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Evaluation of serum prolidase activity as a marker for liver fibrosis in suspected liver disease

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Declaration

I, John Christian Stanfliet, assert that this research report is based on independent work performed by me and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree to any other university. This work has not been published *prior to registration* for the abovementioned degree.

University of Cape Town

Acknowledgements

I, John Christian Stanfliet, hereby thank and acknowledge the invaluable help and encouragement that I received from numerous people, without whom this project could not have been completed.

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For growing the skin fibroblasts and helping with the assay on fibroblast culture

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Who helped design the enzymatic assay and provided useful insights in enzymology and Chemical Pathology. He graciously agreed to be the co-supervisor.

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For his collaboration in this project and analyzing the liver biopsies

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For suggesting the project in the first instance and providing insight and corrections as the project matured.

Dissertation abstract

The diagnostic accuracy of serum prolidase activity as a marker for liver fibrosis in suspected liver disease

Hypothesis

Serum prolidase activity correlates with the degree of hepatic fibrosis and can be used to assess the presence and the degree of fibrosis.

Abstract

Liver dysfunction is common, often unrecognised and likely to increase in incidence in the population in parallel with the obesity and attendant type 2 diabetes mellitus epidemics. Liver fibrosis is a significant finding in liver pathology as it imparts important clinical staging and prognostic information, is a risk marker of adverse clinical outcome and yet, even if advanced, is capable of reversal with appropriate therapy. Histological examination of liver biopsy material is the reference standard in the assessment of liver fibrosis, but it is impractical to biopsy all patients who are suspected of having liver disease and furthermore there are associated drawbacks. No current biomarker or imaging technique performs satisfactorily in diagnosing and/or staging liver fibrosis. Several novel biomarkers have been described for this purpose. This dissertation evaluated the measurement and the diagnostic accuracy of one such biomarker, serum prolidase, in determining the potential presence and degree of liver fibrosis compared with liver biopsy.

Part A describes the scope of the problem, the rationale for the study and the potential impact of the findings. Part B, a literature review, examines the use and limitations of liver biopsy, clinical imaging techniques and other biochemical markers. It provides background on prolidase and discusses important published research findings. Part C details a prospective case control study undertaken by the author, supervisors and collaborators to evaluate the methods used in the measurement of prolidase activity and to determine its diagnostic accuracy compared with liver biopsy. Part D contains supplementary information and appendices such as details of the methodology used, ethics approval letters, the informed consent and the university guidelines *inter alia*..

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Part A Protocol

Part A of this mini-dissertation is written as per the official University of Cape Town guidelines (see Appendix). This section is a protocol which provides background and motivation for the research, the structure of the proposed research and its likely impact.

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Part A

Evaluation of serum prolidase activity as a marker for liver fibrosis in suspected liver disease.

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Summary

Liver injury is very common yet often clinically silent. The identification of such injury will allow for appropriate medical management. The current reference standard for the determination of liver fibrosis is histological examination of liver biopsy material. No current clinical imaging technique or biochemical marker can match the sensitivity and

specificity of biopsy. Serum prolidase is a novel biomarker that has shown promise in several studies in the diagnosis of liver fibrosis.

Aims and objectives

The specific objectives of this project are to:

- a) Establish the methodology for the serum prolidase assay in our laboratory;
- b) Evaluate the prolidase assay method;
- c) Establish whether there is a significant difference in serum prolidase activity when histological fibrosis is present;
- d) Establish whether hepatic fibrosis can be identified early in susceptible patients by means of serum prolidase;
- e) Establish whether serum prolidase activity correlates with the degree of hepatic fibrosis;
- f) Establish a baseline for the future determination of a reference interval for serum prolidase activity in the local population.

Hypothesis

Serum prolidase activity correlates with the degree of hepatic fibrosis and can be used as an indicator of the degree of fibrosis.

Background

The liver is essential for many homeostatic functions, including the maintenance of blood glucose levels, the manufacture of all circulating proteins in blood apart from immunoglobulins, biotransformation of endogenous and exogenous waste products, and the metabolism of many hormones. It is also central to cholesterol and bile acid metabolism.¹

Damage to the liver results in orchestrated healing, but if the damage is chronic, fibrosis may develop.^{2,3} If this continues there is eventual alteration of liver architecture, with islands of hepatocyte regeneration in a background of excessive fibrosis, a condition termed cirrhosis.^{1,3-6} Chronic liver injury comprises a spectrum of steatosis, hepatitis, fibrosis, cirrhosis and hepatocellular carcinoma (HCC). Patients often present late, as the liver has a significant reserve capacity and can regain normal function even in the setting of advanced fibrosis.^{1,7}

The assessment of hepatic fibrosis is a clinical requirement to establish the presence and staging of fibrosis and cirrhosis, to determine the prognosis, available treatment options, and to detect any ensuing complications.⁸⁻¹⁰ Reversal of injury and recovery of function is possible even in the setting of advanced disease with appropriate therapy.^{3,5,11} In addition, monitoring the response to treatment is required if anti-viral and anti-fibrotic therapies are instituted.¹² In reality, there is no current licensed specific treatment for liver fibrosis, and management consists of stopping the

aetiological agent and avoiding further insults to the liver.¹ By inference, the earlier the pathology is detected, the better the outcome will be, given the propensity for the liver to recover.

Several authors estimate the incidence of liver dysfunction at a third of the adult population in the United States of America.¹³⁻¹⁵ Globally, the major aetiological factors causing liver injury are viral infections, alcohol and non-alcoholic fatty liver disease (NAFLD).^{1,6,16} The World Health Organisation (WHO) estimates that 350 million people are infected with chronic hepatitis B and 180 million with chronic hepatitis C and half of patients chronically infected with hepatitis C eventually develop fibrosis and 20% cirrhosis.⁹ A similar proportion of people with alcoholic or non-alcoholic steatohepatitis (NASH) will develop cirrhosis.^{16,17} A recent screening program in France estimated the population prevalence of liver fibrosis at 2.8%.¹¹ The incidence of hepatic fibrosis is likely to increase because of the rapid increase in obesity as well as type 2 diabetes mellitus.^{1,13,14,18}

South Africa suffers the burden of a number of aetiological agents such as hepatitis B infection which is five times more prevalent in people who are positive for human immunodeficiency virus (HIV) -infection compared to those who are HIV-negative;¹⁹ HIV infection is unfortunately particularly prevalent. Liver fibrosis is more common in patients who are HIV-positive.²⁰ Alcohol abuse is also a particular problem in South Africa.²¹ In addition, the prevalence of people classified as being overweight or obese in

South Africa is very high.²² South Africa also has seemingly unique risk factors for liver injury such as elevated dietary iron intake because of brewing traditional beer in iron drums.²³ Kruger *et al.* demonstrated a high level of hepatic fibrosis in patients with NAFLD in the local population in Cape Town.²²

Assessing liver fibrosis

Currently, the gold standard of fibrosis assessment is hepatic biopsy followed by histological examination, with most biochemical sera markers performing less well.¹⁰ Liver biopsy, however, has a number of problems, such as its invasive nature, the problem of obtaining a representative sample, pain and the fact that it is not without risk (see Part B). Very few health services can cope with the current, and anticipated increasing demand for liver biopsy. This is particularly true in the resource-constrained health care system of South Africa. Simply cutting costs by reducing staff or reducing costs across the board are not effective financial strategies.²⁴ Efforts should be made to streamline healthcare, identify and resolve inefficiencies and provide novel and economical solutions, while maintaining and improving the quality of patient care.²⁴ Thus, a simple, accurate and reliable screening test that may exclude the need for biopsy is required.^{10,13,15,18}

Serum biomarkers

Biomarker is a term that refers to any indicator that is measurable and can be used to indicate the presence or degree of injury.²⁵ There are certain ideal characteristics such

as accuracy, precision, sensitivity, specificity, ease and cost-effectiveness of measurement and, in addition, it should guide clinical management.^{14,18,25} The ideal marker of liver fibrosis should be liver specific, should be unaffected by pathology affecting other systems, should measure some aspect of fibrosis, be of value as a screening tool and also be easy to measure.^{14,18} It should be readily available and its assay standardised.²⁶ It should provide reliable prognostic information and should reflect the dynamic process involved in liver pathology with levels that correlate with the degree of injury so that it can be employed to monitor the response to treatment.²⁷ Prior to introduction into routine clinical practice, these characteristics of the biomarker under consideration should be determined. No such marker exists.

The case for prolidase

Early fibrosis is characterized by collagen deposition, and the amino acid proline is a major component of collagen. Thus with early liver damage, and subsequent scar tissue, more collagen will be deposited in the liver. Prolidase is a peptidase which hydrolyses bonds involving proline which are resistant to degradation by most peptidases.²⁸ Thus, with increased fibrosis and collagen deposition, there is an induction of prolidase, which is the enzyme that potentially will help to recycle collagen, and this shall be reflected in enhanced serum prolidase enzyme activity levels.^{27,29}

Recently, interest has been increasing in the use of the serum enzyme prolidase as a marker of hepatic fibrosis and work has been ongoing to investigate the relationship

between the degree of hepatic fibrosis and serum prolidase levels.³⁰ This has been pursued because of the poor correlation of commonly used liver enzyme markers such as transaminases with the degree of fibrosis.¹⁸

Prolidase consists of two subunits and requires Mn^{2+} as a co-factor for full activity.^{28,31} It is found in many tissues and it is most prevalent in the kidney.^{32,33} Levels in plasma and serum are low.³² Its levels are unrelated to age, sex and do not appear to be increased by bone tumours either primary or secondary.³⁴ There are reports that serum prolidase activity is increased in various pathologies characterised by fibrosis (see Part B).³⁵⁻³⁹

Myara *et al.* measured plasma prolidase activity in a population of 338 hospitalised and outpatients with various diagnoses. They established a log-gaussian distribution with a mean value of 900 ± 520 IU/L (2 SD). Five out of 27 patients with cirrhosis had values in excess of 2 000 IU/L and a further three patients who had elevated levels had chronic liver disease. Normal levels were found in patients with myocardial infarction, metabolic bone disease, acute hepatitis and cholelithiasis. There was no correlation with common biochemical markers of liver injury namely transaminases, alkaline phosphatase, gammaglutamyl transferase, total bilirubin and albumin.²⁹ Kayadibi *et al.* have shown that serum prolidase activity is a potential biomarker of liver fibrotic injury and can distinguish steatohepatitis from simple steatosis.³⁰

Detailed methodology

This shall be a prospective cohort study of adult patients at Groote Schuur hospital undergoing liver biopsy. In collaboration with the Department of Anatomical Pathology, patients who are undergoing liver biopsy will be identified, and some of their sera held in the National Health Laboratory Service C17 Core laboratory at Groote Schuur Hospital stored at -80°C until assessment. The degree of liver fibrosis shall be determined and graded using established scoring systems based on histological examination by an experienced histopathologist blinded to the rest of the study. The serum prolidase activity shall be assayed in the stored sera of these patients, with the results of the liver biopsies being blinded so that they are not available to the assayer. The serum prolidase enzyme activity (SPEA) shall be plotted against the degree of liver fibrosis determined at biopsy. The results will be analysed to assess whether serum prolidase is a marker of hepatic fibrosis, and whether the serum prolidase activity correlates with the degree of liver fibrosis. The available liver function and other test results of these patients' in-patient stay will be accessed.

Measuring serum prolidase

The most common way to measure SPEA is the colorimetric method of Chinard as modified by Myara *et al.*²⁹ This involves a 24 hour pre-incubation of sera with Mn^{2+} which results in a large increase in activity, although longer incubation times increase activity further (figure 1), followed by the addition of a substrate, glycyl-proline, which is

cleaved by the enzyme prolidase to yield glycine and proline. Both the glycine and proline so released are free to react with ninhydrin but can be distinguished on the basis of the difference of the absorbance spectra of the chromogen formed. The chromogen that results from proline's reaction with ninhydrin is measured spectrophotometrically at 515nm against a serum blank.³² The equimolar amount of glycine-ninhydrin chromogen formed does not interfere at this wavelength (figure 2). Work on streamlining the assay has continued and the 24 hour incubation step has been reduced.²⁷ Intra-assay and inter-assay CVs of 3.8% and 9.0% respectively are reported.³⁴ Prolidase activity has been determined successfully in patients homozygous for prolidase deficiency using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS),⁴⁰ and a capillary electrophoresis method with electrochemiluminescent detection has been published as well.⁴¹

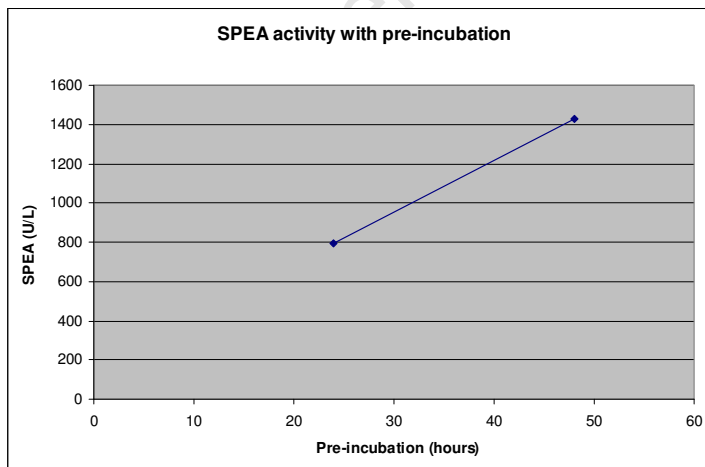


Figure 1: Demonstration of increase in SPEA with incubation time. Aliquots of the same sample were pre-incubated with Mn^{2+} for 24 or 48 hours prior to assay. The x-values represent the incubation time, and the y-values, the SPEA.

