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Evaluation of the Utility of Serum Prolidase as a Marker for Liver Fibrosis

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Background: Liver dysfunction is common and often unrecognized. Liver biopsy is the gold standard in the assessment of liver fibrosis, but has disadvantages. We assessed the diagnostic accuracy of serum prolidase enzyme activity (SPA) in predicting the presence and degree of liver fibrosis, as compared with liver biopsy. Further, we evaluated the effect of hemolysis on measured SPA levels. *Methods:* We undertook a prospective case control study. Thirty eight outpatients without apparent liver illness and 20 patients with liver pathology scheduled to undergo liver biopsy had their SPA levels measured. *Results:* Patients undergoing liver biopsy had higher SPA levels (361 (268) IU/l [median (interquartile range)]) compared with controls (169 (160) ($P < 0.001$)). A SPA cutoff value of 200 IU/l yielded a sensitivity of 89%, specificity of 59%, an odds ratio of 11.5, negative predictive value of 92%, and a positive predictive value of 50%. Hemolysis causes an apparent increase in SPA levels. *Conclusion:* Higher SPA levels in patients undergoing liver biopsies compared with controls may reflect the presence of liver fibrosis. SPA levels could not be used to stage the degree of fibrosis. SPA measurement may be useful in the diagnostic workup of suspected liver disease. J. Clin. Lab. Anal. 29:208–213, 2015.

Key words: diagnostic accuracy; liver pathology; receiver-operator curve; screening; prolidase

INTRODUCTION

Liver disease is common and is showing increasing prevalence with many patients going onto develop advanced disease (1, 2). In suspected liver disease, liver biopsy is the reference standard for investigation but (3,4) owing to its invasive nature (5), it has potential pitfalls and may be inaccurate (6–9). Other investigative methods include clinical imaging techniques (10), measures of liver stiffness using transient elastography (11–13), individual serum biomarkers and combinations thereof. However, all have various shortcomings (1, 2, 5, 6, 8–10, 14, 15).

Prolidase is a cytosolic enzyme that cleaves collagen breakdown products and recycles collagen (16) and is the rate limiting step in fibrosis (17). Its activity is induced in periods of fibrosis (17). Serum levels are low (16, 18), but induction of irreversible liver injury in rats reveals a strong correlation between the degree of resulting fibrosis and the serum prolidase enzyme activity (SPA) levels (18, 19).

Prolidase has been measured in various clinical conditions (19–29). However, there is a paucity of studies in the setting of liver fibrosis. In one study (30) although elevated plasma prolidase was found in cirrhosis, there was no correlation between plasma prolidase activity and the degree of liver fibrosis. In another study, levels of SPA correlated with both cirrhosis and alcoholic hepatitis, but it could not be used to distinguish between early fibrosis and more severe pathology (31). A prospective study of steatosis and steatohepatitis (NASH) in patients with nonalcoholic fatty liver disease (32) showed that SPA levels were significantly higher in patients with NASH (33).

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In view of the relative uncertainty about SPA, we undertook a study to evaluate the usefulness of SPA compared with liver biopsy and to determine whether it could be used as a biomarker to assess the potential presence and degree of liver fibrosis in our local setting.

MATERIALS AND METHODS

Patient Recruitment

Patients were recruited from the inpatient population, which included patients with suspected liver disease. A total of 64 patients were enrolled in the study, which included 28 people undergoing liver biopsy at Groote Schuur Hospital, a tertiary hospital in Cape Town, South Africa. All inpatients scheduled to have liver biopsy from October 2009 to August 2010 were considered. Patients were excluded if the liver biopsy was cancelled or was done as an outpatient procedure. Samples were rejected if patients did not provide informed consent (8 of the 28 patients were thus excluded). Serum and liver biopsy samples were analyzed on the patients undergoing biopsy.

Control Patients

A control group of serum samples from 38 outpatients at the same hospital were used (Fig. 1)(34). These were patients who were attending hospital for nonliver related pathology and did not have liver related tests requested.

