-producing HSCs has been exploited in animal models to reduce fibrosis. The potent fungal metabolic toxin, Gliotoxin, induces apoptosis of activated rat HSCs *in vitro* and *in vivo* and this is associated with a significant reduction in the number of activated HSCs and a reduction in the thickness of the bridging fibrosis and serum ALT without impairment of hepatocyte regeneration [62]. While HSCs are sensitive to apoptosis at low concentrations of Gliotoxin (1.5 uM), hepatocytes are resistant to apoptosis however they will undergo necrosis at high concentrations. Originally thought to act through an NF-kB-dependent mechanism, it is likely that Gliotoxin induces apoptosis via a rapid accumulation of glutathione, which is abrogated by pretreatment with thiol redox active agents such as PDTC [63].

There is mounting evidence suggesting that directly targeting NF-κB is a viable therapeutic option in liver disease. Activated HSCs have high levels of NF-κB activity and an increase in basal expression of NF-κB-regulated antiapoptotic proteins including IL-6, Bcl-2 family members and GADD45β and A20. Blunt inhibition of NF-κB-regulated antiapoptotic genes by treatment with a proteosome inhibitor prevented induction of antiapoptotic genes and caused apoptosis. Treatment of BDL mice with bortezomib was associated with a reduction in α-SMA-positive HSCs and fibrosis [64]. IKK2 inhibition with pharmacological agent AS602868 is also associated with attenuated fibrosis progression in mice with dietary-induced NASH and thought to work by limiting the accumulation of hepatic steatosis and hepatocyte apoptosis [65]. Sulphasalazine is a drug used commonly in humans for treatment of inflammation-type disorders including rheumatoid arthritis and inflammatory bowel disease [66]. Sulphasalazine acts to block NF-κB activity by blocking the activity of inhibitor of κB (IkB) kinases α and β (IKKα and IKKβ) resulting in a downregulation of NF-κB targets. *In vivo* administration of sulphasalazine to rats with CCl_4-induced liver injury was associated with stimulation of HSC apoptosis (and NF-κB-regulated antiapoptotic gene GADD45β) and a concurrent reduction in α-SMA-positive myofibroblasts and Col1a1 [67].

Novo and colleagues noted in cirrhotic human liver that Bcl-2 was highly expressed in α-SMA-positive myofibroblasts, and this staining was particularly strong in areas at the interface between fibrotic septa and regenerative nodule [68]. Upstream from Bcl-2, constitutive expression of P-Ser^{536}-RelA is a key feature of activated human HSCs and is in part regulated in an autocrine fashion by angiotensin II. Inhibition of the angiotensin II receptor (AT1) and/or treatment with ACE inhibitor Captopril was associated with loss of P-Ser^{536}-positive HSCs, a reduction in α-SMA-positive HSCs, and fibrosis regression. Importantly, in patients with hepatitis C viral infection who upon liver biopsy were found to have constitutive expression of P-Ser^{536}, treatment with Losartan was associated with regression of fibrosis. Those patients who did not have P-Ser^{536} expressed on biopsy were not sensitive to Losartan therapy and did not undergo regression of fibrosis. Constitutive expression of P-Ser^{536} may be an important tissue biomarker to assess whether a patient is likely to respond to similar therapy.

Directly targeting hepatocyte apoptosis to reduce liver fibrosis may also be a viable strategy. Male db/db mice fed a methionine/choline-deficient diet (MCD) to induce NASH and liver fibrosis and subsequently treated with a pan-caspase inhibitor, VX-166, showed a reduction in α-SMA-positive HSCs and had reduced expression of Col1a1 mRNA and reduced fibrosis, confirmed with Sirius red staining. However, serum ALT levels were similar in mice fed MCD alone, suggesting that there was no improvement in liver injury despite some reduction in steatosis, reduction in TNF-α production, and formation of nitrotyrosine adducts [69].

Treatment of an injured liver (especially in the presence of ongoing injury) with an apoptosis inhibitor may be detrimental and prevent the normal removal of premalignant hepatocytes and profibrogenic myofibroblasts.

7. Paracrine-Mediated Apoptosis of HSC

Under normal conditions, nitric oxide exerts a paracrine effect on HSC; however, in incidences of severe liver fibrosis and cirrhosis eNOS generation is impaired. This is associated with the typical features of liver disease. *In vitro*, NO donor SNP is associated with an increase in primary rat HSC apoptosis, and overexpression of NO in Lx2 cells sensitized them to apoptosis caused by stimulation with TRAIL [70]. However, the mechanism by which apoptosis occurs is unclear. Langer and colleagues found a significant decrease in mitochondrial membrane potential ($\Delta\Psi_m$) after treatment with SNP, which was not abrogated by pretreatment with pan-caspase inhibitors. It is possible that NO-mediated nitrosylation of the active site of caspase-3 impairs cleavage resulting in caspase independent apoptosis [16]. Enhancing the intracellular oxidative stress further potentiated apoptosis induced by SNP. Importantly, SNP is already approved for use in human, and we await further in vivo data to determine if HSC apoptosis results in regression of fibrogenesis.