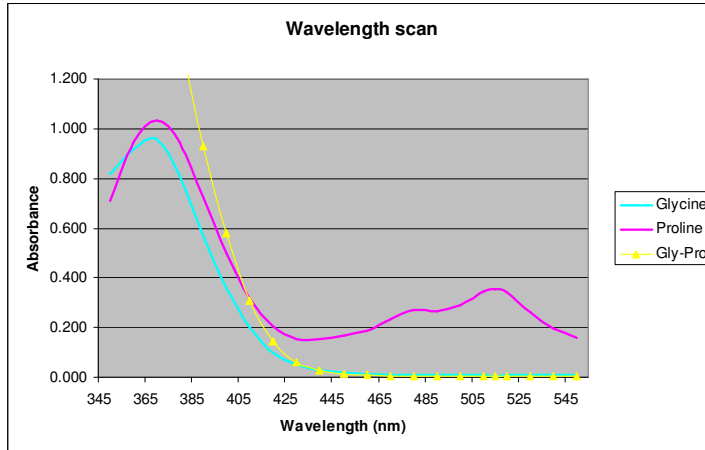


Figure 2: Three individual wavelength scans of 0.65 mmol/l solutions of glycine, proline and glycy-proline respectively after reacting with ninhydrin. This demonstrates that proline produces a peak in absorbance at 515 nm without interference from the chromogens produced by glycine or glycy-proline.

The linearity, CV and stability of prolidase will be examined as well as its ability to withstand freeze-thaw cycles using samples obtained from volunteers. Values reported in the literature for prolidase activity vary widely and a comparison study shall be undertaken to assess the degree of transferability of results and whether reference intervals should be method specific. In addition, as prolidase is present in erythrocyte, the possible interference of haemolysis shall be assessed. Prolidase activity can be measured in cultured fibroblasts; an assessment of the feasibility shall be undertaken.

Envisaged outputs/outcomes

This study will investigate the feasibility of prolidase as a new marker in patients with hepatic disease. The work will be presented at the South African Society of Pathologists annual meeting, the annual conference of the Association for Clinical Biochemistry (UK)

and the American Association of Clinical Chemistry (USA). The study will provide material for Dr Stanfliet's MMed dissertation and will provide Dr Stanfliet with the opportunity to publish in a major peer-reviewed journal such as Journal of Clinical Pathology or Clinical Chemistry.

Impact

The project will establish a base of expertise in a new area of laboratory medicine as it applies to diagnostic clinical chemistry, in the Division of Chemical Pathology at the University of Cape Town. Furthermore, the project will develop capacity in a new and emerging area that is under-researched in both the global, and, the South African context.

Part B Literature Review

This section is a literature review written as per the University's guidelines copied below (see Appendix for the entire set of University guidelines). A single set of references is presented at the end of Part C to prevent repetition and unified numbering is used throughout this mini-dissertation.

Part B: A *structured literature review* appropriate to the subject matter and methods of the dissertation. The literature review must, amongst other things, show that the student is sufficiently acquainted with the relevant literature and is able to perform a critical appraisal and, if appropriate for the topic, show a good understanding of evidence-based medicine.

The review should be between 3 000 and 4 000 words.

A suggested structure for the literature review is as follows:

- a) Objectives of literature review
 - b) Literature search strategy, including inclusion and exclusion criteria
 - c) Quality criteria - some leeway will be allowed here, as candidates will vary in their ability to appraise studies. This will also vary with the nature of the dissertation.
 - d) Summary or interpretation of literature
 - e) Identification of gaps or needs for further research
 - f) References (which will overlap with but will not be the same lists as in the journal article and protocol)
-

Part B Literature review

Evaluation of serum prolidase activity as a marker for liver fibrosis in suspected liver disease

Summary

Liver dysfunction is common, is anticipated to increase, is often silent and early treatment yields the promise of the best outcome (see Part A). This section describes current methods used in the diagnosis of hepatic injury, such as liver biopsy followed by histological examination, clinical imaging techniques, breath tests and current serum biomarkers. Prolidase and diseases associated with alterations in its determined activity levels are detailed. Gaps in the current knowledge base are highlighted and possible areas of future research are suggested.

Search strategy and quality criteria

The literature review was undertaken on PUBMED to identify articles relating to liver dysfunction, fibrosis, prolidase and the local South African situation. The references of selected articles were also searched to identify relevant articles. Review articles from the last five years were given preference. Only articles published in English in peer-reviewed journals were considered. Articles describing the analytical methodology of measuring prolidase activity were specifically sought.

Assessing liver injury

Liver disease is a global epidemic although, surprisingly, it hasn't been identified as such by global agencies such as the World Health Organisation. Irrespective of the aetiology of liver disease, there is a final common pathway involving fibrosis, cirrhosis and further complications if the injury is overwhelming or continuous. Consequently, there are a number of common means to assess the damage inflicted on the liver regardless of the aetiology.

Liver biopsy

Currently, the gold standard of fibrosis assessment is histological examination of hepatic biopsy specimens.^{10,42,43} Liver biopsy, however, is inherently invasive, and is associated with 1 - 5% rate of complications such as pain, hypotension, bleeding and the possibility of needle-track seeding of malignant cells in cancers.^{42,44-46} Rarely, fatalities have occurred at a quoted rate of 1 in 1 000 – 10 000.^{45,47} For these reasons, many patients find it unacceptable and refuse to be biopsied.¹⁷ It is also difficult to standardise histological examination in much the same way that clinical chemistry tests are.^{2,17} Furthermore, it is impractical to use biopsy to screen all patients with suspected liver disease or to use it to routinely follow their clinical course given the very high prevalence of liver injury, the potential risks involved with biopsy and the reality of constrained resources.^{7,48}

Liver biopsy has a coefficient of variance (CV) of 55% with significant intra- and inter-operator variability.² A single blind percutaneous biopsy may miss established cirrhosis in 10 - 30% of cases.^{17,44} This is due to the sampling of a minute fraction (0.002%) of the liver at biopsy and the non-homogeneous nature of the pathology.⁴⁶⁻⁴⁸ A single liver biopsy provides a static picture that gives little information as to the dynamic waxing and waning process.⁴³ Scoring methods used to assess the extent of hepatic fibrosis vary, and may give rise to different scores.⁴⁹⁻⁵¹ In addition, it is argued that although fibrosis assessment is an important determinant of the stage of liver pathology, fibrosis and staging are not equivalent.⁵² Rossi *et al.* state that using a categorical scoring system to determine a continuous variable such as liver fibrosis is flawed, and that the assumption inherent in fibrosis scoring scales that fibrosis increases in a linear fashion between stages is incorrect.²⁶

As liver biopsy cannot be used as a screening tool, and there are difficulties using it to monitor the effectiveness of therapeutic interventions, other possibilities have been explored. There are a number of non-invasive alternative analytical methods in current usage and they can be divided broadly into clinical imaging techniques, specialised tests of liver stiffness, breath tests and serum markers. Their strengths and shortcomings are discussed hereafter.

Non-invasive methods of assessing liver fibrosis

Imaging modalities

The current clinical imaging techniques can identify liver pathology but are unable to distinguish between the degrees of injury and may miss subtle damage.^{13,30,47,49,53}

Generally, current imaging techniques probably should be reserved for the assessment of portal hypertension or the presence of hepatocellular carcinoma in patients with established cirrhosis.⁵⁴

Ultrasound is the usual first-line imaging investigation.⁵⁵ It can detect moderate fatty infiltration of the liver as fat attenuates the sound beam, but is relatively insensitive and cannot detect mild to moderate fibrosis.^{14,45,48} The success of ultrasound is also markedly operator-dependent.^{48,55} Computer tomography and magnetic resonance imaging (MRI) provide semi-quantitative information, may miss mild injury, cannot always differentiate between the degrees of damage and are subject to interference from conditions such as iron overload.^{14,48,54}

Transient elastography

Transient elastography, a commercially patented technique, uses liver stiffness as a surrogate for liver fibrosis. It employs a modified ultrasound probe to send vibrations through the liver and measures the rate of transmission of these waves which is a function of the stiffness of the tissue.⁹ It is rapid, can be done as an outpatient procedure, is painless and allows repeated examinations.⁴⁷ It has been validated in liver

pathology secondary to various pathologies.⁸ It samples an area 100 times larger than does biopsy.⁴⁹ Further, it appears to be operator independent if performed as per the manufacturer's instructions even when comparing novice operators with minimal training with experienced operators.⁸ Generally, it performs excellently and may in time replace biopsy as the gold standard.⁵⁶ However, it is unreliable in obese people, struggles in the presence of even minimal ascites, is very expensive and may not perform well at detecting minimal degrees of fibrosis.^{8,15,45,56} Invalid or uninterpretable results are found in nearly 20% of patients.¹²

Breath tests

There are a number of breath tests employing radioactive isotope ¹³C-labels such as ¹³C-methacetin.¹⁶ These generally perform well but require the use of radio-isotopes and specialized scintillation detectors and are not universally available.⁴⁵ They are not in general clinical practice use.

Serum biomarkers

Serum is an attractive area for biomarker detection because of its low cost and accessibility.⁷ Individual commonly used biochemical screening tests such as alanine transaminase (ALT) levels correlate very weakly with the degree and activity of fibrosis seen at histology although they provide important information on other aspects of liver function.^{6,14,26} It is inaccurate to assume that all people with a normal ALT level are free of liver dysfunction.^{14,15} Surprisingly, data have shown that low levels of dehydroepiandrosterone-sulphate (DHEA-S) are seen in patients with non-alcoholic fatty

liver disease (NAFLD), and patients with DHEA-S levels above a certain cut-off are unlikely to have severe NAFLD.⁷

A number of novel biomarkers of liver injury have been investigated such as procollagen type III amino terminal peptide (P3NP) and hyaluronan.^{13,26,47,57} P3NP is the most widely studied marker and its levels correlate with the degree of fibrosis.^{47,58} However, it can be elevated in pathology affecting other organs such as acromegaly, chronic pancreatitis and lung fibrosis.⁵⁷ None of the other markers investigated to date appears sufficiently reliable for routine use.^{56,57} Most biomarkers that have been studied are good at detecting and quantifying moderate to severe fibrosis but miss subtle fibrotic injury or are unable to distinguish between mild degrees of fibrosis.^{5,15}

The use of test panels, such as liver function tests, has come under increasing scrutiny because of cost and concerns regarding unnecessary testing.¹⁷ However, ratios such as the aspartate transaminase to platelet index (APRI) may offer many benefits not imparted by single tests.^{2,7,20} APRI has been extensively validated in hepatitis C infection and appears to be a reasonable low-cost test suitable for daily practice.⁵⁷ The ratios in common usage generally use analytes that are routinely measured in patients with liver disease.⁴⁵ However, many of the various models are unable to classify correctly almost 25-50% of patients or use patented multi-variable derivatives that require the payment of royalties.^{15,17} Currently, most serum models are used in parallel with, rather than as replacement for liver biopsy.²⁶

Prolidase

Early fibrosis is characterised by the deposition of collagen which is a protein that is particularly rich in the amino acid proline.^{27,28} There is even evidence that the proline component of collagen increases slightly with increasing fibrosis.²⁹ Dipeptides where proline or hydroxyproline constitutes the carboxy-terminal amino acid are resistant to cleavage by non-specific peptidases, and require a dedicated peptidase known as prolidase (peptidase D, imidodipeptidase, proline dipeptidase, aminoacyl-L-proline hydrolase; EC 3.4.13.9) which cleaves such dipeptides which are mainly collagen breakdown products.^{32,59-63} This allows up to 90% of proline to be recycled in collagen biosynthesis.^{32,61,63} In periods of sustained collagen deposition such as fibrosis, prolidase may become the rate-limiting step.^{27,63,64} Prolidase activity is induced in settings of increased fibrosis and this may be reflected in increased serum prolidase enzyme activity (SPEA) levels.^{29,33,65} The exact mechanism of this induction is not clear but it is theorised that type 1 collagen in the extra-cellular matrix binds to and stimulates prolidase activity via cell surface β_1 -integrin receptors.⁶⁵ Further, insulin-like growth factor-1 (IGF-1) can stimulate collagen production on binding the IGF-1 receptor and via the same mechanism can stimulate prolidase activity.⁶⁴

The characteristics of prolidase

Prolidase, a 493 amino acid polypeptide, was first identified in 1923.³² It is a 110 kDa homodimer.^{31,66,67} The 15-exon gene that encodes a 2.2 kb mRNA product is located on

chromosome 19p13.2 and spans a region > 200 kb of DNA.^{63,68} The protein product undergoes post-translational modification via glycosylation and phosphorylation.²⁸ Its three-dimensional structure has been characterized (Figure 1). It is found in many tissues and it is most abundant in the kidney and erythrocytes.^{32,33}

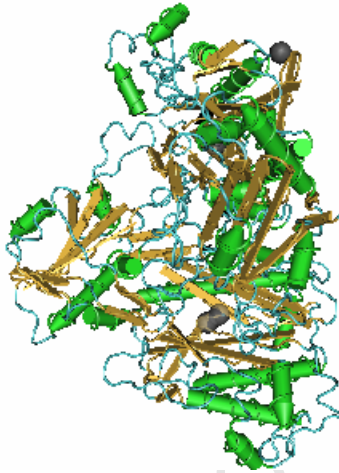


Figure 1: Crystal structure of human prolidase (<http://www.ncbi.nlm.nih.gov/sites/entrez#>)

Studies have shown that prolidase activity is dependent on Mn^{2+} , temperature and pH, with a pH optimum of 8.0 - 9.0 and a temperature optimum of 37 °C.⁶⁰⁻⁶² Colchicine increased the activity when glycyl-proline but not valyl-proline was used as a substrate.³¹ The reason for this observation is not clear. Prolidase is inhibited by various substrates including nickel and the product of its reaction, proline, in a competitive manner.^{66,69}

The catalytic mechanism requires that the proline residue of the substrate is enfolded in a hydrophobic pocket, consisting of six strands with a beta configuration, which ensures the correct spatial orientation of the peptide bond.⁶⁰ Five of these strands bind metal

ions.⁶⁰ A water molecule becomes polarized by interaction with manganese and/or sodium ions, although cobalt and zinc metal ions can also be used, and is positioned for nucleophilic attack on the carbonyl carbon of the peptide bond.^{66,70} A conserved glutamate temporarily extracts a proton from this nucleophile. A histidine residue and one of the metal ions stabilize the intermediate produced. Finally, the glutamate donates the proton it captured from the nucleophile to the amine leaving group, thereby converting the intermediate to the final product.²⁸

