A CORRELATIVE STUDY OF ADVANCED BIOMARKER: KALLISTATIN WITH CONVENTIONAL AND OXIDATIVE MARKERS IN CIRRHOSIS OF LIVER

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MEDICAL BIOCHEMISTRY
Under
Faculty of Medicine

By

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September 2018

DECLARATION BY CANDIDATE

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Study is carried out under the supervision of **Dr Shashidhar KN,** Professor, Department of Biochemistry and Co- supervision of **Dr Lakshmaiah V**, Professor, Department of General Medicine and **Dr Muninarayana C**, Professor, Department of Community Medicine, Sri Devaraj Urs Medical College, A Constituent Institute of Sri Devaraj Urs Academy of Higher Education and Research.

No part of this has formed the basis for the award of any degree of fellowship previously elsewhere.

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Abbreviations

α-SMA	α-Smooth Muscle Actin
γGT	γ Glutamyl Transferase
2DE	2 Dimensional Electrophoresis
ALD	Alcoholic Liver Disease
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
AST	Aspartate Transaminase
ASH	Alcoholic Steato Hepatitis
ATP	Adenosine Tri Phosphate
AUROC	Area Under Receiver's Operating Characteristic curve
bFGF	basic Fibroblast Growth Factor
CAP	Community Acquired Pneumonia
CCL21	C-C Chemokine Ligand – 21
CK	Cyto Keratin
CLD	Chronic Liver Disease
CTGF	Connective Tissue Growth Factor
DAB	3,3` Di- Amino Benzidine
DNA	Deoxyribo Nucleic Acid
DTT	Dithiothreitol
ECM	Extra Cellular Matrix
EGF	Epidermal Growth Factor
ELF Test	Enhanced Liver Fibrosis test
ELISA	Enzyme Linked Immuno Sorbant Assay
EMT	Endothelial Mesenchymal Transition
eNOS	epithelial Nitic Oxide Synthase
ET-1	Endothelin – 1
GOD – POD	Glucose oxidase – Peroxidase
HA	Hyaluronic acid

HBV	Hepatitis B virus
HCC	Hepato Cellular Carcinoma
HCV	Hepatitis C virus
HIV	Human Immuno Virus
HMGB1/RACE	High Mobility Group Box chromosomal protein 1/Receptor for Advanced Glycation Endproduct
HAS	Human Serum Albumin
HSCs	Hepatic Stellate Cells
HUPO	Human Proteomic Organization
IFN-β	Interferon – β
IFN-γ	Interferon – γ
IGF-1	Insulin like Growth Factor – 1
IL	Interleukin
LC-MS	Liquid Chromatography – Mass Spectrometry
LPS	LipoPolySaccharides
miRNA	micro RNA
MALDI	Matrix Assisted Laser Desorption Ionization
MCP-1	Monocyte Chemoattractant Protein – 1
MELD	Model for End stage Liver Disease
MFAP4	Micro Fibrillar Associated Protein – 4
MIP-2	Macrophage
MMP	Matrix Metallo Proteinase
MRI	Magnetic Resonance Imaging
NAD	Nicotinamide Adenine Dinucleotide
NAFLD	Non Alcoholic Fatty Liver Disease
NASH	Non Alcoholic Steato Hepatitis
NF	Necrotic Factor
NF-kB	Nuclear Factor – kB
NO	Nitric Oxide
NS3	Non Structural Protein – 3
NS5	Non Structural Protein – 5

OD	Optical Density
PICP	Procollagen I Carboxy Peptide
PIIINP	Procollagen III Amino Peptide
PAF	Platelet Activating Factor
PAI-1	Plasminogen Activator Inhibitor – 1
PDGF	Platelet Derived Growth Factor
pI	Isoelectric pH
PT-INR	Prothrombin Time – International Normalized Ratio
PUFA	Poly Unsaturated Fatty Acid
PVDF	Poly Vinylidene Difluoride
RCL	Reactive Centre Loop
RNA	Ribo Nucleic Acid
ROS	Reactive Oxygen Species
SDS-PAGE	Sodium Dodecyl Sulphate – Poly Acrylamide Gel Electrophoresis
SELDI	Surface Enhanced Laser Desorption Ionization
SERPIN	Serine Protenase Inhibitor
SNP	Single Nucleotide Polymorphism
SOD	Super Oxide Dismutase
SPARC	Secreto Protein Acidic and Rich in Cysteine
TAC	Total Antioxidant Capacity
TGF-α	Transforming Growth Factor – α
TGF-β	Transforming Growth Factor – β
TIMP	Tissue Inhibitors of Metallo Proteinase
TMB	3,3`,5,5` – Tetra Methyl Benzidine
TNF-α	Tumor Necrotic Factor – α
TOS	Total Oxidative Status
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cell
WHO	World Health Organization

INTRODUCTION

Cirrhosis of liver is a pathological condition characterized by diffuse fibrosis, severe disruption of intrahepatic arterial and venous flow, portal hypertension and finally liver failure (1). Liver fibrosis/cirrhosis is generally the end result of majority of chronic liver insults; major global health concern associated with a significant morbidity and mortality. It is 14th common cause of death globally (2). Deaths due to cirrhosis of liver are increasing exponentially from 0.8 million in 1990 to more than 1.2 million in 2013 globally with a further upward trend. Among the well- known causes alcohol has affected 384,000; hepatitis C 358,000; hepatitis B 317,000 globally (3).

According to Indian statistical estimates it has been documented that liver diseases affect more than 1 in every 10 Indians. Even though alcohol abuse is well documented in Indians, majority of cirrhosis of liver in India is due to hepatitis B (HBV) and hepatitis C (HCV) infections, non alcoholic steatohepatitis (NASH) due to obesity and/or diabetes. World Health Organization (WHO) data published in May 2014 has documented that in India deaths due to liver diseases are 216,865 contributing 2.44% of total recordable deaths (4).

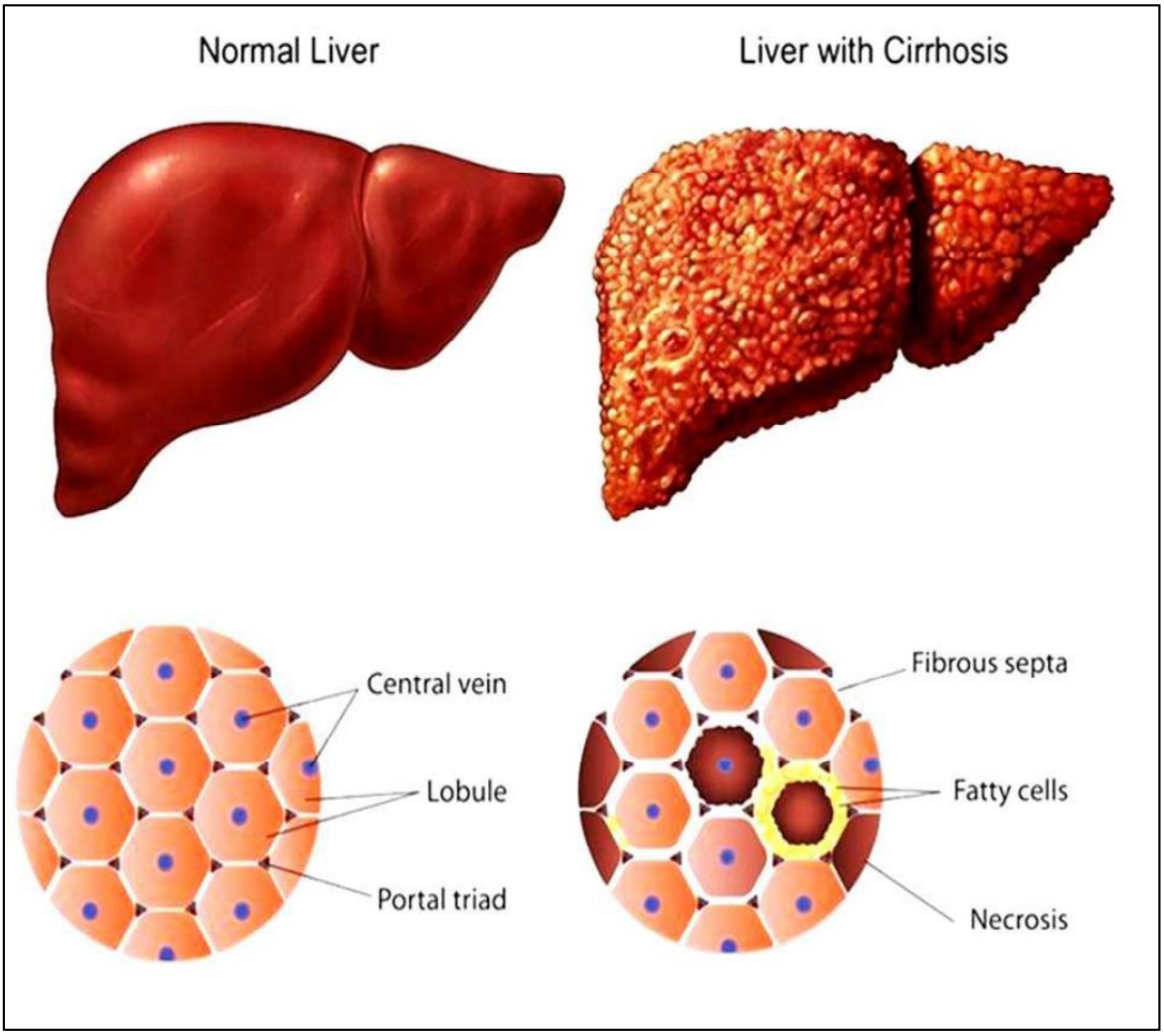
Cirrhosis of liver is reversible natural wound healing response which results in the formation of abnormal continuation of connective tissue production and deposition and regenerative nodular formation in response to chronic liver injury. Cirrhosis of liver is the final pathological result of various chronic liver diseases; fibrosis is the precursor of cirrhosis (5). Causes of liver fibrosis/cirrhosis are multifactorial which include congenital, metabolic, inflammation and toxins. In all these circumstances, replacement of parenchyma by fibrotic tissue, regenerative nodular formation and loss of liver functions are common (6). Recent studies to understand the process of hepatic

fibrogenesis show that treatment aimed at the underlying cause especially in earlier stage of the disease may improve or even reverse fibrosis/cirrhosis. Reasons for resolution may be due to increase in collagenolytic activity and/or increased matrix metalloproteinase (MMP) activity due to decrease in expression of tissue inhibitor of metalloproteinase I (TIMP-I) (7). Studies have reported that cytokine mobilization of bone marrow derived stem cells will restore neutrophil function and promote hepatic regeneration (8). Since, chronic liver disease (CLD) may not present clinically until advanced or cirrhotic stage, the possibility of reversing the fibrosis/cirrhosis is an essential issue for developing therapeutic approaches (9).

In normal liver, extra cellular matrix (ECM) is present in space of Disse in direct contact with low density basal lamina with glycoproteins, proteoglycans and glycosaminoglycans. After an acute liver injury, necrotic or apoptotic cells will be replaced by regenerated parenchymal cells. If the hepatic injury is chronic, there will be failure of regeneration and substitution of hepatocytes with abundant ECM and fibrillar collagen (10) (**Figure 1.1**). Liver fibrosis is associated with major alterations in both quantity and composition of ECM. In advanced stage, fibrotic liver contains 3 to 10 times more ECM than normal liver which includes collagens (I, III and IV), fibronectin, elastin, laminin, hyaluronic acid and proteoglycans (11).

ECM producing cells in the injured liver are hepatic stellate cells (HSCs) which dwell in the space of Disse and are the major storage cells of vitamin A (12). Due to chronic liver injury, activation of HSCs takes place and transdifferentiate into myofibroblast and attains contractile, proinflammatory and fibrogenic property. Chief mitogen for activation of HSCs is platelet derived growth factor (PDGF) which is produced by Kupffer cells.

Activated HSCs migrate and accumulate at tissue repair sites and secrete large amounts of ECM and regulates ECM degradation. HSCs collagen synthesis is regulated at transcription and posttranscriptional levels (12). Replacement of normal low density matrix by high density interstitial matrix disturbs the hepatocyte synthetic and metabolic function and impairs solute transport from sinusoid to hepatocyte. Cellular behavior alterations are mediated by cell membrane receptors termed as Integrins (11, 13).



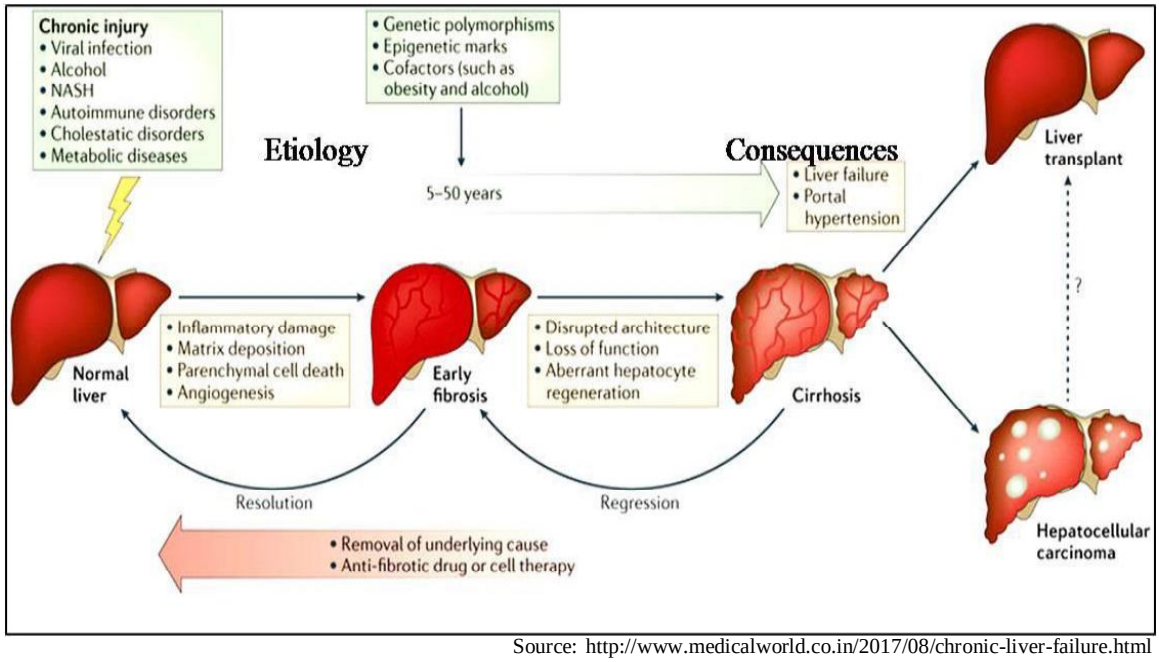
Source: http://www.ezhealthmd.com/medical-condition/cirrhosis

Figure 1.1: Comparative diagrammatic representation of normal liver with cirrhotic liver (modified from 14)

The activation of HSCs takes place in two phases (Initiation and Perpetuation).

Initiation includes early changes in HSCs resulting from paracrine stimuli by neighboring cells *viz.*, sinusoidal endothelium, kupffer cells, hepatocytes and platelets. Inflammatory marker cells stimulate matrix synthesis, cell proliferation and release of vitamin A by HSCs through the action of cytokine transforming growth factor– β (TGF– β), reactive oxygen intermediates and lipid peroxides.

Perpetuation involves seven discrete changes in cell behavior; proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation, retinoid loss and inflammatory signaling and white blood cell (WBC) chemoattraction with cytokine release. Among the discrete changes in cell behavior following the perpetuation of HSCs activation, fibrogenetic factors play a vital role in fibrogenesis (11, 13) (**Figure 1.2**).



Source: http://www.medicalworld.co.in/2017/08/chronic-liver-failure.html

Figure 1.2: Schematic representation of course of chronic liver diseases; etiology to consequence (modified from 15)

Cirrhosis of liver may cause serious complications *viz.*, hemodynamic instability, ascites, renal failure, hepatic encephalopathy, bleeding from esophageal varices and portal hypertension. Development of portal hypertension is the hallmark of cirrhosis of liver. Patients with cirrhosis of liver can remain free of major complications for several years termed as compensated cirrhosis. Decompensated cirrhosis is associated with retention of life of an individual. Cirrhosis of liver is also a risk factor for the development of hepatocellular carcinoma (HCC) (16).

Accurate assessment of extent of the disease is essential to guide clinical management, predict prognosis and for therapeutic decision in patients with CLD. Despite development of potential diagnostic tests, for the past 50 years liver biopsy is considered as a gold standard diagnostic tool to access liver fibrosis/cirrhosis. Disadvantages of liver biopsy are; highly invasive procedure and may obtain poor sample quality and tissue size which make biopsy non reproducible in relation to requirement of the sample. The risk allied for liver biopsy range from pain (84%), bleeding (0.5%) and rest by hypertension and biliary system damage (16, 17).

Scoring system for diagnosis/prognosis of cirrhosis of liver include routine laboratory tests *viz.*, blood counts, hepatic enzymes, bilirubin, prothrombin time and acute phase proteins. However, these scoring systems play a major role only after the effect. Concentration of proteins in serum is directly related to hepatic injury and is used as a surrogate marker. Direct markers are directly involved in ECM turn over whose levels are elevated with progression of the disease and have a tendency to decrease with response to treatment. Assessment of these markers may be useful to plan effective treatment. However, they are neither organ specific nor readily available. Serum levels of cytokines

do not have greater significance and could not add much diagnostic value compared to routine biomarkers (**Table 1.1**) (16, 17).

Table 1.1: Overview of biomarkers and their disadvantages related to cirrhosis of liver

Biomarker	Disadvantages
Serum Albumin	➢ Even though liver specific, concentrations will be decreased in acute and chronic renal failure. ➢ Unable to detect early pathophysiology and compensated liver cirrhosis because of half life
Aminotransferases (AST, ALT)	➢ Activities of both enzymes may reach as high as 100 times the upper reference limit ➢ Peak activities has no relationship to prognosis
Alkaline Phosphatase (ALP)	➢ Elevation tends to be more notable in extrahepatic obstruction than in intrahepatic obstruction ➢ Increase may also be seen in drug therapy
Gamma Glutamyl Transferase (γGT)	➢ Usefulness is limited due to lack of specificity ➢ Increased activity of the enzyme is also found in serum of subjects receiving anticonvulsant drugs example: Phenytoin and Phenobarbital
Serum Bilirubin	➢ Bilirubin peaks after marker enzymes ➢ Unable to detect early pathophysiology
Prothrombin Time (PT) International Normalized Ratio (INR)	➢ Cholestasis will decrease PT ➢ Decrease in PT may be secondary to malabsorption of vitamin K
Direct biomarkers of CLD	➢ Still in research level and needs validation ➢ Do not have greater significance than routine biomarkers
Serum Cytokines	➢ Do not have much diagnostic value ➢ Not organ specific

Hepatic cirrhosis can be proved by imaging techniques *viz.*, ultrasonography, computed tomography and Magnetic Resonance Imaging (MRI). These diagnostic modalities can detect parenchymal changes but require skill; costlier and unaffordable by rural population (17).

Considering these limitations, there is an urgent need to introduce a biomarker which should be organ specific, accurate and precise, freely available in peripheral tissue, easily measurable having diagnostic significance much earlier than the scoring systems or disease onset and eliminate need for invasive liver biopsy.

Biomarker

A biomarker is a measurable indicator of a specific biological state, presence or stage of the disease. Biomarker can be used for diagnosis, monitor the disease, guide targeted therapy or assess therapeutic response. Biomarkers can be gene variants, single nucleotide polymorphisms (SNPs), gene expression products, metabolites, polysaccharides, circulating nucleotides and proteins. The major essential components for a biomarker pipeline are discovery of biomarker candidates, prioritization of candidate, verification, clinical assay optimization, clinical validation and commercialization (18).

Characteristics of an ideal biomarker (19)

1. Should be organ specific, detectable early, prior to histopathological changes
2. Should be sensitive, correlate with the severity of damage; indicator of active damage
3. Capacity to reflect stage of fibrosis/cirrhosis, activity of matrix deposition and matrix removal
4. Possibility to follow progression or regression of the disease

5. Serum levels independent of alterations in renal or reticuloendothelial function
6. Cost effective, easily available, feasible and comparable with gold standard method

Advantages of biomarkers (19)

1. Minimal invasive
2. Analysis can be done without admission, local anesthesia or sedation
3. Biomarkers do not associated with morbidity and mortality
4. Biomarkers are easy to analyze and are reproducible
5. Validated biomarkers with scores may be useful for monitoring therapy
6. Blood markers are less expensive

Disadvantages of biomarkers (19)

1. Direct markers related to matrix turn over are not organ specific, are not readily available; need further validation in large population studies
2. In minimal inflammation, these markers may fail to detect
3. Results of these markers can be influenced by unrelated sites of inflammation
4. Biomarkers depend on clearance rate and influenced by impaired biliary function and renal excretion (extrahepatic factors)
5. None of the biomarker has great accuracy with high sensitivity and specificity to assess liver dysfunction
6. Serum direct as well as indirect biomarkers do not have sensitivity which cannot discriminate intermediate stages of fibrosis/cirrhosis

Evolution of protein biomarker for Cirrhosis of Liver

Proteins that are expressed from liver and enters into circulation reflects the degree of liver dysfunction and may give potential insights for discovery of biomarker candidates. Comparative protein expression analysis between diseased and healthy may generate hundreds of biomarker candidates that are differentially expressed. Discovery phase further progress to prioritization of biomarker candidate and is necessary for further analytical and clinical validation. Prioritization should be based on clinical significance, protein scores and reagents availability. After prioritization, biomarker candidate require verification that demonstrates the differential expression which remain detectable by assay to be used for validation. Validation of biomarker and clinical assay optimization requires measurement of thousands of patient samples with narrow measurement coefficient of variation values (20, 21).

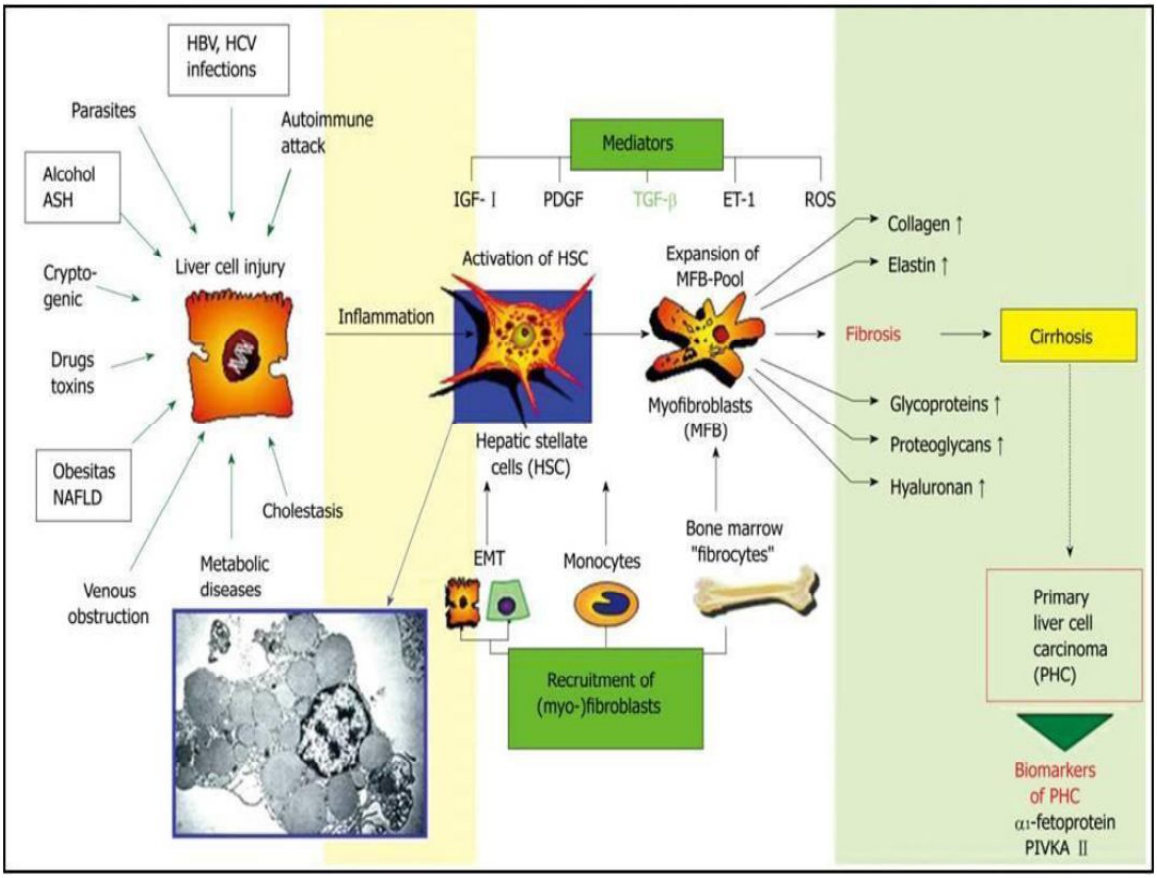
In the present study, SERPINA4/Kallistatin is the newly discovered protein biomarker candidate; known as kallikrein inhibitory protein belongs to serine proteinase inhibitor family, documented to play a vital role in screening, diagnosis and prognosis of cirrhosis of liver. This molecule of research interest needs further research among the population. It is also known to have inhibitory action on tissue kallikrein and a role in inhibiting inflammation, oxidative stress, angiogenesis and apoptotic reactions. Concentrations of SERPINA4/Kallistatin in circulation may vary in different liver diseases *viz.,* fibrosis, cirrhosis and HCC (22). Being antioxidant, any alteration in its level may affect oxidative status and antioxidant capacity. These factors outweigh a need for further research to establish SERPINA4/Kallistatin as a diagnostic/prognostic biomarker for cirrhosis of liver by correlating its serum levels with conventional, direct markers of ECM.

REVIEW OF LITERATURE

Liver is the vital organ which performs crucial functions *viz.,* substrate metabolism, detoxification, protein and digestive enzyme synthesis and immune response for human survival (23). Being highly vascular organ, it is continuously exposed to injury and damage by hepatotoxins *viz.*, viruses, drugs, alcohol, excess fat etc., leading to inflammation and fibrosis (24). Liver fibrosis is natural reversible wound healing response to chronic liver insults which involves deposition of ECM. Accumulation of ECM destroys liver by forming fibrotic scar and subsequent nodular development ultimately leading to cirrhosis of liver. Fibrotic liver contains three to ten times more ECM which in turn distorts liver parenchyma and vascular architecture resulting in liver dysfunction. HSCs are ECM producing cells in fibrotic liver effective after activation and trans-differentiation into myofibroblasts which attains contractile, inflammatory and fibrogenic properties (**Figure 2.1**). HSCs activation results from interactions with damaged hepatocytes, Kupffer cells, disintegrated platelets and sub-fractions of leucocytes (25, 26). Progression of inflammatory and fibrogenic pathways are mediated by cytokines, genetic and epigenetic mechanisms.

In pathophysiology of liver fibrogenesis, TGF– β, PDGF, endothelin-1 and vascular endothelial growth factor (VEGF) play a dominant role (26). Genes regulating hepatocellular damage, inflammatory response to injury and reactive oxygen species (ROS) generation regulates extent of hepatic damage, inflammation and ECM deposition (16). Epigenetic mechanisms [DNA methylation, histone modifications and non-coding micro RNA (miRNA)] have been shown to orchestrate many aspects of fibrogenesis of liver (27). Liver fibrosis is dynamic and potentially bidirectional process; early stage of disease can reverse fibrosis to normal liver architecture by spontaneous resolution of

hepatic scar. Regression of liver fibrosis/cirrhosis is due to decrease in expression of TIMPs which in turn increases activity of MMPs which results in increase in collagenolytic activity. The stage at which disease become irreversible is not well established; but it is hypothesized that irreversibility attains once septal neovascularisation happens and portal pressure increases significantly (28).



Source: Gressner AM, Gao CF, Gressner OA. Non-invasive biomarkers for monitoring the fibrogenic process in liver: A short survey. World J Gastroenterol. 2009; 15(20): 2433-2440.

Figure 2.1: Pathophysiology of cirrhosis of liver (26)

Abbreviations: CTGF: Connective Tissue Growth Factor; PIIINP: Procollagen III amino peptide; MMPs: Matrix metallo proteinases; TIMPs: Tissue inhibitors of metalloproteinases; HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; ASH: Alcoholic Steatohepatitis; NAFLD: Non Alcoholic Fatty Liver Diseases; IGF-1: Insulin like Growth Factor 1; EMT: Epithelial Mesenchymal Transition; TGF-β: Transforming Growth Factor β; PDGF: Platelet Derived Growth Factor; ET-1: Endothelin-1; ROS: Reactive Oxygen Species

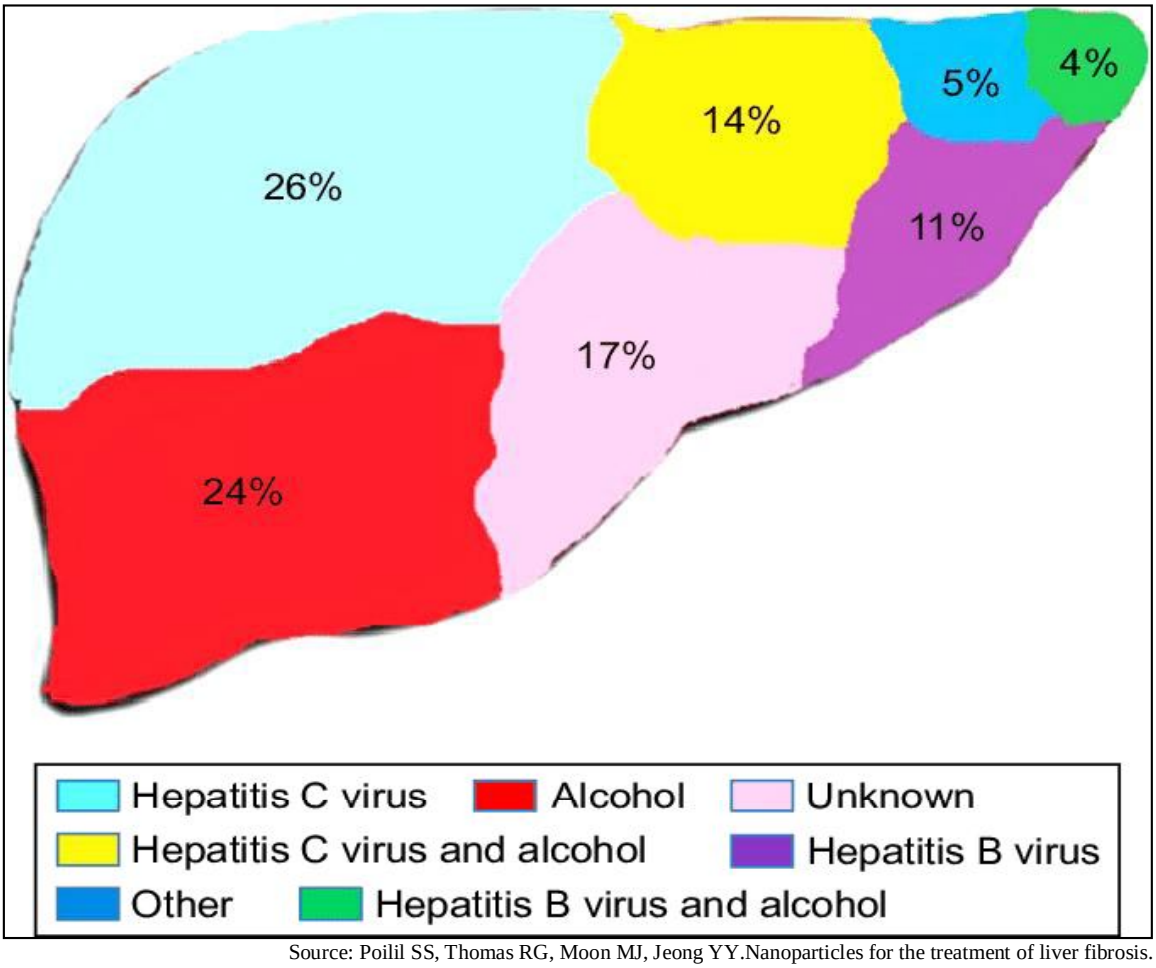
Etiology of Cirrhosis of Liver (**Figure 2.2**) (29, 30)

1) Alcoholism
2) Chronic viral hepatitis
    a. Hepatitis B
    b. Hepatitis C
3) Non-alcoholic steatohepatitis
4) Biliary cirrhosis
    a. Primary biliary cirrhosis
    b. Primary sclerosing cholangitis
    c. Auto immune cholangiopathy
5) Inherited metabolic liver disease
    a. Haemochromatosis
    b. Wilson's disease
    c. α-1antitrypsin deficiency
    d. Cystic fibrosis
6) Autoimmune hepatitis
7) Cryptogenic cirrhosis
8) Cardiac cirrhosis

Symptoms and Signs of Cirrhosis of Liver

Cirrhosis of liver is slow and gradual in its development. Common symptoms of cirrhosis include weakness and loss of weight. Symptoms and signs may be either as a direct result of failure of liver cells or secondary to portal hypertension or both. Spider angiomata,

palmar erythema, gynecomastia, hypogonadism, ascites, fetor hepatics and jaundice are some of the symptoms due to liver dysfunction. Splenomegaly, oesophageal varices, caput medusa and cruveilhier-Baumgarten murmur are due to portal hypertension (29).