8. miRNAs

miRNAs are small noncoding RNAs of 21–25 nucleotide bases thought to regulate gene transcription posttranslationally by changing the stability of mRNA through binding to the 3′ UTR [71]. (mechanism reviewed extensively in He, Nature Reviews Genetics 2004) [72]. Activation of HSCs is associated with a change in the expression profile of mRNAs including miR-16, -15b, -122, -128, -143, and -140 [73]. From this expression profiling, Guo and colleagues then identified a critical role for miR-16 and miR-15b in apoptosis. Transfection of miR-16 and miR-15b into activated HSCs concurrently reduced Bcl2 expression and increased expression of caspase-3, 8, and 9, and this was accompanied by a subsequent increase in apoptosis in activated HSCs [73]. Additionally, transfection of activated HSCs with miR-16 was associated with a reduction in cyclin D1. This was paralleled by a subsequent reduction in proliferation and increased apoptosis [73]. miR-29b is inducible in Lx2 cells by IFN-α in a dose-dependent fashion and was found to suppress Col1a1. This may be part of the mechanism whereby patients with HCV show a regression of fibrosis after treatment with IFN-α. Similarly, inhibition of 27a and 27b has been shown to aid the return of HSC to a lipid-containing phenotype by reducing the expression of retinoid X receptor α and was also associated with a subsequent decrease in proliferation. However, Col1a1 and α-SMA expression levels did not change, nor was there any significant induction of apoptosis [74]. Venugopal and colleagues found a downregulation of miR-150 and miR-194 in hepatic stellate cells isolated from the fibrotic livers from BDL rats compared with sham operated animals. *In vitro* overexpression of miR-150 and miR-194 caused decreased stellate cell activation, inhibition of cell proliferation, and reduction in α-SMA expression and Col1a1 levels, possibly by inhibition of c-myb (miR-150) and rac (miR-194); however, there was no significant increase in apoptosis [75]. Similarly, a decrease in miR-29 expression level is associated with Col1a1 accumulation in activated HSCs. Targeting miRNAs may provide a mechanism to cause apoptosis of activated HSCs. As yet, no serum biomarkers are available to measure miRNA activity; however, tissue biomarkers may be of use to determine if HSCs are reprogrammed to avoid apoptosis.

9. TLR 9 Activation

The injured liver is exposed to high levels of danger-associated molecular patterns (DAMPs) that act as stimuli for members of the Toll-like receptor (TLR) family. Wantanabe et al. hypothesised that debris from apoptotic hepatocytes could modulate the activation of HSC via TLR9 signalling. They showed that expression of the profibrogenic genes Col1a1 and TGFβ1 in LX2 cells and primary mouse HSCs was increased upon exposure to hepatocyte DNA or cytidine-phosphate-guanosine (CPG) oligonucleotides, the ligand for TLR9. Administration of a TLR9 antagonist prevented induction of these genes and also inhibited platelet-derived-growth-factor (PDGF) dependent HSC chemotaxis, an effect also observed in HSCs deficient for either TLR9 or its downstream adaptor molecule MyD88. The authors concluded that DNA released from damaged hepatocytes acts as a signal to "halt" HSC migration, retaining them at the site of injury and promote scar formation [76]. TLR9-mediated activation of HSCs may confer resistance to apoptosis and further promoting fibrogenesis. The role of TLR9 and DAMPS in liver disease needs further investigation.

10. Extracellular Matrix

The link between extracellular matrix and apoptosis of HSCs has previously been reviewed extensively; see Elsharkaway Apoptosis 2005 [77] and *Benyon Seminars* in *Liver Disease* 2001 [78]. In brief, increased activity in matrix degradation enzymes is a key step in resolution of fibrosis [56]. *In vitro* treatment of HSCs with recombinant MMP9 stimulates apoptosis [79], which can be abrogated by pretreatment with an inhibitor [80]. Additionally, under conditions of recovery, interstitial MMPs have an increased collagenolytic activity, degrading the extracellular matrix and leaving the HSC more susceptible to undergo apoptosis [81]. Overexpression of MMP inhibitor TIMP-1 in mice treated with CCl_4, was associated with an inability to undergo apoptosis of activated HSC and no concurrent resolution of fibrosis [82]. The mechanism of action by which TIMP-1 acts to inhibit apoptosis is via activating phosphatidylinositol 3-kinase and ERKs resulting in downregulation of caspases [83]. These studies suggest that in addition to secreting Col1A1, activated HSCs also secrete matrix-related enzymes that act to protect the HSC against proapoptotic signals.