Prolidase deficiency

The congenital absence of prolidase (prolidase deficiency (PD)) was first reported 40 years ago.³² The demonstration of reduced prolidase enzyme activity was published in 1974.⁷¹ To date, only 60 or so case reports have been published, although its apparent rarity might reflect unawareness on the part of physicians.^{28,29,32} PD is inherited in an autosomal recessive manner.³² Mutations in the highly conserved catalytic region appear to be responsible although the pathophysiology remains poorly understood.²⁸ The phenotype is variable but skin ulceration of the lower limbs is the hallmark,^{72,73} along with mental retardation and recurrent respiratory infections.⁶⁰ The mental retardation may arise from cerebral microthrombi identified on MRI in patients with PD.⁷⁴ There is a characteristic facies with frontal bossing, a low hairline, hypertelorism, proptosis and saddle-nose deformity.^{32,73} Immune abnormalities include recurrent infections, defective wound healing, increased serum immunoglobulin and low levels of complement 1q, which contains approximately 25% proline.^{28,32} The clinical

heterogeneity implies that environmental factors are important, and some patients with documented genotypic mutations are apparently asymptomatic.^{67,75}

The biochemical picture is of severe imididopeptiduria.⁷⁵ Chromatographic examination of the urine reveals high levels of glycyl-proline and glycyl-hydroxyproline, and reduced enzyme activity can be shown in leucocytes or fibroblasts.^{28,32} There is no specific treatment, but Mn²⁺ and vitamin C supplementation have been used with varying success, and blood transfusions, apheresis and corticosteroids have also been employed.^{28,71,76} There is no experimental animal model to further the study of PD.⁷⁷

Other uses for prolidase

Prolidase antagonises the toxic effects of organophosphorous compounds, and has been investigated by the military defence industry.⁷⁸ It is used in the food industry to reduce bitterness in cheese.²⁸ High levels of prolidase are found in melanoma and breast cancer cells and there is research to produce a chemotherapeutic prodrug, that is a proline analogue of melphalan, that requires prolidase to cleave it to release the active metabolite, thereby allowing for targeted drug delivery.^{28,79,80}

Prolidase as a marker of hepatic fibrosis

The biological plausibility of using serum prolidase as a marker of liver fibrosis has been established in rat models.^{4,27,30,59,81} SPEA has been reported as being significantly higher in rats that underwent irreversible bile duct ligation compared with those who had a sham operation or controls ($p < 0.001$).²⁷ Following irreversible liver damage, the liver

collagen content, histological grading of fibrosis and SPEA all increased together, rising to a peak, before declining in synchrony once cirrhosis was evident.^{27,58,59} However, Zuyderhoudt *et al.* failed to demonstrate an association between plasma prolidase and rat hepatic fibrosis and ascribed increases in plasma prolidase to leakage from damaged liver cells rather than induction of its activity because of fibrosis.⁸²

There are few clinical studies examining SPEA levels in humans with liver disease. Myara *et al.* measured plasma prolidase activity in a population of patients with liver disease and other disorders.²⁹ Plasma prolidase activity was compared with the degree of liver fibrosis at biopsy in 13 patients in this series and the authors concluded that there was no correlation between the two.²⁹ The statistical handling of the data in this study is a major weakness. A reference distribution that is log-gaussian would not be symmetrical about the mean when back transformed. In addition, the population used to establish the reference interval included patients with liver disease, whereas patients with overt and occult disease should be excluded during the establishment of a reference interval.⁸³ Therefore, this published reference interval needs to be viewed circumspectly, but these values are still referred to in other studies.³⁰ The authors speculated that, as prolidase activity was elevated only in some and not all of the patients with cirrhosis, that enzyme activity varied with the stage of the disease, increasing in the early stages and declining as cirrhosis advanced.²⁹ Importantly, it is not clear what criteria were used to confirm liver disease except for 13 patients who were explicitly stated to have undergone a biopsy.

Brosset *et al.* found a correlation between the findings at biopsy and plasma prolidase in a study of 53 adult “alcoholic” patients with alcohol intake > 60 g/day, but could not use plasma prolidase to distinguish between early fibrosis and more severe pathology.⁸¹ There are a number of limitations to this study which makes it quite difficult to generalise the findings. The means by which patients were identified and selected were not described. Further, although patients were described as “alcoholic”, there was no indication as to how this diagnostic label was reached, or how the quoted daily alcohol consumption was determined or confirmed.

Recently, Kayadibi *et al.* performed a prospective study which enrolled 54 patients and 17 healthy controls and used SPEA to distinguish successfully between steatosis and steatohepatitis.³⁰ Liver biopsy was the gold standard used in the assessment of NAFLD. This study showed a positive correlation between raised SPEA and liver fibrosis ($p < 0.001$) and a negative predictive value of 92% for fibrosis at a SPEA cut-off value of 1 295 IU/L. Significantly, none of the patients had severe fibrosis at biopsy. Other confounding variables such as obesity and other causes of fibrosis were not accounted for.⁷

Horoz *et al.* showed an association between SPEA and the degree of fibrosis in a prospective study in patients with non-alcoholic steatohepatitis (NASH).⁸⁴ Levels were

significantly higher in the 36 consecutive patients with NASH than in the 29 controls ($p = 0.016$). There was no patient documented with cirrhosis in this study.

Prolidase levels in pathology of non-hepatic origin

Collagen is distributed widely in the body and one concern is whether SPEA levels are altered in other situations of increased collagen deposition and/or breakdown. One such circumstance is osteoporosis, as type I collagen comprises ~90% of the bone matrix and post-menopausal osteoporosis is associated with increased degradation of the organic matrix of bone.³⁴ A study of post-menopausal women with osteoporosis found no correlation between SPEA and bone mineral density (BMD), measured with dual energy X-ray absorptiometry ($p > 0.05$).³⁴ This finding has been replicated.⁸⁵ SPEA is unrelated to age and sex, and does not appear to be increased by bone tumours of either primary or secondary origin.³⁴ There is no increase in SPEA in uraemic bone disease. A study involving 44 participants found that end-stage renal disease was not associated with increased levels of serum prolidase.³³ Normal levels have been reported in patients with acute hepatitis and cholelithiasis.²⁹

Aslan *et al.* reported that SPEA levels were significantly higher in patients with *H. pylori* than in those without infection in patients investigated for non-ulcer dyspepsia.³⁵ The authors speculated that this might reflect increased gastric fibrosis caused by chronic infection. There are reports that SPEA may be increased in patients with asthma managed with theophylline, in hypertensive patients and in breast cancer.^{34,38,86} In type

2 diabetes mellitus (T2DM), vascular basement membrane is characterised by the presence of increased connective tissue.⁸⁷ Reports from patients with T2DM are conflicting with both elevated and reduced levels of SPEA being demonstrated.^{34,87} Elevated levels have been shown in fibroblasts obtained from keloid tissue.⁸⁸ A recent report showed elevated levels of prolidase activity in amniotic fluid from patients with neural tube defects.⁸⁹ Intra-uterine growth retardation is postulated to result from increased extra-cellular matrix turnover in the placenta resulting in impedece to vessels and relative hypoxia to the foetus.³⁹ Toy *et al.* demonstrated elevated maternal SPEA levels in a cross-sectional study in mothers with infants who had foetal growth retardation.³⁹ Tuberculosis (TB) is still an unfortunately common condition in the Western Cape province of South Africa with significant morbidity and mortality. The assessment of whether pleural effusions are tuberculous in origin remains invasive and a significant part of laboratories' workload in the developing world. Both serum and pleural fluid prolidase have been reported as being significantly elevated in pleural effusions of tuberculous origin compared with other aetiologies in a cross-sectional study.⁹⁰

Asthma is a chronic airway inflammatory condition, and lower SPEA levels have been described in children with asthma compared with healthy controls.³⁷ The authors speculated that this represented established fibrosis with little collagen turnover.³⁷ Prolidase deficiency has been reported in a few case reports of patients with systemic lupus erythematosus (SLE) and there are suggestions that it may impart increased risk of

developing SLE.^{68,74,91,92} Galicka *et al.* documented low levels of prolidase activity in cultured fibroblasts from a patient with mild osteogenesis imperfecta.⁶⁴

A Turkish group has published a number of studies documenting increased SPEA associated with evidence of increased oxidative stress in diverse clinicopathological conditions.^{35,37,39,89} The significance of these data is unknown.

Measuring and reporting SPEA

The most common way to measure SPEA is the colorimetric method of Chinard as modified by Myara *et al.*,²⁹ but there have been a number of refinements reported and used in the evaluation of SPEA. This has resulted in a wide range of values reported in the literature for SPEA ranging from mean values of 40 to 1 500 IU/L (see table 1).^{27,30,34,35,37} What is much harder to explain is the discrepancy in values obtained using the same methodology from what appears to be the same group in Turkey (Table 1).^{35,37,84,87} A further concern is the methods employed to represent the data. As highlighted earlier, some of the statistical handling of the data and the production of reference intervals are not acceptable. Reporting of data has also been done with mean \pm SD although there is no evidence that the reference distribution for SPEA is gaussian.

Method	SPEA result reported (IU/L)	Reference
Classical method described by Myara with 24 h incubation of serum with 2.5 mM Mn ²⁺ in a 1 in 6 serum dilution, Used 94 mM glycyL-proline substrate concentration	900 (520) (mean (SD))	27
Used a higher concentration of Mn ²⁺ than Myara, also added glutathione to the sample incubation mixture. Pre-incubated for only 3 h. Used much higher gly-pro (144mM) substrate concentration	972 (862 - 1122) (median (IQR))	29
Serum diluted 40-fold with 2.5 mM Mn ²⁺ . Low levels of gly-pro used (30mM). 3 h incubation	80.7 (16) (mean (SD))	84
Serum diluted 40-fold with 2.5 mM Mn ²⁺ . Low levels of gly-pro used (30mM). 2 h incubation	1 613.84 (607.25) (mean (SD))	34
Serum diluted 40-fold with 2.5 mM Mn ²⁺ . Low levels of gly-pro used (30mM). 2 h incubation	39.18 (5.4) (mean (SD))	35
Serum diluted 40-fold with 2.5 mM Mn ²⁺ . Low levels of gly-pro used (30mM). 2 h incubation	1 408 (162) (mean (SD))	37
Serum diluted 40-fold with 2.5 mM Mn ²⁺ . Low levels of gly-pro used (30mM). 2 h incubation	53.3 (2.2) (mean (SEM))	87

Table 1: Description of the differences in the methodology employed, results obtained and the reporting thereof. IQR: interquartile range; mM: millimoles/litre; h: hour; SD: standard deviation; SEM: standard error of the mean

Future research

It is evident from the above literature review that a number of basic issues need to be addressed in the prolidase field. Firstly, evidence of the biological variation of SPEA is required. This would be aid in the assessment as to the potential usefulness of reference intervals.⁸³ Knowledge of biological variation would also guide knowledge as to the required analytical CV which is often targeted at a value half the biological CV to minimize the noise added by the analytical technique to the width of the reference interval. Further, information on biological variation is required when calculating

reference change values which would be useful if SPEA were employed in monitoring.⁹³ There is no information on SPEA levels or characteristics in the paediatric setting. Information on ethnic differences, influence of diet, circadian rhythm if any, and preanalytical factors is lacking. There exists an urgent need to establish a reference interval using appropriate methods which may well be method-specific. Most biomarkers of liver fibrosis have been studied in cross-sectional studies and longitudinal studies are needed to provide data as their role in establishing prognosis, monitoring disease activity and response to therapy.^{12,15,16} The reported increase in SPEA in patients with TB pleural effusions needs to be investigated further, given the high prevalence of TB in the Western Cape. Levels in patients with TB without pleural effusions should be investigated. In summary, the investigation of SPEA is in its infancy.

Any research on a potential biomarker requires a reference standard against which to assess the diagnostic performance of the measurand in question. In liver dysfunction, this remains the liver biopsy which is an imperfect standard as discussed earlier.¹² Its very limitations may result in a falsely low assessment of the diagnostic accuracy of a new method to measure liver fibrosis, even if that method were perfectly sensitive and specific.⁵³ Limiting the sample to patients undergoing liver biopsy may introduce a selection bias as these patients have more severe pathology, which might limit the generalisation of any findings. However, it is questionable whether it would be ethical to biopsy healthy people, given the real, albeit negligible, risks involved. Transient elastography may overcome this issue.

Part C Manuscript

This part is a stand-alone manuscript that is written to be included in a relevant peer-reviewed journal in accordance with the journal's requirements as set out in the "Instructions for Authors". This article is written for the journal, *Annals of Clinical Biochemistry*, in accordance with their requirements (Part D). The manuscript is therefore laid out with an abstract, introduction, methods, results, discussion and the tables and figures thereafter rather than being included in the text. As the journal has a limit as to the number of tables and figures that may be presented, a supplementary data section follows that includes relevant supplemental figures and tables (Supplementary data). However, there is no separate list of references for this section and the common reference list is to be consulted. Unfortunately, as this is a separate manuscript, some of the previous discussion from Part A and B will be repeated here. This manuscript is written in accordance with the Reporting studies of diagnostic accuracy (STARD) guidelines (page 61. 62).⁹⁴

Part C The sensitivity and specificity of serum prolidase activity as a marker for liver fibrosis in suspected liver disease

Liver disease elevates serum prolidase levels

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Declarations

Competing Interests: None.

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Ethical approval: Informed consent was obtained from all liver biopsy patients. All samples were anonymised and confidentiality was maintained. This study was approved by the UCT Research Ethics Committee (*Rec Ref: 435/2009*).

Guarantor: JCS.

Contributorship: JCS obtained the samples, took informed consent, validated the method, analysed the samples, data and prepared the manuscript. ML analysed the liver biopsies. PB validated the method and prepared the manuscript. TSP designed the study and prepared the manuscript.

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Abstract

Background Liver dysfunction is common and often unrecognised. Liver biopsy is the gold standard in the assessment of liver fibrosis, but has disadvantages. Many viable options have been investigated. We assessed the diagnostic accuracy of serum prolidase enzyme activity (SPEA) in predicting the presence and degree of liver fibrosis, as compared with liver biopsy.