Source: Poilil SS, Thomas RG, Moon MJ, Jeong YY.Nanoparticles for the treatment of liver fibrosis. International Journal of Nanomedicine. 2017;12: 6997-7006.

Figure 2.2: Etiology of cirrhosis of liver (30)

Major complications include varices, ascites, hepatic encephalopathy, hepatopulmonary hypertension, hepatocellular carcinoma, hepatorenal syndrome, spontaneous bacterial peritonitis and coagulation disorders. These complications may occur secondary to portal hypertension resulting in abnormal synthetic function. Patients with cirrhosis of liver are at increased risk of numerous complications and decreased life expectancy. Previously,

cirrhosis was thought to be irreversible. However, recent studies have shown that treatment aimed at the underlying cause especially in earlier stages of the disease may improve or even reverse fibrosis/cirrhosis (6).

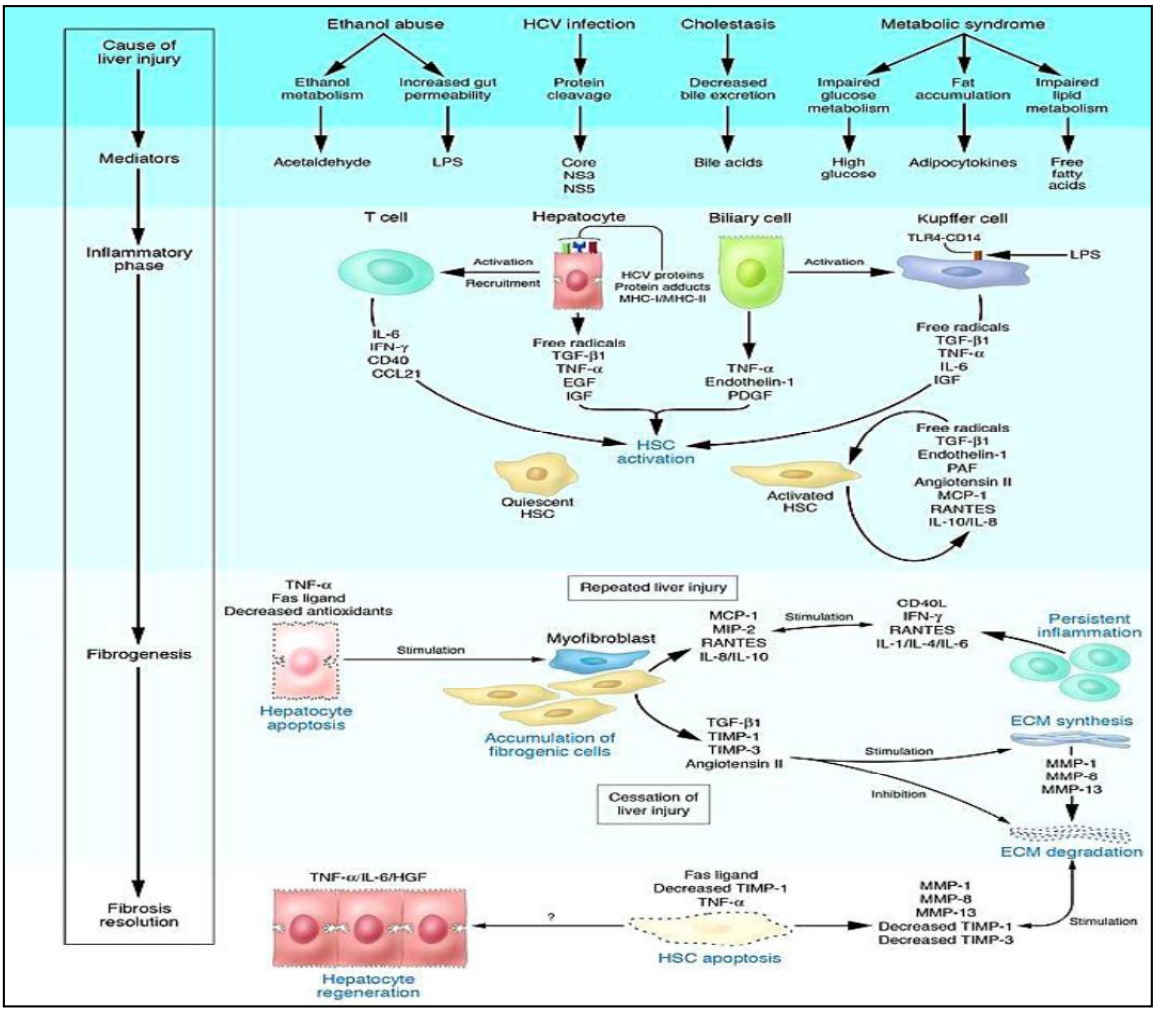
Pathophysiology of Cirrhosis of Liver

Natural reversible wound healing response of liver for chronic liver injury results in formation of hepatic scar leading to fibrosis of liver. After an acute injury, liver parenchymal cells regenerate and replace necrotic and apoptotic cells. If hepatic injury persists, there will be failure in hepatic regeneration and substitution of hepatocytes with abundant ECM having contractile, inflammatory and fibrogenic properties (10). In normal liver, fibril forming collagens are confined to the capsule, around large vessels and portal triad. Subendothelial deposits of Fibronectin, Laminin, **S**ecreto **P**rotein **A**cidic and **R**ich in **C**ysteine (**SPARC**) along with heparin sulphate proteoglycans *viz.*, perlecan, decorin, fibromodulin, aggrecan, lumican and glypican are also present in normal liver (11, 12). Different types of cells (resident innate inflammatory cells, hepatocytes, liver sinusoidal endothelial cells and Kupffer cells) play a role in liver fibrogenesis. Activation of HSCs is a crucial step in inter- linked process of tissue injury and regeneration (31). Quiescent HSCs present in space of Disse will be activated and trans- differentiate into myofibroblasts which are responsible for ECM production and accumulation in injured liver (11). Accumulation of ECM is due to increased synthesis and decreased degradation by over expression of TIMPs which inhibits MMPs (31). Fibrotic liver contains three to ten times more ECM compared to normal liver which includes collagen types, glycoproteins, proteoglycans and glycosaminoglycans (25).

Chief mitogen of HSCs activation is PDGF produced by Kupffer cells; macrophages are source of pro-fibrotic chemokines (11, 32). Activated HSCs activate immune response by secretion of cytokines, chemokines and interacting with immune cells. Complex network of cytokines modify activities of circulating immune cells, HSCs, hepatocytes, liver sinusoidal endothelial cells and Kupffer cells (**Figure 2.3**). Autocrine and paracrine secretions of cytokines activate and trans- differentiate HSCs into myofibroblasts (31). Activated HSCs migrates to tissue repair site and secrete ECM; collagen synthesis is regulated by transcription and post- transcription. Collagen fibrils can be cross- linked by tissue transglutaminase and lysyl oxidase pathways which make collagen susceptible for collagenase activity (33). Low density matrix is replaced by high density interstitial matrix which disturbs metabolic functions and impairs solute transport; altered cellular behavior is mediated by Integrins (12). Damaged hepatocytes release ROS and fibrogenic mediators which stimulate inflammatory cells and fibrogenic action of activated HSCs. Activated HSCs stimulate lymphocytes by secreting inflammatory chemokines. It is a cyclic stimulation process of inflammatory and fibrogenic cells vice versa (33).

Spontaneous resolution of liver fibrosis is possible after successful treatment of causative agent and may take several years depending on cause and severity of the disease (32, 33). Characteristic features of liver fibrosis reversal are decreased inflammation and decreased fibrogenic cytokines, increased collagenase activity and disappearance of myofibroblast and fibrotic scar (7). Regression of liver fibrosis consists of thinning of fibrous septa, regeneration of hepatocytes and recovery of acinal structure (34). Reversal of liver fibrosis can be achieved by inhibition of HSCs activation, neutralization of proliferative, apoptosis or senescence and degradation of scar matrix. Inhibition of HSCs activation

and trans-differentiation into myofibroblasts can be attained by reducing oxidant stress (11). Interferon-β (IFN-β) inactivates HSCs and decrease production of collagen I and α smooth muscle actin (α-SMA) by inhibiting PDGF and TGF-β; Interferon-γ (IFN-γ) has inhibitory action on activation of HSCs (31).



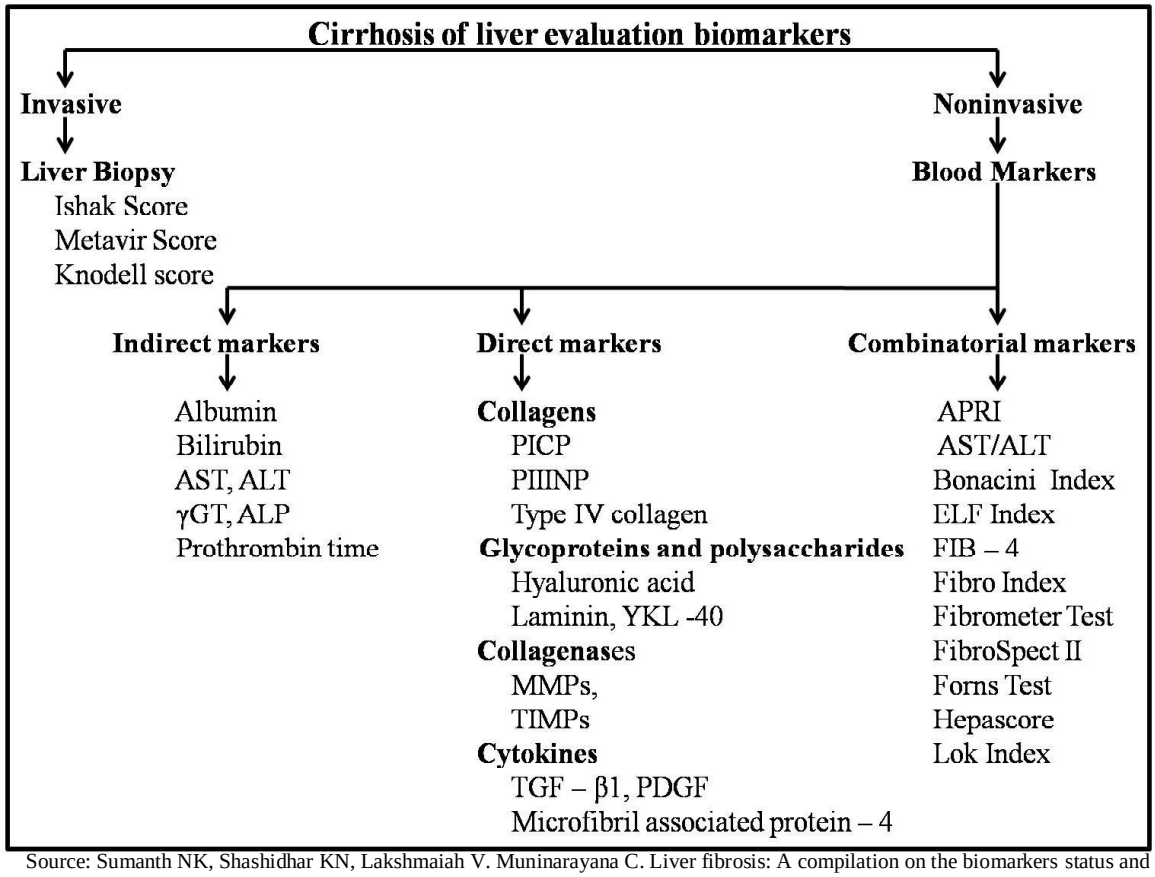
Source: Bataller R, Brenner DA. Liver fibrosis. J Clin Invest. 2005; 115: 209-218.

Figure 2.3: Cellular mechanisms of cirrhosis of liver (16); Abbreviations: LPS: Lipopolysaccharides; IL: Interleukin; INF: Interferon; CCL21: C-C chemokine ligand 21; MCP-1: Monocyte Chemoattractant Protein–1; MIP-2: Macrophage Inflammatory Protein–2; NS3: HCV Nonstructural Protein 3; NS5: HCV Nonstructural Protein 5; TGF-β: Transforming Growth Factor β; TNF-α: Tumor Necrotic Factor α; PDGF: Platelet Derived Growth Factor; ECM: Extra Cellular Matrix; EGF: Epidermal Growth Factor; IGF: Insulin like Growth Factor; PAF: Platelet Activating Factor; MMPs: Matrix Metallo Proteinases; TIMPs: Tissue Inhibitors of Metalloproteinases; HCV: Hepatitis C Virus

Fibrillar collagens are degraded by interstitial MMPs (MMP-1, -8 and -13) which are released in pro-enzyme form and activated by cleavage of inhibitory N- terminal peptide by plasmin. Plasmin synthesis in fibrotic liver is inhibited by synthesis of plasminogen activator inhibitor-1 (PAI-1) expressed from activated HSCs (33). During resolution of fibrosis, MMPs activity is increased due to decreased expression of TIMPs; monocyte/macrophage lineage expresses MMPs (35). After removal of inflammation, macrophages are differentiated into $Ly6c^{low}$ phenotype which produces MMP9 and MMP12 capable of matrix degradation (33). Altered interactions between activated HSCs and ECM favor apoptosis (6, 16). Myofibroblast apoptosis is contributed by activation of death receptor mediated pathway, increased expression of pro-apoptotic proteins (p53, Bax and Bcl-2) and decreased expression of pro-survival proteins (7). After successful removal of causative agent, HSCs undergo caspase-8/caspase-3 dependent apoptosis. Over expression of pro-apoptotic proteins leads to caspase-9 mediated programmed cell death. Over expression of CXCL9 by macrophages and VEGF expression accelerate fibrosis resolution by angiogenesis (35).

Diagnosis of Cirrhosis of Liver

Accurate assessment of the extent of liver cirrhosis is essential for clinical management so as to predict prognosis and therapeutic decision in patients with liver fibrosis/cirrhosis (**Figure 2.4**).



Source: Sumanth NK, Shashidhar KN, Lakshmaiah V. Muninarayana C. Liver fibrosis: A compilation on the biomarkers status and their significance in disease progress. Future Science OA. 2017; doi: 10.4155/fsoa-2017-0083.

Figure 2.4: Algorithm of cirrhosis of liver biomarkers (25)

Liver biopsy

Despite development of potential diagnostic tests for the past 50 years, liver biopsy is considered as gold standard method to classify liver fibrosis/cirrhosis and provides useful information about diagnosis and also other damaging process *viz.*, necrosis, inflammation, and steatosis (36). Three of widely used methods to assess histological fibrosis are: Ishak score, Metavir score and Desmet/Scheuer staging system (37). Each scoring system relies on progressive development of periportal fibrosis followed by septal fibrosis and finally nodule formation (38).

Limitation of liver biopsy is highly invasive. Moreover, poor sample quality and tissue size make biopsy non reproducible and depends on the experience of pathologist which leads to inter observer variations. Risk allied for liver biopsy range from pain (84%), bleeding (0.5%), hypertension, and damage to biliary system with approximately 0.01% mortality rate (39). These limitations of liver biopsy have given urgency for development of non-invasive diagnostic procedure for liver fibrosis/cirrhosis. An ideal Biomarker should be organ specific, sensitive to indicate active damage, easily accessible in peripheral tissue and cost effective (19). Advantages of biomarkers over liver biopsy are that their estimations in serum are by minimal invasive procedure. Further advantages are easy applicability, inter laboratory reproducibility and broad availability.

Serum biomarkers for liver fibrosis/cirrhosis are classified into two categories (26)

1. Direct markers: which reflects ECM turnover
2. Indirect markers**:** molecules released into blood which reflect alterations of hepatic function

Direct markers of cirrhosis of liver

Direct markers are directly involved in deposition and removal of ECM produced by HSC and other hepatic cells. Serum levels of these markers are elevated with progressing disease and have a tendency to decrease with response to treatment (40). Assessment of these markers may be useful for bringing about effective treatment, but they are neither organ specific nor readily available. Direct markers of liver fibrosis/cirrhosis are classified according to their molecular structure (41) (**Table 2.1**).

Table 2.1: Classification of direct biomarkers for Cirrhosis of Liver according to structure (41)

Collagens – PICP – PIIINP – Type IV collagen **Glycoproteins and polysaccharides** – Hyaluronic acid – Laminin – YKL -40	**Collagenases and their inhibitors** – MMPs – TIMPs **Cytokines and proteomic markers** – TGF – β1 – PDGF – Microfibril associated protein – 4
Abbreviations: **PICP**: Procollagen I Carboxy Peptide; **PIIINP**: Procollagen III Amino Peptide; **MMP**: Matrix Metallo Proteinase; **TIMP**: Tissue Inhibitors of Metallo Proteinase; **TGF – β1**: Transforming Growth Factor β1; **PDGF**: Platelet Derived Growth Factor	

Collagens

Procollagen I carboxy peptide (PICP) and Procollagen III amino peptide (PIIINP)

During synthesis of collagen, procollagen undergoes enzymatic cleavage at carboxy and amino terminal ends by procollagen C- peptidase and procollagen N-peptidase and peptides are released into serum whose estimations can be used to assess matrix deposition (42). Fibril forming type I collagen is profuse in healthy liver. During fibrogenesis, type I collagen will be increased up to 8 fold (43). Serum estimations can give an indication regarding the severity of disease. Type III collagen, a fibril forming collagen is an important component of connective tissue. Concentrations of PIIINP in basal membrane are greater during hepatic fibrosis due to chronic liver injury. PIIINP will be correlated with aminotransferase levels in acute hepatitis which reflects degree of fibrosis/cirrhosis (43, 44).

Relatively low sensitivity and specificity (78% and 81%) of these markers have limited their clinical use. There is no correlation between PICP and PIIINP serum levels with histological grading of liver fibrosis. Hence, these are not reliable to establish fibrosis/cirrhosis grading (44, 45).

Type IV collagen

Type IV collagen is a crucial component of hepatic ECM which is deposited integrally in matrix. Serum estimation of type IV collagen is a sign of direct degradation and has positive correlation with grade of liver fibrosis/cirrhosis. Combinatorial use of type IV collagen with PIIINP has a sensitivity and specificity of 88% (44, 46).

Glycoproteins and polysaccharides

Hyaluronic acid (HA)

HA is a glycosaminoglycan synthesized by HSCs and is the main component of ECM. In normal liver, HA uptake and degradation takes place in hepato sinusoidal endothelial cells. Increased concentrations in serum are attributable to increased production and decreased hepatic elimination or both (47). Serum HA levels are related to stage of fibrosis/cirrhosis and degree of necroinflammation. High levels have been detected in liver fibrosis/cirrhosis with varied etiology (48). HA has sensitivity and specificity of 88%-95% and 86%-100% respectively in liver fibrosis/cirrhosis especially in nonalcoholic fatty liver diseases, but positive and negative predictive value of HA has been reported as 61% and 98%-100% respectively (43).

Laminin

Laminin is a non-collagenous glycoprotein deposited in basal membrane of liver, synthesized by HSCs. In liver fibrosis/cirrhosis, laminin increases around the vessels, in perisinusoidal space and portal triad. Serum laminin levels are elevated in liver fibrosis/cirrhosis irrespective of etiology and have a correlation with severity of fibrosis/cirrhosis and liver inflammation (49). Laminin cut off concentration at 1.45 U/ml has sensitivity and specificity of 87% and 74% respectively with positive predictive value of 77% and negative predictive value of 85%. Estimations of serum HA and laminin has good prognostic value for liver fibrosis/cirrhosis complications (50).

YKL-40

YKL -40 (chondrex, human cartilage glycoprotein-39) is a glycoprotein. YKL-40 mRNA is strongly expressed by liver (51). It can be used as a marker to assess liver fibrosis/cirrhosis and helps distinguish between mild stage and extensive stage of liver fibrosis/cirrhosis and has a positive predictive value of 80%. Between HA and YKL-40, HA is a better predictive marker for liver fibrosis/cirrhosis (52).

Collagenases and their inhibitors

Matrix metallo proteinases (MMP)

Tissue inhibitors of metalloproteinases (TIMP)

Degradation of ECM of liver is due to activity of MMP. Three MMPs that are expressed in humans *viz*., MMP- 1 (Collagenases), MMP– 2 (Gelatinase A) and MMP– 9

(Gelatinase B) (53). These enzymes are synthesized intra cellular and secreted as zymogens. MMPs are activated by membrane type matrix metalloproteinase (MT1– MMP) and inhibited by tissue inhibitors of metalloproteinases (40). In liver fibrosis/cirrhosis, there will be inverse correlation between levels of MMP– 1 and histological severity (54). MMP– 2 secreted from hepatic stellate cells in liver disease has a high diagnostic accuracy of 92% to detect liver fibrosis/cirrhosis. There will be a 2.4 fold increase in the levels of MMP– 2 in fibrotic patients when compared to controls (55). MMP– 9 from Kupffer cells has negative correlation with histological severity (56).

ECM degradation by MMPs is inhibited by tissue inhibitors of metalloproteinases (TIMPs), which affect MMPs function. TIMP– 1 will interact with almost all the 3MMPs whereas TIMP– 2 specifically interacts with MMP– 2. With progression of liver disease, serum levels of TIMP's will increase. MMP– 1 /TIMP– 1 ratio is useful for the diagnosis of hepatic fibrosis/cirrhosis and correlates with degree of portal inflammation (57).

Cytokines and Proteomic Markers

Transforming Growth Factor α & β1 (TGF– α &TGF– β1)

In liver fibrosis/cirrhosis, TGF– α enhances proliferation of HSCs and correlates well with progression of the disease (58, 59). Homodimetric polypeptide, TGF– β1, secreted in an inactive form, has pleiotropic effect through membrane receptors. TGF– β1 stimulates production of ECM by HSCs and inhibits hepatocyte growth and proliferation in liver fibrosis/cirrhosis (60). High levels of TGF– β1 correlate with progression of hepatic fibrosis/cirrhosis. TGF– β1 cut off value less than 75 ng/ml is an indicator of

stable disease. Limitation of levels of TGF– β1 is due to contamination of sample by platelet TGF– β (61).

Platelet Derived Growth Factor BB (PDGF– BB)

PDGF– BB is expressed by platelets, fibroblasts, endothelial cells, mast cells, and macrophages (62). It is the main subunit which stimulates HSC proliferation and migration. Serum levels of PDGF– BB have correlation with severity of hepatic fibrosis/cirrhosis. In early studies by Pinzani *et al* and Ikura *et al*; PDGF– BB mRNA expression was found to be markedly elevated in CLD (63, 64). Recent studies by Yoshida *et al* and Jiyuan *et al* showed decreased serum levels of PDGF-BB in liver fibrosis (62, 65).

Microfibrillar associated Protein 4 (MFAP4)

MFAP4 present in ECM including elastin and collagen is a disulfide linked dimer that forms higher oligomeric structure (66). In its C– terminal end, MPAF4 has fibrinogen like domain and in the N– terminal end an integrin binding motif is present (67). Studies conducted by Christian *et al* suggest that MPAF4 has a sensitivity of 91.6% and a specificity of 95.6%. MPAF4 is an ideal serum marker among liver specific proteins (68).

Cytokeratin– 18 fragments (CK– 18)

Cytokeratin– 18 fragments (CK– 18) are the major intermediate filaments present in hepatocytes. Caspase induced apoptosis takes place by cleavage of CK– 18 in different positions and results in the formation of CK– 18 fragments (69). According to Yilmaz *et al* and Yang *et al*; levels of M30 antigen (a neoepitope in CK– 18) and M65 (cytosolic

pool of CK– 18) can distinguish between advanced fibrosis/cirrhosis and early stage fibrosis/cirrhosis (70, 71).

Though single direct marker may serve as an indicator of disease severity; there is growing consensus about combination of multiple markers as an integrated panel which will enhance the performance characteristics in terms of specificity and sensitivity.

Grigorescu in his review documented the best diagnostic accuracy is for HA (86%), laminin (81%), PIIINP (74%) and TGF- β (67%). However, Grigorescu mentioned, the diagnostic advantages over nonspecific markers *viz.*, prothrombin index, γGT, PGA score and α-2 macroglobulin were not reported (40). Murawaki *et al* inferred HA and MMP–2 are useful for diagnosing stages of fibrosis/cirrhosis, but cannot replace liver biopsy as there is an overlap among stages and grades in liver fibrosis/cirrhosis (72). The European Liver Fibrosis Study compared the diagnostic performance of HA, PIIINP and TIMP–1 with liver biopsy with threshold sensitivity greater than 90% and specificity greater than 90% can detect liver fibrosis/cirrhosis (73). Patel *et al* and Hind observed the diagnostic value of HA, TIMP–1 and α2–macroglobulin can differentiate chronic hepatitis C patients with moderate/severe fibrosis from those with no or mild fibrosis (74, 75) (**Table 2.2**).

Indirect markers of cirrhosis of liver

Indirect markers, reflects alteration in hepatic function. These markers are useful in diagnosing, evaluating severity, monitoring therapy and also assessing the prognosis of liver diseases. These include measurement of activity of enzymes *viz.*, aminotransferases, alkaline phosphatase (ALP) and gamma glutamyl transferase (γGT) and estimations of

bilirubin and albumin in blood (76). These indirect markers are for liver injury, not for liver function and should be referred as liver chemistries or liver tests (77).

Table 2.2: AUROC for direct markers in various etiology of CLD (25)

Biomarker	Liver disease evaluated by biochemical marker				AUROC for advanced CLD
	CHC	CHB	NAFLD	ALD	
PICP	NA	_	_	NA	NA
PIIINP	0.69 - 0.78	_	NA	0.67 - .87	0.67 - 0.87
Type IV collagen	0.73 - 0.83	_	0.82	NA	0.58 - 0.83
HA	0.82 - 0.92	0.98	0.97	0.69 - 0.93	0.69 - 0.98
Laminin	0.54 - 0.82	_	NA	NA	0.46 - 0.82
YKL-40	0.7 - 0.81	_	NA	NA	0.7 - 0.81
MMP-2	0.59	_	_	_	0.59

Abbreviations: CLD: Chronic Liver Disease; PICP: Procollagen I Carboxy Peptide; PIIINP: Procollagen III Amino Peptide; HA: Hyaluronic acid; MMP: Matrix Metallo Proteinase; CHC: Chronic Hepatitis C; CHB: Chronic Hepatitis B; NAFLD: Non Alcoholic Fatty Liver Disease; ALD: Alcoholic Liver Disease; AUROC: Area Under Receiver's Operating Curve; NA: AUROC is not available

Aminotransferases

Liver disease is most important cause of increased transaminase activity in serum. Serum activities of aspartate amino transferase (AST) (EC 2.6.1.1) and alanine amino transferase (ALT) (EC 2.6.1.2) are elevated when disease processes affect liver cell integrity. Between these two, ALT is more specific enzyme for liver insult. Alterations of ALT activity persist longer than AST activity. Activities of both enzymes may reach as high as 100 times upper reference limit in liver diseases. Peak activities bear no relationship to prognosis and may fall with worsening of patient's condition (78). AST/ALT ratio >1 is a

prediction of cirrhosis, and has sensitivity and specificity of 81.3% and 55.3% respectively. In some etiologies of chronic hepatitis, the ratio is ≤ 1, whereas ratio >2 suggest alcoholic hepatitis (79).

Alkaline phosphatase (ALP) (EC 3.1.3.1)

Zinc metalloproteinase enzyme, ALP catalyses the hydrolysis of phosphate esters at alkaline pH. Response of liver to any form of biliary tree obstruction induces the synthesis of ALP from canalicular membrane of hepatocytes (77). Thus newly formed enzyme enters the circulation to increase the enzyme activity in serum. Elevation tends to be more notable in extrahepatic obstruction than in intrahepatic obstruction. Serum enzyme activities may reach 10 to 12 times the upper reference limit. Liver diseases that principally affect parenchymal cells *viz.*, infectious hepatitis typically show only moderate increase or even normal serum ALP activity. Increase may also be seen as a consequence of response to drug therapy (80).

Gamma glutamyl transferase (γGT) (EC 2.3.2.2)

Elevated activities of γGT are found in serum of alcoholic hepatitis patients. Moderate elevations occur in infectious hepatitis. Increased activity of γGT is also found in serum of subjects receiving anticonvulsant drugs (Phenytoin and Phenobarbital). γGT is a sensitive indicator and elevated in most of the subjects with liver disease regardless of cause, but its efficacy is limited due to lack of specificity (77, 80).

Albumin

Liver has synthesizing capacity to maintain albumin concentrations until parenchymal damage is more than 50%. Plasma albumin measurements are useful in assessing chronicity and severity of the disease. However, its utility for this purpose is limited, as plasma albumin concentration is also decreased in acute kidney disease (80).

Bilirubin

Sequential measurement of bilirubin is supportive in assessing the severity of liver damage due to different etiology. In acute hepatitis, serum bilirubin peaks later than enzymes but remains elevated for longer time (77, 80).

Prothrombin Time (PT)

Serial PT measurements can be used to differentiate between cholestasis and severe hepatocellular diseases. In severe hepatocellular damage, PT remains elevated for a longer time. Cholestasis will cause a decrease in PT as a result of malabsorption of vitamin K (40, 80).

Combinatorial use of Biomarkers

Combination of different markers can improve sensitivity and specificity of these tests (75).

AST/Platelet Ratio (APRI) (%)

$$\boldsymbol{APRI = \frac{AST\ (upper\ normal\ value)}{Platelet\ count\ (10^9/L)} \times 100}$$

Wai *et* al developed APRI. APRI > 1.5 has area under receiver's operating curve (AUROC) of 80% and 89% for advanced fibrosis F3–F4 and cirrhosis respectively (81). According to Snyder *et al*, APRI cut-off 0.42 or less has high diagnostic accuracy with NPV of 95% (82). In autoimmune hepatitis, Loaeza Del Castillo *et al* showed that APRI does not have any diagnostic value in assessing fibrosis (83).

Lok *et al* enhanced diagnostic accuracy of APRI by incorporating ALT and international normalized (INR) ratio in assessing the progression of liver fibrosis/cirrhosis in post liver transplant patients (84).

Bonacini Index

$$\boldsymbol{Bonacini\ cirrhosis\ discriminant\ Score} = \boldsymbol{Platelet\ score} + \boldsymbol{ALT}: \boldsymbol{AST\ ratio\ score} + \boldsymbol{INR\ score}$$

Bonacini *et al* developed a discriminant score (**Table 2.3**) for diagnosis of advanced fibrosis and cirrhosis by taking three parameters: platelets, ALT/AST ratio and PT which has positive correlation with histological scores and has 98% specificity but 46% sensitivity (85).

FIB – 4 Score

$$\mathbf{FIB-4} = \frac{\mathbf{Age\ (years)} \times \mathbf{AST\ (U/L)}}{\boldsymbol{Plateletcount}\ (\mathbf{10^9/L})} \times \sqrt{\mathbf{ALT\ (U/L)}}$$

Vallet *et al* developed a score to assess liver fibrosis/cirrhosis in HIV/HCV coinfected patients and successfully classified 87% patients at a cut off of 3.25 with an AUROC of

76% (86). Further validation of this score showed AUROCs of 85% and 81% for monoinfected HCV and HBV patient's respectively (87, 88).

Table 2.3: Bonacini cirrhosis discriminant parameters score (25)

Score	Platelets (10^3/µL)	ALT:AST ratio	INR
0	> 340	>1.7	<1.1
1	280 – 340	1.2 – 1.7	1.1 – 1.4
2	220 – 279	0.6 – 1.19	>1.4
3	160 – 219	<0.9	
4	100 – 159	-----	-----
5	40 – 99	-----	-----
6	< 40	-----	-----
Abbreviations: ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; INR: International Normalized Ratio			

Fibro Index

1.738 – 0.064 × platelet count (10^4/mm^3) + 0.005 × AST (IU/L) + 0.463 × gamma globulin (g/dl)

Halfon *et al* developed score from platelet count, AST and γGT to assess fibrosis. A cut off of 2.25 was correlated with F2–F3 fibrosis and has 90% NPV (89). However further validation showed this score has less diagnostic accuracy (90).