Serum levels of MMPs and TIMPs on their own and in combination with liver stiffness tests, enhanced liver fibrosis (ELF), and so forth are useful biomarkers to predict the existence and extent of liver disease and are a useful tool for monitoring resolution. TIMP-1 along with hyaluronic acid (HA) and the N-terminal pro-peptide of collagen type III are the key serum markers of underlying fibrosis on the ELF panel. This panel has been validated in a large cohort of patients with NAFLD and NASH [84, 85] and has been reported to have better diagnostic capabilities than other standard panel of biomarkers including MELD and the Mayo Risk (R) score in instances of primary biliary cirrhosis [86]. To date, most research has been centered around the use of biomarkers in instances of chronic liver disease; however, a recent publication by Dechene and colleagues suggests that they may have some utility in acute liver failure (ALF). Both TIMP-1 (4.2-fold) and TIMP-2 (1.6-fold) were found to be significantly increased in the sera of ALF patients compared with control individuals. Additionally, in the group of patients with ALF, MMP-1 and MMP-2 were significantly upregulated more than two fold, suggesting that these biomarkers of apoptosis and protease activity may be a useful indicator of underlying fibrogenesis. The increase in serum markers was paralleled by an increase in Col1a1 and α-SMA observed on liver biopsy. This research also identified a correlation between serum biomarkers of apoptosis (TIMP-1 and M65) and liver stiffness measured by FibroScan. Additionally, over a one-week observation period, there was a reduction in liver stiffness that corresponded to a reduction in serum markers of apoptosis and fibrogenesis [87]. These studies highlight the important relationship between extracellular matrix and apoptosis identifying fibrogenesis and the potential for using apoptosis biomarkers as part of a panel of markers to longitudinally monitor activity during recovery period.

11. Endogenous Cannabinoid Receptors

Lipidic cannabinoid ligands and receptors CB1 and CB2 have an important role in the pathogenesis of chronic liver injury. Under normal conditions, the endocannabinoid receptors are undetectable; however, expression is slowly increased upon stellate cell activation and remains elevated in later stages of liver disease [88–90]. CB2 receptors are located in HSCs; however, CB1 receptors are also upregulated in vascular endothelium [90]. Protein expression of the CB1 receptor is increased in cirrhotic livers compared with normal human liver and appears to be expressed in nonparenchymal cells located proximal to the fibrotic septa. Immunohistochemical analysis of CB1 receptor showed co-localization with α-SMA-positive cells and activated cultured myofibroblasts express higher levels of the receptor compared with quiescent. In mice injured with CCl_4 or who underwent BDL, selective inhibition of CB1 with antagonist SR141716A decreases TGFβ and α-SMA expression during injury, and this was accompanied by a 37% and 41% decrease in fibrosis respectively. Additionally, in cannabinoid receptor 1 knockout mice ($Cnr1^{-/-}$), there was a 30% and 35% reduction in fibrosis area in thioacetamide and BDL models of fibrosis. In culture, $Cnr1^{-/-}$ HSC are more susceptible to apoptosis mediated by serum deprivation, and there was a 64% increase in apoptosis of α-SMA-positive cells in these animals.

Endocannabinoids mediate apoptosis through CB1, CB2, and also transient receptor potential vanilloid 1 (TRPV1) which acts as the receptor for anandamide (AEA). The mechanism of action of AEA is well understood and reviewed more extensively by Siegmund et al., 2008. HSCs but importantly not hepatocytes are sensitive to apoptosis by both endocannabinoids AEA and 2-AG. Anandamide is the main endogenous agonist against a group of lipid mediators termed endocannabinoid and acts on the cannabinoid receptor CB1 and CB2. Anandamide (AEA) selectively kills HSC by necrosis but not hepatocytes, and this occurs independently of CB1 and CB2 and VR1 receptors. Siegmund and colleagues noted that treatment of activated HSCs with AEA induced necrosis via a Ca2+- and ROS-dependent fashion and that pretreatment with glutathione or a Ca2+ chelator (EDTA, BAPTA-tetrapotassium salt, or BAPTA-AM) significantly abrogated the effects of AEA on HSC necrosis. Additionally, preincubation with membrane cholesterol depleting agent prevented necrosis, suggesting that AEA may not be specific to CB receptors. While causing necrosis of HSC, hepatocytes appear not to be susceptible. This compound needs assessing in vivo and may prove to be a valuable therapeutic to target fibrosis [91].

12. Conclusions

Significant progress has been made in our understanding of the mechanisms of apoptosis and the relative contribution apoptosis plays in disease progression. Pathophysiological role of apoptosis is implicated in a number of liver diseases and contributes directly to fibrogenesis. Serum biomarkers of hepatocyte apoptosis have been well characterized in NAFLD and NASH and provide insight into disease severity. These markers, particularly cleaved CK-18, prove to be sensitive enough to distinguish between patients with simple steatosis and more advanced stages of disease. Further clinical studies are needed to determine CK-18 utility in other liver diseases. While the prospect of treating patients with liver disease with a pharmacological agent to cause the regression of fibrosis is exciting, further investigation is required into the long-term-efficacy. Blanket inhibition of hepatocyte apoptosis with pan-caspase or proteosome inhibitors reduces fibrosis; however, it may not improve liver function. Further studies are needed to determine the long-term effect of these inhibitors on liver function. Inhibition of the normal physiological process of removing damaged hepatocytes may prove to be detrimental and leave patients susceptible to developing hepatocellular carcinomas.