Methods We undertook a prospective case control study. 38 outpatients without apparent liver illness and 20 patients with liver pathology scheduled to undergo liver biopsy had their SPEA levels measured. We evaluated a SPEA assay for linearity, imprecision, stability of the measurand and the effect of haemolysis. We used the data from the control group to establish a reference interval.

Results The SPEA assay is linear and has reasonable imprecision. It is affected by haemolysis and freeze-thaw cycles should be avoided. A reference interval of 22 - 504 IU/l was obtained. Patients undergoing liver biopsy had higher SPEA levels (361 (268) IU/L [median (interquartile range)]) compared with controls (169 (160) (p < 0.001)). A SPEA cut-off 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%.

Conclusions Higher SPEA levels in patients undergoing liver biopsies compared with controls may reflect the presence of liver fibrosis. SPEA levels could not be used to stage the degree of fibrosis. The utility of SPEA as a biomarker of liver injury should be investigated further.

Introduction

Liver disease is common,^{1,13} likely to increase in prevalence,¹ but often silent with many patients developing advanced disease.¹⁸ Ongoing injury may result in fibrosis which is associated with significant morbidity and mortality but is treatable even when advanced.^{4,11}

Liver biopsy followed by histological examination is the accepted reference standard in the investigation of patients with suspected liver disease.^{10,43} However, it has multiple potential problems owing to its invasive nature,⁴² and it may not be as accurate as often believed.^{2,17,47,48} Other methods that have been investigated such as clinical imaging techniques,^{13,14,30,48} measures of liver stiffness such as transient elastography,^{9,11,49,95} individual serum biomarkers and combinations thereof all have various shortcomings.^{2,11,13,14,18,42,47,48,96}

Prolidase is a cytosolic enzyme that cleaves collagen breakdown products,³² is important in collagen recycling and is the rate limiting step in fibrosis;⁶⁴ moreover, its activity is induced in periods of fibrosis.^{29,64} Levels in the serum are low,³² but experiments that induced irreversible liver injury in rats, showed a strong correlation between the degree of resulting fibrosis and the serum prolidase enzyme activity (SPEA) levels.^{4,27,59}

Although a number of cross-sectional studies have measured prolidase in various clinical situations such as gastritis,³⁵ asthma,³⁷ inter-uterine growth retardation,³⁹ left ventricular hypertrophy,³⁸ osteoporosis,^{85,87} bone disease seen in chronic kidney disease,³³ tuberculosis,⁹⁰ breast cancer,⁸⁶ and neural tube defects,⁸⁹ there is a paucity of studies in the setting of its main indication, liver fibrosis. Myara *et al.* measured plasma prolidase activity in 338 hospitalised and outpatient French patients with various disorders.²⁹ 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. Brosset *et al.* measured plasma prolidase enzyme activity and compared these to the liver histology results on 53 adult patients with alcoholism who had alcohol intakes in excess of 60 g/day. They found that levels of SPEA correlated with both cirrhosis and alcoholic hepatitis, but could not use it to distinguish between early fibrosis and more severe pathology.⁸¹ A recent prospective study of 54 patients and 17 healthy controls used SPEA to distinguish between steatosis and steatohepatitis (NASH) in patients with non-alcoholic fatty liver disease (NAFLD) ($p < 0.001$).³⁰ Horoz *et al.* showed that SPEA levels were significantly higher in 36 consecutive patients with NASH, compared with controls ($p = 0.016$).⁸⁴ Therefore, we undertook a study to ascertain the diagnostic accuracy of SPEA compared with liver biopsy and whether it could be used as a biomarker to assess the potential presence and degree of liver fibrosis in our local setting.

Methods and Materials

The study was approved by the University of Cape Town's Research Ethics Committee.

Written informed consent to be included in the study was obtained from all participants undergoing liver biopsy.

A total of 64 patients were enrolled in the study, which included 28 people undergoing liver biopsy examination 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 analysed on the patients undergoing biopsy. A control group of serum samples from 38 outpatients at the same hospital were used (Figure 1 and Table1).⁹⁴ These were patients who were attending hospital for non-liver related pathology and did not have liver related tests requested. Demographic data to ensure that the samples were from adults were checked, but no other data were captured.

Prolidase Assay

Serum samples were stored at -80°C before being analysed using a well-described spectrophotometric method based on the reaction of proline with Chinard's reagent.²⁹

5 μl of serum was incubated for 2 h 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. Colour 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 proline standard curve of increasing concentrations of proline (Sigma Aldrich) in 6.7 % (w/v) TCA. SPEA levels were reported as µmol proline formed per min per litre serum (IU/l). Quality control was performed via frozen aliquots of a single serum analysed in each run. This colorimetric method has a very low coefficient of variation.³⁰ The person performing the SPEA assay (JCS) was blinded as to the results of the liver biopsy.

Methods used to investigate prolidase assay

The linearity of the assay as well as an assessment of the stability of SPEA at room temperature, freezing and with repeated freeze-thaw cycles were performed. Intra-assay CV was performed by measuring a pooled serum sample 10 times. The effect of haemolysis was assessed on a pooled serum sample using a published modified osmotic shock method.⁹⁷ Briefly, a haemolysate was made by centrifuging whole blood obtained from a volunteer and thereafter the plasma was removed. The red cells were washed five times with equal volume normal saline, and then stored at -20° C overnight with an

equal volume of distilled water. This was then centrifuged and the haemoglobin (Hb) in the supernatant measured. A range of haemolysates 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. 100 μ l of increasing haemolysate (100 μ l saline was used as a blank) was added to 900 μ l pooled serum. In addition, to ascertain the comparability of published methods, 10 pooled serum samples were analysed using two well-described assays of Myara and Kayadibi and a comparison was done.^{29,30} A reference distribution was established using the control samples (n = 38).

Cultured fibroblasts

5 cell lines (n = 10) of skin fibroblasts used as controls in routine investigations and that were close to their expiry date and due to be discarded were used. The fibroblasts were grown in duplicate under standard conditions (see Appendix). Briefly, the fibroblasts were grown in Dulbecco's modified eagle's medium and foetal calf serum with added glutamine and antibiotics. They underwent two passages of tryptinisation before being stored. Confluent fibroblasts were harvested and washed, before being resuspended in 0.3 mL 50 mmol/l Tris-HCl (pH 7.8). The fibroblasts were sonicated for three cycles of 5 bursts each at 30% power and duty cycle, and placed on ice between cycles. The supernatant was used for protein determination and 0.1 ml supernatant fluid was pre-incubated with 0.1 ml 0.05 mmol/l Tris HCL (pH 7.8) containing 2 mmol/l $MnCl_2$ for 2 h at 37° C before proceeding using the method described above.

Histopathological assessment of liver biopsy

The liver biopsies were stained with haematoxylin and eosin, and further slides were made with bile sirius red stains. The degree of fibrosis at biopsy was assessed using a well-characterised scoring system by one pathologist who was blinded to the results of SPEA analysis but who had access to the patient's records if required (ML).⁵⁰

Statistical analysis

Statistical analysis was undertaken on Microsoft Excel and Statistica 9.0. Data became gaussianly distributed 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 box-and-whiskers plots drawn. $P < 0.05$ was taken as indicative of statistical significance.

Results

Prolidase assay

Linearity of the prolidase assay was demonstrated by assaying a serum sample neat and at increasing dilutions (Supplemental data Figure 1S). This agreed with previous reports.²⁹ SPEA activity appeared stable at room temperature for one week and at -20° C for two months, but repeated freezing and thawing resulted in decreases in activity, and showed a decrease of 15% with the first cycle (Figure 2 & supplemental data Figure 2S & 3S). We obtained an intra-assay co-efficient of variance (CV) of 11% which

was higher than that reported by Kayadibi *et al.*³⁰ Haemolysis was a significant interferant in the prolidase assay and even low levels of haemolysis affected the obtained results (Figure 3). Haemolysed samples should be rejected. Comparison of SPEA levels obtained using the methods described by Myara and Kayadibi provided different results which were not comparable ($R^2 = 0.38$) (Figure 4, supplemental data Figure 4S).

Reference interval

The SPEA value for one participant in the control group was removed as an obvious outlier. The reference distribution became gaussian when the square root of the data was taken (Figure 5). A mean value of 174 IU/l was established with a range of 22 – 504 (mean \pm 2SD) when the data were back-transformed.

Fibroblast assay

The 5 fibroblast cell lines yielded prolidase activity of 342 (43) nmol/min/mg protein (mean (SD)). This is somewhat higher than the results reported by Galicka *et al.*⁶⁴

Liver biopsy and SPEA assay

No adverse events were reported in any of the patients. The characteristics of the patients who underwent liver biopsy are shown (Table 2). Some patients did not have all tests performed during this admission. In those cases, all available data were

analysed. The fibrosis assessment at biopsy and accompanying SPEA are shown (Table 2, supplemental data Figure 6S).

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 patients who had a liver biopsy tested positive for HIV, hepatitis B and/or syphilis. No patient returned a positive test for hepatitis A or C infection.

The patients who underwent liver biopsy had higher SPEA levels than the controls that did not ($p < 0.001$) (Figure 6). SPEA values were: control group 166 IU/L (22-504) (mean \pm 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 SPEA levels between the groups with minimal and extensive fibrosis ($p = 0.59$) (Figure 11).

Receiver operating characteristics curve analysis revealed an optimum cut-off of 200 IU/L for the detection of fibrosis (Figure 12). This SPEA 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%. 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%,¹¹ this translates into a NPV of 99.5% and a PPV of 5.9% (Table 3).

Discussion

This study demonstrated that SPEA levels were significantly higher in patients undergoing liver biopsy than in control patients who were attending outpatients and assumed to be free of liver pathology. The lack of liver biopsies on the group of controls is problematic, but ethically it is difficult to justify biopsy of apparently healthy people given the real, albeit small, risks involved.⁴² Therefore, there may be individuals in the control group with liver fibrosis. If this is the case, this would serve to reduce the apparent discriminatory power of SPEA analysis in this study. Setting this study in a tertiary institution introduces spectrum bias that may make the findings less generalisable.⁴⁹

We established that the reference distribution is square root-gaussian in appearance and using a group of outpatients established a reference interval that is different to that previously published.²⁹ Myara *et al.* established a reference interval from a group of patients many of whom had established liver disease and it is not clear whether appropriate statistical techniques were employed in generating the reference interval;²⁹ this would not be an acceptable means to establish reference intervals today.⁸³ The use of outpatients may be questioned but has been proposed as a proxy of healthy people

and has been used to establish reference intervals in other studies.⁹⁸ We have shown that results are not interchangeable between methods and therefore suggest that reference intervals are method dependent.

Fibrosis is often, but not inevitably, found in chronic liver injury and this may reflect the apparent lack of resolving power of SPEA analysis when examining the sensitivity, specificity and related data.¹⁸ At a cut-off SPEA 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 mirrors the findings of other researchers.³⁰ A negative likelihood ratio at this cut-off is 0.19 which is above the suggested value < 0.1 for an excellent test.⁴⁹ Liver biopsy is the current gold standard but it has certain limitations that should be considered when assessing the diagnostic accuracy of a new marker.^{42,53} The use of transient elastography as the reference method may obviate this problem in future studies.

There was no significant difference in SPEA in the group with minimal compared with extensive fibrosis as determined by liver biopsy. This is apparently at odds with the results reported by Kayadibi *et al.* but they had no patients with moderate to severe fibrosis in their study.³⁰ However, there appears to be an increase in SPEA with mild-moderate fibrosis, with a reduction once fibrosis becomes more severe. This trend is not statistically significant but replicates other published data.^{27,37,59} It has been suggested

that more advanced fibrosis is characterised by a slower turn-over of collagen than states of moderate fibrosis and this may lessen the induction of prolydase activity.²⁹

This study demonstrated that SPEA reportable range is linear, has a reasonable CV for a manual test and is quite stable but that freeze-thaw cycles should be avoided. The values that were obtained for SPEA in this study were lower than those reported in several other studies but this appears to be due to differences produced by the analytical methods used.^{29,30,34,37} There is no agreed reference method or standard reference material available.

This study has shown that haemolysis is a positive interferant in SPEA analysis because prolydase levels are high in erythrocytes. No haemolysed samples were used in this study. It is not clear how any haemolysed samples, if any, were handled in previous studies.^{29,30,34,37}

Cultured fibroblast prolydase activity was reduced in one case report of a family with osteogenesis imperfecta;⁶⁴ expression of prolydase in fibroblast cultures should be more thoroughly investigated. To this end this paper has demonstrated the feasibility of measuring prolydase activity under local conditions, and provided values that may be of use for other researchers.

There is as yet little knowledge as to the biology of SPEA. Future areas of research include further diagnostic accuracy studies, SPEA levels in various clinicopathological 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 people who are infected with HIV, SPEA should be investigated in this setting.²⁰ Tuberculosis remains a significant source of morbidity and pathology locally and early promising reports that SPEA can be used to distinguish correctly pleural effusions of tuberculous from non-tuberculous origin should be investigated further.⁹⁰ As yet, we can find no published longitudinal studies looking at the clinical outcomes with prolidase.

There are issues with the current assay that should be addressed. Although the turn-around time of the prolidase analysis has improved dramatically with a reduction in the length of time for the pre-incubation step, this remains extended at two hours and would not fit modern work flow practices and expectations for a routine screening test. Further refinements of the assay have been described which should be investigated.²⁷ Further evolution of the assay may involve the use of an artificial substrate which, when cleaved by prolidase, produces a chromogen directly without the need for further manipulation. The role of other well-known possible interferants, including bilirubin which interferes in numerous assays and may be increased in the population of interest, remains to be elucidated.