Ethics Committee Permission

Demographic data to ensure that the samples were from adults were checked, but no other data were captured. This study was approved by the UCT Research Ethics Committee (Rec Ref: 435/2009).Written informed consent to be included in the study was obtained from all participants undergoing liver biopsy. All samples were anonymized and confidentiality was maintained.

Prolidase Assay

Serum samples were taken as part of the routine inpatient work-up for liver biopsy; all patients therefore had blood drawn on the day prior to, or on the day of the biopsy. Informed consent to use this left over serum for SPA analysis was obtained from patients. Serum was stored at −80°C prior to analysis. SPA was assayed using a spectrophotometric method based on the reaction of proline with Chinard's reagent (30). Five microliters of serum was incubated for 2 hr with 15 μl 5 mmol/l $MnCl_2$ in 100 mmol/l Tris HCl (pH 8.0) in a 37°C water bath. Thereafter, 60 μl 100 mmol/l glycyl-proline (Sigma-Aldrich), 80 μl 100 mmol/l Tris (pH 8.0), and 40 μl water was added and the mixture was incubated for 30 min in a 37°C water bath. The reaction was stopped by the addition of 100 μl 20% w/v trichloroacetic acid (TCA). The mixture was centrifuged for 5 min and 200 μl supernatant fluid was mixed with 400 μl glacial acetic acid and 400 μl Chinard's reagent (2.5 g of ninhydrin dissolved at 70°C in 60 ml glacial acetic acid, 23.7 ml water, and 16.3 ml 85% orthophosphoric acid ($d = 1.7$) and heated for 10 min at 90°C. Color development at 515 nm was read against a blank treated similarly, except for the addition of TCA prior to substrate. Proline concentration was calculated using a standard curve of increasing concentrations of proline (Sigma-Aldrich) in 6.7% w/v TCA. SPA levels were reported as micromole proline formed per minute per liter serum (IU/l). Quality control was performed via frozen aliquots of a single serum analyzed in each run. The person performing the SPA assay was blinded as to the results of the liver biopsy.

The Effect of Hemolysis on the Prolidase Assay

The effect of hemolysis was assessed on a pooled serum sample using a published modified osmotic shock method (35). Briefly, a hemolysate was made by centrifuging whole blood obtained from a volunteer after which the plasma was removed. The red cells were washed five times with an equal volume of 0.9% saline, and then stored at -20°C overnight with an equal volume of distilled water. Thereafter, this was centrifuged and the hemoglobin (Hb) in the supernatant measured. A range of hemolysates were then prepared using dilutions of this stock solution in saline to provide a range of Hb 0–100 g/l in increments of 5 g/l. A 100 μl of increasing hemolysate (100 μl saline was used as a blank) was added to 900 μl pooled serum.

Histological Assessment of Liver Biopsy

The liver biopsies were stained with hematoxylin and eosin, and further sections were stained with bile sirius red stains. The degree of fibrosis at biopsy was assessed using a well-characterized scoring system by one pathologist (ML) who was blinded to the results of SPA analysis but who had access to the patient's records if required (ML) (36).

Statistical Analysis

Statistical analysis was undertaken on Microsoft Excel and Statistica 9.0. Data showed a Gaussian distribution after being transformed by taking square roots. The F-test of variance was used on the square-root transformed data to establish that variances were equal, and then Student's unpaired *t*-test assuming equal variances was used. Back-transformed data are shown and scatter