Ideally, next-generation antifibrotic therapies will target apoptosis-inducing mechanisms specific for activated HSC or benefit from targeted delivery systems. New insights into mechanisms of apoptosis have highlighted HSC specific involvement of NF-κB signaling, cannabinoid receptor

signaling, and miRNAs important in regulating apoptosis and may provide further pharmacological targets. Perhaps most importantly, HSC-specific biomarkers of apoptosis may not only provide further clinically relevant information regarding underlying disease but also predict the likelihood of a patient's response to therapy.

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Review Article

Flow Injection/Sequential Injection Analysis Systems: Potential Use as Tools for Rapid Liver Diseases Biomarker Study

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Flow injection/sequential injection analysis (FIA/SIA) systems are suitable for carrying out automatic wet chemical/biochemical reactions with reduced volume and time consumption. Various parts of the system such as pump, valve, and reactor may be built or adapted from available materials. Therefore the systems can be at lower cost as compared to other instrumentation-based analysis systems. Their applications for determination of biomarkers for liver diseases have been demonstrated in various formats of operation but only a few and limited types of biomarkers have been used as model analytes. This paper summarizes these applications for different types of reactions as a guide for using flow-based systems in more biomarker and/or multibiomarker studies.

1. Introduction

Liver diseases (hepatic diseases) cover the broad range of liver disorders. The liver has many important functions including breaking down toxic substances in the body and excreting them into urine, producing and secreting bile to aid in food digestion, converting glucose into stored glycogen and *vice versa*, metabolizing ingested medicines to obtain active ingredients, and producing blood-clotting factors, amino acids and cholesterol to transport fat. Damage to the liver will cause disruption of these functions which can cause serious damage to the body. Even though the liver is considered the only organ in the body that has exceptional capability in replacing damaged cells, if too many liver cells are damaged, the liver may fail to perform properly. Symptoms caused from liver failure may not be obvious until many liver cells (up to 75%) have malfunctioned [1]. Detection of liver problems at an early stage would increase the chances of curing them. Liver biopsy is currently the best method for diagnosis of chronic liver diseases [2, 3]. However, biopsy procedure involves the rather invasive method of taking a small piece of liver tissue to be examined under a microscope. The repetition of liver biopsy during diagnosis and treatment is hard on patients. Since liver diseases can also affect almost all other body systems, many substances/chemicals in the body may respond to the malfunctioning of the liver. The use of these substances/chemicals as biomarkers has become an area of high interest in research as scientists look for alternative noninvasive diagnostic approaches [2, 4, 5]. Even though none of the biomarkers alone nor the liver biopsy is perfect, they can be used for indication whether further investigation is required [2].

Studies for the effectiveness of biomarkers involve the collection of numerous samples which may be available at different periods of time. Then the analyses of those samples are usually carried out using the same method for comparison. Most of the possible biomarkers being proposed and under study are biomolecules such as proteins and enzymes [6–9]. Analysis of biomarkers in samples with complicated matrices like body fluids requires highly specific and sensitive techniques. Immunoassay is one of the most widely used techniques for these purposes [10]. It can be used to quantify proteins, enzymes, and other biomolecules owing to the flexibility of the immunoassay format and the available antibodies. Fluorescence- and chemiluminescence-based techniques are also of interest due to their high sensitivity [11, 12]. However, these techniques are time consuming and require a skillful operator. From the many

possible biomarkers proposed for liver diseases, only a few are accepted for use and only one which is called fucosylated Alpha-fetoprotein (AFP) is a United States Food and Drug Administration approved biomarker [13, 14]. The study of biomarkers for any disease seems to have the same common problem of unreliable results. This is mainly due to having insufficient numbers of sample subjects and insufficient diverse population as well as the lack of reliable and easy methods to manage various sets of samples and to validate analytical methods [13, 15]. On the other hand, since each research group around the world has access to different small sets of samples, it may be useful for the various groups to conduct their studies using different methods. Even though the results cannot be compared directly, the accumulated studies of those results/conclusions about the same biomarker obtained from different analysis methods should help the medical community in drawing conclusions about the trends and effectiveness of a particular biomarker. The effectiveness of the biomarkers should be revealed with similar significance, no matter which methods were used to quantify them.

Analysis technology has evolved into more rapid and low volume operations. The automatic features of the analysis system that can handle tedious and time-consuming analysis protocols are in demand. This paper is focused on the flow-based analysis techniques called flow injection/sequential injection analysis (FIA/SIA) for use as alternative systems for automatic and rapid quantification of biomarkers for liver diseases. The relatively low cost of these semi to fully automatic systems may help broaden opportunities for medical care/clinical studies in remote areas where medical personnel are limited and also provide additional information/results about the effectiveness of the candidate biomarkers.