Fibro Test

4.467 x $\log_{10}$ [alpha2 macroglobulin (g/L)] – 1.357 × $\log_{10}$ [haptoglobin (g/L)] + 1.017 × $\log_{10}$ [γGT (IU/L)] + 0.0281 × [age (years)] + 1.737 × $\log_{10}$ [bilirubin (μ mol/L)] – 1.184 × [apolipoprotein A1 (g/L)] + 0.301 × sex (female=0, male=1) – 5.54

Fibro Test (Fibro Sure in USA) was patented since 2001 by APHP (Assistance publique - Hopitaux de Paris), the Parisian public hospital system. Fibro test is the most validated test and based on age, gender, serum haptoglobin, α2 macroglobulin, apolipoprotein A1, γGT and bilirubin. However, it is less significant in detection of intermediate stages of fibrosis (91). Poynard *et al* established high accuracy of Fibro test in steato hepatitis with AUC of 85% (92).

Forns Index

7.811 – 3.131 × ln (platelet count [10^9/L]) + 0.781 × ln (γGT [IU/L]) + 3.467 × ln (age) – 0.014 × cholesterol (mg/dl)

In 2002, Forns *et al* developed this score by calculating age, platelet count, serum cholesterol and γGT which can differentiate mild fibrosis with advanced fibrosis at a cut off value of 6.9 (93). Further Adams *et al* validated this index and showed sensitivity of 94%, specificity 51% with AUROC 81% to 86% (94).

PGA Index

1. **P**rothrombin Time (control): ≥ 80% = 0; 70% – 79% = 1; 60% – 69% = 2;

   50% – 59% = 3; < 50% = 4

2. γGT (IU/L): < 20 = 0: 20 – 49 = 1; 50 – 99 = 2; 100 – 199 = 3; ≥ 200 = 4

3. **A**polipoprotein A1 (mg/dL): ≥ 200 = 0; 175 – 199 = 1; 150 – 174 = 2;

   125 – 149 = 3; < 125 = 4

4. **α**2 macroglobulin (g/L): < 1.25 = 0; 1.25 - 1.74 = 1; 1.75 - 2.24 = 2;

   2.25 - 2.74 = 3; ≥ 2.75 = 4

PGAA index is the sum of the above

Poynard *et al* anticipated PGA index in combination with γGT, Prothrombin index and apolipoprotein A to assess alcoholic liver disease (95). The accuracy of this index has been increased from 65% to 70% by addition of α2 macroglobulin (PGAA) (96).

Calculating such score greatly improves sensitivity and specificity and can avoid limitations of individual marker. Combinations of direct and indirect markers may increase diagnostic accuracy, but has not been implemented in clinical practice (**Table 2.4**) (97). Scores may give clear positive or negative prediction only at early stages of fibrosis/cirrhosis. In acute hepatic injury, there may be false positive results in scores such as APRI, Forns index and FIB-4. False positive results may be possible for Fibro test with respect to haemolytic and hyper bilirubinemia (46). WHO 2015 report documented APRI and Fibro test are preferred non-invasive tests to assess the presence of cirrhosis caused by hepatitis B (98). APRI has low performance when compared to FIB-4 and Fibro test in liver disease caused by HBV and HCV (99). FIB-4 cut offs were

initially validated only for F3 and F4, and needs specific validation before comparing with Fibro test and APRI (86).

Table 2.4: Main scoring system for CLD with sensitivity and specificity (25)

Test	Parameters	Sensitivity %	Specificity %
APRI	AST/platelet count	57	93
AST/ALT	AST/ALT	51	71
Bonacini Index	ALT/AST, INR, platelet count	46	98
ELF Index	Age, HA, PIIINP and TIMP-1	90	69
FIB – 4	Platelet count, AST, ALT and age	65	97
Fibro Index	Platelet count, AST and γ globulin	35	97
Fibrometer Test	Platelet count, INR, AST, α2 macroglobulin, HA, urea and age	80	84
FibroSpect II	HA, TIMP-II and α2 macroglobulin	76	73
Forns Test	Age, platelet count, γGT and cholesterol	30	95
Globulin - albumin Ratio	Globulin and albumin	43	98
GUCI	Platelet count, AST and INR	80	78
Hepascore	age, gender, bilirubin, γGT, HA and α2 macroglobulin	84	71
Lok Index	Platelet count, AST, ALT and INR	68	72

Abbreviations: ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; INR: International Normalized Ratio; HA: Hyaluronic acid; PIIINP: Procollagen III amino peptide; TIMP-1: Tissue inhibitor of metalloproteinase I; TIMP-II: Tissue inhibitor of metalloproteinase II; γGT: Gamma glutamyl transferase

Grading and Prognosis of Cirrhosis of Liver

Poor prognosis is associated with prolonged PT, high serum bilirubin and ALP, elevated serum creatinine and also low albumin values, encephalopathy, marked ascites, gastrointestinal bleeding and advanced age.

Child- Pugh score (100)

Dr C.G. Child and Dr J.G. Turcotte, University of Michigan first proposed the scoring system in 1964 and it has been modified by Pugh *et al* in 1972 by addition of Prothrombin time International Normalized Ratio (PT INR) (**Table 2.5**). Severity of cirrhosis of liver is classified as class A, B and C based on Child-Pug score. Child pug score includes serum bilirubin, serum albumin, plasma INR, presence and severity of ascites and encephalopathy (**Table 2.6**).

Table 2.5: Calculation of Child- Pugh score

Parameter	1 point	2 point	3 point
Total bilirubin (mg/dL)	< 2	2 – 3	> 3
Serum albumin (g/dL)	> 3.5	2.8 - 3.49	< 2.79
PT – INR	< 4.0	4.1 - 6.0	> 6.1
Ascites	None	Mild	Moderate/ Severe
Hepatic encephalopathy	None	Grade I & II	Grade III & IV
Abbreviations: PT-INR: Prothrombin time International Normalized Ratio			

Table 2.6: Interpretation of cirrhosis of Liver: Child-Pugh Class A to C (100)

Points	Class	One year survival	Two year survival	Comments
5 – 6	A	100%	85 %	Favorable prognosis
7 – 9	B	81 %	57 %	Moderate risk
10 – 15	C	45 %	35 %	Very high risk resulting in death

Model for End Stage Liver Disease score (MELD) (100)

MELD was developed at the Mayo Clinic by Dr. Patrick Kamath as "Mayo End-stage Liver Disease" score; later modified as Model for End Stage Liver Disease. MELD scoring system was developed to determine the prognosis in patients with cirrhosis of liver.

MELD = 3.78 × ln [serum bilirubin (mg/dL)] + 11.2 × ln [INR] + 9.57 × ln [serum creatinine (mg/dL)] + 6.43

Need for the Study

Existing biomarkers for cirrhosis of liver in clinical practice have narrow applicability due to lack of specificity (predict etiology) and lack of sensitivity (distinguish intermediate stages). The scoring systems play a major role only after the effect; early diagnosis of cirrhosis of liver is neither conclusive nor clear. Thus the need of the hour for early diagnosis and management should include a biomarker which should be organ specific, sensitive, predictive, rapid, simple, accurate, cost effective and easily available (25).

A biomarker is a molecule that is analytically measured with well-established performance characteristics in an established scientific frame work of evidence that elucidates physiological, toxicological, pharmacological or clinical outcome (101). Validation of a biomarker includes assessing the biomarker, its measurement performance characteristics, determining the range of conditions for reproducibility and accuracy. Biomarker validation relates biomarker with biological process and clinical end point and is necessary for fit-for-purpose. Validation helps research data for better patient care. An ideal biomarker for CLD should be tissue specific, sensitive to indicate active damage, able to detect pathology prior to histological changes, easily accessible in peripheral tissue and cost effective (25, 101).

A biological marker is objectively measures and evaluates normal biological, pathogenic process or pharmacological response to a drug. Surrogate markers serve as a substitute for a clinically meaningful endpoint. Prognostic biomarker indicates likely outcome of a disease irrespective of treatment. Predictive biomarker helps to assess response to a

particular treatment. Pharmacodynamic biomarker gives interaction between drug and target (101, 102).

Biomarker development by proteomic approach

Establishment of correlation between disease state and biomarker alterations will help clinician for diagnosis and tailored therapy (103). In CLD, protein domain will have alterations where the amount of protein from liver enters into circulation and serves as an indicator for degree of liver dysfunction; holds good for discovery of novel protein biomarkers using proteomics (20). Proteins have more structural diversity and stability than DNA and RNA; carry more information than nucleic acids which are dynamic and reflection of cellular physiology (104). Advances in proteomic approach help discover and identify clinically significant protein biomarker candidates for CLD. Protein biomarker pipeline include series of essential components viz., discovery, research assay optimization, analytical and clinical validation and clinical utility (105, 106) (**Figure 2.5**).

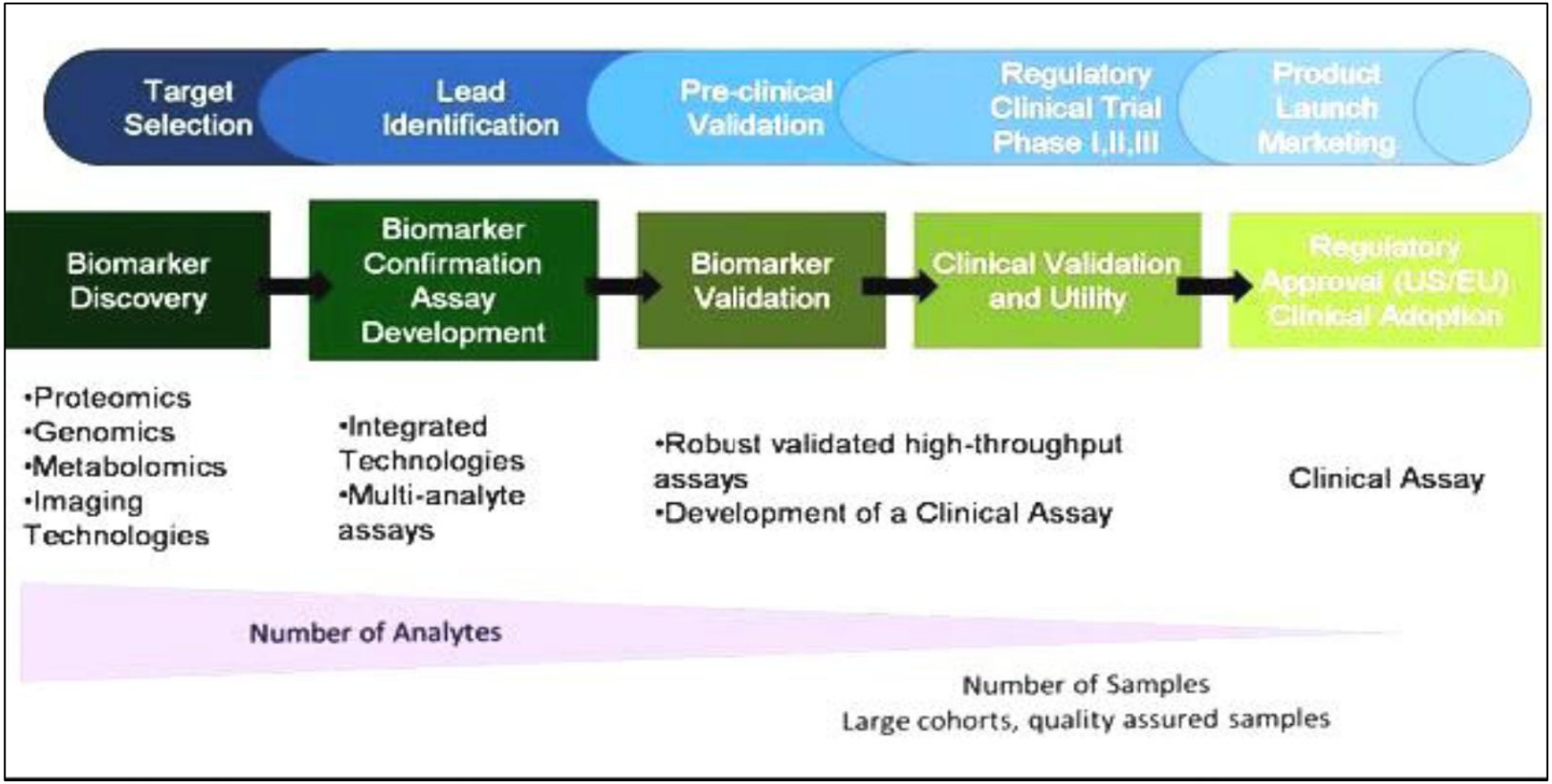
Protein biomarker candidate discovery

Protein biomarker discovery is a simplified, semi quantitative, unbiased binary comparison between diseased and normal by using biological samples for maximal detection of significant protein expression differences. It needs avoiding contamination of other diseases and confounding factors (25, 107). Proteins that are differentially expressed between CLD and normal are due to changes in translation, post translational modifications and degradation or that are involved in pathophysiological changes which are good sources of biomarker candidates (104). Comparative analysis between diseased

and healthy generates hundreds of protein biomarker candidates that are differentially expressed. There is an inverse relationship between number of samples analyzed and number of proteins quantified (107, 108) (**Figure 2.6**).

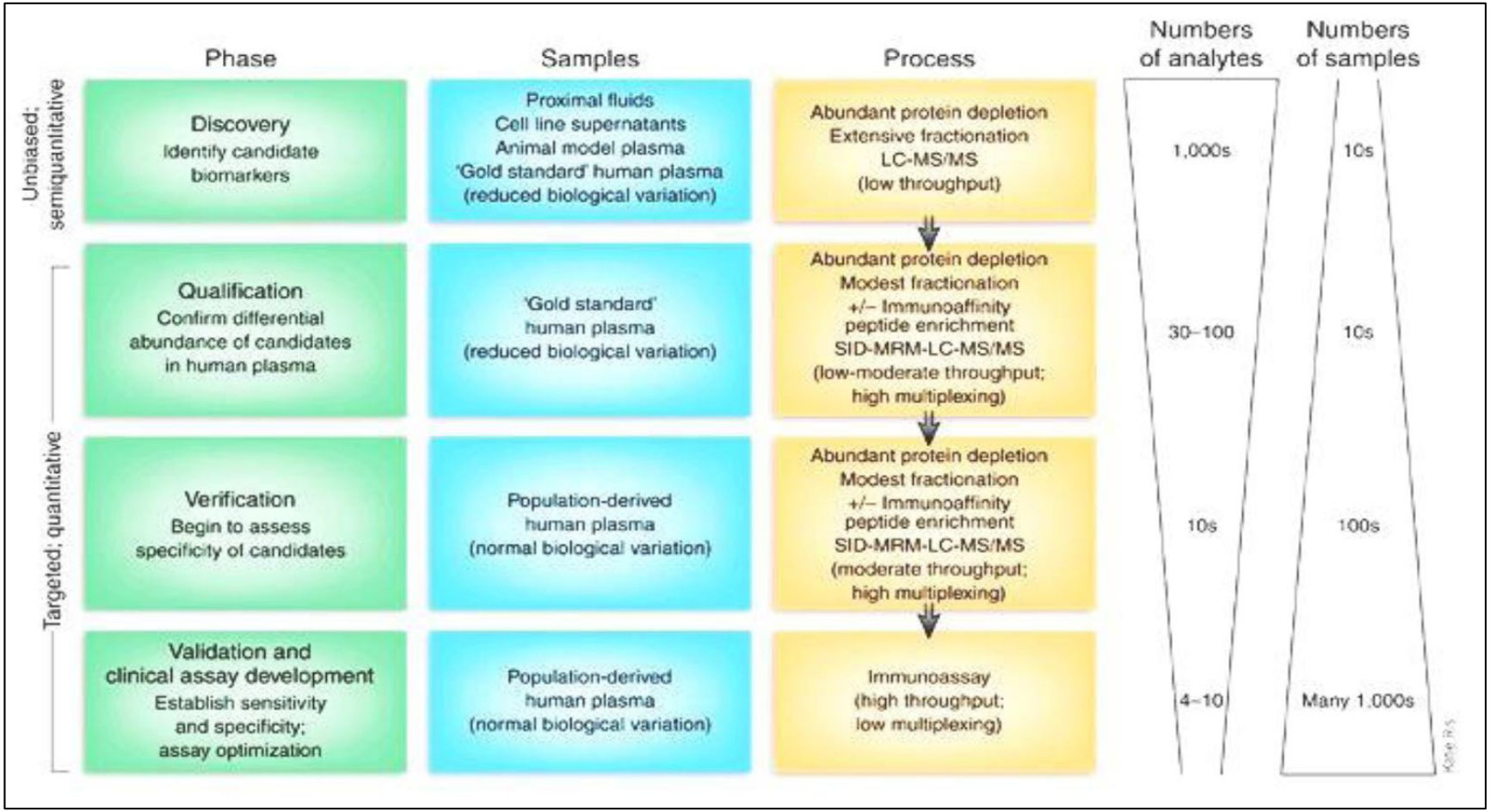
Discovery of protein biomarker candidates for CLD; plasma/serum is the best choice among other body fluids and represents physiological and pathological process (109). During discovery phase, the variables (study design, preanalytical and analytical) which affect precision should be minimized. Study population should be selected from a well-defined study design with definite inclusion and exclusion criteria to minimize bias. Case control study or cohort study usually considered as a better study design for discovery phase of biomarker. Multiple sources of bias could be seen in retrospective and observational studies (110). Preanalytical variables *viz.*, type and manufacture of collection tubes, phlebotomy device, patient's posture, time of sample collection, type of sample to be collected, storage conditions and sample preparation should be controlled in order to get significant observation. Analytical variables; mass resolution and collision energy needs to be controlled to minimize the source of variations (107, 108).

In candidate discovery for CLD, to obtain significant protein expressional difference, use of gold standard sample is recommended. Plasma is the biofluid of choice [Human Proteomic Organization (HUPO)]; contains proteins that reflect a variety of human diseases (111). Anticoagulants, (EDTA or citrate) cause osmotic shifting of fluid from cell to plasma which gives 10% less values when compared to serum and are known to chelate cations, give negative results in case protein of interest has cations in its structure. Antigenic epitope mask might happen which reduces immunoreactivity because of heparin (107, 112).



Source: Nies KM, Ivy DD, Everett DA. The untapped potential of proteomic analysis in pediatric pulmonary hypertension. Proteomics Clin Appl. 2014; 8: 862-874.

Figure 2.5: FDA protocol for biomarker discovery, verification and validation (106)



Source: Rifai N, Gillette AM, Carr AS. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nature Biotechnology. 2006; 24(8): 971-983

Figure 2.6: Protocol for the development of protein biomarker (107)

Compared to plasma, proteins are more stable in serum. For large studies, serum is the preferred sample by clinicians since it is the simplest matrix (108). Although individual sample analysis is recommended, pooling strategy with definite criteria from multiple individuals reduces sample number and cost (113).

Protein biomarker discovery in plasma/serum is complicated. Around 99% of protein content is comprised of 20 abundant proteins which interfere in identification and characterization of low abundant proteins by mass spectral and electrophoretic analysis (114, 115, 116, 117). Depletion of high abundant proteins allows detection of low abundant proteins and which may further remove low abundant proteins that are bound to high abundant proteins (108). For accurate protein biomarker candidate discovery for CLD, depletion of albumin (55% of total protein) and immunoglobulins may be achieved using high affinity columns (115, 116). In two-dimensional gel electrophoresis (2-DE), depletion dilemma can be rectified using narrow pH (3-5.6) range which avoids interference of abundant proteins (albumin, transferrin and immunoglobulins) (20, 108).

After depletion, discovery may be carried out by fractionation and purification using different analytical methods: 2-DE for separation of proteins followed by identification of significant protein spots using software tools. Identified spots are subjected for in-gel digestion to identify peptides and proteins either by Surface-Enhanced Laser Desorption-Ionization (SELDI) or Matrix-Assisted Laser Desorption-Ionization (MALDI) or Liquid Chromatography (LC) - Mass Spectrometry (MS) and proteomics search engines viz., MASCOT or SEQUEST (118, 119, 120). Unlike gel based discovery, LC-MS carried out before or after enrichment of proteins by trypsin digestion, splitting long proteins into

short peptides followed by chromatographic separation in addition to mass to charge ratio (108, 121, 122).

2-DE has limited sensitivity, reproducibility compared to LC-MS. The main disadvantage of SELDI/MALDI is difficulty in detection of differential pattern and identification of peaks. Automated LC-MS is suitable for protein biomarker discovery. Secondary ions collected from chromatographic profiles from MS spectra are subjected to proteomic search engines (123, 124). Identified peptides are used to determine differential expression between CLD and normal. Use of parametric statistical tools prior to peptide identification is recommended. Biomarker candidates reported and identified for CLD by one group of researchers are not identified by another group because of lack of standardization of multi-step procedures. Selection of specific criteria during LC-MS gives complexity and errors for reproducibility between laboratories. Biomarker discovery and validation should be performed in a blinded fashion, free from bias and performed in a similar fashion which remove all confounding factors and generates significant biomarker candidates (124).

Biomarker validation

Biomarker validation is necessary to deliver high quality research data for effective use of biomarker for better patient care. Great interest and technological advancement in biomarker discovery results in identification of protein biomarker candidates for CLD. Biomarker candidates require verification that demonstrates the differential expression which remains detectable by assay to be used for validation (107). Despite, numerous biomarker candidates identification, verification may be done only for few qualified

candidates in terms of marker performance and reagent availability (104). Proteins which act in cellular pathways and deregulated in CLD should be considered for further validation (113). Validation of biomarker and clinical assay optimization requires measurement of thousands of patient samples with narrow measurement coefficient of variation values (107).

Assay optimization

As MS is unable to achieve high measurement accuracy and precision, it is necessary to develop antibodies for quantification of biomarker candidates. Concentration of protein in serum or plasma range from picogram to nanograms per milliliter, highly sensitive immunotechniques are required for quantification. Enzyme Linked Immuno Sorbent Assay (ELISA) is the best alternative for quantification of these proteins compared to sophisticated non immune based techniques (125). Capture and detection antibodies (monoclonal or polyclonal) which detect distinct epitope of the protein are needed to form sandwich reaction. Specificity of antibodies is established by using western blot or immunostaining. During development of ELISA, care should be taken to minimize the effect of variables viz., avidity, concentration of antibodies (monoclonal capture/detection 0.5-4/0.25-2 µg/ml, polyclonal capture/detection 0.2-0.8/0.05-0.4 µg/ml respectively), incubation time and temperature, sample volume, dilution of sample, pH, composition and concentration of diluents, enzyme, substrate and quality of detector which affect performance characteristics. Fluorescent or chemiluminescent are other alternatives for better sensitivity (107, 126).

Analytical evaluation

Newly developed assay requires analytical validation before evaluating clinical utility in terms of performance characteristics viz., outcome studies, clinical requirement, proficiency testing and goals set by regulatory agencies (125). Preanalytical variables should be characterized and controlled in various physiological and pathological conditions. Time of collection of sample (fasting or fed state) should be defined. In fed state, chylomicrons do not affect ELISA. Selection of appropriate sample (plasma or serum) and use of anticoagulants should be determined. Storage conditions and duration of storage should be confirmed appropriately. Physiological factors viz., age, gender and ethnicity significantly affect protein concentrations along with lifestyle factors. Pathological conditions and drugs which influence protein concentrations should be examined before estimation (107).

Indicators of accuracy, precision, analytical measurement range and reference intervals should be defined. Trueness is the closeness of agreement between average measured values of different samples which reflect bias (systemic error). Accuracy is the closeness of agreement between the values measured and true concentration of analyte (127). Newly discovered methods usually do not have reference materials and methods, should use alternative protocols viz., spike, recovery and linearity. Use of specific antibodies should be necessary to have no cross reactivity with other proteins. Care should be taken during ELISA development to minimize the errors because of exogenous and endogenous substances. The factors (buffer components, sample matrix, compliment and rheumatoid factor) can impact antibody binding in natural samples and therefore influence the accuracy of results should be ruled out (128). Repeatability is the measurement

performed in same condition, reproducibility is measurement performed in different conditions. To assess precision, two replicates per sample per run, and two runs per day for least 20 days is recommended (129).

Reference intervals must be defined for protein of interest and new methodology by comparing healthy individual values similar to those of patient values (129). Reference values should be subdivided into groups based on age, gender, race and physiological states. Normal distribution of reference intervals for protein of interest for parametric analysis is presented as mean ± 2SD and for non-parametric analysis will be presented as percentiles. Limits of detection and quantification must be defined with acceptable accuracy and precision. Limit of detection is the lowest value that exceeds the measurand value against blank sample which does not have protein of interest. Linearity gives the relation between observed value and expected value which is above the range of measurand values (107, 129).

Clinical validation

After analytical validation of new methodology for protein of interest, biomarker candidate should confirm the performance characteristics in terms of consistency and accuracy in clinical evaluation to diagnose or predict the clinical outcome of CLD. The newly identified biomarker candidate should satisfy following criteria (**Table 2. 7**) (130).

Table 2.7: Criteria to be satisfied by newly identified biomarker candidate

Sensitivity of biomarker	The ability of a biomarker or change in magnitude of a biomarker with precision which is sensitive enough to reflect a meaningful change in clinical endpoint of CLD
Specificity of biomarker	The ability of a biomarker or change in magnitude of a biomarker which distinguish patients who are responders and non-responders in terms of change in clinical endpoint of CLD
Probability of false positive	Desired change of biomarker is not reflected by positive change in clinical endpoint or even worse is associated with negative change in a clinical endpoint of CLD
Probability of false negative	No change or small change is observed in magnitude of biomarker which fails to signal positive and meaningful change in a clinical endpoint of CLD
Pharmacokinetic/ Pharmacodynamic model	Correlation between changes in biomarker and drug exposure, to predict future outcome or standardization of dose adjustments based on biomarker measurements

Likelihood ratio of biomarker indicates certainty of the diagnosis of disease prevalence and calculates post test odds of having a disease as the prevalence changes. Receiver operating characteristic (ROC) curve is the comparison of diagnostic accuracy of two or more test and to define appropriate cut off for clinical utility of test. Likelihood ratio and ROC curve are derived from sensitivity and specificity values (25, 131).

Clinical utility

Clinical utility predict positive outcome of drug in selected and unselected groups. Novel biomarker candidate needs to be evaluated in a series of human population (sub and stratified).

a) **Phase I** (exploratory phase): test results should be different from patients with confirmed CLD and those of control population without CLD. Area under ROC curve should be > 0.5 for newly identified biomarker candidate to proceed further
b) **Phase II** (challenge phase): different cut off values for sensitivity and specificity should be defined with diagnostic accuracy to predict the presence or absence of CLD
c) **Phase III** (advanced clinical phase): is to establish diagnostic accuracy of biomarker in target population in different geographical regions independently
d) **Phase IV** (outcome phase): gives the positive influence of test to get healthy outcome of CLD by evaluating the both tested and untested patients with respect to diagnostic and therapeutic intervention (107, 132, 133)

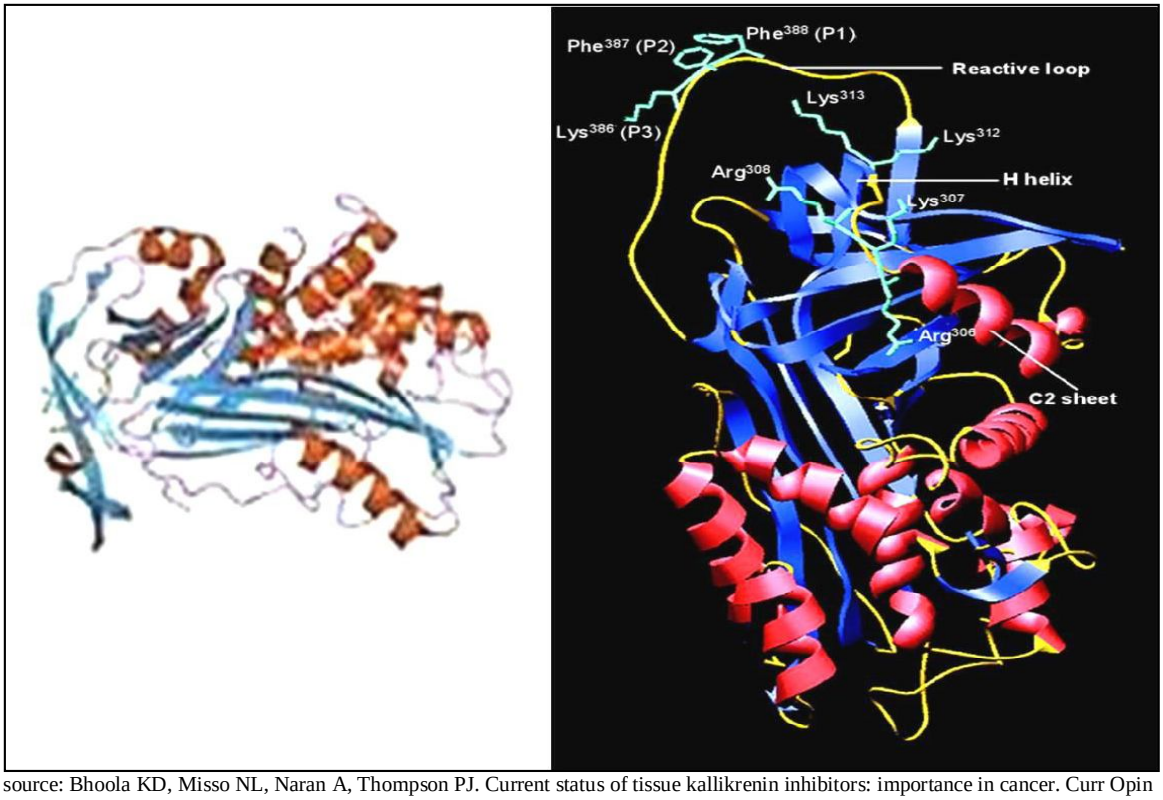
In this regard, proteins which are expressed from liver and enters into circulation reflects degree of liver dysfunction and holds good for biomarker candidate discovery using technological advancement in proteomics (134). Proteins have more stability than DNA and RNA; carry more information than nucleic acids which are dynamic and reflection of cellular physiology (104). Protein biomarker pipeline include series of essential components *viz.*, discovery, analytical and clinical validation and clinical utility.

Thus, in our study the newly discovered protein biomarker candidate by proteomic approach, SERPINA4/Kallistatin has documented to play a role for early diagnosis and prognosis of cirrhosis of liver and needs to be studied in our population.

SERPINA4/Kallistatin is a novel, promising new tissue kallikrein binding plasma protein; is a member of serine proteinase inhibitor super family (22, 135). SERPINA4/Kallistatin forms a specific and covalently linked complex with tissue kallikrein (136). Inhibition of SERPINA4/Kallistatin is accompanied by formation of an equimolar, heat and SDS-stable complex between tissue kallikrein and SERPINA4/Kallistatin and by generation of a small carboxy- terminal fragment from the inhibitor due to cleavage at reactive site by tissue kallikrein. Heparin blocks SERPINA4/Kallistatin's complex formation with tissue kallikrein and abolishes its inhibitory effect on tissue kallikrein's activity (137).

SERPINA4/Kallistatin is an acidic glycoprotein with a mol. wt. of 54kD which is expressed from liver cells (Hep G2 and Hep 3B). The isoelectric pH of SERPINA4/Kallistatin ranges from 4.6 to 5.2. Serine proteinase inhibitor, SERPINA4/Kallistatin has a strong inhibitory action on tissue kallikrein and weak inhibitory action on other proteinases *viz.,* chymotrypsin and elastase (138). SERPINA4/Kallistatin [Serpin Peptidase Inhibitor, Clade A (α1 Antiproteinase, Antitrypsin) member 4], is a protein that in humans is encoded by the *SERPINA4* gene. *SERPINA4* gene is mapped to chromosome 14q31-32.1, which shows 5 exon and 4 intron serpin gene structures. The cDNA encoding human liver SERPINA4/Kallistatin has 1284 bp and encodes 427 amino acid residues of which 26 residues are signal peptide and 401 residues are mature peptide. Genomic southern blot using the full length

SERPINA4/Kallistatin cDNA probe revealed simple banding patterns suggesting the gene encoding SERPINA4/Kallistatin is a single copied (139, 140).



source: Bhoola KD, Misso NL, Naran A, Thompson PJ. Current status of tissue kallikrenin inhibitors: importance in cancer. Curr Opin Investig Drugs. 2007; 8(6): 462-468.