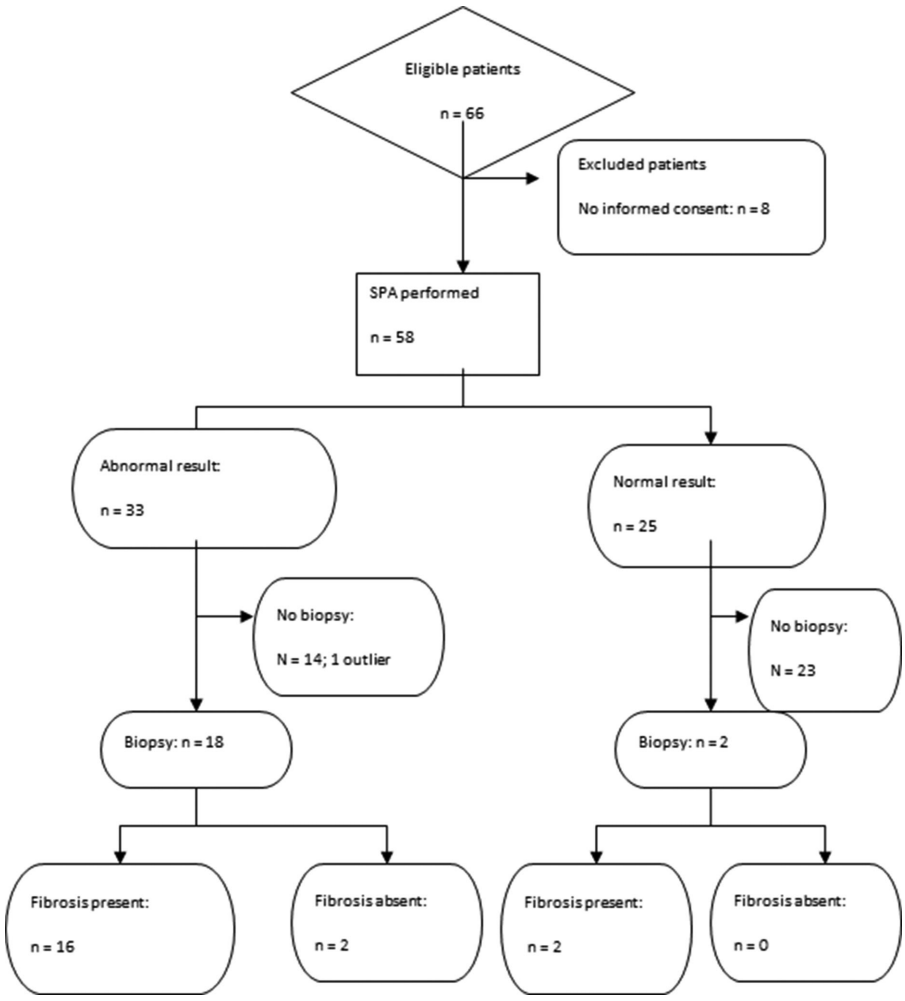

Fig. 1. STARD flow diagram illustrating the testing process.

plots drawn. $P < 0.05$ was taken as indicative of statistical significance.

RESULTS

Prolidase Assay

Linearity and precision of the prolidase assay concurred with published reports (data not shown)(30, 32). Hemolysis was a significant interferent in the prolidase assay and even low levels of hemolysis affected the results obtained (Fig. 2). Therefore, hemolyzed samples should not be used.

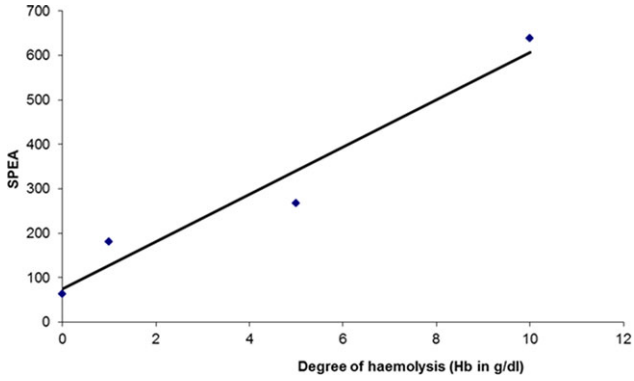

Fig. 2. Effect of hemolysis on serum prolidase activity.

Liver Biopsy and SPA Assay

No adverse events were reported in any of the patients. Some patients did not have all tests performed during this admission. In such instances, all available data were analyzed.