Tables and figures

Tables

Category	Mean (SD)	Reference interval
Sex male/female	7/13	
Age (years)	39.9 (16.0)	
AST (U/L)	255 (368)	5-40
ALT (U/L)	387 (688)	5-40
AST:ALT	0.9 (0.5)	
ALP (U/L)	329 (440)	40-120
GGT (U/L)	455 (732)	0-35
Albumin (g/l)	34 (8)	35-52
Bilirubin (total) ($\mu\text{mol/L}$)	122 (147)	0-21
Bilirubin (conjugated) ($\mu\text{mol/L}$)	85 (108)	0-6
HbA1C (%)	9.8 (3.3)	< 6% normal non-diabetic range
Glucose - random (mmol/L)	5.8 (2.7)	< 7.8
Creatinine ($\mu\text{mol/L}$)	63 (16)	Male/Female 64-104/49-90
INR	1.2 (0.4)	
Ferritin ($\mu\text{g/L}$)	4483 (6392)	Male/Female 30-400/15-150
AFP ($\mu\text{g/L}$)	17 (22)	<8
CRP (mg/L)	15.4 (23.3)	0.0-5.0
ESR (mm/hour)	20 (1)	0-15

Table 1: Characteristics of patients who underwent liver biopsy. Values reported are the mean, one standard deviation in brackets, and the local reported adult reference interval. AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; GGT, gamma glutamyl transferase; HbA1C, glycated haemoglobin A1; INR, international normalised ratio; AFP, alpha fetoprotein; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

Patient	Fibrosis score	SPEA (IU/l)
1	0	582
2	0	324
3	1	101
4	1	464
5	1	201
6	1	284
7	1	363
8	1	887
9	2	206
10	2	360
11	2	865
12	2	457
13	2	498
14	3	204
15	3	528
16	3	84
17	3	554
18	3	473
19	3	323
20	4	337

Table 2: Results of fibrosis score at biopsy and serum prolidase enzyme activity (SPEA)

		SPEA		Total
		positive	negative	
Liver biopsy	positive	16	2	18
	negative	16	23	39
Total		32	25	57

Table 3: Results of serum prolidase enzyme activity (SPEA) discriminatory power at a SPEA cut-off level of 200 IU/L

Figures

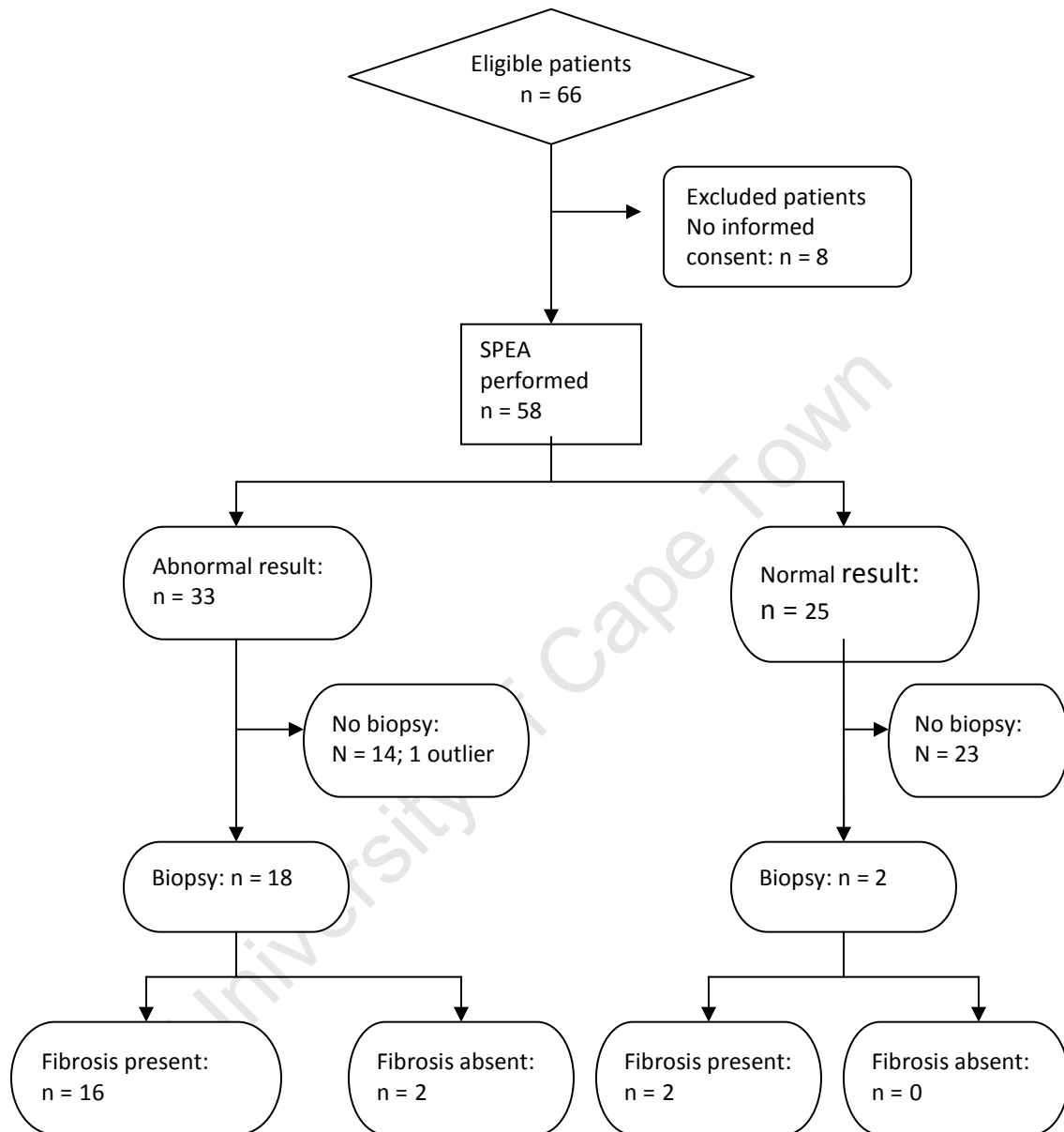


Figure 1: STARD flow diagram detailing the testing process

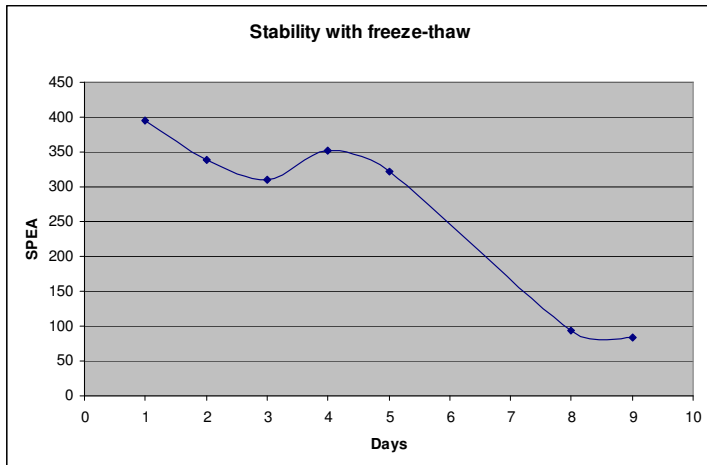


Figure 2: SPEA activity measured on one sample from a volunteer which was subjected to repeated freeze-thaw cycles over a number of days. Freezing was done at -20°C .

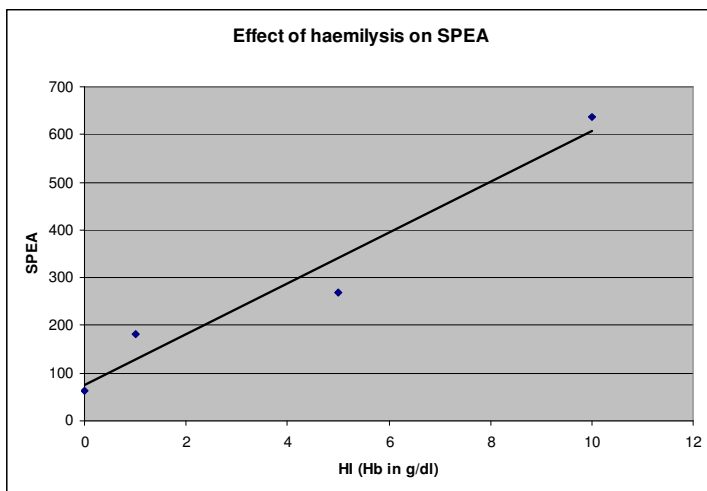


Figure 3: Haemolysis affects apparent SPEA levels, even at low levels of haemolysis

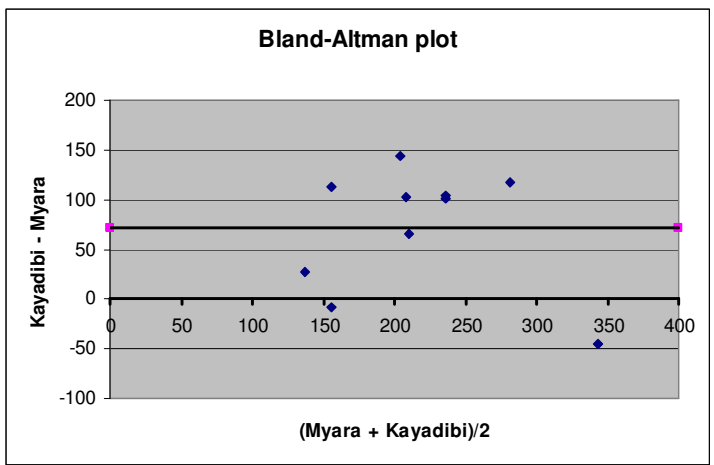


Figure 4: Bland-Altman plot comparing values on 10 samples measured using both Kayadibi and Myara’s method. The solid horizontal line represents the average difference between the two methods.

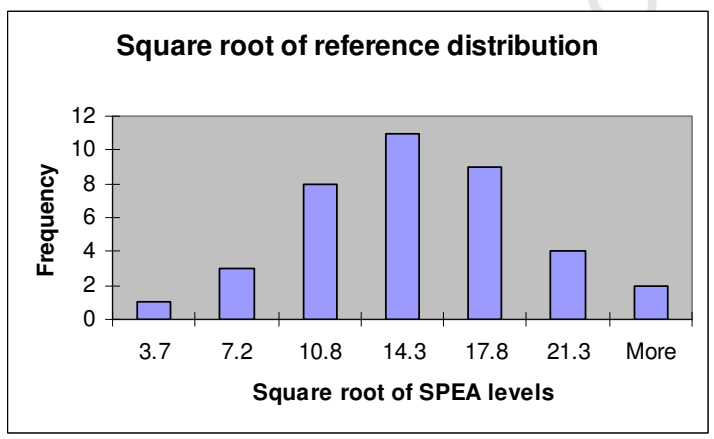


Figure 5: Reference distribution of the SPEA values in the control group (n=38) after the square root of the SPEA values was taken

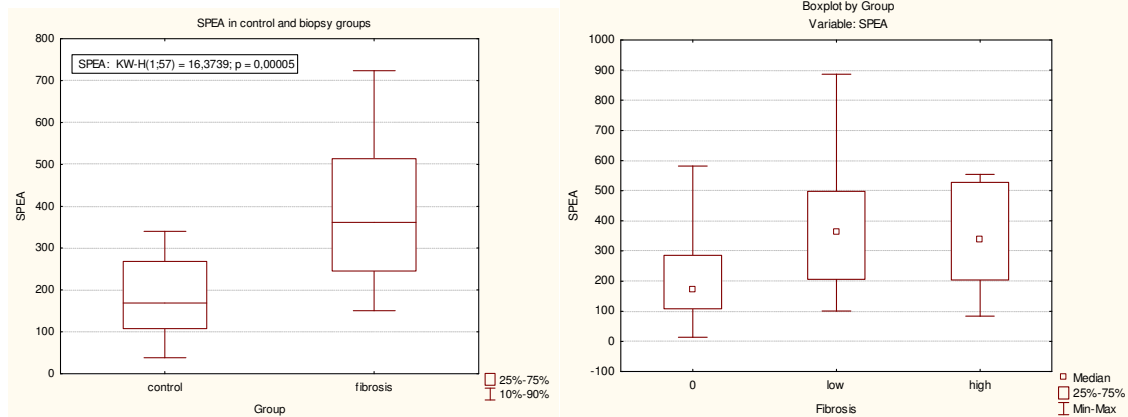


Figure 6A: Box and whiskers plot for serum prolidase enzyme activity (SPEA) in the control group versus the patients undergoing liver biopsy. The horizontal line represents the median, the box 25-75th centiles and the whiskers 10-90th centiles.

Figure 6B: Box and whiskers plot for serum prolidase enzyme activity (SPEA) stratified on the basis of increasing degree of fibrosis. The small box represents the median, the box 25-75th centiles and the whiskers 10-90th centiles.