Figure 2.7: Structure of SERPINA4/Kallistatin (141)

Human SERPINA4/Kallistatin has a unique cleavage site with Phe- Phe- Ser at P2-P1-P1[1]. The basic residues, Lys (312) - Lys (313), in the region between the H helix and C2 sheet of Kallistatin, comprise a major heparin binding site responsible for its heparin suppressed tissue kallikrein binding (140). Crystallized SERPINA4/Kallistatin exists in relaxed confirmation, with its reactive center loop inserted in the central β sheet (142). SERPINA4/Kallistatin mRNA is found in cells of liver, stomach, pancreas, aorta, prostate, atrium, ventricle and lungs with unclear sub-cellular site. SERPINA4/Kallistatin is present in tissues of eye, kidney, liver, heart, arteries and veins, atheroma, blood cells

and body fluids. Liver is the major site for the synthesis and secretion of SERPINA4/Kallistatin (22). SERPINA4/Kallistatin is known to play a role in prevention of various diseases *viz.*, cancer, cardiovascular disease and arthritis through the effects of anti-angiogenic, anti-inflammatory, anti-apoptotic and antioxidative process (143).

SERPINA4/Kallistatin may be involved in regulation of the process of inflammation and coagulation in severe community acquired pneumonia (CAP) patients. Lower levels are associated with more severe illness and increased mortality. Level of SERPINA4/Kallistatin is positively correlated with the levels of anticoagulation factors and inversely correlated with the levels of inflammatory mediators. These findings indicate that SERPINA4/Kallistatin may be protective against severe CAP which implies possible therapeutic benefits of SERPINA4/Kallistatin in these patients (143).

Animal experiments have shown that SERPINA4/Kallistatin gene therapy has a prophylactic effect in inhibiting arthritis. SERPINA4/Kallistatin inhibits arthritis through its anti-angiogenesis and anti-inflammatory activity. This implies that the therapeutic application of suppression of arthritis by SERPINA4/Kallistatin gene therapy (144). SERPINA4/Kallistatin cures fibrosis of organs by suppressing TGF-β. TGF-β is an enhancer for endothelial mesenchymal transition (EndMT) which plays an important role in organ fibrosis and cancer. SERPINA4/Kallistatin treatment blocks TGF-β induced EndMT by morphological changes, increases endothelial and reduces mesenchymal expression (145).

SERPINA4/Kallistatin has two structural elements namely “active site” and “heparin binding domain”. SERPINA4/Kallistatin inhibits formation of ROS, expression and

activity of NADPH oxidase. It blocks TGF-β induced miR-21, snail synthesis, necrotic factor (NF) activation, MMP 2 synthesis and activation, ROS formation by its heparin binding site. SERPINA4/Kallistatin stimulates the synthesis of endothelium nitric oxide synthase by its active site; these effects can be blocked by tyrosine kinase inhibitor "Genistein". SERPINA4/Kallistatin's heparin binding site is crucial in preventing TGF-β induced miR-21 and oxidative stress, while its active site is key region for stimulating the expression of antioxidant genes via interaction with an endothelial surface tyrosine kinase. This implies that SERPINA4/Kallistatin has a protective activity against fibrosis and cancer (145).

SERPINA4/Kallistatin inhibits VEGF, basic fibroblast growth factor (bFGF) induced proliferation, migration and adhesion of endothelial cells, implies the novel role of SERPINA4/Kallistatin in inhibition of angiogenesis and tumor growth (146). SERPINA4/Kallistatin is a pleiotropic cytokine which has therapeutic uses in prevention of cardio- metabolic disorders, vascular injury, arthritis, cancer, kidney fibrosis, cardiac hypertrophy and fibrosis. It is well documented that prolonged inflammation and oxidative stress are emerging as key causes of pathological wound healing and development of liver fibrosis/cirrhosis. SERPINA4/Kallistatin scavenges H_2O_2 induced ROS and suppresses the activation of primary HSCs (147).

Oxidative stress

Free radicals are molecular species capable of independent existence that contains unpaired electron in an atomic orbital; unstable and highly reactive. They can donate an electron or accept an electron from other molecules. Free radicals form continuously in

the cell as a consequence of both enzymatic and non-enzymatic reactions. Because of their unstable and highly reactive nature, free radicals adversely alter lipids, proteins and DNA and trigger a number of human diseases (148). Oxidative damage to proteins occurs: Oxidative modification of specific amino acids, free radical mediated peptide cleavage and formation of protein cross linkage due to reaction with lipid peroxidation products (148)

Lipid peroxidation is a free radical process which involves a source of secondary free radicals that occurs on poly unsaturated fatty acids (PUFA) located on cell membrane. DNA may be damaged by strand breaks. Thus DNA damage may directly cause inhibition of protein and enzyme synthesis leading to mutation or cell death (148). Total cellular damage produced by ROS is prevented by antioxidants. An antioxidant is a stable molecule which can donate electron to neutralize the free radical and delay or inhibit cellular damage (149).

Antioxidants based on biochemical reaction are classified into: Preventive and Chain breaking (149)

a) Preventive antioxidants *viz.*, catalase, glutathione peroxidase inhibit production of free radicals
b) Chain breaking antioxidants *viz.*, superoxide dismutase, uric acid, vitamin E etc., inhibits propagative phase

Oxidative stress is end result of an imbalance between free radical production and anti-oxidant defense leading to damage of lipids, proteins and nucleic acids. Oxidative stress significantly contributes to all inflammatory diseases, ischemic diseases, haemochromatosis, neurological disorders, alcoholism and smoking related

diseases (150). Metabolism of exogenous and endogenous substances and viral load leads to generation of ROS which causes oxidative stress in pathogenesis of liver diseases. Enzymes *viz.*, superoxide dismutase (SOD), glutathione peroxidase and catalase are essential components of the antioxidant system which require inorganic elements *viz.*, zinc (Zn^{2+}) and copper (Cu^{2+}) for their synthesis. Along with these, ascorbic acid and glutathione are essential to reduce the effects of oxidative stress and they are defective in chronic hepatic diseases (151).

Thus, in the present study, a multifunctional protein, SERPINA4/Kallistatin discovered in discovery phase of biomarker pipeline needs to be validated in terms of analytical and clinical validation. Optimization of research assay for quantification of SERPINA4/Kallistatin needs to be done to rule out interfering substances which may give false diagnostic values and can mislead diagnosis. Correlative analysis with direct biomarkers of ECM, conventional markers, oxidative stress and antioxidant capacity will give clinical validation and can prove SERPINA4/Kallistatin as a diagnostic marker for cirrhosis of liver.

OBJECTIVES

- To estimate the concentration of SERPINA4/Kallistatin in cirrhosis of liver and healthy subjects
- To correlate SERPINA4/Kallistatin levels with hyaluronic acid, YKL-40, conventional biomarkers, oxidative status and antioxidant capacity in cirrhosis of liver

To fulfill the primary objectives, we considered add on secondary objectives

- To discover protein biomarker candidates for cirrhosis of liver by proteomic approach
- To prioritize and identify protein biomarker candidate for analytical and clinical validation
- To check cross reactivity of SERPINs in quantification of SERPINA4/Kallistatin
- To develop ELISA quantitative diagnostic kit for SERPINA4/Kallistatin (research use) with accuracy and precision

MATERIALS and METHODS

4.1. Type of study

Laboratory based case control study

4.2. Materials

4.2.1. Individuals with Cirrhosis of Liver

Individuals attended to Department of Medicine, R. L. Jalappa Hospital and Research Centre attached to Sri Devaraj Urs Medical College, A constituent of Sri Devaraj Urs Academy of Higher Education and Research with diagnosed cirrhosis of liver aged between 18 to 60 years.

4.2.1.1. Inclusion criteria

Individuals diagnosed with cirrhosis of liver based on clinical symptoms *viz.*, ascites, encephalopathy, jaundice, splenomegaly and altered biochemical parameters; caused by different etiologies *viz.*, ALD, viral hepatitis (HBV and HCV), NAFLD, biliary cirrhosis, inherited metabolic liver diseases, autoimmune hepatitis, cryptogenic cirrhosis and cardiac cirrhosis were included in the study

4.2.1.2. Exclusion criteria

a) Physiological conditions e.g., pregnancy

b) Individuals with diabetes and/or complications of diabetes, myocardial infarction, acute and chronic renal failure, pneumonia and cancer

c) Individuals on thrombolytic therapy

4.2.2. Healthy subjects (Control group)

Age and gender matched healthy subjects, not having a history of acute and chronic diseases *viz.,* diabetes, myocardial infarction, acute and chronic renal failure, cancer and pneumonia. Women who are pregnant and subjects on thrombolytic therapy were excluded from the study. Healthy subjects were selected from the patient relatives/attendees and employees of the hospital who are aged between 18 to 60 years. This helps in prevention of selection bias and confounding factors.

Study was approved by the Institutional Ethical Committee (**Ref. No: DMC/KLR/IEC/61/2016-17; dated: 08-08-2016)**. After obtaining informed consent from cirrhosis of liver and healthy subjects; data regarding the health status was collected in semi structured proforma. Venous blood sample of 5ml was collected from antecubital vein in comfortable position of patient and healthy under aseptic precautions; 2ml of blood was transferred to sodium citrate tube for plasma parameters and 3 ml into serum separator tube for serum parameters analysis. All standard precautions were taken so that haemolysis and pre examination errors were minimized. Parameters which can be analyzed later and whose values do not alter with proper storage were stored at – 20^{o}C until further analysis.

4.2.3. For discovery of protein biomarker candidates

Blood samples were collected from 20 subjects: 10 clinically and diagnostically proven alcoholic cirrhotic liver with varying degree and age and gender matched 10 healthy from Department of Medicine, R. L. Jalappa Hospital and Research Centre attached to Sri

Devaraj Urs Medical College, A constituent of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India (**Table 4.2.3.1**).

Individuals diagnosed with cirrhosis of liver caused by ALD based on clinical history and symptoms *viz.*, ascites, encephalopathy, jaundice and altered biochemical parameters were included in the study. Individuals with diabetes and/or its complications, myocardial infarction, acute and chronic renal failure, pneumonia and cancer were excluded from the study.

Table 4.2.3.1: Details of 20 subjects (10 liver cirrhotic cases, 10 healthy age and gender matched controls) used for discovery of biomarker candidates by proteomic approach

Controls				**Cases**			
Sample ID	**Gender**	**Age**	**Etiology**	**Sample ID**	**Gender**	**Age**	**Etiology**
C1	M	36	NA	D1	M	36	ALD
C2	M	28	NA	D2	M	28	ALD
C3	M	60	NA	D3	M	60	ALD
C4	M	36	NA	D4	M	36	ALD
C5	M	35	NA	D5	M	35	ALD
C6	M	40	NA	D6	M	40	ALD
C7	M	58	NA	D7	M	58	ALD
C8	M	30	NA	D8	M	30	ALD
C9	M	55	NA	D9	M	55	ALD
C10	M	30	NA	D10	M	30	ALD

Abbreviations: C: Control; D: Diseased (Alcoholic Liver Cirrhosis); M: Male; NA: Not Applicable; ALD: Alcoholic Liver Disease

4.2.4. For cross reactivity analysis

Blood samples were collected from 20 subjects: 10 clinically and diagnostically proven cirrhotic liver with varying degree and varied etiology and age and gender matched 10 healthy from Department of Medicine, R. L. Jalappa Hospital and Research Centre attached to Sri Devaraj Urs Medical College, A constituent of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India (**Table 4.2.4.1**).

Table 4.2.4.1: Details of 20 subjects (10 liver cirrhotic cases, 10 healthy age and gender matched controls) used for cross reactivity analysis

Controls				**Cases**			
Sample ID	**Gender**	**Age**	**Etiology**	**Sample ID**	**Gender**	**Age**	**Etiology**
C1	M	36	NA	D1	M	36	ALD
C2	M	28	NA	D2	M	28	ALD
C3	M	60	NA	D3	M	60	ALD
C4	F	26	NA	D4	F	26	NAFLD
C5	M	35	NA	D5	M	35	ALD
C6	F	26	NA	D6	F	26	NAFLD
C7	M	58	NA	D7	M	58	ALD
C8	M	30	NA	D8	M	30	ALD
C9	M	55	NA	D9	M	55	ALD
C10	M	30	NA	D10	M	30	ALD

Abbreviations: C: Control; D: Diseased; M: Male; F: Female; NA: Not Applicable; ALD: Alcoholic Liver Disease; NAFLD: Non Alcoholic Fatty Liver Disease

Individuals diagnosed with cirrhosis of liver caused by varied etiology *viz.*, ALD and NAFLD based on clinical history and symptoms *viz.*, ascites, encephalopathy, jaundice and altered biochemical parameters were included in the study. Individuals with diabetes

and/or its complications, myocardial infarction, acute and chronic renal failure, pneumonia and cancer were excluded from the study.

4.2.5. For estimation of SERPINA4/Kallistatin and correlation analysis

Sample size estimation was based on mean difference observed in SERPINA4/Kallistatin levels between cirrhosis of liver and healthy. To detect a reduction of 15% in SERPINA4/Kallistatin levels with 90% power at 95% confidence interval, the required sample size estimated to be 78 per group, expecting a dropout rate of 20%, thus total estimated sample size per group was 96.

4.2.5.1. Sample size was calculated using formula (22)

$N= 2\sigma^2 [Z_\alpha+Z_{1-\beta}]^2/ (d)^2$

σ^2 = Variance

Z_α = critical value of normal distribution at 5% interval of significance = 1.96

$Z_{1-\beta}$ = critical value of normal distribution at 90% power = 0.842

d = difference would like to detect

Blood samples were collected from clinically and diagnostically proven cirrhotic liver subjects with varying degree (n=96) and age and gender matched healthy subjects from registered in the Department of Medicine, R. L. Jalappa Hospital and Research Centre attached to Sri Devaraj Urs Medical College, A constituent of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India. Individuals diagnosed with cirrhosis of liver caused by different etiologies *viz.,* ALD, viral hepatitis (HCV & HBV), NAFLD based on clinical symptoms *viz.*, ascites, encephalopathy, jaundice and altered

biochemical parameters were included. Individuals with diabetes and/or its complications, myocardial infarction, acute and chronic renal failure, pneumonia and cancer and individuals on thrombolytic therapy were excluded from the study (**Figure 4.2.5.1.1**).

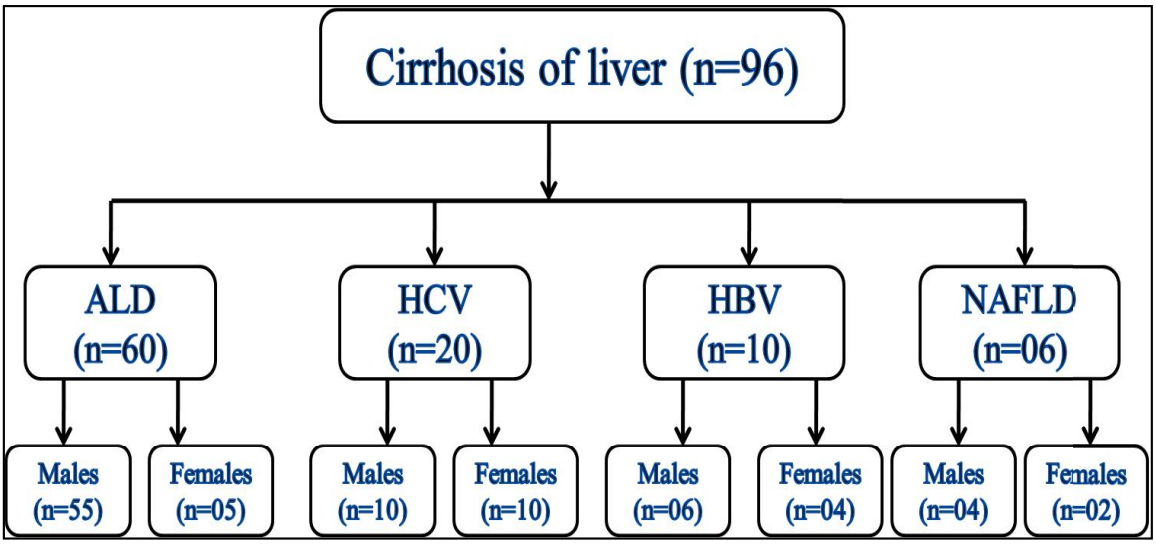


Figure 4.2.5.1.1: Schematic representation of cirrhotic liver subjects based on varied etiology and gender

4.2.5.2. Statistical analysis

Collected data was coded and entered into MS-excel form. The quantitative variables were presented by mean, standard deviation. Statistical analysis was done by using institutional licensed version of IBM SPSS 22. Comparison for quantitative variables between groups for significance was done by using Student t-test. Pearson's correlation analysis was applied to find correlation between quantitative variables. One way ANOVA test was performed to find out difference of variables (SERPINA4/Kallistatin, hyaluronic acid and YKL-40) between cirrhotic liver subjects with varied etiology. Area

Under Receiver's Operating Characteristic (AUROC) curve was analyzed to find out the diagnostic accuracy of SERPINA4/Kallistatin, hyaluronic acid and YKL-40.

4.3. Methods

4.3.1. For discovery of protein biomarker candidates

4.3.1.1. Serum separation

Serum was collected from clotted blood using serum separator tubes centrifuged at 4000 rpm for 10 min. Serum was stored at – 20^{o}C till further analysis. All the samples were used for discovery of biomarker candidates after depletion of abundant albumin. Desalting was carried out by acetone precipitation. 2DE was carried out to find differentially expressed proteins between cirrhotic and healthy subjects. Identified spots were characterized by LC-MS after in-gel and in-solution trypsin digestion.

4.3.1.2. Reagents

Dye based (cibacron blue dye) pre fractionation albumin depletion kit was procured from Thermo Fisher Scientific, USA. Precast gels and other chemicals of analytical grade for SDS-PAGE, 2DE, in gel trypsin digestion and LC-MS were procured from Bio-Rad and Sigma Aldrich, USA.

4.3.1.3. Depletion of albumin

Re-suspended resin (200µL aliquot of resin) was transferred into spin column (column volume: 900µL; 10µm pore size polyethylene filter). Bottom of column was twisted off and placed in 1.5 ml collecting tube. Centrifuged at 12,000g for 1 min and discarded the

flow-through and placed spin column back into the same collection tube. Around 200µL of binding/wash buffer was added to the spin column. Centrifuged at 12,000g for 1 min and discarded flow-through and placed spin column into a new collection tube. About 50µL of pooled serum sample (cirrhotic and healthy in separate columns) was added into resin and incubated for 2 min at room temperature. Centrifuged at 12,000g for 1 min and reapplied flow-through to spin column and incubated for 2 min at room temperature. Centrifuged at 12,000g for 1 min and retained flow-through. Spin column was placed in a new collection tube. Washed resin to release unbound proteins by adding 50µL of binding/wash buffer for each 200µL of resin used. Retained fractions of cirrhotic and healthy samples were run on SDS-PAGE for confirmation of albumin depletion (152).

4.3.1.4. Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

SDS gel was prepared as per standard protocol. Cirrhotic liver and healthy subject's samples after depletion of albumin along with un-depleted samples were loaded in gel and SDS-PAGE was carried out at 25 mA in 1X SDS running buffer for confirmation of depletion along with molecular weight marker. After electrophoresis, gel was incubated in fixing solution (40% methanol, 10% acetic acid) at room temperature for 20 min. Gel was subjected for staining with silver stain (0.1% silver nitrate and 36% formaldehyde) at room temperature for 20 min. Excess staining solution was removed and the gel was washed with 5% acetic acid (153 – 155).

4.3.1.5. Acetone precipitation

Acetone precipitation was carried out to remove excess salts which interferes electrophoretic run. Protein samples after depletion of albumin (cirrhotic and healthy separately) were placed in acetone compatible tubes. Four times the sample volume of cold acetone (-20°C) was added into both tubes. Vortex tube and incubated for 60 min at -20°C. Centrifuged at 13,000g for 10 min and disposed the supernatant carefully for the retention of protein pellet (156).

4.3.1.6. Two-dimensional poly acrylamide gel electrophoresis

Albumin depleted and desalted protein pellet (200µg) from cirrhotic and healthy subjects were diluted with rehydration buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol, 0.2% w/v Bio-Lyte 3/10 ampholyte, bromophenol blue) and used separately for 2DE with 7 cm pH 4-7 nonlinear immobilized pH gradient dry strips (Bio-Rad). Samples were left over night for rehydration on 7 cm pH 4-7 dry strips. Iso electric focusing was carried out at 250 v for 20 min followed by 4000 v for 5 hrs at 20ºC. Proteins were separated by 8% - 16% precast polyacrylamide gels at 200 V for 40 min (**Figure 4.3.1.6.1)**. After electrophoresis, gels were subjected for staining with silver stain (0.1% silver nitrate and 36% formaldehyde) at room temperature for 20 min. Excess staining solution was removed and the gel was washed with 5% acetic acid (157).

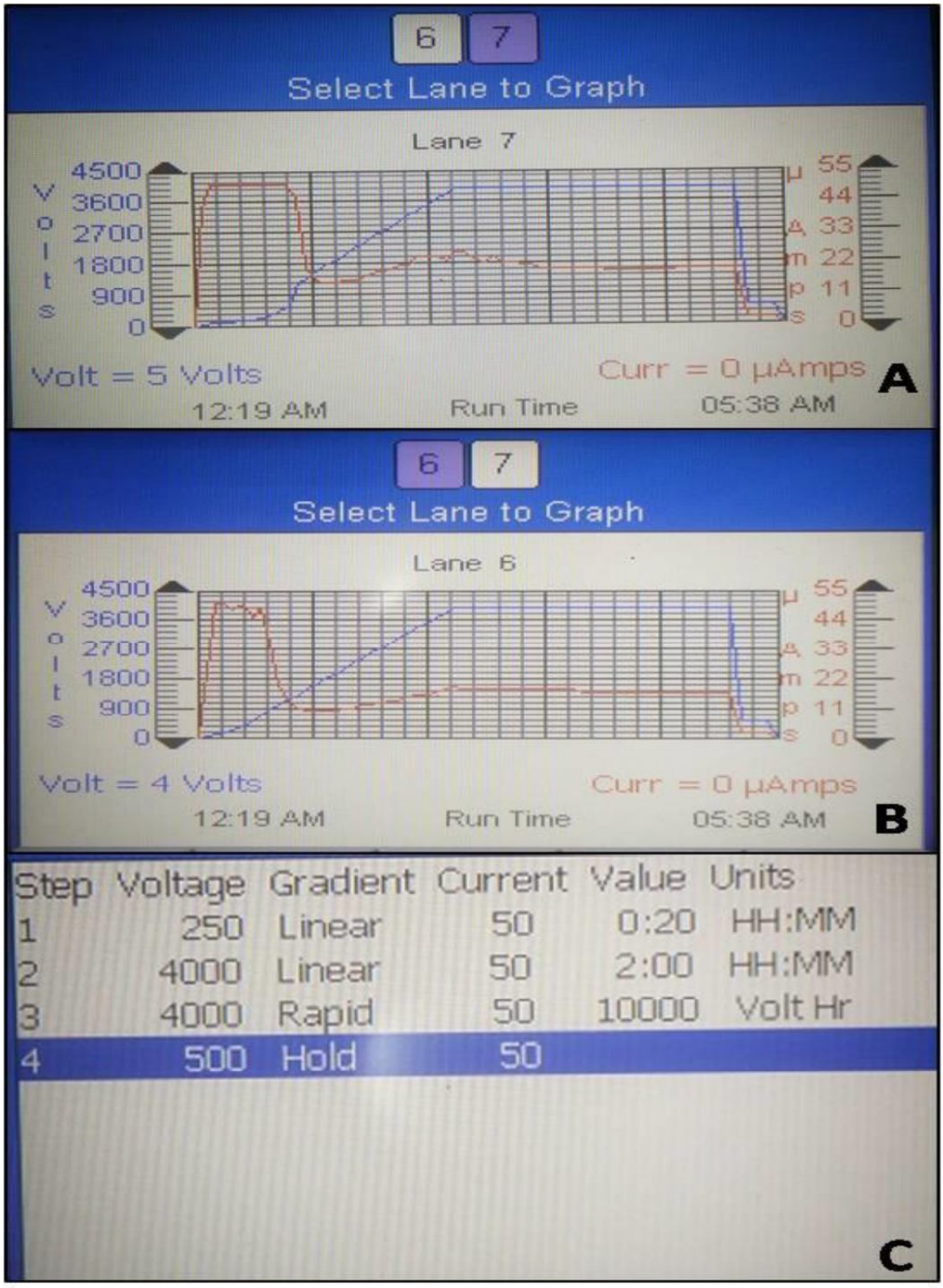


Figure 4.3.1.6.1: Protocol for Two Dimensional Electrophoresis; A: Graph – Diseased; B: Graph – Normal; C: Protocol

4.3.1.7. Image analysis

For image analysis, scanned gels were processed by using PDQuest 2-D analysis software (Bio-Rad). For differential analysis, cirrhotic gel was compared with that of healthy gel. Differential expression of proteins present in both cirrhotic and healthy gels was considered significant when the fold change was least 2 and $p \leq 0.05$ with 95% confidence with the application of rank-sum test.

4.3.1.8. In-gel digestion and peptide extraction

Excised spots were cut into cubes and transferred into a micro centrifuge tube and 100µl of destaining solution (100mM ammonium bicarbonate/acetonitrile [1:1 vol/vol]) was added and incubated for 30 min. 500µl of neat acetonitrile was added and the tubes were incubated for 10 min until gel pieces shrink. 50µl of DTT solution (10mM DTT in 100mM ammonium bicarbonate buffer) was added to cover the gel pieces and incubated in 56^0C thermostat for 30 min. 500µl of acetonitrile was added to the tubes and further incubated for 10 min. All the liquid was removed from the tube. Following DTT treatment, to get reduction and alkylation of cytines and cysteines in the protein, 50µl of iodoacetamide solution (55mM iodoacetamide in 100mM ammonium bicarbonate solution) was added to tubes and incubated for 20 min at room temperature in dark. The gel pieces were again treated with acetonitrile for 10 min and the entire liquid was removed from the tube. The gel pieces were saturated with trypsin buffer (13ng/µL of trypsin in 10mM ammonium bicarbonate in 10% acetonitrile) for 30 min. Cold trypsin (20µg of trypsin in 1.5 ml of ice cold 1mM HCl) was added to the tubes and incubated overnight at 37^0C. Tubes were cooled to room temperature and gel pieces were

centrifuged at 10000rpm for 1min and peptides were extracted in 100µl of extraction buffer (1:2, 5% formic acid/acetonitrile) by incubating for 15 min at 37^0C shaker and the supernatant was withdrawn directly for LC-MS analysis (158).

4.3.1.9. In-solution peptide extraction

The pH of protein samples was adjusted to 8.5 by adding 50mM ammonium bicarbonate. Samples were mixed well. Ice cold trypsin of 13ng/µl was added to protein samples (1:30). Placed the tubes in thermostat and incubated at 55^{o}C for 2hr. Tubes were cooled to room temperature and 5% of formic acid was added until pH comes to 3. Samples were mixed well and subjected for MS analysis (159).

4.3.1.10. Mass spectrometric analysis

Mass spectrometric analysis of extracted peptides was performed using Nano LCMS-LTQ-Orbitrap Discovery (Thermoscientific) coupled to Nano LC (Agilent 1200). The samples were reconstituted in 0.1% formic acid prior to injection. Peptides extracted from in-gel trypsin digestion, 70 min gradient run was setup using acetonitrile and water with formic acid as mobile phase. Peptides extracted from in-solution trypsin digestion, 110 min gradient run was setup using acetonitrile and water with formic acid as mobile phase. LTQ Orbitrap Discovery is a hybrid type MS system with the ability to determine accurate m/z of intact precursors. Raw files post MS run was analyzed using Proteome discover software and MASCOT as search engine against human database (160).

4.3.2. For cross reactivity analysis

4.3.2.1. Serum separation

Serum was collected from clotted blood using serum separator tubes centrifuged at 4000 rpm for 10 minutes. Serum was stored at – 20^{o}C for further analysis. All the samples were used to find cross reactivity of other serpins with SERPINA4/Kallistatin by western blot after protein segregation by SDS-PAGE.

4.3.2.2. Reagents

Primary monoclonal, polyclonal and monospecific antibodies (monoclonal antibody alternative) specific for SERPINA4/Kallistatin along with secondary antibodies and recombinant SERPINA4/Kallistatin were procured from R&D systems, USA. Other chemicals of analytical grade were procured from Bio-Rad and Sigma Aldrich, USA.

4.3.2.3. SDS-PAGE

SDS gels were prepared as per standard protocol. Cirrhotic liver and healthy subject's serum samples were loaded in different gels and SDS-PAGE was carried with duplication in triplets at 25 mA (2 gels run @ 50 mA) in 1x SDS running buffer. After electrophoresis, gels were incubated in fixing solution (7% acetic acid and 10% methanol) at room temperature for 20 minutes. At this point, the gels were transferred onto a PVDF (polyvinylidene diflouride) membrane for western blot and duplicate gels were subjected for staining with colloidal Coomassie brilliant blue in a shaker at room temperature for 2 hours. Excess staining solution was removed and the gels were washed

with 10% acetic acid and placed in deionized water for destaining till the appearance of bands (153, 154).

4.3.2.4. Western blot

Proteins separated by SDS-PAGE were transferred onto PVDF membranes using a Transblot-Blot SD semi dry transfer cell (Bio-Rad, USA) at 15 V for 2 hours (1x transfer buffer: Tris/Glycine with 20% Methanol). After transfer, PVDF membranes were kept for blocking using blocking buffer (5% skimmed milk powder in 1x PBST) and incubated over night at 4^{o}C. After overnight blocking, PVDF membranes were washed with 1x PBST thrice for 3 minutes each. Primary antibodies (monoclonal, polyclonal and monospecific antibody separately) were diluted (1:100) and PVDF membranes were incubated in diluted primary antibody solution at room temperature with slow shaking on rocker for 2 to 3 hours. PVDF membranes were washed with 1x PBST thrice for 3 minutes each (161, 162).

Secondary antibody was diluted (1:5000) and PVDF membranes were incubated in diluted secondary antibody solution at room temperature with slow shaking on rocker for 2 to 3 hours. After incubation, PVDF membranes were washed with 1x PBST thrice for 3 minutes each. Around 12.5 mL Tris buffer (pH 7.35), 30 µl of 30% H_2O_2, a pinch of DAB were added into detection tray, mixed well and PVDF membranes were kept into the tray. The tray was gently shaken for a period of 10 minutes until the color developed in the control lane. SDS-PAGE and western blot were repeated with pooled and concentrated cirrhotic liver and healthy serum samples along with recombinant SERPINA4/Kallistatin (161, 162).

4.3.2.5. Concentration of serum proteins by dialysis using solid sucrose

Dialyzing tube containing serum to be concentrated is coiled up in a beaker and covered with commercial sucrose for 4 hours. The liquid accumulated outside the dialyzing bag was poured off. Tubing was removed from the sugar at the end of 4 hours and is tied off above the solution placed in water to dialyze away the sugar (163).

4.3.3. For In House ELISA quantification kit development

4.3.3.1. Reagents

Monospecific antibody (monoclonal antibody alternative) specific for SERPINA4/Kallistatin, secondary antibodies specific for primary antibodies, streptavidin HRP conjugate along with substrate and empty ELISA wells and recombinant SERPINA4/Kallistatin were procured from R&D systems, USA.

4.3.3.2. Plate preparation

Capture antibody diluted to working dilution with PBS and coated immediately to a 96-well micro plate with 100µL per well. Plate was sealed and incubated overnight at room temperature. Each well was aspirated and washed with 400µL diluted wash buffer; repeated the process two times for a total of three washes. After the last wash, remaining wash buffer was removed by aspirating or by inverting the plate and blotting it against clean paper towels. Plates were blocked by adding 300µL reagent diluent to each well and incubated at room temperature for 1 hour. Washing process was repeated 3 times (164).

4.3.3.3. Assay procedure

Sample or standard of 100µL was added in each well. Plate was covered with an adhesive strip and incubated for 2 hours at room temperature. Washing process was repeated 3 times. Around 100µL of diluted detection antibody was added in each well. Plate was covered with an adhesive strip and incubated for 2 hours at room temperature. Washing process was repeated 3 times. Around 100µL of diluted Streptavidin- HRP was added in each well. Plate was covered with an adhesive strip and incubated for 20 min at room temperature. Washing process was repeated 3 times. Around 100µL of substrate solution was added in each well. Plate was covered with an adhesive strip and incubated for 20 min at room temperature. Around 50µL of stop solution was added to each well. The optical density of each well was determined immediately using a microplate reader set to 450 nm (164).