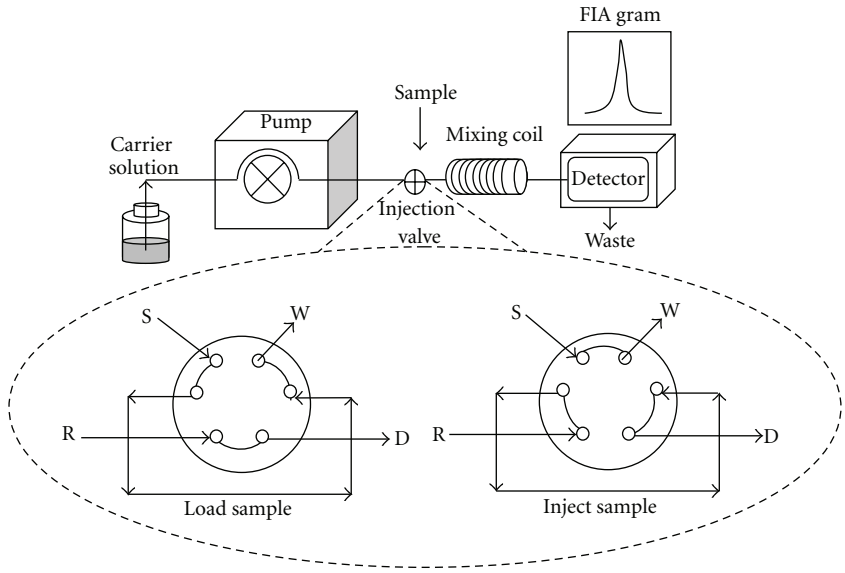
2. Flow Injection/Sequential Injection Analysis

Flow injection analysis technique has been employed to automate a wide variety of chemical/biochemical analyses since its invention in the early 1970s [16]. Owing to its feasibility in coupling with various types of detectors, the applications are numerous. Since parts of the systems can be made in house or replaced as suitable, the cost of the systems is relatively lower than many other commercial instrumentation-based analysis systems. This makes flow injection/sequential injection analysis systems especially suitable for low-budget laboratories. A simple flow injection system, see Figure 1, is composed of a pump for drawing solutions (reagents) into the system via pump tubing. An injection valve is used as an introduction port for sample to merge into the stream of continuously flowing reagent. Detectable product is formed while flowing and simultaneously it enters the detector and then proceeds out to waste. Various types of pump and valve may be used. The most common types are peristaltic pump and a six-port switching valve (similar to one used in HPLC system). Unlike most commercial instrumentation-based analysis systems, the FIA and SIA systems may be built/assembled in house. The beneficial feature of flow injection systems is the flexibility of the formats and designs that can be created based on applications. Various companies offer parts (i.e., pumps, valve, detector, tubing, nuts, and ferrule) for various purposes. These parts can be adapted for use in flow-based systems depending on budget and the flow system design. For example, in the early years of FI development and application in low-budget laboratories, an IV bag hung at a certain height and a cheap aquarium pump were reported as successfully used in place of a peristaltic pump [17]. Lower-cost solenoid valves or plastic 3-way valves and tubing may also be used to construct a hydrodynamic injection system instead of using a more expensive commercial 6-port valve [18].

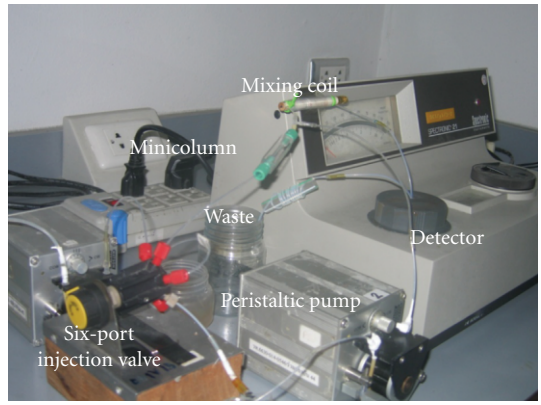
Reagent may be used as a carrier, where the sample is injected into the reagent stream. However, if the reagent is high cost and the sample is abundant, reverse FIA [19] may be carried out by using the sample solution as the carrier stream and introducing reagent at the injection point. The product is formed while reagent and sample flow together in the small tubing after merging at the sample injection point. The product zone, see Figure 2, has concentrated product in the middle of the zone, and it is more diluted on both sides of the zone due to dispersion of the solution plug in the carrier stream. Therefore, when the product zone flows through the detector, the beginning of the zone (part A) with low product concentration will enter first, followed by the high product concentration middle zone (part B) and the end of zone with low product concentration (part C). The resultant signal shows as peak signal, called FIA gram, where the highest point resulted from the highest product concentration in the middle of the zone. Detection is done much earlier before steady state; therefore the analysis time is dramatically reduced as compared to conventional batch-wise analyses where detection is normally done after the reaction is completed or has reached steady state. The constant flowrate enables the detection of repetitive analyses to be done at the same point of time. Thus, even though not at steady state, the resultant FIA gram (peak height or peak area) can be precisely related to the quantity of sample.

Later generations of flow injection analysis technique incorporate many pumps, valves, and tubing to accommodate more complicated chemical reactions that need many reagents. The latest generation, called sequential injection analysis [20], see Figure 3, has a downscaled system that consumes even smaller volumes of reagents and samples in a few μL level with the use of a bidirectional syringe pump and multiports selection valve. Reagents and sample can be drawn sequentially and stacked into the mixing coil before mixing while being pushed in reverse direction into the detector. The operational steps from sample introduction, chemical reaction, to detection are fully automated and precisely controlled with computer software. The system can be programmed to stop for a desired period of time; therefore, the study of slow reactions and those that require incubation time such as immunoassay is possible. Accessories such as lab-on-valve (LOV) unit with ports for attaching a fiber optic spectrophotometric detector introduce more areas of applications with real time detection [21–23].