Part C Supplemental Data

Tables

Section and Topic	Item #		On page #
TITLE/ABSTRACT/ KEYWORDS	1	Identify the article as a study of diagnostic accuracy (recommend MeSH heading 'sensitivity and specificity').	46
INTRODUCTION	2	State the research questions or study aims, such as estimating diagnostic accuracy or comparing accuracy between tests or across participant groups.	47
METHODS			
<i>Participants</i>	3	The study population: The inclusion and exclusion criteria, setting and locations where data were collected.	50, 51
	4	Participant recruitment: Was recruitment based on presenting symptoms, results from previous tests, or the fact that the participants had received the index tests or the reference standard?	50
	5	Participant sampling: Was the study population a consecutive series of participants defined by the selection criteria in item 3 and 4? If not, specify how participants were further selected.	50
	6	Data collection: Was data collection planned before the index test and reference standard were performed (prospective study) or after (retrospective study)?	50, 51
<i>Test methods</i>	7	The reference standard and its rationale.	50
	8	Technical specifications of material and methods involved including how and when measurements were taken, and/or cite references for index tests and reference standard.	51-54
	9	Definition of and rationale for the units, cut-offs and/or categories of the results of the index tests and the reference standard.	64,72
	10	The number, training and expertise of the persons executing and reading the index tests and the reference standard.	53,54
	11	Whether or not the readers of the index tests and reference standard were blind (masked) to the results of the other test and describe any other clinical information available to the readers.	53,54
<i>Statistical methods</i>	12	Methods for calculating or comparing measures of diagnostic accuracy, and the statistical methods used to quantify uncertainty (e.g. 95% confidence intervals).	64
	13	Methods for calculating test reproducibility, if done.	52
RESULTS			
<i>Participants</i>	14	When study was performed, including beginning and end dates of recruitment.	50
	15	Clinical and demographic characteristics of the study population (at least information on age, gender, spectrum of presenting symptoms).	50, 63
	16	The number of participants satisfying the criteria for inclusion who did or did not undergo the index tests and/or the reference standard; describe why participants failed to undergo either test (a flow diagram is strongly recommended).	65
<i>Test results</i>	17	Time-interval between the index tests and the reference standard, and any treatment administered in between.	50
	18	Distribution of severity of disease (define criteria) in those with the target condition; other diagnoses in participants without the target condition.	50
	19	A cross tabulation of the results of the index tests (including indeterminate and missing results) by the results of the reference standard; for continuous results, the distribution of the test results by the results of the reference standard.	64
	20	Any adverse events from performing the index tests or the reference standard.	55

<i>Estimates</i>	21	Estimates of diagnostic accuracy and measures of statistical uncertainty (e.g. 95% confidence intervals).	56,57
	22	How indeterminate results, missing data and outliers of the index tests were handled.	65
	23	Estimates of variability of diagnostic accuracy between subgroups of participants, readers or centers, if done.	
	24	Estimates of test reproducibility, if done.	53
DISCUSSION	25	Discuss the clinical applicability of the study findings.	57-61

Table 1S: STARD checklist for reporting of studies of diagnostic accuracy (*version Jan 2003*). www.stard-statement.org/ (accessed January 2011)

Figures

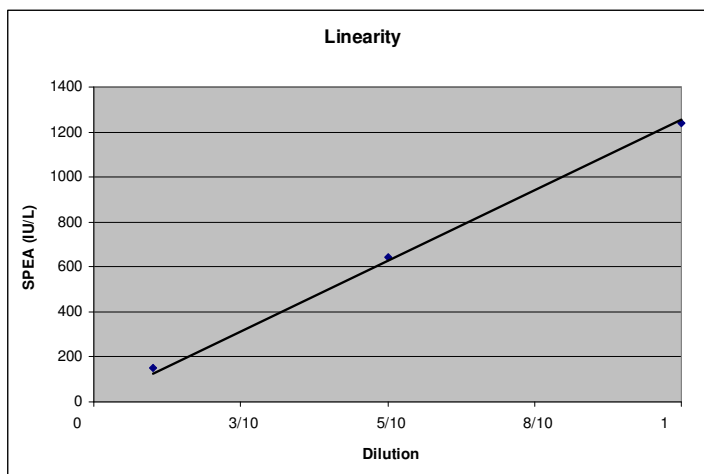


Figure 1S: Linearity of the prolidase assay established by measuring dilutions of a sample with a high SPEA level

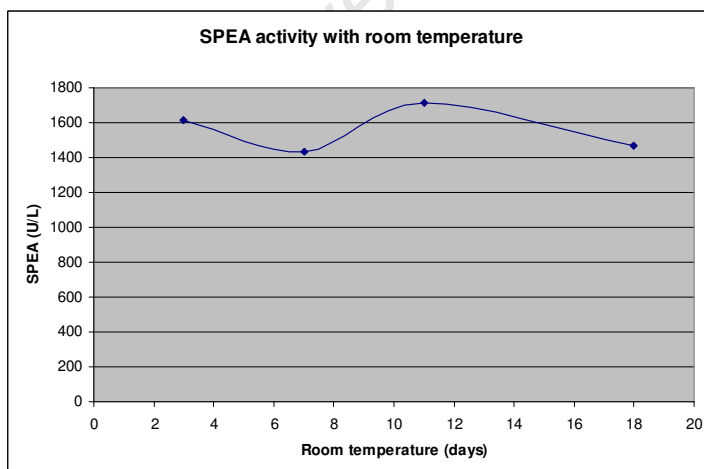


Figure 2S: SPEA activity of a pooled sample aliquotted and stored at 25°C and assayed on various days

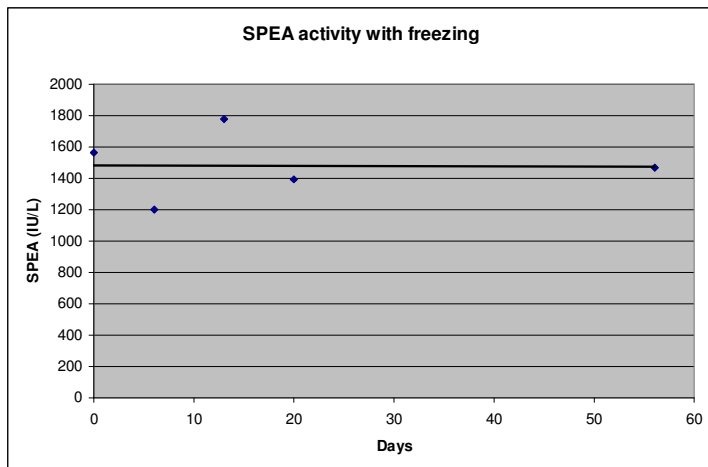


Figure 3S: SPEA levels in a pooled sample aliquotted and stored at -20°C

University of Cape Town

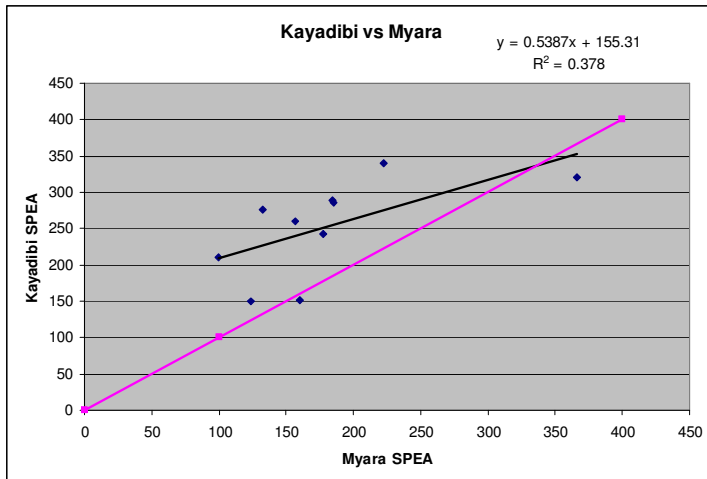


Figure 4S: Linear regression plot of 10 samples analysed using Kayadibi's method compared with Myara's method

University of Cape Town

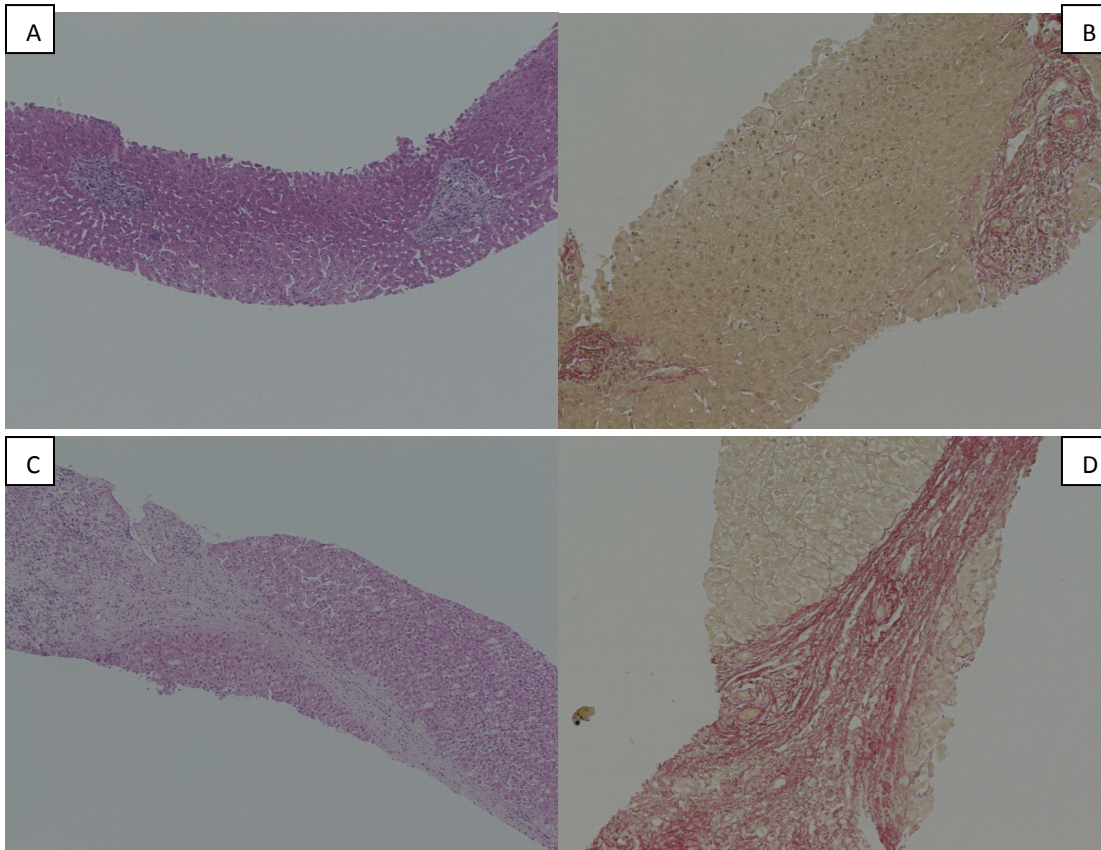


Figure 6S: Photomicrographs of histological examination of liver biopsies. A: Low power haematoxylin and eosin (H&E) stain of a liver biopsy showing no fibrosis; B: the same biopsy at medium power stained with bile sirius red (BSR) which stains for fibrosis; C (collagen stains red): Low power H&E stain of extensive (4+) fibrosis; D: same biopsy as C but stained with BSR at medium power demonstrating the fibrosis.

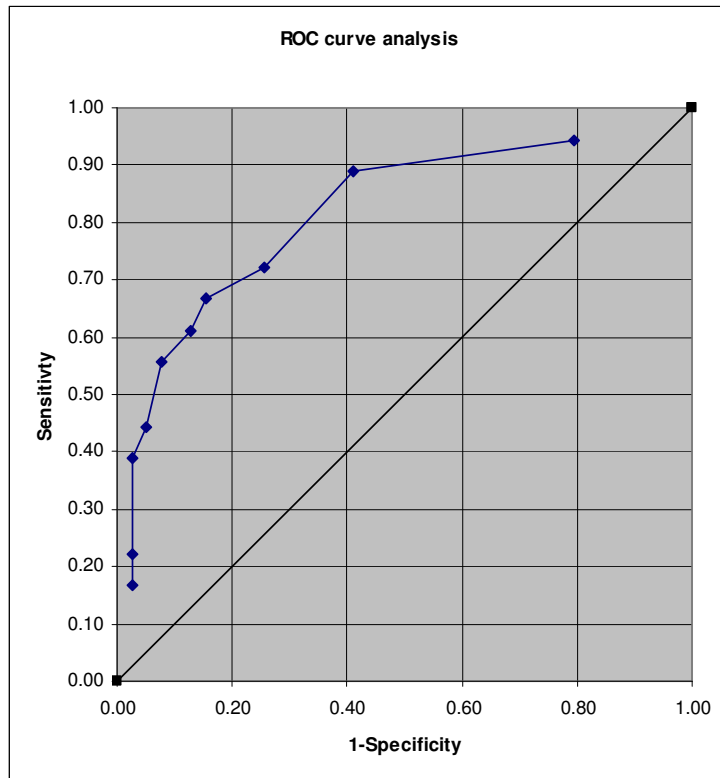


Figure 6S: Receiver operating characteristics curve analysis showing serum prolidase activity cut-off levels of 529, 500, 463, 450, 360, 330, 321, 280, 200 and 100 IU/L

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<http://www.ncbi.nlm.nih.gov/sites/entrez#>

Part D Appendix

This part contains information that provides supplementary detail. This section contains a copy of the University's official guidelines which mandates the layout of this minor dissertation, the method used in the determination of prolidase as well as the classical method described by Myara *et al.*²⁹ Further information not included in Part A - C but that will be of value in future research are Michaelis-Menten and Lineweaver-Burk plots, therefore there are included here. In addition, the approval from the Ethics Review board is included, along with the informed consent form. Finally, the "Instructions for Authors" downloaded from the Annals of Clinical Biochemistry are also appended.

University of Cape Town

Part D Appendix

University of Cape Town's MMED minor dissertation guidelines

7. Minimum Requirements for Dissertations for MMed and MPhil for Subspecialties Degrees

Following extensive discussion with Heads of Divisions, Dr S Kalula and Prof S Kidson recommend the following minimum criteria for dissertations for MMed and MPhil (subspeciality) degrees:

	UNIVERSITY OF CAPE TOWN FACULTY OF HEALTH SCIENCES	
MMed/MPhil Part III (minor dissertation) Guidelines for candidates, supervisors and examiners		

The MMed minor dissertation (or the MPhil dissertation in the case of sub-specialities) is one of three examination components of the MMed/MPhil degree. This minor dissertation carries one third of the weight of a full master's dissertation in terms of its credit weighting.

The dissertation must be a study containing the results of an analytical, quantitative, or epidemiological study carried out by the candidate (for certain disciplines, the candidate may choose instead to do a qualitative study, an audit cycle or a formal systemic review). A case report is not acceptable for the dissertation.

The dissertation must be the result of independent work of the candidate conducted under the guidance and direction of a supervisor(s) and should demonstrate evidence of an ability to undertake research, to adequately interpret results and to comprehensively and critically review the relevant literature. Although the findings of the research need not necessarily be original, they must be seen to advance scientific understanding. The topic and scope of research will depend on the particular disciplines and must be agreed upon in consultation with the supervisor(s).

Research protocol

Candidates intending to register for the MMed/MPhil Part III are required to submit a full research protocol for approval to their respective Departmental Research Committee (DRC). The candidate must also obtain FHS UCT Ethics approval prior to conducting their research. This full research protocol (together with a copy of the ethics approval letter) must be submitted to the postgraduate administration for approval by the Board of the Faculty of Health Sciences, prior to commencement of the research. For most disciplines, submission of the research protocol should be made no later than the end of year 2.

The research protocol should outline the scope and content of the dissertation and must include the title of the proposed dissertation, name of the supervisor(s) and their brief curriculum vitae.

Submission of dissertations

On completion, the dissertation should be submitted to the Faculty Postgraduate Officer. The candidate should inform the Faculty Officer one month in advance of the intention to submit.