4.3.4. For estimations of biochemical parameters and correlative analysis

4.3.4.1. Serum separation

Serum was collected from clotted blood using serum separator tubes centrifuged at 4000 rpm for 10 min. Serum and citrate blood were stored at – 20^{o}C for further analysis. Serum was used to estimate biochemical parameters and plasma was used to estimate PT. INR was calculated.

4.3.4.2. Methodology

4.3.4.2.1. SERPINA4/Kallistatin (In-House Double Sandwich ELISA)

In-House ELISA was prepared for quantification of SERPINA4/Kallistatin as described in **section 4.3.3**. Wells were coated with Monospecific (Monoclonal alternative) antibodies (Mouse Anti-Human SERPINA4 capture antibody) specific for SERPINA4/Kallistatin. Standards and test samples were added to the wells. After incubation, wells were washed and detection antibody (Biotinylated Goat Anti-Human SERPINA4/Kallistatin detection antibody) was added in all wells. After incubation, wells were washed and a Streptavidin- HRP was added in all wells.

Wells were washed and substrate TMB was added to all wells. TMB was catalyzed by HRP to produce a blue color product that changed yellow after adding acidic stop solution. The density of yellow colored compound formed was directly proportional to the human SERPINA4/Kallistatin concentration in the sample which was captured on the plate (164). OD values were red at 450 nm by using microplate reader (**Table 4.3.4.2.1.1**).

All standards and samples were run in duplicates. Standard graph was prepared by using recombinant SERPINA4/Kallistatin range 125 pg/mL - 8000 pg/mL (**Figure 4.3.4.2.1.1**). Concentration of SERPINA4/Kallistatin in samples (cirrhotic and healthy) was calculated by using formula $\mathbf{y = 551.6x^2 - 187.2x + 277.9\ (R^2 = 0.997).}$ The sample concentration obtained from an ELISA is dependent upon the interaction between the protein of interest and the ELISA's antibody and comparison of this interaction relative to a recombinant protein standard curve (165, 166).

Spike and Recovery protocol was performed with known concentrations of recombinant SERPINA4/Kallistatin and natural sample (cirrhotic liver and healthy) to rule out interference of other factors *viz.*, buffer components, sample matrix, compliment, rheumatoid factor and heterophilic antibodies which can impact antibody binding in the natural sample and therefore influence the accuracy of ELISA results. Linearity protocol was performed to define linearity range of diagnostic kit (167).

Spiking stock solution concentration was approximately 10 times to the recommended high standard concentration.

Sample and Control Spike preparation

1. Labeled 3 tubes: Neat, Spiked and Control
2. From a well-mixed sample, prepared two aliquots:
    a. Pipette 1.0 mL into a "Neat" tube: Neat sample
    b. Pipette 0.98 mL into a "Spiked" tube: Spiked sample
3. Pipette 0.98 mL reagent diluents into "control" tube: Control spike
4. Added 20 µL of spiking stock into Spiked sample and control spike tubes
5. Mixed samples by vortexing

Preparing Sample/Control Spike Serial Dilutions for Sample Linearity

1. Spiked Sample (Neat): 2000 pg/mL
2. **1:2 dilution**: added 0.5 mL of Sample Spike, Control Spike or Neat sample to 0.5 mL reagent diluents; concentration: 1000 pg/mL
3. **1:4 dilution**: added 0.5 mL of 1:2 dilution to 0.5 mL reagent diluents; concentration: 500 pg/mL
4. **1:8 dilution**: added 0.5 mL of 1:4 dilution to 0.5 mL reagent diluents; concentration: 250 pg/mL

If "Neat" sample measures above the standard curve range, dilute it until the value reads within the standard curve range

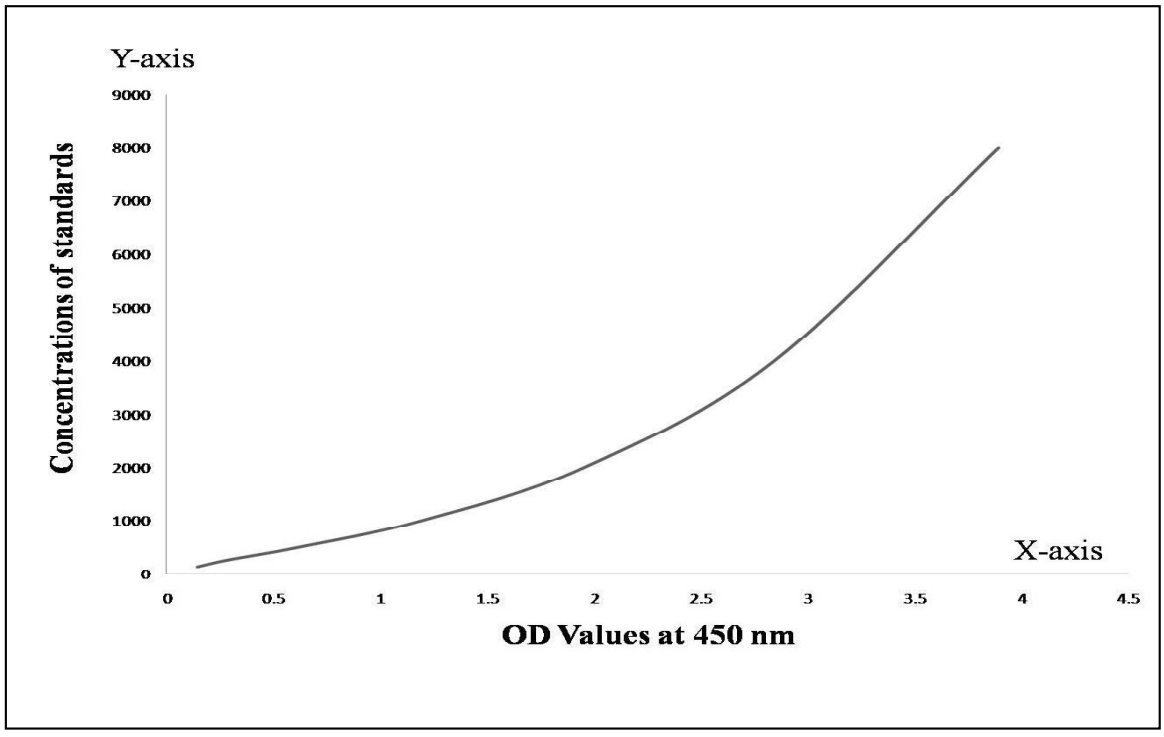


Figure 4.3.4.2.1.1: SERPINA4/Kallistatin standard curve

Table 4.3.4.2.1.1: Concentrations and optical density values of Recombinant SERPINA4/Kallistatin standards

Standard	Concentration of Standard (pg/mL)	OD Values at 450 nm		Mean OD value	Mean OD Value after subtracting average zero OD value (0.016)
		First trail	Second trail		
S1	125	0.164	0.160	0.162	0.146
S2	250	0.295	0.303	0.299	0.283
S3	500	0.636	0.647	0.642	0.626
S4	1000	1.207	1.216	1.212	1.196
S5	2000	1.975	1.962	1.968	1.952
S6	4000	2.856	2.862	2.859	2.843
S7	8000	3.914	3.903	3.908	3.892

Calculations

1. **Spike/Recovery**

   % Recovery = {(Observed – Neat)/Expected} X 100

   Observed = Spiked sample concentration

   Neat = Unspiked sample concentration

   Expected = Amount spiked into sample (calculated based on assigned concentration of spiking stock and volume spiked into sample)

2. Linearity

$$\% \text{ Recovery (1:2)} = \left(\frac{\text{Observed value of 1:2 dilution}}{\text{Expected value/2}} \right) \times 100$$

$$\% \text{ Recovery (1:4)} = \left(\frac{\text{Observed value of 1:4 dilution}}{\text{Expected value/2}} \right) \times 100$$

$$\% \text{ Recovery (1:8)} = \left(\frac{\text{Observed value of 1:8 dilution}}{\text{Expected value/2}} \right) \times 100$$

4.3.4.2.2. Hyaluronic acid (Double Sandwich ELISA) (168)

The assay employs the quantitative double sandwich enzyme immunoassay technique. Recombinant human (rh) Aggrecan has been pre-coated onto a microplate. Standards, controls and samples (cirrhotic and healthy subjects) were pipette into wells and any hyaluronic acid present was bound by the immobilized rh Aggrecan. After washing away any unbound substances, enzyme linked rh Aggrecan-enzyme reagent was added to all wells. After wash to remove unbound rh- Aggrecan-enzyme reagent, a substrate solution was added to all wells. The developed color was directly proportional to the concentration of hyaluronic acid present in samples. The color developed is stopped by adding acidic stop solution and intensity of color was measured at 450 nm.

Hyaluronic acid standards preparation

Reconstitute the hyaluronic acid (recombinant) standard with Calibrator Diluent RD5-18. This reconstitution produced a stock solution of 40 ng/mL. Mixed well to ensure complete reconstitution and allowed the standard for 15 min. Serial dilutions of standard were prepared from 40 ng/mL to 0.625 ng/mL by diluting with Calibrator Diluent RD5-18. Calibrator Diluent RD5-18 served as the zero standard (0 ng/mL).

Assay procedure

1. Prepare all reagents, working standards, and samples as directed in the previous sections.

2. Add 50 µL of Assay Diluent RD1-14 to each well (may contain a precipitate). Mix well before and during use.

3. Add 50 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.

4. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with wash buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

5. Add 100 µL of hyaluronan Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.

6. Repeat the aspiration/wash as in step 4.

7. Add 100 µL of substrate solution to each well. Incubate for 30 minutes at room temperature on the bench top. Protect from light.

8. Add 100 µL of stop solution to each well. Gently tap the plate to ensure thorough mixing.

9. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Hyaluronic acid Standard Curve

Standard curve (**Figure 4.3.4.2.2.1**) was plotted against OD values of serial standards from S0 to S7 (**Table 4.3.4.2.2.1**). Concentration of hyaluronic acid present in samples (cirrhotic and healthy) was calculated by using formula

$y = 3.839x^2 + 3.388x + 0.326$ ($R^2 = 0.998$)

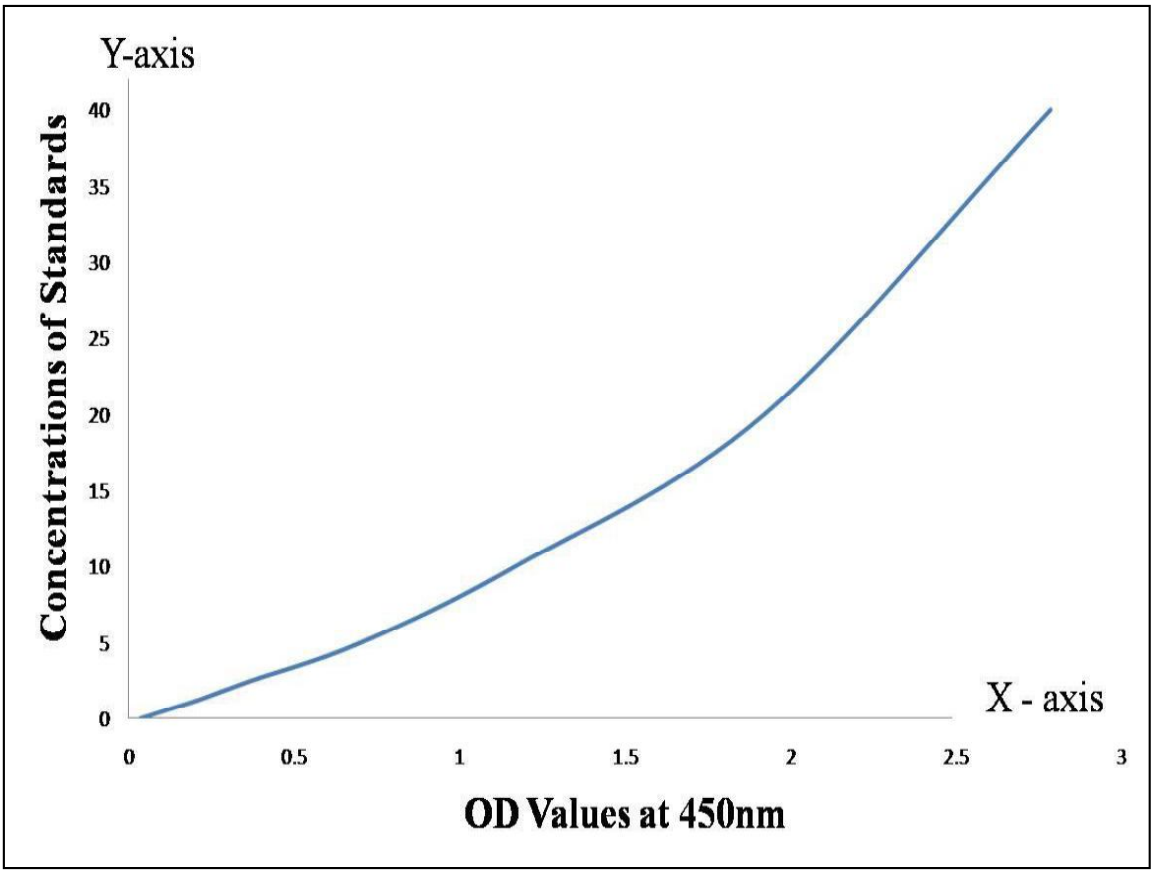


Figure 4.3.4.2.2.1: Hyaluronic acid standard curve

Table 4.3.4.2.2.1: Concentrations and optical density values of hyaluronic acid standards

Standard	Concentration of Standard (ng/mL)	OD Value at 450 nm
S0	0	0.035
S1	0.625	0.122
S2	1.25	0.213
S3	2.5	0.371
S4	5.0	0.702
S5	10.0	1.169
S6	20.0	1.923
S7	40.0	2.788

4.3.4.2.3. YKL-40 (Double Sandwich ELISA) (169)

The assay employs the quantitative double sandwich enzyme immunoassay technique. A monoclonal antibody specific for human YKL-40 (CH3L1) has been pre-coated onto a microplate. Standards and samples (cirrhotic and healthy subjects) were pipette into wells and any YKL-40 (CH3L1) present was bound by the immobilized monoclonal antibody. After washing away any unbound substances, enzyme linked polyclonal antibody specific for human YKL-40 (CH3L1) was added to all wells. After wash to remove unbound enzyme-linked polyclonal antibody reagent, a substrate solution was added to all wells. The developed color was directly proportional to the concentration of hyaluronic acid present in samples. The color developed is stopped by adding acidic stop solution and intensity of color was measured at 450 nm.

YKL-40 standards preparation

Reconstitute the human YKL-40 (CH3L1) (recombinant) standard with deionized or distilled water and Calibrator Diluent RD5-18. This reconstitution produced a stock solution of 40 ng/mL. Mixed well to ensure complete reconstitution and allowed the standard for 15 min. Serial dilutions of standard were prepared from 40 ng/mL to 0.625 ng/mL by diluting with Calibrator Diluent RD5P. Calibrator Diluent RD5P served as the zero standard (0 ng/mL).

Assay procedure

1. Prepare all reagents, samples, and working standards as directed in the previous sections.

2. Add 100 µL of Assay Diluent RD1-34 to each well.

3. Add 50 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.

4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with wash buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper toweling.

5. Add 200 µL of Human CHI3L1 Conjugate to each well. Cover with a new adhesive strip.

6. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 4.

8. Add 200 µL of substrate solution to each well. Incubate 30 minutes at room temperature. Protect from light.

9. Add 50 µL of stop solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform than gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from

the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

YKL-40 Standard Curve

Standard curve (**Figure 4.3.4.2.3.1**) was plotted against OD values of serial standards from S0 to S7 (**Table 4.3.4.2.3.1**). Concentration of YKL-40 present in samples (cirrhotic and healthy) was calculated by using formula **$y = 260.8x^2 + 689.3x + 22.56$ ($R^2 = 0.999$)**

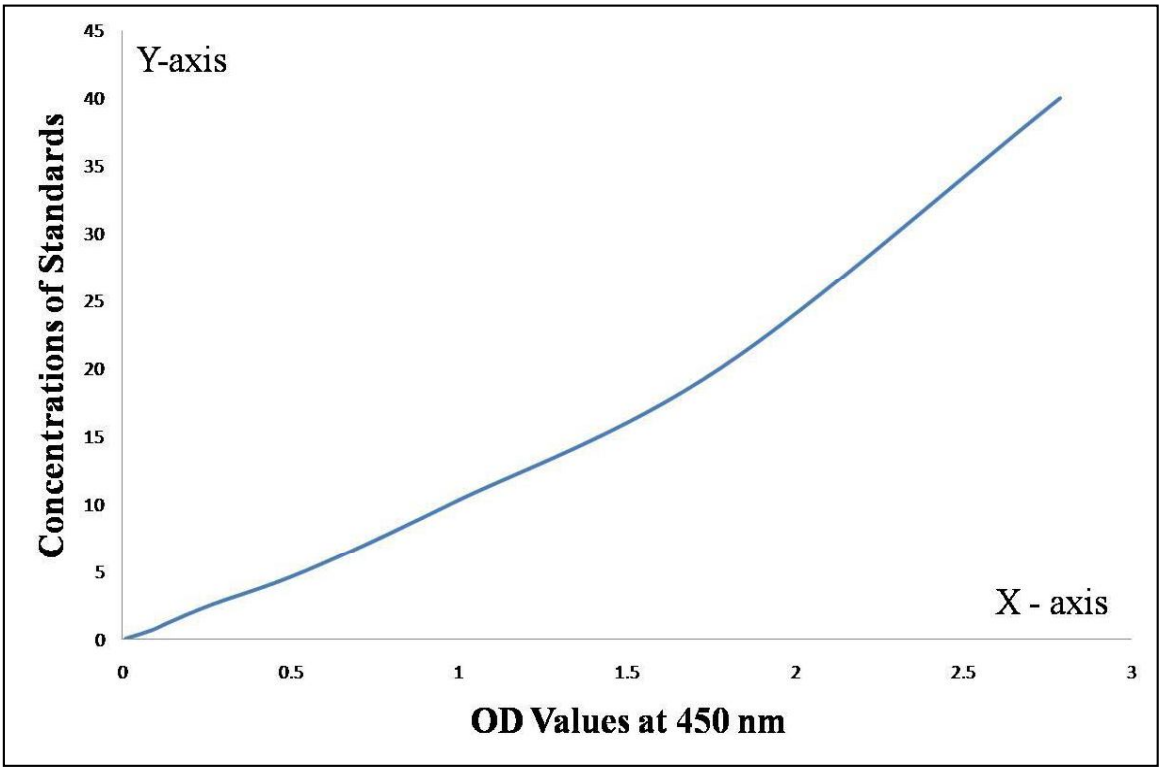


Figure 4.3.4.2.3.1: YKL-40 standard curve

Table 4.3.4.2.3.1: Concentrations and optical density values of YKL-40 standards

Standard	Concentration of Standard (ng/mL)	OD Value at 450 nm
S0	0	0.035
S1	0.625	0.122
S2	1.25	0.213
S3	2.5	0.371
S4	5.0	0.702
S5	10.0	1.169
S6	20.0	1.923
S7	40.0	2.788

4.3.4.2.4. Total Antioxidant Capacity (TAC) (Colorimetric) (170)

Antioxidants play an important role in preventing the formation of free radicals and scavenging of free radicals and other potentially toxic oxidizing species. There are three categories of antioxidant species: enzyme systems (GSH reductase, catalase, peroxidase, etc.), small molecules (ascorbate, uric acid, GSH, vitamin E, etc.) and proteins (albumin, transferrin etc.). Different antioxidants vary in their reducing power. Trolox is used to standardize antioxidants, with all other antioxidants being measured in Trolox equivalents. Measurement of the combined non-enzymatic antioxidant capacity of biological fluids and other samples provides an indication of the overall capability to counteract ROS, resist oxidative damage and combat oxidative stress related diseases. The Total Antioxidant Capacity Assay Kit can measure either the combination of both small molecule antioxidants and proteins or small molecules alone in the presence of our protein mask. Cu^{2+} ion is converted to Cu^{+} by both small molecule and protein. The

proteins mask prevents Cu^{2+} reduction by protein, enabling the analysis of only the small molecule antioxidants. The reduced Cu^{+} ionis chelated with a colorimetric probe giving a broad absorbance peak around 570 nm which is directly proportional to the total antioxidant capacity.

Assay procedure

Measurement of Antioxidants

Preparation of sample

The kit has been tested with serum, urine, culture media, food and drinks. No sample purification from these sources is necessary. If only small molecule TAC is desired, samples should be diluted 1:1 with protein mask. Sample volumes between 0 – 100 µL can be assayed per well and should be done in duplicate. For serum samples, we suggest to assay 0.01-0.1µL without Protein Mask, or 1 – 10 µL with Protein Mask. All well volumes should be adjusted to 100 µL with ddH_2O. The absorbance of samples should be in the linear range of the standard curve (0 – 20 nmol/well). If they fall outside of this range, they should be rediluted and rerun. The detection limit of the assay is approximately 0.1 nmoL per well (or 1µM) of Trolox.

Preparation of working solutions

Dilute one part Cu^{2+} reagent with 49 parts of Assay diluent. Dilute enough working solution for the number of assays. Each well requires 100 µL of Cu^{2+} working solution.

Assay procedure

1. Add 100 µL Cu^{2+} working solution to all standard and sample wells.

2. Cover the plate and incubate at room temperature for 1.5 hours

3. Read the absorbance at 570 nm using the plate reader

TAC Standard Curve

Standard curve (**Figure 4.3.4.2.4.1**) was plotted against OD values of serial standards from S0 to S5 (**Table 4.3.4.2.4.1**). Samples (cirrhotic and healthy) antioxidant capacity was calculated by using formula **$y = 15.34x^2 + 8.431x + 0.649$ ($R^2 = 0.997$)**

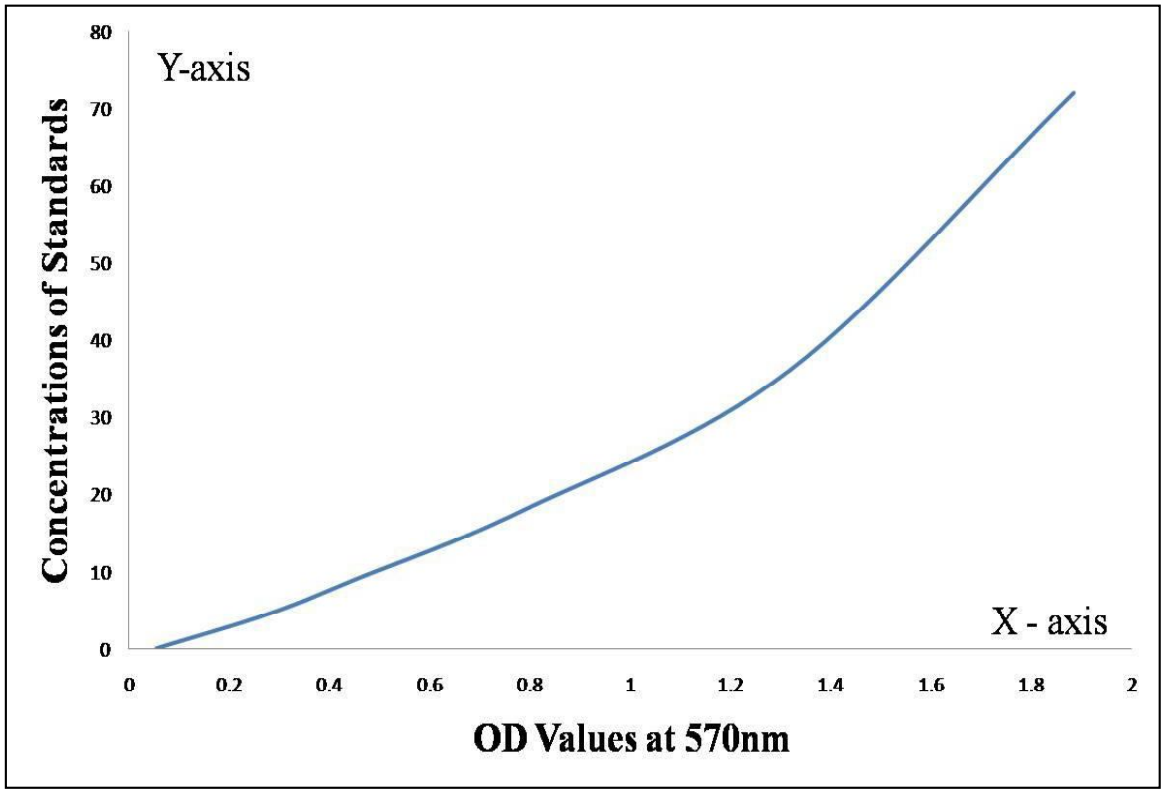


Figure 4.3.4.2.4.1: Total antioxidant capacity (TAC) standard curve

Table 4.3.4.2.4.1: Concentrations and optical density values of TAC standards

Standard	Concentration of Standard (nmol/µL)	OD Value at 570 nm
S0	0	0.0561
S1	4.5	0.2812
S2	9.0	0.4557
S3	18.0	0.7907
S4	36.0	1.3178
S5	72.0	1.8853

4.3.4.2.5. Total Oxidative Status (TOS) (Colorimetric) (171)

Oxidants present in the sample oxidize the ferrous ion chelat or complex to ferric ion. The oxidation reaction is prolonged by enhancer molecules, which are abundantly present in the reaction medium. The ferric ion makes a colored complex with chromogen in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of µmol H_2O_2Equiv/L.

Assay procedure

Prepare working standard solution. Stock standard solution is diluted with deionised water.

First step dilution: A liquid of 50 µL stock standard solution is added to 10 mL deionised water and vortexed.

Second step dilution: A liquid of 50 µL of the prepared solution is added to 10mL deionised water and vortexed.

The final concentration of the working standard is 20 µmol H_2O_2.

Prepare working solution daily.

Around 500 µL assay buffer is added in all wells and 75 µL the prepared standard and samples are added to individual wells. The initial absorbance is estimated at 530 nm for the first absorbance point. Around 25 µL prochromogen solution is added to all wells and incubates 10 min at room temperature or 5 min at 37ºC. The initial absorbance is estimated at 530 nm.

TOS Standard Curve

Standard curve (**Figure 4.3.4.2.5.1**) was plotted against OD values of serial standards from S0 to S5 (**Table 4.3.4.2.5.1**). Samples (cirrhotic and healthy) total oxidative status was calculated by using formula $\mathbf{y = 26.66x^2 + 11.24x - 0.725\ (R^2 = 0.999)}$

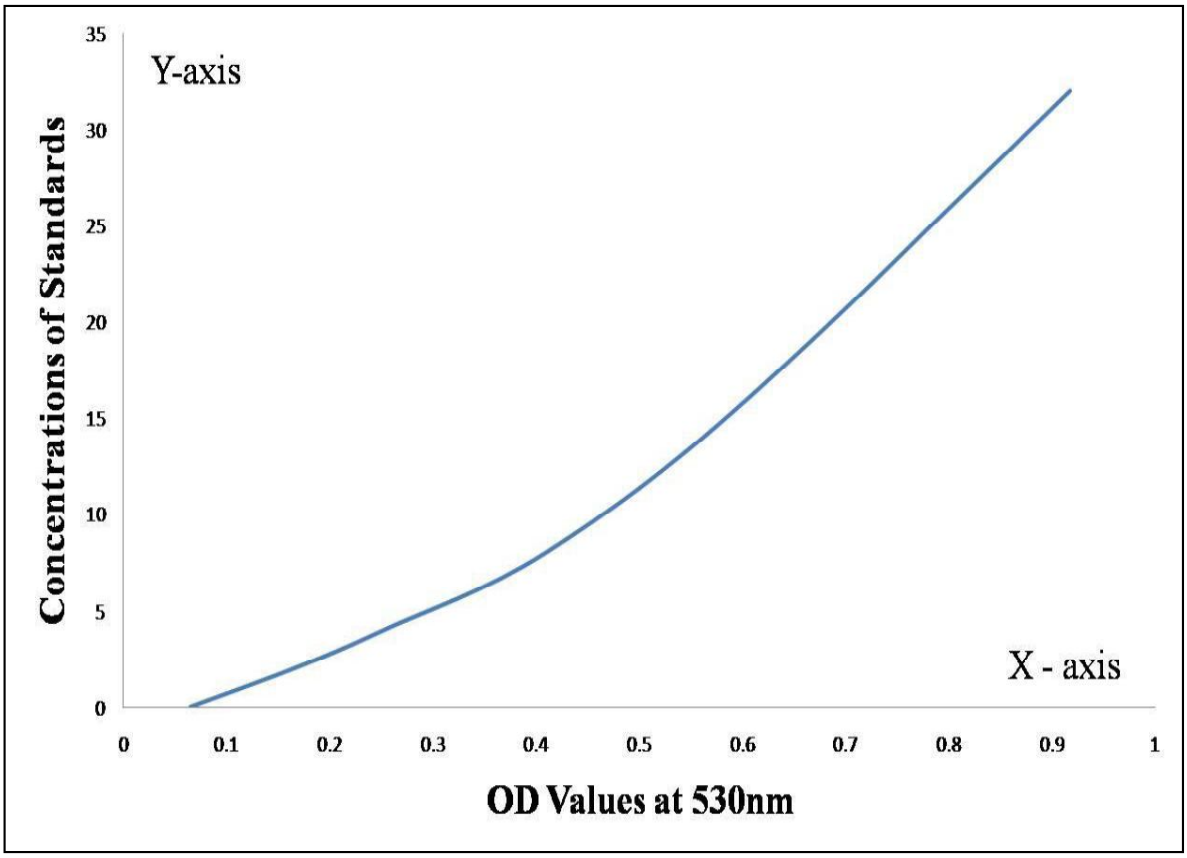


Figure 4.3.4.2.5.1: Total oxidant staus (TOS) standard curve

Table 4.3.4.2.5.1: Concentrations and optical density values of TOS standards

Standard	**Concentration of Standard (µmol H_2O_2 Equiv/L)**	**OD Value at 530 nm**
S0	0	0.0651
S1	2	0.1652
S2	4	0.2526
S3	8.0	0.4096
S4	16.0	0.605
S5	32.0	0.9173

4.3.4.2.6. Serum Glucose (Glucose Oxidase Peroxidase [GOD-POD]) (172)

Glucose present in blood is determined after enzymatic oxidation in the presence of glucose- oxidase. The hydrogen peroxide formed, reacts under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoeimine dye as indicator. Absorbance is read at 550 nm.

Reagent composition

Reagent 1: GOD-POD solution

Standard: Glucose standard (100 mg/dL)

Wave length	550 nm
Reaction type	End point
Cuvette	1 cm light path
Reaction type	Increasing
Measurement	Against reagent blank
Sample volume	10 µL
Reagent volume	1000 µL
Incubation	05 min
Blank absorbance limit	< 0.300
Low normal	60 mg/dL
High normal	110 mg/dL
Linearity	400 mg/dL

Assay procedure

	Blank	Standard	Test
Sample	--------	--------	10 µL
Standard	--------	10 µL	--------
Reagent	1000 µL	1000 µL	1000 µL

Mix and incubate for 05 minutes at 37ºC. Measure the absorbance of sample (AT) and standard (AS) against reagent blank at 550 nm. Intensity of color is directly proportional to concentration of glucose present in samples. Color produced is stable for 30 minutes at room temperature.

Calculation

Concentration of glucose (mg/dL) = (AT/AS) x concentration of standard

4.3.4.2.7. Urea (Urease) (173)

Serum urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide. The ammonia produced combines with α- oxo glutarate and NADH in the presence of glutamate dehydrogenase to yield glutamate and NAD. Absorbance is read at 340 nm (39).

Reagent composition

Reagent 1: Urease solution

Standard: Urea standard (50 mg/dL)

Wave length	340 nm
Reaction type	Fixed time kinetic
Cuvette	1 cm light path
Reaction type	Decreasing
Measurement	Against distilled water
Sample volume	10 µL
Reagent volume	1000 µL
Number of readings	01
Blank absorbance limit	> 0.800
Low normal	15 mg/dL
High normal	50 mg/dL
Linearity	300 mg/dL

Assay procedure

	Standard	**Test**
Sample	--------	10 µL
Standard	10 µL	--------
Reagent	1000 µL	1000 µL

Mix well and read after 30 seconds initial absorbance of sample (A1s) and standard (A1 std) and start timer simultaneously. Read again after 60 seconds of sample (A2s) and standard (A2std).

Calculation

Concentration of Urea (mg/dL) = (A2s-A1s)/(A2std-A1std) X concentration of standard

4.3.4.2.8. Creatinine (Modified Jaffe's; Decreases Bilirubin Interference)(173)

Serum creatinine in alkaline solution reacts with picrate to form yellow colored complex whose absorbs is read at 520 nm. The amount of complex formed is directly proportional to creatinine concentration.