Two patients tested positive for hepatitis B virus (HBV) infection and two for human immunodeficiency virus (HIV) infection. Two further patients tested positive for two infectious agents: one for HBV and HIV, and the other for HIV and syphilis. Thus, in total six (30%) of

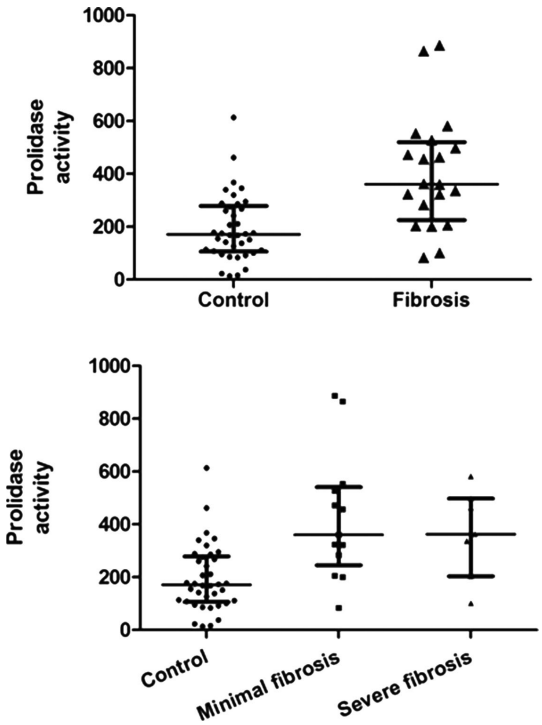

Fig. 3. (A) Scatter plots for serum prolidase enzyme activity (SPA) in the control group versus the patients undergoing liver biopsy. The horizontal lines represent the median and the interquartile range. (B) Scatter plots for serum prolidase enzyme activity (SPA) stratified on the basis of increasing degree of fibrosis. The horizontal lines represent the median and the interquartile range.

patients who had a liver biopsy tested positive for HIV, hepatitis B and/or syphilis. None of the patients tested positive for hepatitis A or C infection.

The patients who underwent liver biopsy had higher SPA levels than the controls that did not ($P < 0.001$) (Fig. 3). SPA values were as follows: control group 166 IU/l (22–504) (mean (mean ± 2SD)), the entire fibrosis group 376 ($P < 0.001$ vs. controls), minimal fibrosis group 392 ($P < 0.001$ vs. controls), extensive fibrosis group 335 ($P < 0.001$ vs. controls). There was no significant difference in SPA levels between the groups with minimal and extensive fibrosis ($P = 0.59$) (Fig. 3).

TABLE 1. Results of Serum Prolidase Enzyme Activity (SPA) Discriminatory Power at a SPA Cut-Off Level of 200 IU/l

	SPA		
	Positive	Negative	Total
Liver biopsy			
Positive	16	23	39
Negative	16	23	39
Total	32	25	57

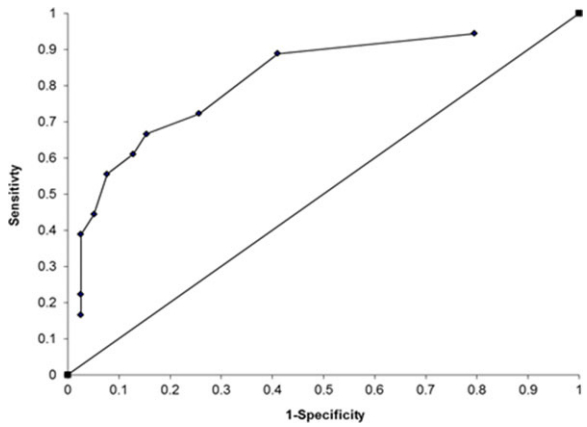

Fig. 4. Receiver operating characteristics curve analysis showing serum prolidase activity cut-off levels of 529, 500, 463, 450, 360, 330, 321, 280, 200, and 100 IU/l.

Receiver operating characteristics curve analysis revealed an optimum cut-off of 200 IU/l for the detection of fibrosis (Table 1 and Fig. 4).

This SPA cut-off value yielded a sensitivity of 89%, specificity of 59%, and a diagnostic odds ratio of 11.5. This assay had a negative predictive value (NPV) of 92% and a positive predictive value (PPV) of 50%. The area under the ROC curve was 0.96. If these results were translated into a screening test in a general unselected population, then using a quoted population prevalence of liver fibrosis of 2.8% (14, 16) this would translate into a NPV of 99.5% and a PPV of 5.9%.