Most research groups have reported that the flow-based systems not only increase sample throughput but also reduce



(a)



(b)

Figure 1: (a) Diagram of a simple flow injection system (S is sample, W is waste, R is reagent, D is detector) and (b) a picture of a simple flow injection system setup showing a peristaltic pump with pump tubing, a six-port injection valve, a minicolumn chemical reactor, a mixing coil, and a detector.

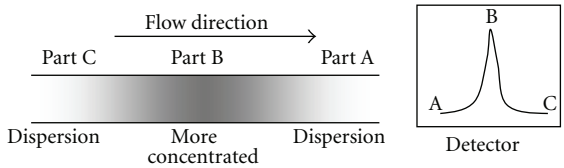


Figure 2: Product zone in the flow line.

the consumption of sample and reagents. This may be a suitable approach for cases where body fluid/blood samples are limited or need to be divided for various other tests. As compared to most conventional bench top wet chemistry, flow injection requires a lot less sample volume. For example, in titration, sample volume in batch method is in mL whereas in flow-based titration, sample volume injected is in μL [24]. A direct comparison between volumes used can only be made when considering the same analyte and detection methods. Some downscaled batch methods are able to reduce the volume to μL, but in general, FI usually requires relatively less sample volume for a particular analyte or sample being studied. For example, the osmotic fragility test (OFT) of red blood cells normally requires 20 μL of undiluted blood sample in batch spectrometric method whereas only 1 μL of undiluted sample is required in the FI system where it is tested in 100-fold dilution [25]. As compared to standard bioassay technique such as ELISA, the volume required by flow-based systems is also usually lower. For example, the assay of hyaluronan in serum using SI required 10 μL of serum sample, as compared to the conventional routine microplate assay that requires 120 μL of serum sample [26]. For some commercially available ELISA kits such as the cytokeratin 18 (CK18) biomarker kit which requires 50 μL of sample [27], no direct comparison of sample volume usage can be made unless those samples have been tested

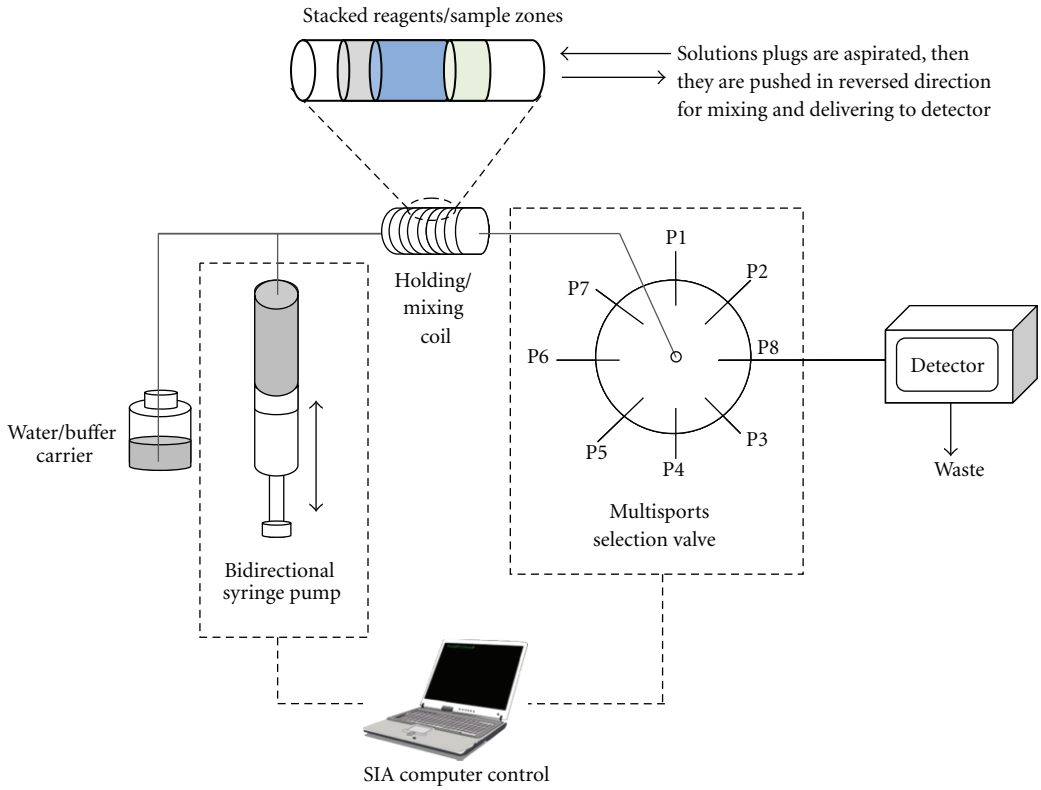


Figure 3: Diagram of a sequential injection analysis system (not to scale). P1–8 are ports on a multiports selection valve for transportation of various reagents.

within the flow-based system, but lower sample volume would be expected in the flow-based system. Most lateral flow chromatography kits may require only a drop of sample, but they normally only yield a simple yes/no answer without any detail of quantity.