Reagent composition

Reagent 1: Picric acid solution

Standard: Creatinine standard (2 mg/dL)

Wave length	520 nm
Reaction type	Fixed time kinetic
Cuvette	1 cm light path
Reaction type	Increasing
Measurement	Against distilled water
Sample volume	100 µL
Reagent volume	1000 µL
Number of readings	01
Blank absorbance limit	> 0.800
Low normal	0.8 mg/dL
High normal	1.4 mg/dL
Linearity	25 mg/dL

Assay procedure

	Standard	**Test**
Sample	--------	100 µL
Standard	100 µL	--------
Reagent	1000 µL	1000 µL

Mix well and read after 30 seconds initial absorbance of sample (A1s) and standard (A1std) and start timer simultaneously. Read after 1 minute determines Δ A/min of standard (As) and sample (Ac) against reagent blank.

CALCULATION

Concentration of Creatinine (mg/dL) = (ΔA/ ΔA1) X Concentration of Standard

4.3.4.2.9. Uric acid (Uricase Enzymatic End Point) (173)

Uric acid is converted by Uricase to allantoin and hydrogen peroxide which under the catalytic influence of peroxidase, oxidizes 3, 5-dichloro-2-hydroxy benzene sulfonic acid and 4-aminophenazone to form a red violet quinoeimine compound. Absorbance is measured at 520 nm.

Reagent composition

Reagent 1: Uricase enzyme solution

Standard: Uric acid standard (5 mg/dL)

Assay procedure

	Blank	**Standard**	**Test**
Sample	--------	--------	25 µL
Standard	--------	25 µL	--------
Reagent	1000 µL	1000 µL	1000 µL

Wave length	520 nm
Reaction type	End point
Cuvette	1 cm light path
Reaction type	Increasing
Measurement	Against reagent blank
Sample volume	25 µL
Reagent volume	1000 µL
Incubation	10 min
Blank absorbance limit	< 0.200
Low normal	2.4 mg/dL
High normal	7.2 mg/dL
Linearity	25.0 mg/dL

Mix and incubate for 10 minutes at 37ºC. Measure the absorbance of sample (AT) and Standard (AS) against reagent blank at 520 nm. Intensity of color is directly proportional to concentration of uric acid present in samples. Color produced is stable for 30 minutes at room temperature.

Calculation

Concentration of uric acid (mg/dL) = (AT/AS) X concentration of standard

4.3.4.2.10. Total Protein (Biuret method) (174)

Cupric ions in alkaline medium react with protein peptide bonds resulting in the formation of a violet colored complex. Absorbance is read at 540 nm (40).

Reagent composition

Reagent 1: Biuret solution

Standard: protein standard (8 g/dL)

Wave length	540 nm
Reaction type	End point
Cuvette	1 cm light path
Reaction type	Increasing
Measurement	Against sample blank
Sample volume	20 µL
Reagent volume	1000 µL
Incubation	5 min
Blank absorbance limit	< 0.200
Low normal	6.2 g/dL
High normal	8.5 g/dL
Linearity	1.0 – 15.0 g/dL

Assay procedure

	Blank	**Standard**	**Test**
Sample	--------	--------	20 µL
Standard	--------	20 µL	--------
Reagent	1000 µL	1000 µL	1000 µL

Mix and incubate for 05 minutes at 37ºC. Measure the absorbance of sample (AT) and Standard (AS) against reagent blank at 540 nm. Intensity of color is directly proportional

to concentration of total protein present in samples. Color produced is stable for 30 minutes at room temperature.

Calculation

Concentration of total protein (g/dL) = (AT/AS) X concentration of standard

4.3.4.2.11. Albumin (BCG) (174)

Measurement of serum albumin is based on its quantitative binding to the indicator 3, 3'5, 5-tetra bromo cresol sulphophthalein (bromocresol green, BCG). The albumin BCG complex absorbs maximally at 620 nm.

Reagent composition

Reagent 1: BCG solution

Standard: albumin standard (4 g/dL)

Wave length	620 nm
Reaction type	End point
Cuvette	1 cm light path
Reaction type	Increasing
Measurement	Against sample blank
Sample volume	10 µL
Reagent volume	2500 µL
Incubation	5 min
Blank absorbance limit	< 0.200
Low normal	3.5 g/dL
High normal	5.5 g/dL
Linearity	1.0 – 7.0 g/dL

Assay procedure

	Blank	Standard	Test
Sample	--------	--------	10 µL
Standard	--------	10 µL	--------
Reagent	2500 µL	2500 µL	2500 µL

Mix and incubate for 05 minutes at 37ºC. Measure the absorbance of sample (AT) and Standard (AS) against reagent blank at 620 nm. Intensity of color is directly proportional to concentration of albumin present in samples. Color produced is stable for 30 minutes at room temperature.

Calculation

Concentration of albumin (g/dL) = (AT/AS) X concentration of standard

4.3.4.2.12. Total Bilirubin (Diazo) (174)

Bilirubin Assay Kit utilizes the Jendrassik- Grof principle to detect bilirubin. Total bilirubin (Unconjugated + conjugated) concentration is determined in the presence of a catalyst, where bilirubin reacts with a diazo- salt to form azobilirubin, which absorbs light at 560 nm.

Reagent composition

Reagent 1: Sulfanilic acid in HCl

Reagent 2: Sodium nitrite

Reagent 3: Caffeine in Sodium benzoate

Reagent 4: Tartarate in Sodium hydroxide

Wave length	560 nm
Reaction type	End point
Cuvette	1 cm light path
Reaction type	Increasing
Measurement	Against sample blank
Sample volume	200 µL
Reagent volume	2200 µL
Incubation	15 min
Factor	10.8
Low normal	0.1 mg/dL
High normal	1.0 mg/dL
Linearity	0.1 – 30.0 mg/dL

Assay procedure

	Sample blank	**Test**
Reagent 1	200 µL	200 µL
Reagent 2	--------	1 drop
Reagent 3	1000 µL	1000 µL
Mix and incubate for 10 min at 25^0C		
Reagent 4	1000 µL	1000 µL

Mix and incubate for 05 minutes at 25ºC. Measure the absorbance of sample (AS) against sample blank at 560 nm. Intensity of color is directly proportional to concentration of bilirubin present in samples. Color produced is stable for 30 minutes at room temperature.

Calculation

Concentration of total bilirubin (mg/dL) = AS X 10.8

4.3.4.2.13. Alanine Transaminase (ALT) activity (IFCC) (174)

Enzyme catalyses the transamination reaction between alanine and α- ketoglutarate to obtain pyruvate and glutamate. Pyruvate that is produced is then reacts with lactate dehydrogenase in the presence of NADH to produce lactate. The decrease in absorbance of NADH at 340 nm is measured which will give activity of ALT.

Reagent composition

Reagent 1: TRIS, L-Alanine, Lactate dehydogenase (LDH) pH: 7.5

Reagent 2: 2- oxoglutarate, NADH, Azide

Wave length	340 nm
Reaction type	Kinetic
Flow cell temperature	37^0C
Reaction direction	Decreasing
Zero setting with	Distilled water
Sample volume	100 µL
Reagent volume	1000 µL
Delay time	60 sec
Kinetic interval	60 sec
Number of readings	04
Factor	1746
Low normal	Zero U/L
High normal	48 U/L
Linearity	Up to 500 U/L

Assay procedure

	Test
Working reagent	1000 µL
Sample	100 µL

Mix well and after 60 sec incubation at 37^0C, measure the change in optical density per 60 sec during 180 sec against distilled water at 340 nm.

A0 = Exactly after 60 sec

A1, A2, A3 = Exactly after every 60 sec for 180 sec

Calculation

Calculate the average change in absorbance/min = Δ Abs/min

Activity of ALT = (Δ Abs/min) X 1746

4.3.4.2.14. Aspartate Transaminase (AST) activity (IFCC) (174)

Enzyme catalyses the transamination reaction between aspartic acid and α- ketoglutarate to obtain oxaloacetic acid and glutamate. Oxaloacetic acid that is produced is then reacts with malate dehydrogenase in the presence of NADH to produce malate. The decrease in absorbance of NADH at 340 nm is measured will give activity of AST.

Reagent composition

Reagent 1: TRIS, L-Aspartate, Lactate dehydogenase (LDH) pH: 7.5

Reagent 2: α-ketoglutarate, NADH, Azide

Working reagent	1000 µL
Sample	100 µL
Wave length	340 nm
Reaction type	Kinetic
Flow cell temperature	37^0C
Reaction direction	Decreasing
Zero setting with	Distilled water
Sample volume	100 µL
Reagent volume	1000 µL
Delay time	60 sec
Kinetic interval	60 sec
Number of readings	04
Factor	1746
Low normal	Zero U/L
High normal	48 U/L
Linearity	Up to 500 U/L

Assay procedure

	Test
Working reagent	1000 µL
Sample	100 µL

Mix well and after 60 sec incubation at 37^0C, measure the change in optical density per 60 sec during 180 sec against distilled water at 340 nm.

A0 = Exactly after 60 sec

A1, A2, A3 = Exactly after every 60 sec for 180 sec

Calculation

Calculate the average change in absorbance/min = Δ Abs/min

Activity of AST = (Δ Abs/min) X 1746

4.3.4.2.15. Alkaline Phosphatase (174)

ALP activity is measured using p- nitro phenyl phosphate as substrate at alkaline pH. Hydrolysis of this phosphate ester yields inorganic phosphate and a highly coloured para-nitrophenoxide anion.

Reagent composition

Reagent 1: Substrate solution

Wave length	405 nm
Reaction type	Kinetic
Flow cell temperature	37^0C
Reaction direction	Increasing
Zero setting with	Distilled water
Sample volume	10 µL
Reagent volume	1000 µL
Delay time	60 sec
Kinetic interval	30 sec
Number of readings	04
Factor	5454
Low normal	53 U/L
High normal	141 U/L
Linearity	Up to 2000 U/L

Assay procedure

	Test
Reagent	1000 µL
Sample	10 µL

Mix well and after 60 sec incubation at 37^0C, measure the change in optical density per 30 sec during 120 sec against distilled water at 405 nm.

A0=Exactly after 60 sec

A1, A2, A3 = Exactly after every 30 sec for 120 sec

Calculation

Calculate the average change in absorbance/min = Δ Abs/min

Activity of ALP = (Δ Abs/min) X 5454

4.3.4.2.16. γ – Glutamyl Transferase (174)

γGT transfers γ- glutamyl residue from the synthetic substrate L- γ- glutamyl-3-carboxy-4- nitroanilide to glycylglycine to yield γ- glutamyl- glycylglycine and free 5- amino- 2 -nitro benzoate (ANB). The rate of formation of ANB is measured at 410 nm which gives the activity of γGT (40).

Reagent composition

Reagent 1: TRIS, Glycylglycine pH: 8.28

Reagent 2: L-γ-Glutamyl-3-Carboxy-4-Nitroanilide pH: 6.0

Wave length	405 nm
Reaction type	Kinetic
Flow cell temperature	37^0C
Reaction direction	Increasing
Zero setting with	Distilled water
Sample volume	100 µL
Reagent volume	1000 µL
Delay time	60 sec
Kinetic interval	60 sec
Number of readings	04
Factor	1158
Low normal	9 U/L
High normal	55 U/L
Linearity	Up to 300 U/L

Assay procedure

	Test
Working reagent	1000 µL
Sample	100 µL

Mix well and after 60 sec incubation at 37^0C, measure the change in optical density per 60 sec during 180 sec against distilled water at 405 nm.

A0 = Exactly after 60 sec

A1, A2, A3 = Exactly after every 60 sec for 180 sec

Calculation

Calculate the average change in absorbance/min = Δ Abs/min

Activity of γGT = (Δ Abs/min) X 1158

4.3.4.2.17. Prothrombin Time International Normalized Ratio (PT INR) (175)

To perform this common coagulation assay, tissue thromboplastin and patients plasma are incubated for several minutes, later the citrated plasma mixture is recalcified by the addition of excess $CaCl_2$ and the time required for clot formation is measured using analyzer. INR was calculated.

4.3.4.2.18. Total Leukocyte and Platelet Count

Complete blood count is done by 5 part fully automated hematology analyzer.

RESULTS

5.1. Discovery of protein biomarker candidates

5.1.1. SDS-PAGE analysis for depletion of Albumin

Immobilized resin form of cibacron blue was effective in binding abundant albumin from plasma/serum samples for depletion. Human serum albumin (HSA) from pooled serum samples of cirrhotic and healthy subjects was depleted using cibacron blue dye loaded resin columns. Proteins in flow-through were analyzed by SDS-PAGE (**Figure 5.1.1.1**) along with pre-stained molecular weight marker to investigate efficient depletion of HSA. Sensitive stain, silver staining was helpful for the detection of low nanogram proteins compared to Coomassie Brilliant Blue. Silver stained gel demonstrated significant amount of abundant albumin depletion from serum samples of both cirrhotic and healthy subjects.

5.1.2. Identification of biomarker candidates by In-Gel trypsin digestion

Synthetic gel image representative of all features in the differential analysis comparing samples from cirrhotic and healthy are shown in **Figure 5.1.2.1**. Image analysis software and statistical analysis found 46 spots in cirrhotic gel and 69 spots in control gel of which 14 spots were identified with significant altered expression levels between cirrhotic and healthy subjects. In-Gel trypsin digestion followed by LC-MS analysis of these 14 spots was identified by MASCOT against human database revealed 68 proteins with significant differential analysis. Among 68 proteins, we identified 46 candidate biomarkers for liver cirrhosis. Among 46 candidate biomarkers, 28 were identified based on protein score and clinical significance (**Table 5.1.2.1**). Among 28 protein biomarker candidates, 13 with

increased expression and 15 with decreased expression were identified in cirrhotic liver when compared to healthy subjects.

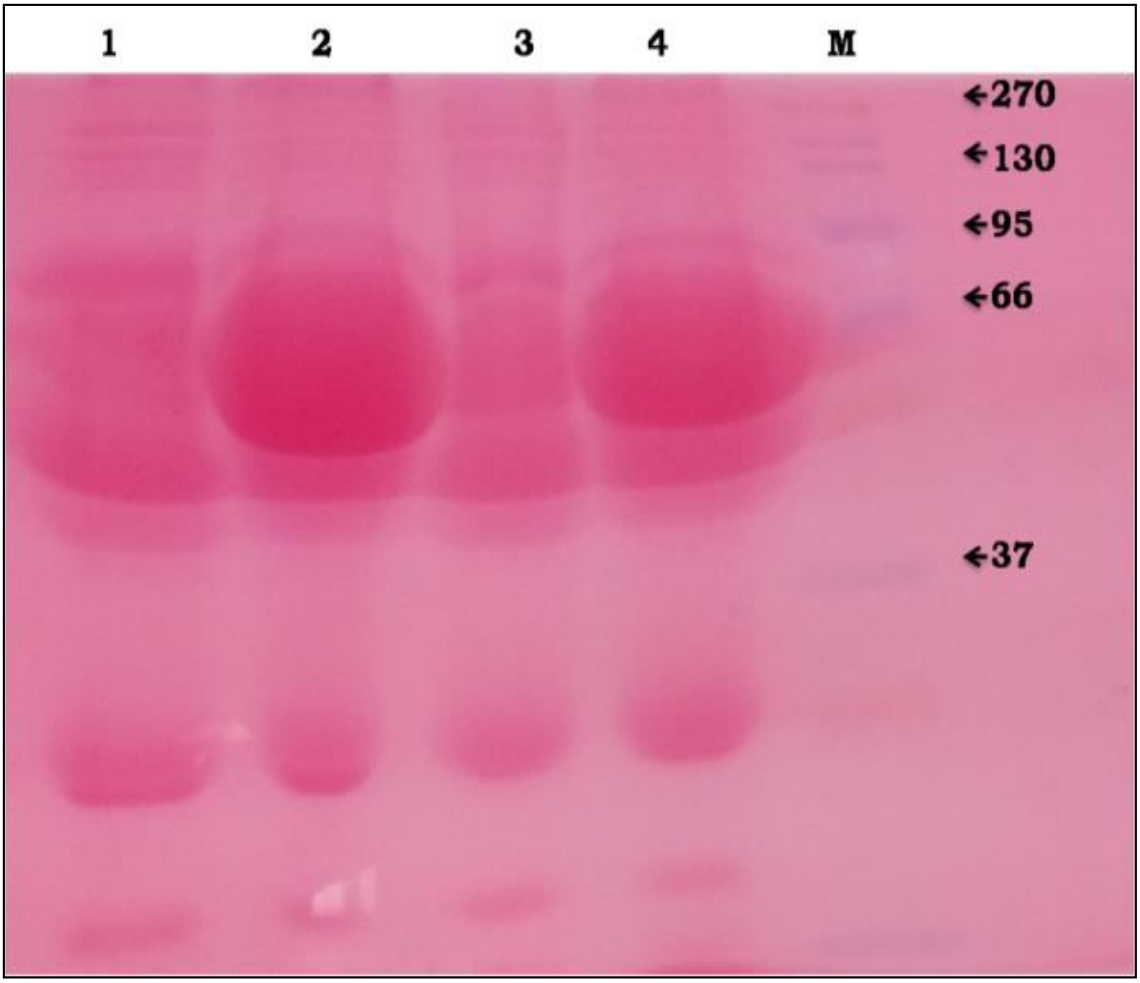


Figure 5.1.1.1: SDS-PAGE analysis for confirmation of albumin depletion (silver stained gel)

1: Normal pooled albumin depleted serum; **2**: Normal pooled serum; **3**: Cirrhotic liver pooled albumin depleted serum; **4**: Cirrhotic liver pooled serum; **M**: Pre-stained molecular weight marker

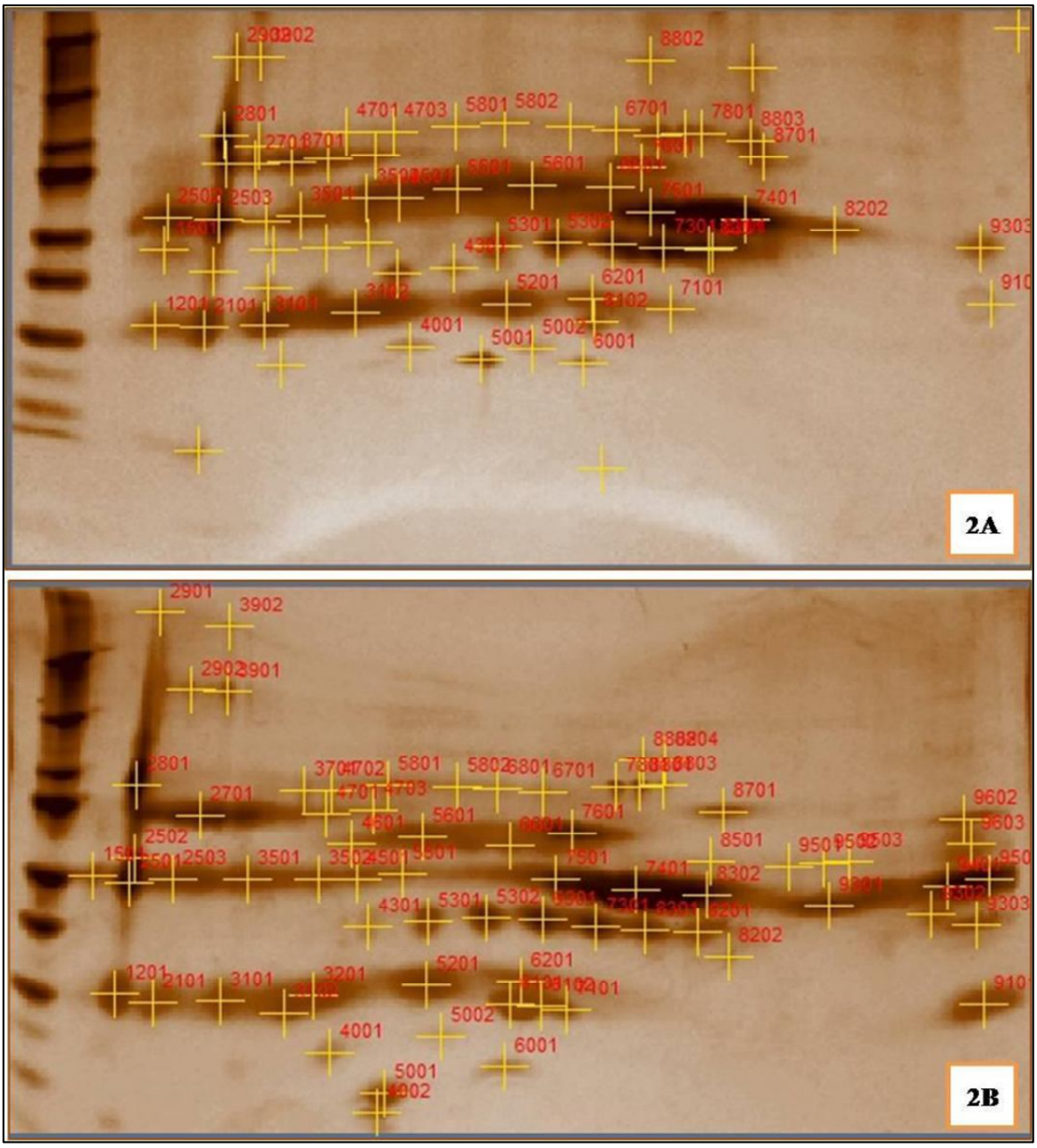


Figure 5.1.2.1: Comparison of Two dimensional electrophoresis (2DE) gel images representative of all features in differential analysis from alcoholic cirrhotic and healthy subjects

2A: 2DE gel image of liver cirrhosis

2B: 2DE gel image of healthy

Table 5.1.2.1: Protein biomarker candidates identified by 2DE after depletion of albumin followed by LC-MS for cirrhosis of liver

Biomarker candidates identified	Expression in LC	Mol. Wt	Calc. pI
Keratin type II cuticular Hb6 isoform X1	Increases	62	6.37
Keratin type I cuticular Ha1	Increases	47.2	4.88
Keratin type II cuticular Hb5 isoform 1	Increases	55.8	6.55
Keratin type II cytoskeletal 6C	Increases	60	8
Keratin type II cytoskeletal 2 epidermal	Increases	65.4	8
Keratin type I cytoskeletal 9	Increases	62	5.24
Lumican precursor	Increases	38.4	6.61
Polymeric Ig receptor isoform X1	Increases	83.2	5.74
Serotransferrin precursor	Increases	77	7.12
Ig lambda like polypeptide 5 isoform 1	Increases	23	8.84
Vitamin D binding protein isoform 3	Increases	55	5.63
Haptoglobin isoform 1 preproprotein	Increases	45.2	6.58
Transmembrane protein 201 isoform 1	Increases	72.2	9.22
α-1-antitrypsin	Decreases	46.7	5.59
Hemopexin precursor	Decreases	51.6	7.02
Apolipoprotein A-IV precursor	Decreases	45.3	5.38
CD5 antigen like isoform X1	Decreases	38.7	5.66
Zinc-α2-glycoprotein precursor	Decreases	34.2	6.05
Dermcidin isoform 1 preproprotein	Decreases	11.3	6.54
α1-B-glycoprotein precursor	Decreases	54.2	5.86
Glycerol kinase isoform X1	Decreases	63.6	6.54
α2-HS-glycoprotein preproprotein	Decreases	39.3	5.72
Kininogen-1 isoform 1 precursor	Decreases	71.9	6.81
Sex hormone binding globulin isoform1 precursor	Decreases	43.8	6.71
α1-acid glycoprotein 1 precursor	Decreases	23.5	5.11
Leucine rich α2 glycoprotein precursor	Decreases	38.2	6.95
α2-antiplasmin isoform X1	Decreases	56.6	6.89
Antithrombin-III precursor	Decreases	52.6	6.71

Abbreviations: **2DE**: Two Dimensional Electrophoresis; **LC-MS**: Liquid Chromatography-Mass Spectrometry; **LC**: Liver Cirrhosis; **Mol. Wt**: Molecular Weight; **Calc. pI**: Calculated Iso Electric pH; **Igs**: Immunoglobulins

5.1.3. Identification of biomarker candidates by In-Solution trypsin digestion

Identification of proteins present in 23 spots which were not present in cirrhotic gel was carried out by In-Solution trypsin digestion analysis and compared between cirrhotic and healthy subjects. In-Solution trypsin digestion analysis followed by characterization of proteins using MASCOT database revealed 38 protein biomarker candidates of which 14 were selected based on clinical significance and protein scores (**Table 5.1.3.1**). Among 14 protein biomarker candidates identified; 08 with increased expression and 06 with decreased expression were identified in liver cirrhotic when compared to healthy subjects.

Table 5.1.3.1: Proteomic biomarker candidates identified by comparative protein expression analysis by In-Solution trypsin digestion followed by LC-MS for cirrhosis of liver

Biomarker candidates identified	Expression in LC	Mol. Wt	Calc. pI
Keratin associated protein 9-3	Increases	16.8	7.53
Keratin type I cytoskeleton 10 isoform X1	Increases	63.3	5.26
Keratin type II cytoskeleton 5	Increases	62.3	7.7
Keratin type II cytoskeleton 2 epidermal	Increases	65.4	8
Keratin associated protein 3-3	Increases	10.4	5.69
Ig Fc binding protein precursor	Increases	571.6	7.02
Cytoplsamic Actin 1	Increases	42	5.48
Putative V set Ig domain protein	Increases	23.8	8.94
Serum albumin prepro protein	Decreases	69.3	6.28
Angiotensinogen preproprotein	Decreases	53	6.32
SERPINA4/Kallistatin	**Decreases**	**54**	**5.0**
Ig lamda like polypeptide 5 isoform 1	Decreases	23	8.8
Histone H1.3	Decreases	22.3	11.02
α1-antichymotrypsin precursor	Decreases	47.6	5.52

Abbreviations: **LC**: Liver Cirrhosis; **Mol. Wt**: Molecular Weight; **Calc. pI**: Calculated Iso Electric pH

5.2. Prioritization of protein biomarker candidates

Technological advancement in proteomic approach for biomarker candidate discovery for cirrhosis of liver revealed 42 biomarker candidates; 28 protein biomarker candidates by In-Gel trypsin digestion followed by LC-MS characterization and identification after depletion of abundant albumin and 14 biomarker candidates by In-Solution trypsin digestion followed by LC-MS characterization and identification of protein based on clinical significance, protein scores and reagent availability. Protein biomarker candidates discovered in the present study needs verification and validation.

Proteins which are expressed from liver, act in cellular pathways and deregulated in disease progression should be considered for further analytical and clinical validation. Validation of biomarker and clinical assay optimization requires measurement of thousands of patient samples with narrow measurement coefficient of variation values.

Prioritization of protein biomarker candidates identified with the help of technological advancement in –omics approach in discovery phase is a prerequisite for validation regimen. Along with clinical significance; availability of reagents also plays an important role in prioritization. Based on clinical significance and reagent availability, we considered **SERPINA4/Kallistatin**; a multi-functional protein belongs to SERPIN super family for further analytical and clinical validation. SERPINA4/Kallistatin is expressed from liver cells (Hep G2 and Hep 3B) and strong inhibitor of tissue kallikrein. Concentration of SERPINA4/Kallistatin in circulation may reflect degree of liver dysfunction which shall give potential insights for assessment of extent of liver disease. Analytical and clinical validation of SERPINA4/Kallistatin may prove it as a noninvasive biomarker for cirrhosis of liver with varied etiology.

5.3. Cross reactivity of SERPINs with SERPINA4/Kallistatin

5.3.1. Cross reactivity analysis by using monoclonal antibodies

Since SDS-PAGE is an efficient tool for separation of proteins based on molecular weight, proteins in serum of both diseased (D1 to D10) and healthy (C1 to C10) were separated in gels along with corresponding molecular weight marker. Recombinant kallistatin was spotted on another SDS-PAGE with pooled and concentrated samples of cirrhotic liver and healthy subjects.

Western blot analysis allowed identification of cross reactivity of SERPINs with SERPINA4/Kallistatin in diseased and healthy samples by using monoclonal antibodies specific for SERPINA4/Kallistatin followed by secondary antibodies conjugated with HRP.

No bands were observed on PVDF membranes of diseased as well as healthy **(Figure 5.3.1.1)**. Absence of bands revealed that other SERPINs will not cross react with monoclonal antibodies specific for SERPINA4/Kallistatin. Even though monoclonal antibodies are more specific for SERPINA4/Kallistatin, they failed to capture protein of interest on PVDF membranes of both diseased and healthy. These findings might be due to less sensitivity of monoclonal antibodies who failed to capture protein of interest and whose concentrations are in pg/mL in samples.

However, a significant band was observed with recombinant SERPINA4/Kallistatin. There were no band detection with pooled and concentrated samples of diseased and healthy indicating that there is no cross reactivity of other serpins with

SERPINA4/Kallistatin for monoclonal antibodies. There was a significant band with recombinant SERPINA4/Kallistatin.

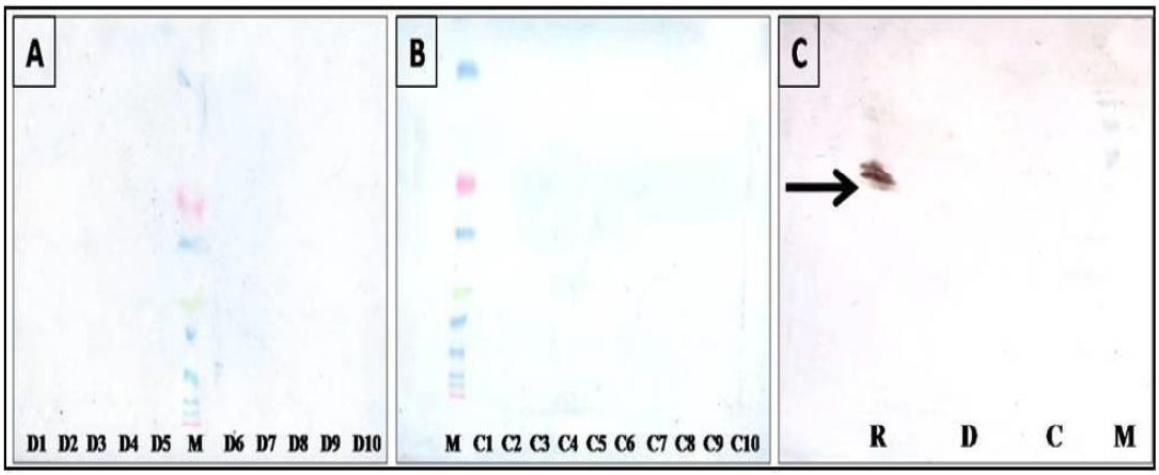


Figure 5.3.1.1: Western blot analysis for cross reactivity with monoclonal antibodies
A: Western blot with diseased serum; **M**: Pre stained marker; **D**: Diseased subjects (Cirrhosis of Liver; D1 to D10);

B: Western blot with control serum; **C**: Healthy subjects (C1 to C10); **C:** Westernblot with Recombinant kallistatin; **R**: Recombinant SERPINA4/Kallistatin; **D**: Pooled and concentrated diseased serum;

C: Pooled and concentrated control serum; **Arrow:** Detection of band with recombinant SERPINA4/Kallistatin

5.3.2. Cross reactivity analysis by using polyclonal antibodies

Proteins were separated according to molecular weight in both diseased (D1 to D10) and healthy (C1 to C10) by using SDS-PAGE along with corresponding molecular weight marker. Western blot analysis allowed identification of cross reactivity of SERPINs with SERPINA4/Kallistatin in diseased and healthy samples by using polyclonal antibodies

specific for SERPINA4/Kallistatin followed by secondary antibodies conjugated with HRP.

PVDF membranes of both cirrhotic liver and healthy did not show any band which revealed that there will not be any cross reactivity of other SERPINs even with polyclonal antibodies specific for SERPINA4/Kallistastin (**Figure 5.3.2.1**). However, even polyclonal antibodies with more sensitivity were failed to capture SERPINA4/Kallistatin in both cirrhotic and healthy subjects like monoclonal antibodies.