DISCUSSION

This study has demonstrated that in patients undergoing liver biopsy, SPA levels were significantly higher than in control patients who were attending the outpatient clinic and who were assumed to be free of liver disease. The lack of liver biopsies from the group of controls is problematic, but owing to ethical considerations, it would not be possible to have liver histology data from disease-free individuals. Given the prevalence of liver disease, there may well have been individuals in the control group with unsuspected liver fibrosis. If so, this would have reduced the apparent discriminatory power of SPA analysis. Furthermore, the fact that this study was performed in a referral hospital will bias the findings because of the selected population (12). The small size and the case control design are limitations of the study.

Fibrosis is often, but not always, found in chronic liver injury and this may reflect the apparent lack of resolving power of SPA when examining the sensitivity, specificity, and related data (2). At a cut-off SPA value of 200 IU/l a negative predictive value of 92% can be obtained but is

at the expense of multiple false positives as the specificity is 59%. This concurs with previous findings (32, 37). A negative likelihood ratio at this cut-off is 0.19 which is above the suggested value <0.1 for an excellent test (12).

There was no significant difference in SPA in the group with minimal compared with extensive fibrosis as determined by liver biopsy. This contrasts with a previous study however, there were patients with moderate to severe fibrosis included (32). In another study, plasma prolidase activity was measured in 338 patients with various disorders (30). The authors found elevated levels of plasma prolidase in 5 out of 27 patients known to have cirrhosis. However, there was no correlation between plasma prolidase activity and the degree of liver fibrosis determined at liver biopsy in 13 patients in this study. In another study, plasma prolidase enzyme activity and liver histology were compared in 53 adult patients who had alcohol intake in excess of 60 g/day. In this study, levels of SPA correlated with both cirrhosis and alcoholic hepatitis, but it could not be used to distinguish between early fibrosis and more severe pathology (31). A prospective study of 54 patients and 17 healthy controls used SPA to distinguish between NASH in patients with nonalcoholic fatty liver disease ($P < 0.001$)(32). This showed that SPA levels were significantly higher in 36 consecutive patients with NASH, compared with controls. However in the present study, there appears to be an increase in SPA with mild-moderate fibrosis, with a reduction once fibrosis becomes more severe. This trend is not statistically significant but has been observed (18, 21, 22). More advanced fibrosis may be characterized by a slower turn-over of collagen than states of moderate fibrosis and this may lessen the induction of prolidase activity (30).

We also demonstrate that hemolysis is a positive interferent in SPA because prolidase levels are high in erythrocytes. No hemolyzed samples were used in this study. It is not clear how any hemolyzed samples, if any, were handled in previous studies (21, 30, 32, 38).

Future areas of research should include more diagnostic accuracy studies and analysis of SPA levels in other conditions such as Paget's disease and an examination of the biological variation in healthy people including pregnant women and children. In view of the HIV epidemic in South Africa and reports that liver fibrosis is more common in HIV infection, SPA should be investigated in this setting (39). Tuberculosis remains a significant source of morbidity and pathology locally and early reports that SPA can be used to distinguish pleural effusions of tuberculous from nontuberculous origin should be investigated (27).

The values that were obtained for SPA in this study were lower than those reported in several other studies but this may be due to differences produced by the analytical methods used (21, 30, 32, 38). There is no reference method or standard reference material available. Although the turn-around time of the prolidase analysis has improved dramatically with a reduction in the length of time for the preincubation step, this remains extended at 2 hr and would not fulfill expectations for a routine screening test. Further refinements of the assay have been described which should be investigated (18). Future advances may involve the use of an artificial substrate which, when cleaved by prolidase, produces a chromogen directly without the need for further manipulation. Immunoassays that measure the prolidase mass rather than activity will be useful. The effects of other possible interferents, including bilirubin, which may be increased in liver disease, remains to be elucidated. We conclude that measurement of SPA may be useful in diagnostic investigation of suspected fibrotic liver disease.

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