3. Flow Injection/Sequential Injection Systems as Alternative Tools for Rapid Determination of Biomarkers for Liver Diseases

Table 1 summarizes the works that employed flow injection/sequential injection systems and microfluidics devices for rapid quantitative analysis of some substances that have been reported as candidate biomarkers for liver diseases. Most works emphasize the improved sample throughput. Most works also demonstrated very high precision, reported as percent relative standard deviation (%RSD), as shown in Table 1. Various possible ways of operation and detection using flow-based systems are described.

3.1. Flow-Based Analysis System for Simple Reactions with Various Types of Detectors. A flow injection analysis system can be used simply as an automatic system to carry out the mixing of sample and reagent(s). The product formed simultaneously flows into the detector**.** Depending on the reaction involved, product can be detected by coupling the flow injection system with suitable detectors (e.g., fluorescence spectrometer [28–30], UV-Vis spectrometer [31, 32], Rayleigh light scattering [33–35], or amperometer [36]). Normally, the detection cells for the detectors are modified to be compatible with the continuous flow of solution in the flow systems by having inlet and outlet tubings, and they are commercially available with various volumes and formats [37, 38]. Fluorescence is highly sensitive; therefore, it has been employed as a detector for the analysis systems that involve very low product volume such as in a nanofluidic system [30]. Sakai et al. demonstrated the formation of micelle in the flow line using nonionic surfactant Triton X-100 (amphipathic molecules that arrange themselves in spherical form in aqueous solution with hydrophilic ends outwards and hydrophobic ends inward) [31] and also the successive determination of multianalytes (e.g., human serum albumin and glucose) from the same sample [39]. Even though the samples used in the report were from diabetic subjects, it is clear that the same key idea of multianalytes detection can be adapted for liver diseases cases as well.

3.2. Flow-Based Analysis System for Multisteps Bioassay. A microcolumn packed with specific reagent-coated microbeads can be used as a reactor to accommodate the chemical/biochemical reaction. For example, Gao et al. [40] utilized packed columns with enzyme-coated beads to carry out multisteps enzymatic reactions. Another reported type

Table 1: Summarization of works reported on liver diseases biomarkers using flow-based analysis systems. FI: flow injection; FI-BI: flow injection-bead injection; SI: sequential injection; LOC: lab on chip.

Flow-based system	Detector	Reagent (s)	Biomarker sample	Detection Limit	Working range	Sample throughput	% RSD	Reference no.
FI	Florescence spectrometer	Fluorescein, sodium hypochlorite and surfactant	Albumin in urine	0.03 μg/mL	0.05–24 μg/mL	—	0.8	[28]
FI	Rayleigh light scattering	Amide Black -10B	Albumin in serum	0.11 μg/mL	—	—	<3	[33]
		Dye acid chrome blue K	Total protein in serum	85 ng/mL	2–40 μg/mL	60/h	<2	[34]
		Eriochrome black T		0.8 μg/mL	7–36 μg/mL	90/h	0.76	[35]
FI	Visible spectrometer	Tetrabromophenolphthalein Et ester triton x-100 (micelle formation reagent)	Albumin in urine	0.05 mg/dL	0.15–12 mg/dL	30/h	1.2	[31]
		Sulfate sulfatase enzyme immobilized on beads packed in reactor	Sulfate bile acid	—	1–75 μM	15/h	<1	[40]
FI	Surface Plasmon resonance spectrometer	Gold surface	Albumin in serum	500 μg/dL	—	90 s/sample	—	[46]
FI	Biolumines-cence spectrometer	coimmobilized luciferase and NADH:FMN oxidoreductase on hollow fiber reactor	3-alpha hydroxyl bile acid in serum	—	1–7.5 μM	>20/h	6–8	[41]
FI-BI	Visible spectrometer	Wheat germ lectin-coated beads and para-nitro phenyl phosphate (PNPP)	Alkaline phosphatase in serum	10 U/L	10–1000 U/L	30 min/sample	5-6	[32]
SI	Visible spectrometer	Hyaluronan standard coated glass capillary, biotinylated HA binding proteins, anti-biotin-HRP and Tetra-methyl benzidine substrate for immunoassay	Albumin in serum	9 ng/mL	Linear 25–500 ng/mL	20 min/sample	3–5.5	[26]
Nanofluidic (LOC)	Fluorescence spectrometer	Fluorescein label	Albumin in serum	0.3 pM	0.3–3 pM	200 s/sample	—	[30]
Microfluidic	Amperometer Glass chip	Substrate conjugated albumin packed in microflow channel	Activity of enzymes (glutamic oxaloacetictransaminase, glutamic pyruvic transaminase, γ-glutamyl transpeptidase)	—	Up to 100 −300 U/L	—	—	[36]

of reactor [41] is a commercial hollow fiber reactor with cuprammonium rayon membrane for immobilization of enzyme. These works sought detection of bile acids in urine and serum, respectively. Flow injection facilitated the introduction of sample solution and reagent into the reactor and simultaneously transported the colored/luminescent product to the detector.