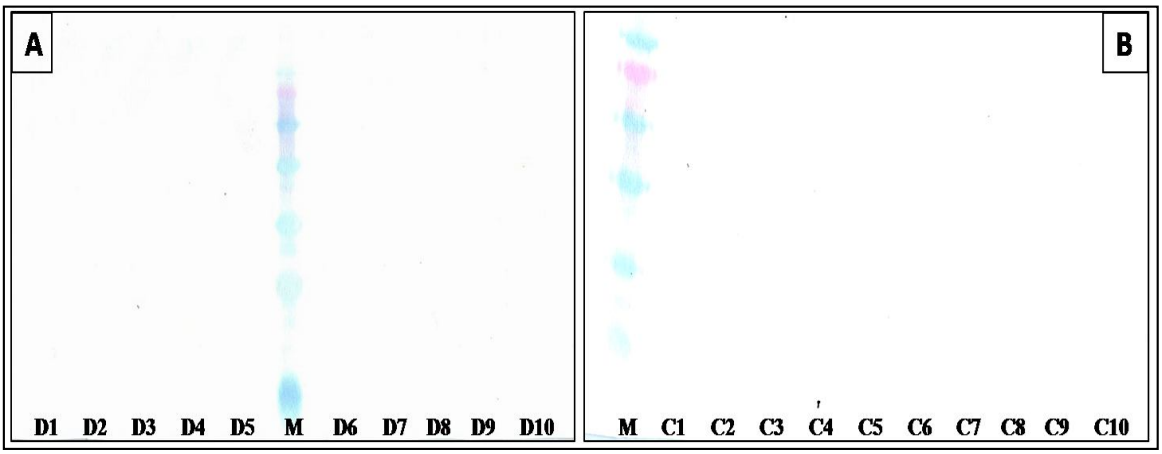


Figure 5.3.2.1: Western blot analysis for cross reactivity with polyclonal antibodies
A: Western blot with diseased serum; **M**: Pre stained marker; **D**: Diseased subjects (D1 to D10; Cirrhosis of Liver);

B: Western blot with control serum; **C**: Healthy subjects (C1 to C10)

5.3.3. Cross reactivity analysis by using monospecific antibodies

Separation of proteins according to molecular weight was achieved by using SDS-PAGE along with pre-stained molecular marker in both healthy (C1 to C10) and diseased (D1 to D10) samples. Western blot analysis allowed identification of cross reactivity of SERPINs with SERPINA4/Kallistatin in diseased and healthy samples using

monospecific (Monoclonal alternative) antibodies specific for SERPINA4/Kallistatin followed by secondary antibodies conjugated with HRP.

PVDF membrane of healthy (**Figure 5.3.3.1**) showed bands corresponding to SERPINA4/Kallistatin molecular weight revealed that monospecific antibodies have ability to capture protein of interest which is in pg/mL. However, no other bands were observed in healthy PVDF membranes which indicate that there will not be any cross reactivity of other SERPINs with monospecific (monoclonal alternative) antibodies.

Cirrhotic liver PVDF membrane did not show any band corresponding to molecular weight of SERPINA4/Kallistatin which revealed that there will be decreased concentration of SERPINA4/Kallistatin in cirrhotic liver subjects when compared to healthy subjects. There were no other bands in cirrhotic liver PVDF membrane that showed no cross reactivity of other SERPINs with SERPINA4/Kallistatin.

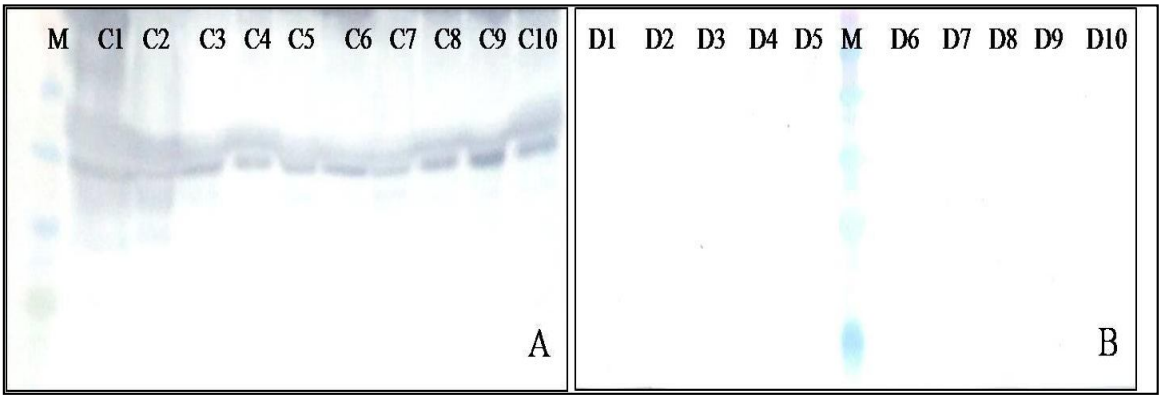


Figure 5.3.3.1: Western blot analysis for cross reactivity with monospecific (monoclonal alternative) antibodies

A: Western blot with healthy serum; **M**: Pre stained marker; **C**: Healthy subjects (C1 to C10);

B: Western blot with control serum; **D**: Diseased subjects (D1 to D10; Cirrhosis of Liver)

Comparison of cross reactivity analysis with monoclonal, polyclonal and monospecific (Monoclonal alternative) concluded that SERPINs do not cross reactive with antibodies specific for SERPINA4/Kallistatin. Monospecific (monoclonal alternative) antibodies are more sensitive and more specific to form double sandwich ELISA than monoclonal and polyclonal antibodies. Monospecific antibodies are well characterized antibodies for protein studies especially in clinical diagnosis to capture protein of interest whose concentrations will be in pg/mL.

5.4. In-House quantitative ELISA for quantification of SERPINA4/Kallistatin

In-House quantitative ELISA was developed by using mouse anti-human monospecific (monoclonal alternative) antibodies specific for SERPINA4/Kallistatin as capture antibodies and biotinylated goat anti-human SERPINA4/Kallistatin polyclonal antibodies as detection antibodies. Streptavidin conjugated with horseradish peroxidase was used as an enzyme. Stabilized 3,3`,5,5`-tetramethylbenzidine (TMB) diluted in hydrogen peroxide was used as substrate for the enzyme horseradish peroxidase. Acidic (2NSulfuric acid) stop solution was used to stop the color development.

Recombinant SERPINA4/Kallistatin was used for preparation of serial standards and standard curve was plotted against OD values at 450 nm (**Section 4.3.4.2.1**). Known concentrations of recombinant SERPINA4/Kallistatin and natural samples were used to define Intra-assay and the Inter-assay precision of In-House developed ELISA quantitative kit.

Buffer components, sample matrix, complement rheumatoid factor and heterophilic antibodies can impact antibody binding in the natural samples and therefore influence the accuracy of ELISA. During ELISA assay development, two tests were performed to determine if the value obtained from a sample was accurate or was there any factor in the sample matrix interfere the measurements.

In **Spike/Recovery** assays, a known amount of recombinant protein was spiked into a sample and run in ELISA. If the recovered value differs significantly from expected value this can be a sign that some factor in the sample matrix might be causing a falsely elevated or falsely depressed value. The resulting concentration/recovery of spiked material in present study demonstrated that expected value could be measured accurately by In-House developed quantitative ELISA for quantification of SERPINA4/Kallistatin.

In **Linearity** assay, a spiked sample was serial-diluted *viz.*, 1:2, 1:4, and 1:8. If a sample does not exhibit linear dilution this indicates that a matrix component is interfering with accurate detection of a specific analyte at a given dilution. Spiked sample in the present study exhibited linear dilution which indicates that a matrix component did not interfere in accurate detection of SERPINA4/Kallistatin in sample.

5.4.1. Precision

5.4.1.1. Intra-assay precision

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision (**Table 5.4.1.1.1**)

Table 5.4.1.1.1: Intra-assay Precision

Sample	**1**	**2**	**3**
N	20	20	20
Mean (pg/dL)	566	1031	2202
Standard deviation	26.7	44.4	103
Coefficient of Variation (%)	5.1	4.8	5.0

5.4.1.2. Inter-assay precision

Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision (**Table 5.4.1.2.1**).

Table 5.4.1.2.1: Inter-assay Precision

Sample	**1**	**2**	**3**
N	20	20	20
Mean (pg/dL)	606	1051	2178
Standard deviation	34.3	50.9	123
Coefficient of Variation (%)	5.7	5.3	5.9

5.4.2. Spike and Recovery

Recovery of human SERPINA4/Kallistatin spiked to levels throughout the range of the assay was evaluated (**Table 5.4.2.1**).

Table 5.4.2.1: Spike and recovery

Sample	Average % recovery	Range
Serum	95	91 % – 102 %

5.4.3. Linearity

To assess the linearity of the assay, samples containing high concentrations of human SERPINA4/Kallistatin were serially diluted with calibrator diluents to produce samples with values within dynamic range of the assay (**Table 5.4.3.1**).

Table 5.4.3.1: Linearity (serum)

		Serum
1:2	**Average % expected**	96
	Range (%)	93 – 101
1:4	**Average % expected**	102
	Range (%)	98 – 102
1:8	**Average % expected**	97
	Range (%)	89 – 107

5.5. Correlative analysis of SERPINA4/Kallistatin with direct markers of ECM (hyaluronic acid and YKL-40), conventional, oxidative and antioxidant status

In the present study, cirrhotic liver subjects were in the age group of 25 to 60 years with a mean age ± SD of **43.15 ± 8.34**; 78% (**n=75**) were males while 22% were females (**n=21**) with varying degree and different etiological factors (**Figure 5.5.1**).

All the variables were expressed in mean ± SD (**Table 5.5.1a, 5.5.1b & 5.5.1c**). Significant reduction of SERPINA4/Kallistatin was observed in cirrhotic liver subjects compared to healthy subjects (**1768.54 ± 471.41** vs. **3989.66 ± 546.47**). Significant elevation was observed for the variables serum uric acid (**6.11 ± 0.67** vs. **3.56 ± 0.50**), hyaluronic acid (**22.84 ± 6.11** vs. **7.99 ± 3.02**) and YKL-40 (**140.71 ± 23.93** vs. **29.93 ± 6.64**) in cirrhotic liver compared to healthy subjects (**Figure 5.5.2**). Significant increase in activities of AST (**210.97 ± 55.03** vs. **37.93 ± 14.41**), ALT (**267.78 ± 53.58** vs. **33.82 ± 15.96**), ALP (**332.76 ± 48.14** vs. **110.57 ± 25.32**) and γGT (**269.54 ± 69.06** vs. **37.68 ± 14.01**) with elevated serum levels of total bilirubin (**5.40 ± 1.49** vs. **0.76 ± 0.24**) and prolonged PT INR (**2.57 ± 0.50** vs. **1.08 ± 0.14**) were observed in cirrhotic liver subjects. Serum total protein (**4.97 ± 0.62** vs. **6.93 ± 0.34**) and albumin (**2.56 ± 0.41** vs. **3.94 ± 0.35**) were reduced significantly in cirrhotic liver subjects compared to healthy subjects. In cirrhotic liver subjects there was an increased total oxidative stress (**33.27 ± 6.05** vs. **12.18 ± 3.59**) with decreased total antioxidant capacity (**20.75 ± 4.97** vs. **33.37 ± 5.95**) compared to healthy subjects.

Pearson correlation analysis showed positive correlation between serum SERPINA4/Kallistatin levels with serum levels of total proteins, albumin and total antioxidant capacity. A negative correlation was observed between serum levels of SERPINA4/Kallistatin with total oxidant status, direct markers of ECM *viz.,* hyaluronic acid and YKL-40 with, AST, ALT, ALP and γGT, total bilirubin and PT INR (**Table 5.5.2, 5.5.3, 5.5.4**).

A positive correlation was observed between serum levels of hyaluronic acid with those of AST, ALT, ALP and γGT with total bilirubin, PT INR and total oxidant status. A negative correlation was observed between serum levels of uric acid, hyaluronic acid and YKL-40 between total protein, albumin and total antioxidant capacity (**Table 5.5.5, 5.5.6**).

A positive correlation was observed between serum levels of YKL-40 with AST, ALT, ALP and γGT with total bilirubin, PT INR and total antioxidant capacity. A negative correlation was observed between serum levels of uric acid, hyaluronic acid and YKL-40 between total protein, albumin and total antioxidant capacity (**Table 5.5.7, 5.5.8**).

Among cirrhotic liver subjects, one way ANOVA analysis showed high reduction of SERPINA4/Kallistatin in ALD (**1592.35 ± 451.56**) compared to HCV (**2099.30 ± 371.84**), HBV (**1993.80 ± 370.79**) and NAFLD (**2051.50 ± 187.25**) subjects. High levels of serum HA (**24.41 ± 6.46**) in ALD subjects was observed compared to HCV (**21.26 ± 5.00**), HBV (**18.00 ± 2.84**) and NAFLD (**20.49 ± 3.94**) subjects. YKL-40 was expressed high in ALD (**149.80 ± 20.53**) compared to HCV (**129.71 ± 19.59**), HBV (**115.41 ± 22.19**) and NAFLD (**128.79 ± 25.21**) subjects (**Table 5.5.9**).

ROC analysis was carried out to define the diagnostic accuracy for serum SERPINA4/Kallistatin, hyaluronic acid and YKL-40 in cirrhotic liver and healthy subjects; serum SERPINA4/Kallistatin showed an AUROC of 0.969 (95% CI; 0.934 - 0.989) with sensitivity 94.06% and specificity 96.70%, serum hyaluronic acid with an AUROC of 0.961 (95% CI; 0.923 – 0.984) and YKL-40 with an AUROC of 0.973 (95% CI, 0.939 – 0.991) (**Figure 5.5.3&5.5.4**).

Table 5.5.1a: Reference range for established biomarkers

Parameter	Our lab Reference Range	Methodology	Linearity
Glucose	75 – 140 mg/dL	GOD-POD	400 mg/dL
Urea	12 – 40 mg/dL	Urease	300 mg/dL
Creatinine	0.6 – 1.3 mg/dL	Modified Jaffe's	25.0 mg/dL
Uric aid	2.6 – 7.2 mg/dL	Uricase	25.0 mg/dL
ALT	Up to 45 U/L	IFCC	Up to 500 U/L
AST	Up to 35 U/L	IFCC	Up to 500 U/L
ALP	42 – 128 U/L	IFCC	Up to 2000 U/L
γGT	Up to 55 U/L	IFCC	Up to 300 U/L
Total Protein	6.4 – 8.3 g/dL	Biuert	1.0 – 15.0 g/dL
Albumin	3.5 – 5.2 g/dL	BCG	1.0 – 7.0 g/dL
Total Bilirubin	0.2 – 1.3 mg/dL	Diazo	0.1-30.0 mg/dL

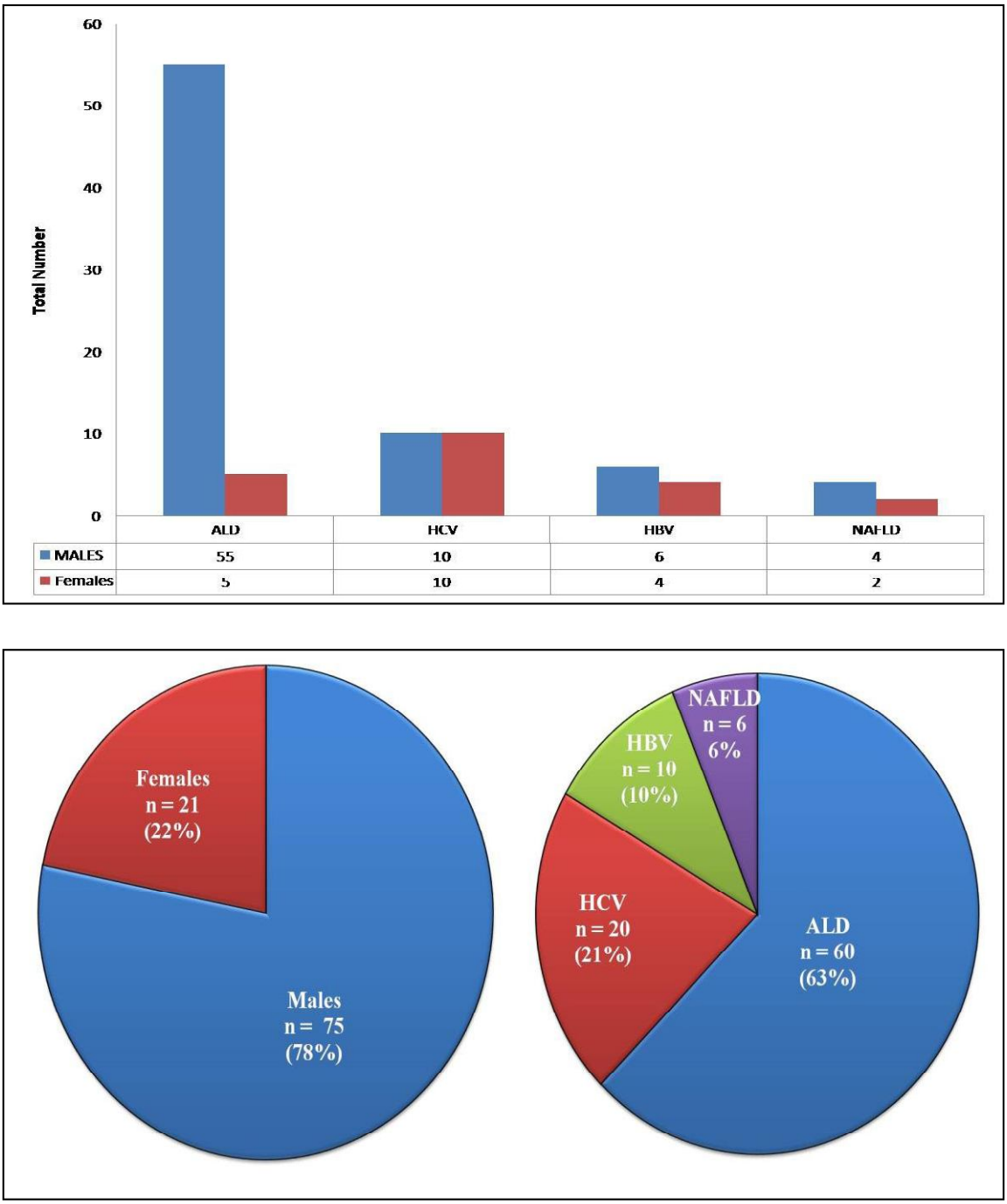


Figure 5.5.1: Demographic representation of cirrhosis of liver subjects based on gender & etiology

Table 5.5.1b: Statistical analysis of biochemical parameters (n=96 cases and controls)				
Variables	**Groups**	**Mean ± SD**	**t-Value**	**p-Value**
RBS (mg/dL)	II	101.21 ± 10.96	-0.68	0.49
	I	100.12 ± 11.08	-0.68	
Blood Urea (mg/dL)	II	33.16 ± 6.25	-2.2	0.23
	I	31.19 ± 5.94	-2.2	
Creatinine (mg/dL)	II	1.09 ± 0.19	-0.72	0.47
	I	1.07 ± 0.22	-0.72	
Kallistatin (pg/mL)	II	1768.54 ± 471.41	30.15	0. 01*
	I	3989.66 ± 546.47	30.15	
Hyaluronic acid (ng/mL)	II	22.84 ± 6.11	-21.32	0.01*
	I	7.99 ± 3.02	-21.32	
YKL-40 (ng/mL)	II	140.71 ± 23.93	-43.70	0.01*
	I	29.93 ± 6.64	-43.70	
AST (U/L)	II	210.97 ± 55.03	-29.80	0.01*
	I	37.93 ± 14.41	-29.80	
ALT (U/L)	II	267.78 ± 53.58	-40.99	0.01*
	I	33.82 ± 15.96	-40.99	
ALP (U/L)	II	332.76 ± 48.14	-40.01	0.01*
	I	110.57 ± 25.32	-40.01	
γGT (U/L)	II	269.54 ± 69.06	-32.23	0.01*
	I	37.68 ± 14.01	-32.23	
Total Protein (g/L)	II	4.97 ± 0.62	26.86	0.01*
	I	6.93 ± 0.34	26.86	
Albumin (g/L)	II	2.56 ± 0.41	24.58	0.01*
	I	3.94 ± 0.35	24.58	
Total Bilirubin (mg/dL)	II	5.40 ± 1.49	-30.05	0.01*
	I	0.76 ± 0.24	-30.05	
Uric acid (mg/dL)	II	6.11±0.67	-29.58	0.01*
	I	3.56 ± 0.50	-29.58	
PT-INR	II	2.57 ± 0.50	-28.11	0.01*
	I	1.08 ± 0.14	-28.11	
TAC (nmol/µL)	II	20.75 ± 4.97	15.94	0.01*
	I	33.37 ± 5.95	15.94	
TOS(µmol H_2O_2 Equiv/L)	II	33.27 ± 6.05	-29.33	0.01*
	I	12.18 ± 3.59	-29.33	

*p<0.05: significant; **Group II**: Clinically& diagnostically proven Cirrhotic liver subjects; **Group I**: Healthy subjects; **SD**: Standard Deviation; **AST**: Aspartate Transaminase; **ALT**: Alanine Transaminase; **ALP**: Alkaline Phosphatase; **γGT**: Gamma Glutamyl Transferase: **PT INR**: Prothrombin Time-International Normalized Ratio; **TAC**: Total Anti-oxidant Capacity; **TOS**: Total Oxidative Status

Table 5.5.1c: Statistical comparison of biochemical parameters in cirrhosis of liver with varied etiology (II) Vs healthy (I)					
Variable (*p<0.05 considered as significant)	**Groups**	**Varied etiology**			
		Alcoholic Liver Disease (n=60)	**Hepatitis C Virus (n=20)**	**Hepatitis B Virus (n=10)**	**Non Alcoholic Fatty Liver Disease (n=6)**
Kallistatin (pg/mL) 0.01*	II	1592.35 ± 451.56	2099.30 ± 371.84	1993.80 ± 370.79	2052.50 ± 187.25
	I	4000.63 ± 633.21	3974. 90 ± 376.24	3899.30 ± 410.61	4079.83 ± 269.79
Hyaluronic acid (ng/mL) 0.01*	II	24.41 ± 6.46	21.26 ± 5.00	18.00 ± 2.84	20.49 ± 3.94
	I	8.17 ± 3.08	7.57 ± 3.00	7.89 ± 2.92	7.67 ± 3.21
YKL-40 (ng/mL) 0.01*	II	149.80 ± 20.53	129.70 ± 19.59	115.40 ± 22.19	128.79 ± 25.21
	I	29.20 ± 6.42	31.25 ± 6.11	29.53 ± 7.85	33.43 ± 8.50
Aspartate Transaminase (U/L)	II	216.13 ± 60.53	188.75 ± 41.09	205.10 ± 29.94	243.33 ± 51.50
	I	38.90 ± 15.39	35.75 ± 15.59	35.60 ± 7.22	39.50 ± 9.35
Alanine Transaminase (U/L)	II	268.25 ± 61.38	260.90 ± 38.79	268.50 ± 23.32	284.83 ± 53.46
	I	33.25 ± 13.50	36.65 ± 23.59	33.40 ± 16.64	30.83 ± 5.70
Alkaline Phosphatase (U/L)	II	331.58 ± 46.25	317.25 ± 54.48	355.70 ± 41.36	358.00 ± 40.59
	I	110.36 ± 20.49	116.25 ± 39.41	102.30 ± 16.83	107.50 ± 23.12
γ Glutamyl Transferase (U/L)	II	259.03 ± 66.67	272.05 ± 60.94	293.10 ± 90.19	327.00 ± 54.75
	I	37.10 ± 14.12	42.00 ± 17.14	36.20 ± 6.35	31.66 ± 7.78
Total Protein (g/dL)	II	4.83 ± 0.47	5.10 ± 0.72	5.46 ± 0.95	5.18 ± 0.59
	I	6.97 ± 0.34	6.91 ± 0.29	6.78 ± 0.37	6.90 ± 0.42
Albumin (g/dL)	II	2.47 ± 0.35	2.63 ± 0.40	2.89 ± 0.63	2.65 ± 0.33
	I	3.94 ± 0.35	4.01 ± 0.34	3.76 ± 0.32	4.10 ± 0.44
Total Bilirubin (mg/dL)	II	5.88 ± 1.32	4.50 ± 1.29	4.24 ± 1.09	5.58 ± 2.09
	I	0.80 ± 0.25	0.71 ± 0.19	0.63 ± 0.18	0.81 ± 0.24
Uric acid (mg/dL) 0.01*	II	6.30 ± 0.59	5.86 ± 0.75	5.77 ± 0.62	5.56 ± 0.57
	I	3.66 ± 0.49	3.32 ± 0.42	3.33 ± 0.39	3.68 ± 0.76
Prothrombin Time International Normalized Ratio	II	2.63 ± 0.54	2.55 ± 0.42	2.50 ± 0.36	2.26 ± 0.45
	I	1.09 ± 0.13	1.03 ± 0.15	1.06 ± 0.17	1.13 ± 0.10
Total Antioxidant Capacity (nmol/µL) 0.01*	II	20.15 ± 4.43	20.81 ± 6.59	22.28 ± 4.60	24.02 ± 3.52
	I	33.70 ± 6.21	32.83 ± 5.32	32.93 ± 6.22	32.60 ± 5.95
Total Oxidative Status (µmol H_2O_2 Equiv/L) 0.01*	II	32.82 ± 4.94	31.83 ± 6.17	37.90 ± 10.34	34.82 ± 3.91
	I	12.48 ± 3.55	12.36 ± 4.05	9.93 ± 3.33	12.30 ± 1.65

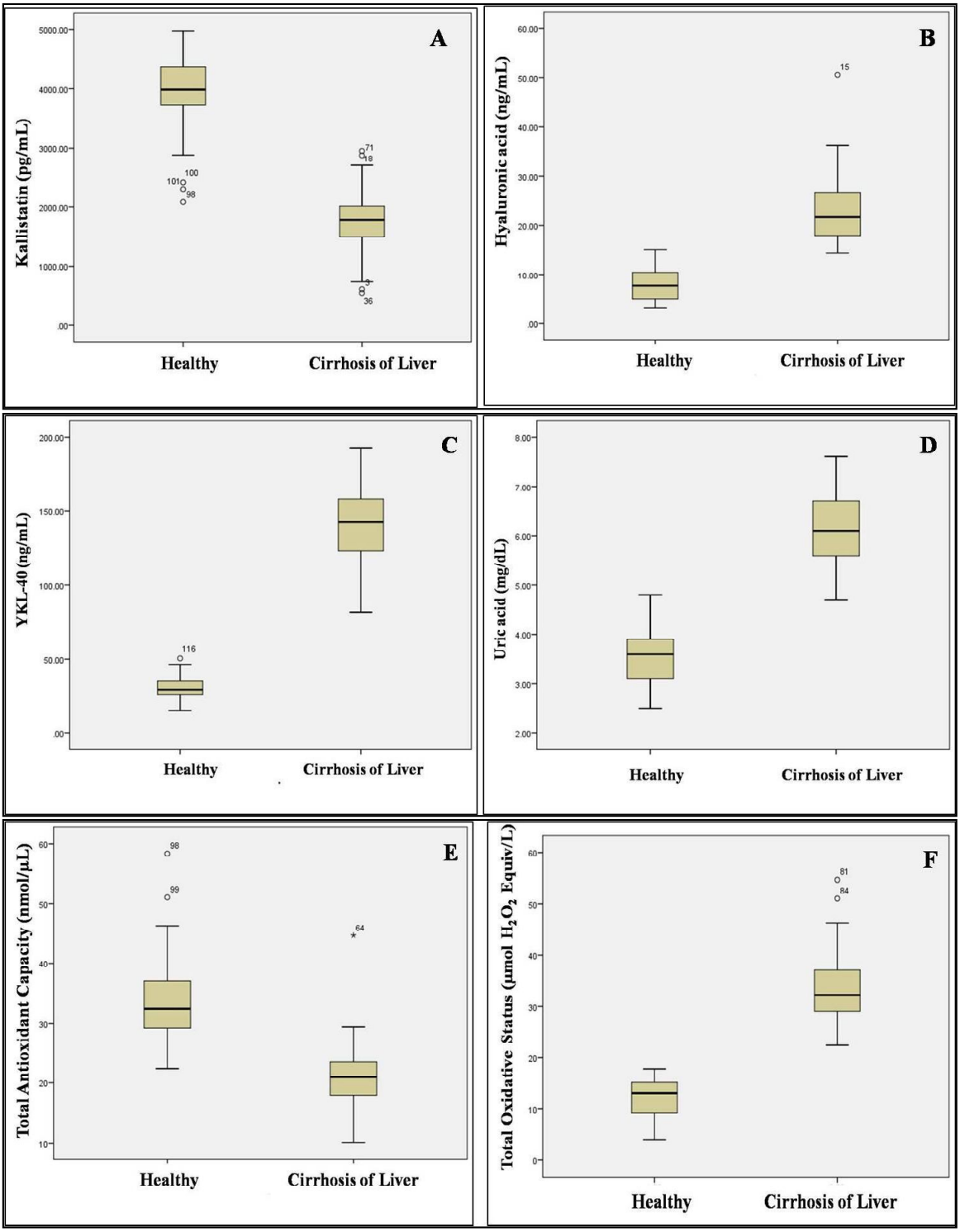


Figure 5.5.2: Box and Whisker plot; comparison of serum SERPINA4/Kallistatin (**A**), hyaluronic acid (**B**), YKL-40 (**C**), uric acid (**D**), total antioxidant capacity (**E**) and total oxidative status (**F**) concentrations in healthy subjects and cirrhotic liver subjects

Table 5.5.2: Correlation of SERPINA4/Kallistatin with direct markers of ECM for Cirrhosis of Liver *viz.*, Hyaluronic acid and YKL-40

Correlations			
		Hyaluronic acid	**YKL-40**
Kallistatin (pg/mL)	Pearson Correlation	-0.75**	-0.893**
	Sig. (2-tailed)	0.01	0. 01
	n	192	192
** Correlation is significant at the 0.01 level (2-tailed).			
* Correlation is significant at the 0.05 level (2-tailed).			

Table 5.5.3: Correlation of kallistatin with serum uric acid and conventional markers of cirrhosis of liver

Correlations										
		AST	**ALT**	**ALP**	**γGT**	**Total Protein**	**Albumin**	**Total Bilirubin**	**Uric acid**	**PT INR**
Kallistatin (pg/mL)	Pearson Correlation	-0.84**	-0.87**	-0.87**	-0.81**	0.84**	0.82**	-0.84**	-0.85**	-0.82**
	Sig. (2-tailed)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
	N	192	192	192	192	192	192	192	192	192
** Correlation is significant at the 0.01 level (2-tailed).										
* Correlation is significant at the 0.05 level (2-tailed).										

Table 5.5.4: Correlation of SERPINA4/Kallistatin with total antioxidant capacity and total oxidant status of cirrhosis of liver

Correlations			
		TAC	**TOS**
Kallistatin (pg/mL)	Pearson Correlation	0.67**	-0.81**
	Sig. (2-tailed)	0.01	0. 01
	n	192	192
** Correlation is significant at the 0.01 level (2-tailed).			
* Correlation is significant at the 0.05 level (2-tailed).			

Table 5.5.5: Correlation of hyaluronic acid with uric acid and conventional markers of cirrhosis of liver

Correlations										
		AST	**ALT**	**ALP**	**γGT**	**Total Protein**	**Albumin**	**Total Bilirubin**	**Uric acid**	**PT INR**
Hyaluronic acid (ng/mL)	Pearson Correlation	0.75**	0.78**	0.77**	0.78**	-0.79**	-0.78**	0.81**	0.79**	0.75**
	Sig. (2-tailed)	0.01	0.01	0. 01	0. 01	0. 01	0. 01	0. 01	0.01	0. 01
	n	192	192	192	192	192	192	192	192	192
** Correlation is significant at the 0.01 level (2-tailed).										
* Correlation is significant at the 0.05 level (2-tailed).										

Table 5.5.6: Correlation of hyaluronic acid with total antioxidant capacity and total oxidant status of cirrhosis of liver

Correlations			
		TAC	**TOS**
Hyaluronic acid (ng/mL)	Pearson Correlation	-0.67**	0.77**
	Sig. (2-tailed)	0.01	0.01
	n	192	192
** Correlation is significant at the 0.01 level (2-tailed).			
* Correlation is significant at the 0.05 level (2-tailed).			

Table 5.5.7: Correlation of YKL-40 with serum uric acid and conventional markers of cirrhosis of liver

Correlations										
		AST	**ALT**	**ALP**	**γGT**	**Total Protein**	**Albumin**	**Total Bilirubin**	**Uric acid**	**PTINR**
YKL-40 (ng/mL)	Pearson Correlation	0.87**	0.91**	0.91**	0.87**	-0.88**	-0.86**	0.93**	0.92**	0.86**
	Sig. (2-tailed)	0.01	0.01	0.01	0.01	0.01	0.01	0. 01	0. 01	0. 01
	n	192	192	192	192	192	192	192	192	192
** Correlation is significant at the 0.01 level (2-tailed).										
* Correlation is significant at the 0.05 level (2-tailed).										