Submission deadlines:

1. March 15th for June graduation
2. August 15th for December graduation

Supervisors will be requested by the Faculty Postgraduate Officer to submit a letter supporting submission. This letter should be supplied by the primary supervisor. If this supervisor is external, the internal supervisor must be kept informed at every stage of the process. Specific submission requirements may be set by individual disciplines.²

Note on fees: To avoid attracting fees, dissertations need to be submitted before the beginning of the first quarter (first day of academic year), and before the start of the second semester (mid July) to qualify for a 50% fee rebate.

Supervisors

One cannot overemphasize the importance of identifying a dissertation supervisor as early as possible. The supervisor should be an individual who can relate to the candidate's research project, be available for frequent and regular discussion and advice, and someone with whom the candidate can develop a good working relationship. Where specialised equipment and/or laboratory work is required for the study, the supervisor should assist in facilitating such access to such facilities. Supervisors may assist candidates in developing scientific communication skills but they are not required to do detailed editing or correction of spelling, grammar, or style. They may refer candidates to the UCT Writing Centre for this purpose.

The primary supervisor may be based outside the candidate's home department, faculty or university. In such a case, an internal (or secondary) supervisor will be required in addition to the primary supervisor, to serve as a guide and link to discipline-specific procedures. Primary supervisors retain responsibilities to the candidate and the university until the dissertation process is complete.

Please note: in order to assist a candidate with a master's research topic the supervisor needs to hold a master's degree or higher, or have relevant research experience. If the primary supervisor does not hold a higher degree or equivalent (such as a Fellowship of The College of Medicine of South Africa), then a secondary supervisor who has a higher degree will need to be appointed in addition to the primary supervisor.

Candidates are strongly encouraged to publish the study with the supervisor(s) as co-author(s). This may require work beyond the graduation date. Such arrangements should be discussed and documented in advance.

- 2 For Public Health Medicine and Occupational Medicine the dissertation must be submitted for examination at least 4 months prior to the deadline for registration for the examinations of the relevant College. This is in order to ensure that a final examination mark for the dissertation can be submitted by the candidate to the College of Medicine of South Africa (CMSA) at the time of registration as required by CMSA examination regulations.

The dissertation

Submission of the dissertation should satisfy the following criteria:

1. The title page should contain the candidate's name, dissertation title and the name of the university. It must also state the degree, e.g. Master of Medicine (MMed) in Public Health Medicine, Occupational Medicine, Family Medicine, Surgery, etc. The title page should also include a statement to the effect that the research report is based on independent work performed by the candidate and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree to any other university. It must also state that this work has not been published prior to registration for the abovementioned degree.
2. The body of the dissertation, which must be structured in 4 parts, should include the following:

Part A: The *protocol* (as approved by the Departmental Research Committee and Faculty Research Ethics Committee). The protocol should not exceed 4000 words.

Part B: A *structured literature review* appropriate to the subject matter and methods of the dissertation. The literature review must, amongst other things, show that the student is sufficiently acquainted with the relevant literature and is able to perform a critical appraisal and, if appropriate for the topic, show a good understanding of evidence-based medicine.

The review should be between 3 000 and 4 000 words.

A suggested structure for the literature review is as follows:

- a) Objectives of literature review
- b) Literature search strategy, including inclusion and exclusion criteria
- c) Quality criteria - some leeway will be allowed here, as candidates will vary in their ability to appraise studies. This will also vary with the nature of the dissertation.
- d) Summary or interpretation of literature
- e) Identification of gaps or needs for further research
- f) References (which will overlap with but will not be the same lists as in the journal article and protocol)

Part C: The results of the study must be presented in the form of a *manuscript* of an article for a

named peer reviewed journal, meeting all the requirements set out in the "Instructions for Authors" of that journal, including the word count and referencing style. (Unless specially motivated, the journal chosen will need to allow for *at least* 3000 words excluding abstract, tables, figures and references). The "Instructions to Authors" of the journal must be appended. The journal chosen for publication must be appropriate to the subject matter of the dissertation and accredited by the Department of Education or listed in the citation index of the Institute for Scientific Information (ISI). Important note: the candidate need not have submitted the article, not is the acceptance of the article and requirement for passing the degree. The norm of practise is to publish the study with the supervisor(s) as co-author(s) and candidates are strongly encouraged to submit their manuscript either before or after examination of the mini-dissertation.

Part D: All supporting documents including:

- Questionnaire/data capture instrument
 - Consent forms and any related participant information sheets
 - Technical appendices, including, if considered necessary, any additional tables not included in the main manuscript for the examiner to have available. These should be accompanied by a brief narrative.
 - Official Ethics approval letter from the Faculty Research Ethics Committee
3. The article does *not* have to be submitted to the journal in order to meet academic requirements.
 4. A candidate must submit 2 copies of the dissertation in temporary binding, and an electronic copy on compact disc in a universally readable format (e.g. pdf).

Examiners

The full dissertation will be submitted for examination through the Postgraduate office of our Faculty to two external examiners (nominated by the supervisors and HOD). Three examiners will be nominated, two of which are invited to examine, and one held as an alternate. All examiners must be external to UCT. These nominations are circulated to the Faculty Dissertation Committee. It is the *supervisor's (or co-supervisor's)* responsibility to submit names of potential examiners to the Faculty Officer when the candidate is ready to submit.

The examiners will be well briefed regarding the specific requirements and criteria for submission and examination of the mini-dissertation. Such criteria will clearly explain the difference between the mini-dissertation and a Master's degree by dissertation alone.

Details required for each examiner are: academic qualifications, postal and/or physical address, telephone and fax numbers and e-mail address, and one paragraph description of their standing in the relevant field (drawn from their CV if need be.)

The candidate may not be informed of the identity of the examiners. After the outcome of the mini-dissertation has been finalised, the examiners' identities are made known if the examiners have indicated that they do not object to this.

[Heads of Departments]

Michaelis-Menten analysis

Michaelis-Menten analysis was undertaken using a pooled serum sample and a wide range of glycyL-proline substrate concentrations (10 – 1 000 mmol/l). SPEA activity was determined and plotted against substrate concentration as shown. A double reciprocal (Lineweaver-Burk) plot was also done to ascertain a K_m value of 24.8 mmol/l and a V_{max} of 1 668 IU/l.

SPEA	Gly-Pro conc. (mM)
482	10
835	50
1914	500
1766	1000

Table 1: SPEA values obtained at various glycyL-proline concentrations. SPEA: serum prolidase enzyme activity; gly-pro: glycyL-proline.

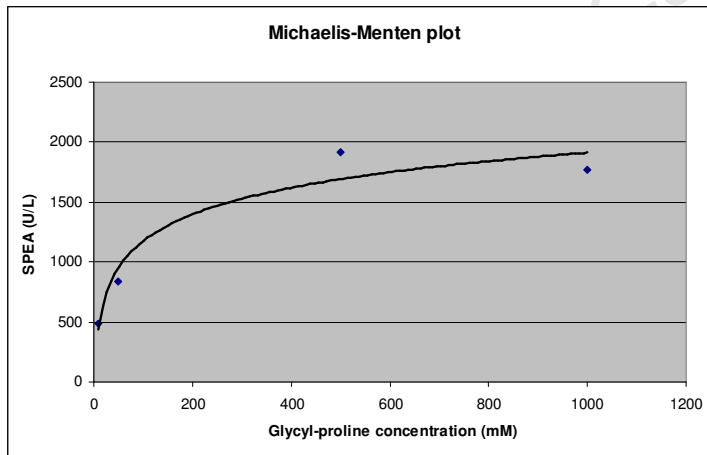


Figure 1: Michaelis-Menten plot of reaction rate versus substrate concentration using the data presented in Table 1

Lineweaver-Burk plot

1/v	1/s
0.002	0.1
0.001	0.02
0.001	0.002
0.001	0.001
0	-0.04027

Table 2: The reciprocals of the values presented in Table 1

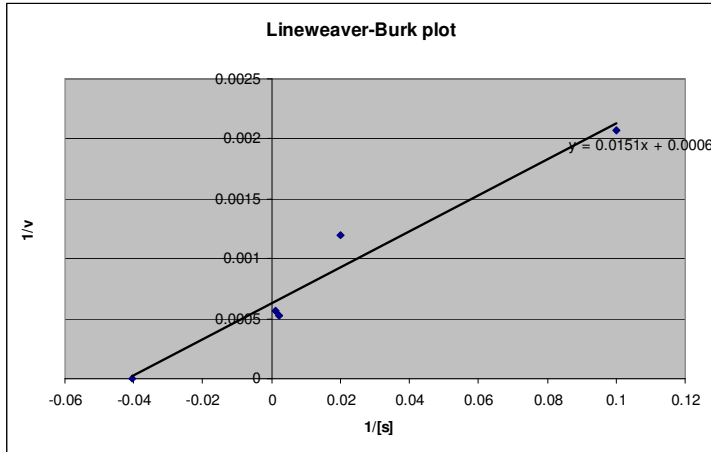


Figure 2: The reciprocals shown in Table 2 were plotted in a Lineweaver-Burk (double reciprocal) plot.

Thus from the Lineweaver-Burke plot

K _m	24.83 mmol/L
V _{max}	1666.67 IU/L

Table 3: The y-intercept of Figure 2 yielded a value for $1/V_{\max}$; likewise, the x-intercept yielded $-K_m$. Therefore, V_{\max} and K_m can be determined.

Serum prolidase assay standard operating procedure (SOP)

Reagents:

Serum diluent: 40mM Tris HCl pH 8.0 containing 2.5mM MnCl₂

Substrate: 100mM gly-pro (17.2mg/ml)- dissolve 86mg in 5ml water, (stored frozen in 1ml aliquots)

Buffer: Tris HCl 100mM pH 8.0; dissolve 0.24g Tris (free base) in ±15ml water, pH to 8.0 then make up to 20ml. Store at 4°C.

Stopping reagent: 20% TCA (w/v)

Proline stock standard: 10mM proline in 6.7% TCA. Dissolve 5.8mg in 5ml 6.7% TCA

Proline standard diluent: 6.7% TCA

Chinard's reagent: Mix 23.7ml water, 60ml glacial acetic acid and 16.3ml 85% H₃PO₄ (d=1.7). Add 2.5g ninhydrin and heat at 70°C to dissolve.

Method:

Prepare 2 microfuge tubes for each serum to be assayed thus (volumes in µl)

	blank	test
serum	5	5
serum diluent	15	15

Incubate 2h at 37°C

20% TCA	100	-
#gly-pro 100mM	60	60
#Tris 100mM pH 8.0	80	80
#water	40	40

[Note: these last 3 reagents can be pre-mixed as a 'cocktail' immediately before use in a ratio of gly-pro:Tris:water of 3:4:2, and add 180µl per tube]

Mix well and incubate at 37°C for 30min

20% TCA	-	100
---------	---	-----

Mix and spin down protein pellet. Assay supernatant fluid (snf) proline content alongside proline standards by pipetting into fresh microfuges as follows (volumes in µl):

Standard Curve

<i>Proline standards (mM)</i>	0	0.5	1
6.7% TCA	200	190	180
10mM Proline in 6.7% TCA	0	10	20

Blank and tests

Assay snf's (blanks and tests)	-	-	-	200
Glacial acetic acid	400	400	400	400
Chinard's reagent	400	400	400	400

Heat 10min at 90°C transfer to disposable plastic 1ml cuvettes and read absorbance at 515nm against the zero proline standard.

Calculation

Determine proline concentration in the sample tests and blanks in mmol/l off the standard curve.

Subtract blanks from test (T-B).

Serum prolidase activity

$$= (T-B) \times 200/5 \times 1/30 \times 1000 = (T-B) \times 1333 \mu\text{mol}/\text{min}/\text{litre}$$

SOP for Myara's modification prolidase assay²⁹

MW

- Glycyl-L-proline = 410.28
- Glacial acetic acid [CH₃COOH] = 60
- Orthophosphoric acid [H₃PO₄] = 98
- Ninhydrin = 178.14 requires no special care or attention and absorbs maximally @ 440nm.
- TCA = 163.38
- Tris HCl [C₄H₁₁NO₃ClH] Tris= 121, Tris HCl = 158
- MnCl₂ = 126
- Proline = 115

Reagents

Chinard's reagent:

Mix:

- 600 mL of glacial acetic acid
- 400 mL of 6 mol/L orthophosphoric acid (407 mL of orthophosphoric acid 85%, $d = 1.7$, and 593 mL of water)
- Dissolve 25g of Ninhydrin in this mixture at 70° C.

Standard proline solution:

This is a 650 μ mol/L solution in 0.45 mol/L trichloroacetic acid.

Mix:

- proline 74.75 mg
- TCA 73.35g
- 1 L water

Buffer Mixture

0.05 mol/L Tris HCl buffer, pH 7.8, containing 1 mmol of MnCl₂ per litre.

- Tris 5.93 g
- Tris HCl 0.078 g
- MnCl₂ 126 mg
- 1 litre water

Glycyl-L-proline:

94 mmol/L glycyl-proline in 0.05 mol/L Tris HCl buffer, pH 7.8, containing 1 mmol of MnCl₂ per litre.

Mix:

- glycyl-proline 38.54g
- 1 Litre buffer mixture

Sample preparation

Collect venous blood into heparinized tubes (EDTA, fluoride, and sodium citrate are all inhibitors) and separate the plasma by centrifugation at 2300 x g for 15 mm. Discard haemolysed samples because erythrocyte prolidase activity is relatively high. Store plasma samples at -20° C until assay.

Method

	Blank	Control	Standard	Sample
<i>Plasma</i>				100
<i>Buffer mixture</i>				500
<i>Incubate x 24 hours @ 37 degrees C</i>				
<i>SNF</i>		-		100
<i>Glycyl-proline solution</i>		100		100
<i>0.45 mmol TCA</i>		1 000		-
<i>Incubate x 30 minutes at 37 degrees C</i>				
<i>0.45 mmol TCA</i>		-		1 000
<i>SNF</i>		100		
<i>centrifuge at 10,000g at 4 °C for 10 min. Then in lockable eppendorf tubes</i>				
<i>SNF</i>	250 µl of 0.45 mmol TCA	250 SNF	250 proline standard	250
<i>Glacial acetic acid</i>	500	500	500	500
<i>Chinard's reagent</i>	500	500	500	500
<i>Heating block x 10 minutes at 90 degrees C</i>				
<i>Read the absorbance at 515 nm against the blank.</i>				

Calculate the enzyme activities as follows:

For proline determination, we took 0.5 mL from a total volume of 1.2 ml

$\frac{E - C}{S} \times [S] \times \frac{12}{5} \times \frac{0.5}{1000}$ = millimoles of proline formed per 0.1 mL of plasma, during 30 min where

E is the experimental tube absorbance

C is the control absorbance

S is the absorbance of the standard

[S] is the concentration of the substrate in mmol/L.