Many biomarkers are protein or enzyme which normally can be determined using immunoassay technique. Conventional immunoassay technique is carried out in microplate format where multisteps incubations and washing are done in an array of small plastic wells, each accommodating 100–500 μL volume of solution. The test requires skillful lab personnel to obtain precise and accurate results from

handling the microvolume solution and ensuring precise incubation/washing time and volume for each well. The immunoassay process usually takes 3–8 hours depending on detailed steps. The good point of microplate immunoassay format is that many samples can be analyzed in parallel. However, in many circumstances, a small number of samples may need to be analyzed with the demand of quick results. In addition, in many areas of the world, skilled medical personnel are not available to conduct such a complicated test. More automated immunoassay systems where the volume and time are controlled by using a constant flow rate pump to introduce and to draw solutions from the reaction cell have been reported in various formats [42].

To change the format from conventional static immunoassay system to dynamic flow formats, the reaction cell has to be changed from being the wall of a microwell plate to other forms of solid surfaces that can be accommodated easily in the flow of solution. Microbeads and capillary are the main types reported. An example for the application in biomarkers research is sequential injection-glass capillary immunoassay [26, 43]. The sequential injection system was used to precisely control the incubation time and small volume of solution in the range of 10–80 μL which is even smaller than some of those used in conventional microwell plate format. A glass capillary was easily connected to the system as part of the tubing that the solution conveniently flows through without any back pressure which may occur when using beads. The wall of the glass capillary was used as the solid surface for immobilization of biomolecules to be employed in subsequent steps of competitive immunoassay.

Microbeads are used in immunoassay with the capability to increase surface area to improve sensitivity. However, beads that are packed or trapped in the reactor can cause back pressure inside the flow system, so a slow flow rate should be used. Example of sequential injection bead-based immunoassay system was reported for determination of hyaluronan, a possible biomarker for liver diseases [44]. The lab-on-valve (LOV) unit with on-valve fiber optic spectrometer can also be used in conjunction with functionalized beads that are trapped in the LOV unit to obtain direct, real time detection [45]. Although, there is no work reported on its application for study of liver disease biomarkers, the possibility exists for employing LOV bead immunoassay for such a task. Nevertheless, employing beads in the flow system would require more sophisticated control systems than most low-budget laboratories could devise unless preexisting equipment were to be adapted.

3.3. Flow-Based Analysis System with Preconcentration Capability. The use of microbeads in another aspect, other than using them as a solid surface for immobilization of biomolecules as used in immunoassay, is reported as a preconcentration surface to accumulate analyte of interest before detection. The flow of solution within an easily designed cell for trapping beads and releasing beads when needed is called a flow injection-bead injection system. An example of such a system for alkaline phosphatase in human serum was demonstrated [32]. Even though the work used beads coated with wheat germ lectin for specific binding for bone-alkaline phosphatase, it should be able to combine with total alkaline phosphatase test to estimate for liver alkaline phosphatase. The use of membrane for the same purpose of improvement of sensitivity was also reported. With-state-of-the art development in nanotechnology, nanopore membrane used with electrokinetic fluid flow as a nanofluidic protein accumulator was claimed to offer a much higher sensitivity in the analysis of human serum albumin than other methods [30].

By using a detection method that can measure the differences of surface properties before and after binding to the target analyte, such as surface plasmon resonance (SPR) technique, the amount of bound analyte on the surface could be quantitated directly without the need to add any reagents. Aoki and Toyama [46] demonstrated this system by determination of human serum albumin in urine using gold as an adsorption surface. A flow injection system was employed for continuously feeding the sample containing uric protein onto the gold surface.

4. Remarks

As can be observed from Table 1, most published works that demonstrated flow-based systems for liver diseases biomarkers used the same limited types of target analytes, that is, serum albumin and total protein. There are many substances in body fluids that have been reported as potential biomarkers for liver diseases. Determination of these different biomarkers at the same time may provide better conclusion about the existence of the diseases. Therefore, future studies using flow injection/sequential injection systems should focus on various other possible biomarkers as well as applications for conducting simultaneous detection of multibiomarkers from one shot of sample. Trends in development of analytical devices have also been gearing toward a point of care purpose. The main challenges are to develop the system for solution introduction with controllable flow rates, effective reagent mixing, and detection unit in downscaled format that can be integrated into compact stand-alone devices.

5. Conclusion

Various formats of flow injection/sequential injection analysis and micro-/nanofluidic systems can be set up to study biomarkers. Even though few works have reported on the study of liver disease biomarkers using flow-based systems, works related to determination of protein and enzymes are numerous and should be adaptable for studies of liver disease biomarkers. These flow-based systems are versatile and can be used as an alternative method for rapid screening of biomarkers to aid in disease diagnosis. Their low-volume consumption is particularly suitable for the study of a biomarker, in which samples may be divided for many other tests, either to evaluate different biomarkers or to accompany the initial biomarker test.

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