If we express the result per litre of sixfold diluted plasma, during one minute, we obtain:

$$\frac{E - C}{S} \times [S] \times \frac{12}{5} \times \frac{0.5}{1000} \times 10\,000 \times 6 \times \frac{1}{30}$$

Millimoles of proline formed per litre of plasma per minute at 37 ° C at pH 7.8.

Simplifying the equation:

$$\frac{E - C}{S} \times [S] \times 2.4 = \text{mmol} \cdot \text{min}^{-1} \cdot \text{L}^{-1}, \text{ or}$$

$$\frac{E - C}{S} \times [S] \times 2400 = \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{L}^{-1} \text{ at } 37\text{ }^\circ\text{C and pH } 7.8, \text{ or}$$

$$\frac{E - C}{S} \times [S] \times 40 = \mu\text{kat per litre at } 37\text{ }^\circ\text{C and pH } 7.8$$

Fibroblast culture SOP

Objective

Fibroblasts are grown from skin biopsies for diagnostic purposes. They provide a source of enzymes to diagnose diverse inherited metabolic diseases.

Materials and Reagents

All reagents are stored in the tissue culture fridge/freezer

- Gibco Dulbecco's modified eagle's medium (DMEM), stored at 4°C
- Lonza DMEM, stored at 4°C
- Sigma Foetal calf serum, stored at -20°C
- Gibco foetal calf serum, stored at -20°C
- Gibco 200mM Glutamine, stored at -20°C
- Antibiotics - Add streptomycin 2 g, penicillin 2 g and neomycin 2 g to 100 ml sterile normal saline (0.9%). Filter sterilize and aliquot 2.5ml into 5ml sterilin containers, store at -20°C
- Trypsin - Add EDTA 0.4 g, NaCl 16.0 g, KCl 0.4 g, Na₂ HPO₄ 2.3 g, KH₂PO₄ 0.4 g, glucose 0.4 g and trypsin 2.5 g to 200 ml sterile water. Check and adjust pH 7.2 - 7.4. Filter sterilize and store at -20°C in 10ml aliquots. Working solution - add 10ml of stock trypsin to 90ml distilled sterile H₂O; final concentration: trypsin 0.125 % in 0.5 mmol/l EDTA, Phosphate 10mM

Complete tissue culture medium

Add 50ml 10% Sigma or Gibco Foetal calf serum (FCS), and 5 ml 200 mM glutamine to 500ml Gibco or Lonza Dulbecco's modified eagle's medium +; bottles are labelled A or B.

Add 2.5ml of the combined antibiotics to 500ml of culture medium to obtain a final concentration of 100 units/ml. If glutamine or antibiotics are added this must be noted on the bottle as well as the date the medium was made up. Cell lines are cultured in duplicate using two different manufacturers, the medium must only be used by one

person, no sharing of medium should be allowed. Before using the culture medium, transfer 5ml into a 25cm² sterile flask and incubate for 3 days to check for contamination in the medium.

Disposables

- 5ml sterilin containers
- Scalpel Blades No. 23
- 22cm x 22cm glass cover slips dipped in 70% alcohol and flamed.
- Forceps
- 35mm Petri dishes
- 25cm² flasks
- 75cm² flasks
- 2ml cryotubes
- 15ml conical centrifuge tubes
- Individually wrapped sterile pipettes
 - 5ml pipettes
 - 10ml pipettes
 - 25ml pipettes
- 0.22µm sterile filters

Specimens

Skin biopsy is taken sterilely and placed in 2.5ml culture medium containing antibiotics.

The specimen should ideally be processed on the same day as taken. It is nevertheless possible to get cell growth after 3-4 days later provided the specimen is placed in cell culture medium and kept at 4° C. If culture medium is not available the biopsy can be put in sterile saline, but then it must be processed within 24hrs.

Preparation

- Using two scalpel blades finely slice the tissue into little pieces in a 35mm Petri dish and cover with a drop of culture medium containing antibiotics.
- Dispense the tissue fragments into several 35mm Petri dishes, cover with a flamed cover slip and add 2.5 ml culture medium containing antibiotics. Incubate at 37⁰C in a 10% CO₂ incubator.
- When there is a fairly confluent growth of fibroblasts (not epithelial cells), invert the coverslip, cell side up, into a clean 35mm Petri dish. Add 2.5ml culture medium. Once the fibroblasts are reasonably confluent, they can be trypsinised and seeded into 25cm² flasks (one 35mm Petri dish per one 25cm² flask).
- This is the 1st passage.
- When the 25cm² flask is confluent, it is trypsinised and seeded into a 75cm² flask, this is the 2nd passage. Two confluent 75cm² flasks are harvested and frozen in liquid Nitrogen for further studies.

Maintenance

- *35mm Petri dishes*: 2.5ml culture medium containing antibiotics removed and replaced twice a week until cells are confluent.
- *25cm² and 75cm² flasks*: culture medium without antibiotics is removed and replaced twice a week (5ml and 12ml respectively) until cells are confluent. Once fibroblasts are confluent, they must be trypsinised and seeded into fresh flasks. If left for too long the fibroblasts will lift off the bottom of the flask and die.

Trypsinization

Petri dish

Remove medium and wash twice with 1ml trypsin working solution. After the 2nd wash, add 0.5 ml trypsin solution and put in incubator until cells detach (generally 2-3min), but NOT longer than 15mins, as prolonged exposure to trypsin can damage the cell membrane. Transfer the trypsin and fibroblasts into a 25cm² flask and add 5ml culture medium immediately to neutralize trypsin. The FCS in the tissue culture medium stops the action of trypsin.

25cm² Flask

Pour off medium and wash twice with 2ml trypsin. Add 1ml trypsin and put in incubator until cells are off the surface. Tap side of flask to dislodge cells and transfer cells as follows:

1 x 25cm² flask into 3 x 25cm² flasks add 5ml culture medium into each flask

1 x 25cm² flask into 75cm² flask add 12ml culture medium to flask.

75cm² Flask

Pour off medium and wash twice with 4ml trypsin. Add 2ml trypsin to dislodge cells, transfer the cells into 3 x 75cm²Flasks and add 12ml of culture medium to each flask.

Freezing procedure

- Freezing Medium: DMEM with 20% FCS and 10% glycerol

- Two 75cm² confluent flasks are trypsinized, the cells pooled and transferred into a 15ml sterile conical tube and topped up with culture medium. Centrifuge for 10min at 1000rpm. Medium is discarded, 5ml culture medium added and centrifuged as above. The medium is discarded and cell pellet resuspended in 7ml of freezing medium.
- Aliquot 1 ml of cell suspension into 7 x 2ml sterile cryotubes.
- Place a drop of the cell suspension into two 35mm Petri dishes containing a sterile cover slip, add 2.5ml of culture medium without antibiotics and incubate for two to three days for mycoplasma testing.
- The 2ml cryotubes are placed in the nalgene freezing container, containing 250ml Propyl alcohol and placed in the -80°C freezer for a minimum of 4h. The tubes are then carried in liquid nitrogen to the liquid nitrogen storage containers and placed in the boxes. One vial is thawed, put into a 25cm² flask containing 5ml culture medium and incubated for three days as a control to check for viability and exclude contamination.

Quality Control

Culture each cell line in duplicate using two different manufacturers of DMEM and Foetal Calf serum. At the time of freezing the cells are plated for mycoplasma testing and tested immediately. Cells are grown in antibiotic-free medium as the presence of

antibiotics can mask contamination with mycoplasma. If bacterial contamination is noted, cells are immediately discarded.

Reference:

R. Ian Freshney: Culture of animal cells. A manual of Basic techniques. Wiley 2005

(ISBN: 0471453293)

University of Cape Town

Ethics Approval

UNIVERSITY OF CAPE TOWN



Health Sciences Faculty
 Research Ethics Committee
 Room E52-24 Groote Schuur Hospital Old Main Building
 Observatory 7925
 Telephone [021] 406 6338 • Facsimile [021] 406 6411
 e-mail: nosi.tywabi@uct.ac.za

09 October 2009

REC REF: 435/2009

Dr JC Stanfliet
 C17
 NGSB

Dear Dr Stanfliet

PROTOCOL TITLE: EVALUATION OF SERUM PROLIDASE ACTIVITY AS A MARKER FOR LIVER FIBROSIS IN SUSPECTED LIVER DISEASE

Thank you for submitting your study to the Research Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above mentioned study.

Approval is granted for one year until 16 October 2010.

Please submit an annual progress report if the study extends beyond the approval period. Alternatively, please submit a brief summary of your findings so that we can close our records.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

PP **PROFESSOR M BLOCKMAN**
CHAIRPERSON, HSF HUMAN ETHICS

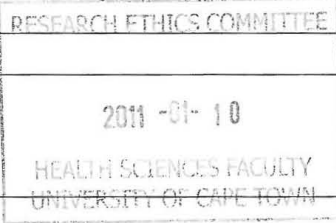
This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

lemjedi

FHS017: Annual progress and study closure report

Record Reviews/Audits/Collection of Biological Specimens/Repositories/Databases/Registries

1. Protocol information

Date	7 January 2011		
HREC REF Number	435/2009		
Protocol number (if applicable) & Protocol title			
Principal Investigator	Dr John Stanfliet		
Department / Office Internal Mail Address	NHLS C17 Core Laboratory		
1.1 Does this protocol receive US Federal funding?		<input type="checkbox"/> Yes	<input checked="" type="checkbox"/> No


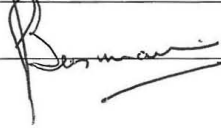
2. Protocol status (tick ✓)

<input type="checkbox"/>	Research-related activities are ongoing
<input checked="" type="checkbox"/>	Data collection is complete, data analysis only
<input type="checkbox"/>	All research-related activities are complete (i.e. final report)

3. Protocol summary

Total number of records or specimens collected, reviewed or stored since the original approval	68
Total number of records or specimens collected, reviewed or stored since last progress	N/A
Have any research-related outputs (e.g. publications, abstracts, conference presentations) resulted from this research? If yes, please list and attach with this report.	<input type="checkbox"/> Yes <input checked="" type="checkbox"/> No

My signature certifies that I will maintain the anonymity and/ or confidentiality of information collected in this research. If at any time I want to share or re-use the information for purposes other than those disclosed in the original approval, I will seek further approval from the HREC.

Signature of PI		Date	7 January 2011
Signature of Supervisor (if PI is a student)		Date	7/1/2011

Consent Form

DEPARTMENT OF CHEMICAL PATHOLOGY
UNIVERSITY OF CAPE TOWN

CONSENT FORM

Investigators: Prof Tahir Pillay, Dr John Stanfliet

EVALUATION OF SERUM PROLIDASE ACTIVITY AS A MARKER FOR LIVER FIBROSIS IN SUSPECTED LIVER DISEASE

Currently, when someone is thought to have scarring of the liver, the only way to tell for sure is to do a biopsy; that is, to insert a needle into the liver, and look at the cells under a microscope. However, this can be a problem because of pain, fear and the need for specialist pathologists to examine the cells. We are looking for an enzyme in the blood that may indicate that there is scarring of the liver, and also indicate the degree of scarring.

The blood that is being taken from you will be tested for the activity of this enzyme called prolidase and then analyzed for different protein and enzyme concentrations.

I,, agree to participate in the above study and hereby give permission that blood may be taken and serum analysed and/or stored.

I understand that I will undergo the following investigations:

- i. Venous blood will be taken for a serum prolidase activity test and the measurement of serum proteins and enzyme concentrations.
- ii. I further understand that a portion of these blood samples will be stored for:
 - a) possible reanalysis
 - b) analysis when the study is completed, if additional information has become available
 - c) further research purposes, subject to the approval of the University of Cape Town Research Ethics Committee and that any information from such research will remain confidential
 - d) I understand the results of these tests will not become available to me

I have been informed that:

- a) the investigators are under the obligation to respect medical confidentiality
- b) No DNA will be stored and no genetic/DNA tests will be done on these samples
- c) All samples will be anonymised prior to being sent to the laboratories.

I UNDERSTAND THAT THIS STUDY WILL BE OF NO BENEFIT TO ME AND ONCE MY BLOOD HAS BEEN TAKEN, THERE WILL BE NO WAY TO TRACE IT BACK TO ME.

ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS HAVE BEEN ADEQUATELY ANSWERED

Participant: Name Date:

Participant: Sign Date:

Informed consent obtained by: Name
Sign

Witness: Sign:

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Please **do not** submit the patient's actual written informed consent with your article, as this in itself breaches the patient's confidentiality. The Journal requests that you confirm to us, in writing, that you have obtained written informed consent but the written consent itself should be held by the authors/investigators themselves, for example in a patient's hospital record. The confirmatory letter may be uploaded with your submission as a supplementary file.

Should you require a template form for obtaining written informed consent, this can be supplied by e-mailing

provides consultative advice to Lab Tests Ltd.

Funding: This research was funded by the University of York

Ethical approval: The ethics committee of Northern Yorkshire approved this study (REC number: GH23335H)

Guarantor: EF

Contributorship: LM and HG researched literature and conceived the study. EF was involved in protocol development, gaining ethical approval, patient recruitment and data analysis. AB wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version of the manuscript

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A structured abstract of no more than 250 words must accompany all Review Articles, Original Articles and Short Reports. The abstract should normally use four headings: Background (context and rationale); Methods (type of study, patients, materials, techniques); Results (main numerical data and statistical information); and Conclusions (main objective and verifiable conclusions). Personal Views and Case Reports should be accompanied by an unstructured abstract of up to 250 words. Letters to the Editor do not require an abstract.

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