Table 5.5.8: Correlation of YKL-40 with total antioxidant capacity and total oxidative status of cirrhosis of liver

Correlations			
		TAC	**TOS**
YKL40 (ng/mL)	Pearson Correlation	-0.73**	0.86**
	Sig. (2-tailed)	0.01	0.01
	n	192	192
** Correlation is significant at the 0.01 level (2-tailed).			
* Correlation is significant at the 0.05 level (2-tailed).			

Table 5.5.9: Post Hoc and Bonferroni test for ANOVA Comparison; SERPINA4/Kallistatin, Hyaluronic acid and YKL-40 between cirrhotic liver groups with varied etiology

Variables	**Groups**	**N**	**Mean±SD**	**Sig**
Kallistatin (pg/mL)	1	60	**1592.35±451.56**	0.01*
	2	20	2099.30±371.84	
	3	10	1993.80±370.79	
	4	6	2052.50±187.25	
	Total	96	1768.54±471.41	
Hyaluronic acid (ng/mL)	1	60	**24.41±6.46**	0.05*
	2	20	21.26±5.01	
	3	10	18.00±2.84	
	4	6	20.49±3.94	
	Total	96	22.84±6.11	
YKL40 (ng/mL)	1	60	**149.80±20.53**	0.01*
	2	20	129.70±19.59	
	3	10	115.40±22.19	
	4	6	128.79±25.21	
	Total	96	140.71±23.93	
* The mean difference is significant at the 0.05 level				

Abbreviations: **n**: Sample number; **Group 1**: Alcoholic Liver Disease; **Group 2**: Hepatitis C; **Group 3**: Hepatitis B; **Group 4**: Non Alcoholic Fatty Liver Disease

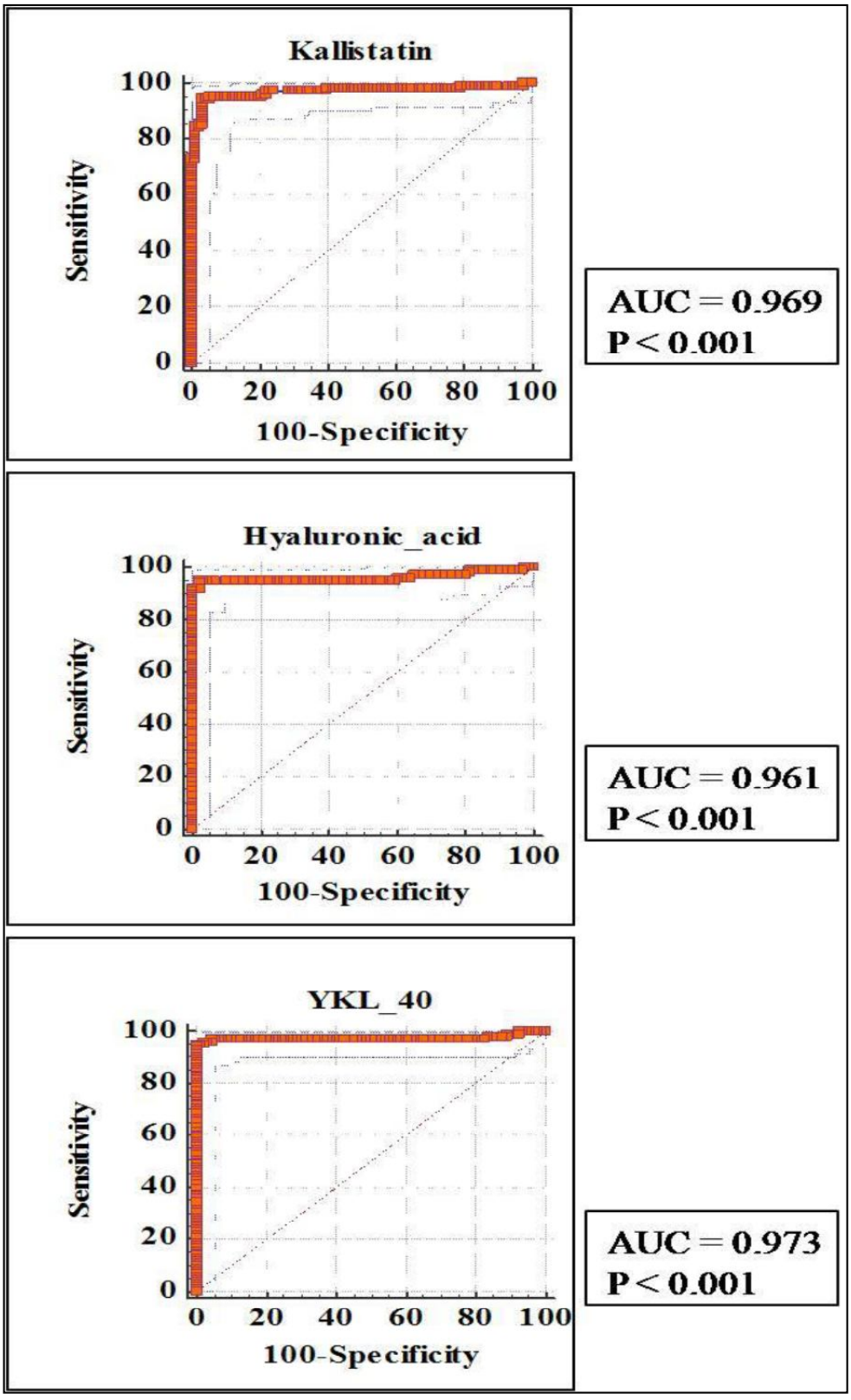


Figure 5.5.3: AUROC analysis; AUROC for SERPINA4/Kallistatin, Hyaluronic acid and YKL-40

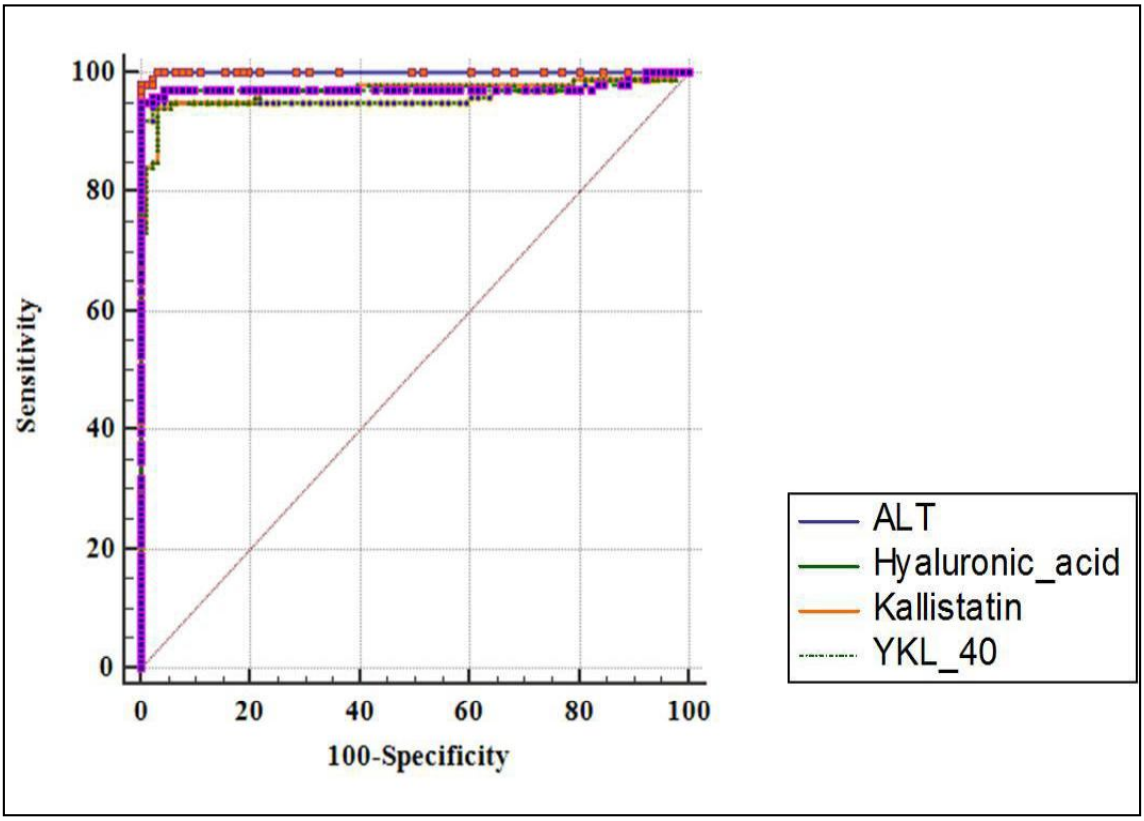


Figure 5.5.4: AUROC analysis; comparison of AUROC curve for SERPINA4/Kallistatin (0.969), Hyaluronic acid (0.961) and YKL-40 (0.973) with ALT (0.999)

DISCUSSION

Cirrhosis of liver, a global health hazard needs to be addressed early to prevent casuality particularly in middle age group and in developing countries. Early detection of the progression of cirrhosis of liver is a crucial step for preventing further complications of CLD. Even though invasive liver biopsy is a "gold standard" diagnostic tool for liver fibrosis/cirrhosis with varied etiology which distinguishes between intermediate stages. However the risk of clinical complications, poor acceptance and sampling errors are some of its remarkable limitations. Recently, several new noninvasive biomarkers have been evaluated as potential alternatives to liver biopsy. Identification of hepatocellular damage at early stage remains unclear probably, due to poor diagnostic accuracy of circulating biomarkers and algorithms in early and mild stages of cirrhosis of liver compared to advanced cirrhosis. The slow and asymptomatic progression of the disease is a major drawback to adapt noninvasive biomarker specific for liver (25).

Reliable noninvasive biomarker with sensitivity and specificity is needed for diagnosis/prognosis and effective management of the disease (25). In the present study, we used 2DE after albumin depletion followed In-Gel trypsin digestion and LC-MS characterization and In-Solution trypsin digestion followed by LC-MS characterization against human data base for identification of biomarker candidates for cirrhosis of liver. For maximal detection of meaningful protein expression difference; cases and controls should differ absolutely in terms of disease of interest. Simplified, unbiased binary comparison between diseased and healthy avoids contamination by other diseases and confounding factors which may alter expression of proteins which results in false discovery of biomarker candidates (107).

Discovery of biomarker candidates by proteomic approach is difficult particularly in the pH range of blood between 3-7 as abundant albumin probably interfere in identification and characterization of low abundant proteins by mass spectral and electrophoretic analysis. In our study, we achieved accurate protein biomarker candidate discovery after depletion of albumin by using dye based affinity column. Antibody based immunoprecipitation is more robust for depletion of abundant proteins from plasma/serum and is suitable for identification of novel biomarker candidates (114, 115, 116). Depletion dilemma can be rectified by using narrow pH (3-5.6) range; avoids interference of abundant proteins (albumin, transferrin and immunoglobulins) but chance to miss proteins whose pI is in alkaline range which was documented by Bevin *et al* (134). However in our study, we observed 42 protein biomarker candidates mislead the albumin band in electrophoresis confirmed by SDS-PAGE with silver staining. May be this confuse in the early identification of biomarker candidates by proteomic approach and delay in the management of cirrhosis of liver.

Technological advancement in biomarker candidate discovery resulted in identification of protein biomarker candidates for CLD with varied etiology (**Table 6.1**). However, we did 2DE and LC-MS and arrived at a conclusion in identifying the advanced protein biomarker SERPINA4/Kallistatin. Biomarker candidates identified require verification which demonstrates that the differential expression should remain detectable by assay to be used for validation (107). This was proved till date in Caucasians and now proved to be true even with Indian population confirmed by our study. Despite, numerous biomarker candidates identified, verification may be done only for few qualified candidates in terms of marker performance and reagent availability (104).

Table 6.1: Protein biomarker candidates identified by chronic liver diseases with varied etiology in different studies by proteomic approach

Authors	Etiology of liver disease	Type of sample	Proteomic techniques	Protein biomarker candidates identified
White *et al* (2007)(176)	HCV	Serum	2DE, LC-MS	α2 macroglobulin Haptoglobin Complement C4 Serum retinol binding protein Apolipoprotein A1 Apolipoprotein A-IV
Bevin G *et al* (2007) (177)	HCV	Serum	2DE, LC-MS	α2 macroglobulin Inter- α-trypsin inhibitor heavy chain H4 α1 antichymotrypsin Apolipoprotein L1 Paraoxonase/aryleserase 1 Zinc- α2-glycoprotein CD5 antigen like protein β2 glycoprotein I
Bevin G *et al* (2011) (20)	HCV	Serum	2DE, LC-MS In-solution isoelectric focusing	Beta chains of C3 and C4
Bevin G *et al* (2012) (134)	HCV	Serum	2DE, LC-MS	Adiponectin, Sex hormone binding protein 14-3-3 protein zeta/delta, Compliement C3dg Immunoglobulin J chain Apolipoprotein CIII Corticosteroid binding globulin, α2 HS glycoprotein Lipid transfer inhibitor protein, Haptoglobin related protein
Katrinli *et al* (2017) (178)	HBV	Liver tissue	2DE, LC-MS	Apolipoprotein A1 Pyruvate kinase Glyceraldehyde 3-phosphate dehydrogenase Glutamate dehydrogenase Alcohol dehydrogenase Transferrin, Peroxiredoxin 3 Keratin 5, Annexin

Abbreviations: CLD: Chronic Liver Disease; HCV: Hepatitis C Virus; HBV: Hepatitis B Virus; 2DE: 2 dimensional electrophoresis; LC-MS: Liquid chromatography- Mass spectrometry

In our study with 2DE and LC-MS we also observed similar findings. Biomarker candidates that show significant expressional differences between diseased and healthy in discovery phase are prioritized. Proteins that are secreted and/or present on cell surface and which act in cellular pathways and deregulated in liver diseases should be considered for further validation as observed by Paulovich *et al*, our study also tried to approach this (113).

In 2007, White *et al.,* in their proteomic approach for biomarker candidate discovery for mild liver fibrosis by HCV infection; identified seven individual proteins in the blood pH range of 3-10 with either increased expression (α2-macroglobulin and haptoglobin) or decreased expression (complement C-4, serum retinol binding protein, apolipoprotein A-1 and two isoforms of apolipoprotein A-IV) with advanced fibrosis and protein expression profile was performed in a blinded fashion using 2DE after abundant albumin depletion followed by LC-MS/MS protein characterization (176). In our study, the novel biomarkers SERPINA4/Kallistatin, hyaluronic acid and YKL-40 are observed to be altered *viz.,* decreased SERPINA4/Kallistatin and inversely correlated with hyaluronic acid and YKL-40.

Bevin *et al* in their study identified inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) fragments, α1-antichymotrypsin, apolipoprotein L1 (Apo L1), prealbumin, albumin, paraoxonase/arylesterase 1, and zinc-α 2-glycoprotein with decreased expression; CD5 antigen-like protein (CD5L) and β2 glycoprotein I (β2GPI) with increased expression in cirrhotic liver subjects by HCV infection compared to healthy subjects; protein expression profile was performed using 2DE without abundant albumin depletion followed by LC-MS/MS protein characterization in the blood pH range of 3-10 (177).

Our study, has observed the much advanced biomarkers SERPINA4/Kallistatin decreased, and increased hyaluronic acid and YKL-40 and also decreased total antioxidant capacity with increased total oxidative status.

To overcome depletion dilemma, in 2011, Bevin *et al* used 2DE over a narrow blood pH 3-5.6 range since this lies outside the range of highly abundant albumin, transferrin and immunoglobulins. In addition to this, Bevin *et al* used In-Solution isoelectric focusing followed by SDS-PAGE to find biomarkers in HCV induced liver cirrhosis. Using the blood pH 3-5.6 range for 2DE, they achieved improved representation of low abundance features and enhanced separation. They found In-Solution isoelectric focusing to be beneficial for analyzing basic, high molecular weight proteins. Using this method, the β chains of both complement C3 and C4 were found to decrease in serum from HCV patients with cirrhosis, a change not observed by 2-DE. By this two proteomics approaches they discovered 23 novel biomarker candidates for hepatic fibrosis by HCV infection (20). In our study with 2DE and LC-MS and In-Solution isoelectric focusing, we observed 42 proteins confuse albumin band in electrophoresis in cirrhosis of liver.

Bevin *et al* in 2012 identified 20 novel biomarker candidates for liver fibrosis with HCV infection. Proteins in plasma samples from healthy individuals and patients with HCV induced cirrhosis were analyzed using 2-DE. Identified markers were validated across all Ishak fibrosis stages and compared to the markers used in FibroTest, Enhanced Liver Fibrosis (ELF) test, Hepascore and FIBROSpect by Western blotting. Western blot validation of all candidate markers using plasma samples from patients across all Ishak fibrosis scores showed that the markers which changed with increasing fibrosis most consistently included lipid transfer inhibitor protein, complement C3d, corticosteroid-

binding globulin, apolipoprotein J and apolipoprotein L1. These five novel fibrosis markers which are secreted into blood sream showed a promising consistent change with increasing fibrosis stage when compared to the markers used for the FibroTest, ELF test, Hepascore and FIBROSpect (134). However, in our study further advanced marker for cirrhosis of liver viz., SERPINA/Kallistatin quantitatively validated using ELISA by comparing with conventional and direct biomarkers of cirrhosis of liver and we construed this marker is a better biomarker in early management of cirrhosis of liver.

Katrinli *et al* in 2017 enrolled 47 HBV infected patients with different fibrotic stages (F1 to F6) and used tissue samples for two dimensional difference gel electrophoresis (2D-DIGE) proteomic screening. Differentially expressed proteins were identified by mass spectrometry and verified by western blotting. Functional proteomic associations were analyzed by Enrich Net application. Fibrotic stage variations were observed for apolipoprotein A1 (APOA1), pyruvate kinase PKM (KPYM), glyceraldehyde 3-phospahate dehydrogenase (GAPDH), glutamate dehydrogenase (DHE3), aldehyde dehydrogenase (ALDH2), alcohol dehydrogenase (ALDH1A1), transferrin (TRFE), peroxiredoxin 3 (PRDX3), phenazine biosynthesis-like domain-containing protein (PBLD), immuglobulin kappa chain C region (IGKC), annexin A4 (ANXA4), keratin 5 (KRT5) (178). However with 2DE followed by LC-MS for cirrhosis of liver, we observed 13 biomarker candidates with increased expression in cirrhosis of liver and 15 protein biomarker candidates with decreased expression in cirrhosis of liver and majority of our samples included males and had history of chronic alcoholism.

Further, Katrinli *et al* (178), in their study with enrichment analysis with Reactome and Kegg databases highlighted the possible involvement of platelet release, glycolysis and

HDL mediated lipid transport pathways. Moreover, string analysis revealed that HIF-1α (Hypoxia-inducible factor 1-alpha), one of the interacting partners of HBx (Hepatitis Bx protein) may play a role in the altered glycolytic response and oxidative stress observed in liver fibrosis. Observed changes in the glycolytic pathway caused by HBx presence and therefore its interactions with HIF-1α can be a target pathway for novel therapeutic purposes (178).

However in our study, we observed blood glucose levels, blood urea, serum creatinine were not altered between healthy controls and cirrhotic liver subjects. This proved that the blood glucose levels to get altered the percentage of liver damage needs to be advanced. Since, we have collected the blood samples in early stage of cirrhosis of liver and majority of the cases were alcoholics with male preponderance; genetics might have taken care to resist the diabetic status. Basic renal parameter, uric acid was almost doubled in clinically and diagnostically proven cirrhotic liver subjects compared to healthy controls probably due to altered nucleotide metabolism.

In the present study, keratin isoforms with a molecular weight ranging from 47.2 kDa to 65.4 kDa and calculated pI 4.88 to 8.0 showed up-regulation in cirrhosis of liver. Keratin is a fibrous structural protein which protects epithelial cells from damage and stress; regulate key cellular activities *viz.*, cell growth and protein synthesis (179). However in our study we observed increased keratin isoforms expression in cirrhosis of liver which was identified by 2DE followed by LC-MS.

Lumican, leucine-rich repeat proteoglycan constitute an important fraction of non-collagenous ECM proteins. It plays a major role in tissue homeostasis and modulates

cellular functions *viz.*, cell proliferation, migration, and differentiation (180). In our study the lumican precursor with a molecular weight of 38.4 kDa and calculated pI 6.61 is increased in cirrhosis of liver.

Polymeric Ig receptor (pIgR) isoform X1 is type I transmembrane protein expressed from glandular epithelial cells of liver and breast. It mediates transcellular transport of polymeric immunoglobulins. The key regulators of pIgR expression are pro-inflammatory cytokines, *viz.,* interferon-γ (INF-γ), tumor necrosis factor (TNF) and interleukin-1 (IL-1); up-regulate in cirrhosis of liver (25, 181, 182). In our present study, pIgR X1 with molecular weight 83.2 kDa and calculated pI 5.74 is increased in cirrhosis of liver which was identified by 2DE followed by LC-MS.

Vitamin D-binding protein, multi-functional protein belongs to the albumin gene family, can bind various forms of vitamin D (ergocalciferol, cholecalciferol and calcifediol) for the transport. It is synthesized by hepatic parenchymal cells which were documented by Norman (183). In our study, vitamin D binding protein isoform 3, molecular weight 55 kDa and calculated pI 5.63 was increased in cirrhosis of liver.

Haptoglobin which is included in existing noninvasive marker panel has showed increased expression in the present study as it is an acute phase protein. Studies conducted by White *et a*l, Vang *et al* and Trayhurn *et al* has documented haptoglobin is synthesized majorly from liver and hepatic expression is stimulated by up-regulation of IL-6 in HCV (176, 184, 185). However in present study, majority of the cases were ALD, haptlobin isoform 1 preprotein, molecular weight 45.2 kDa and calculated pI 6.58 was

increased. These findings are correlated well with the findings documented by White *et a*l, Vang *et al* and Trayhurn *et al* (176, 184, 185).

Studies conducted by Heit *et al* has observed transmembrane protein 201 is involved in nuclear movement during fibroblast polarization and migration; actin-dependent nuclear movement through association with transmembrane actin-associated nuclear (TAN) lines (186). Our study observed transmembrane protein 201 isoform 1 with molecular weight 72.2 kDa and calculated pI 9.22 has increased expression in cirrhosis of liver.

Studies conducted by Janciauskiene *et al*, Tyagi *et al* and Yoon *et al* has observed α-1-antitrypsin (SERPINA1) and α-2-antichymotrypsin (SERPINA3), serine protease inhibitors produced primarily in liver hepatocytes and are released directly into blood stream and showed down regulation in cirrhosis of liver compared to healthy (187, 188, 189). Our findings are compared positively with their findings.

Studies conducted by Smith A *et al* documented hemopexin is a single polypeptide chain of 439 amino acids residues with molecular weight of 63 kDa which is expressed from liver and it acts as heme scavenging protein (190). In our study, we observed hemopexin precursor protein with molecular weight 51.6 kDa and calculated pI 7.02 has decreased expression in cirrhosis of liver.

Apolipoprotein A-IV, even though not evident from liver, its expression was decreased in ALD. Down regulation of Apolipoprotein A-IV was reported in hepatic fibrosis in rat models (176, 191). In present study, Apolipoprotein A-IV precursor molecular weight 45.3 kDa and calculated pI 5.38 has decreased expression in cirrhosis of liver.

CD5 antigen like isoform X1, key regulator of lipid synthesis was down regulated in cirrhosis of liver, whereas up regulation was noted in liver cirrhotic patients in HCV infection (177). In present study, CD5 antigen like isoform X1 molecular weight 38.7 kDa and calculated pI 5.66 has decreased expression in cirrhosis of liver and majority of our cases were ALD.

Zinc-α2-glycoprotein, adipokine plays an important role in fat catabolism and reduces insulin resistance was down regulated in cirrhosis of liver (177, 192). However zinc-α2-glycoprotein precursor molecular weight 34.2 kDa and calculated pI 6.05 was decreased in cirrhosis of liver despite unaltered blood glucose levels measured randomly.

Studies conducted by Sindhu *et al* have proposed the key enzyme in the regulation of glycerol uptake and metabolism, glycerol kinase, a phosphotransferase involves in triglycerides and glycerophospholipids synthesis in individuals with type2 diabetes and correlates negatively with the plasma fetuin-A/α2-HS/glycoprotein with inflammatory cytokines, chemokines and activation biomarkers. Glycerol kinase converts glycerol, waste product of lipolysis to glucose in the liver. In our study, the glycerol kinase isoform X1, molecular weight 63.6 kDa and calculated pI 6.54 was down regulation in liver cirrhosis (193).

Sindhu *et al* documented α2-HS-glycoprotein, secretory protein expressed from liver and key regulator in inhibition of vascular calcification, bone metabolism regulation, control of protease activity, insulin resistance and breast tumor cell proliferative signaling (193). In present study α-1-acid glycoprotein preprotein, molecular weight 39.3 kDa and calculated pI 5.72 was down regulated in cirrhosis of liver indicating liver metabolism

directly or indirectly inhibits vascular calcification, bone metabolism regulation, control of protease activity, insulin resistance and breast tumor cell proliferative signaling.

Colomba *et al* has observed the orosomucoid (α1-acid glycoprtoien) plasma concentration and genetic variants effects on human immunodeficiency virus protease inhibitor clearance and cellular accumulation (194). Our study noted α1-acid glycoprtoien precursor molecular weight 23.5 kDa, caluletd pI 5.11 has decreased expression in cirrhosis of liver compared to healthy controls.

Carpenter *et al* has documented α2-anti plasmin and its deficiency, fibrinolysis out of balance in liver diseases. Our observation with respect to α2-anti plasmin isoform XI (SERPINA2), molecular weight 56.6 kDa calculated pI 6.89 has decreased expression in cirrhosis of liver (195).

Pereyra *et al* has documeneted early predition of post operative liver dysfunction and clinical outcome by using activity of antithrombin III. Antithrombin III, member of the serpin family is inhibitor of proteinases *viz.*, thrombin and factor Xa; primarily synthesized by hepatocytes. (196). In present study we observed Antithrombin III precursor, molecular weight 52.6 kDa and calculated pI 6.71 was decreased in expression in cirrhosis of liver.

In addition to these documented observations, the novelty of our study, we have observed increased expression of lumican precursor (molecular weight 38.4 kDa and calculated pI 6.61), polymeric Ig receptor isoform X1 (molecular weight 83.2 kDa and calculated pI 5.74), serotrnaferrin precursor (molecular weight 77 kDa and calculated pI 7.12) and Ig lamda like polypeptide 5 isoform 1 (molecular weight 23 kDa and calculated pI 8.84) in

liver cirrhosis. We also documented decreased expression of kininogen-1 isoform 1 precursor (molecular weight 71.9 kDa and calculated pI 6.81) and sex hormone binding globulin isoform 1 precursor (molecular weight 43.8 kDa and calculated pI 6.71) in liver cirrhosis. Clearly showing newer biomarkers candidates unnoticed, masked and non documented were documented in our study and may prove beneficial in early management of cirrhosis of liver.

Prioritization of protein biomarker candidate is a prerequisite for validation in terms of performance characteristics, analytical validation, accuracy, precision and clinical utility (100). Proteins that are expressed from liver and/or present on cell surface and which act in cellular pathways and deregulated in cirrhosis of liver should be considered for further analytical and clinical validation (113). Based on physiological role and reagent availability, **SERPINA4/Kallistatin**, a multi-functional protein expressed from liver cells (Hep G2 and Hep 3B) might give potential insights for diagnosis of cirrhosis of liver (138). In present study we followed prioritization of protein biomarker candidate as a prerequisite for validation, performance characteristics, accuracy, precision and clinical utility for SERPINA4/Kallistatin and with development of In-House ELISA quantitative kit and correlated the observed values with conventional and direct markers and with total antioxidant capacity and total oxidative status.

Study of human biology and disease in respect to proteomic studies is a major practical challenge due to lack of well validated antibodies to many of the human proteins (197). Protein affinity reagents are fundamental tools for both basic and wide range of applications in biomedical research (198). In human protein atlas, 80% of the antibodies are polyclonal antibodies which imply binding to multiple epitopes which increase the

risk of cross specificity towards other proteins. Cross reactivity for antibodies is a major problem in diagnostic and therapeutic application; essential to measure cross reactivity of given antibody against full proteome by using western blot, immunohistochemistry, immunofluorescence or sandwich immunoassays (199). However, with the development of In-House SERPINA4/Kallistatin kit, these flaws are proved.

Proteins share stretches of their primary structure which is identical or differ only by few amino acid residues and some proteins with similar functions have domains with surface patches of high similarities. An antibody targeting an epitope in one of these regions shows cross reactivity to other proteins than the intended target, making the results from an assay with this antibody unreliable and hard to interpret. Availability of well characterized antibodies provides valuable resource for diagnostic studies of the corresponding protein (200). Mapping of linear epitopes of a polyclonal antibody followed by sequential epitope specific capture using synthetic peptides generates single epitope specific antibodies (199). Since monospecific (monoclonal alternative) antibodies are more specific with high sensitivity due to presence of single linear specific epitope for the immunological binding towards protein of interest, we used monospecific (monoclonal alternative) antibodies for the development of In-House SERPINA4/Kallistatin ELISA quantitative kit.

Serpins are broadly distributed family of protease inhibitors which circulates in blood and are mainly expressed from liver (201). Highly conserved similar structure (native, monomeric, active, latent, cleaved, delta and polymeric proteins) of serpins are crucial for their inhibitory function and play an important role in haemostasis and fibrinolysis (202, 203). These proteins are suicide or single use inhibitors that use conformational changes

to inhibit target enzymes (204). Inhibitor binds tightly to a protease by incorporating reactive centre loop (RCL) of inhibitor into β sheet of the enzyme by forming SDS and heat stable complex (203).

As shown in **Table 6.2**; SERPINA7 and SERPINA8 are derived from Xq22.3 and 1q42.2 respectively. Rest, SERPINA1, 2, 3, 4, 5, 6, 9, 10, 11and 12 are all derived from 14q32.1 but with different organ specificity.

However, present study we concentrated on SERPINA4 symbol P14, synonyms KST, KAL, KLST, Kallistatin with chromosome specific 14q32.1.

SERPINA1, (α1-antitrypsin) is an inhibitor of neutrophil elastase (202).

Pseudogene SERPINA2 indicates an ongoing process of pseudogenization (205).

Antichymotrypsin, SERPINA3 is an inhibitor of chymotrypsin and cathepsin G found in blood, liver, kidney and lungs (202).

SERPINA5 inhibits active C protein and are expressed from liver (206).

Non inhibitory hormone binding protein, SERPINA6 is a cortisol transporter (201).

SERPINA9 expressed from liver plays important role in maintaining native B cell (206).

The inhibitory protein of activated coagulation factors Z and XI is SERPINA10 (202).

SERPINA11 is a pseudogene and uncharacterized (206).

SERPINA12 is an inhibitory of kallikrein and plays a role in insulin sensitivity (208).

SERPINA4/Kallistatin (serpin family A member 4, tissue kallikrein inhibitor), belongs to clade A serpins encoded by the *SERPINA4* gene with 5 exons and 4 introns mapped to chromosome 14q31-32.1 in humans and expressed from liver.

Serpins (Serine Protease Inhibitors) are class of plasma proteins that have similar structure and diverse functions. Serpins are divided into clades based on sequence similarities.

In humans (clades A to I), 36 serpin coding genes and 5 pseudogenes are identified based on phylogenetic relationship (206).

Extracellular clade A molecules are localized on chromosomes 1, 14 and X.

Intracellular clade B serpins are localized on chromosome 6 and 18 (202).

Serpins are interrelated due to highly conserved core structure. Majority of clade A serpins are localized on chromosome 14 which are expressed from liver (203).

A highly conserved secondary and tertiary structure is the main criteria for the classification with modest amino acid similarities (206). Despite chromosomal proximity, these genes have divergent function (209). Serpin genes are present in clusters on same chromosome with common precursor. The human genes encoding α1-antitrypsin, corticosteroid-binding globulin, α1-antichymotrypsin and protein C inhibitor are mapped to the chromosome 14q32.1. SERPINA4/Kallistatin is also mapped within the region on the same chromosome (210, 211).

Despite similarity in chemical properties having minor amino acids sequence resemblance and mapped on same gene, our study did not show any cross reactivity

between serpin class proteins in cirrhotic liver and healthy subjects which may be attributed due to absence of identical epitope among serpins. Cross reactivity occurs when two different serpins share an identical epitope. Epitope comprises approximately 15 amino acids of which 5 amino acids influence strongly for binding to definite paratope of *Fab* region on variable domain of antibody (212). Due to the absence of identical epitope among serpins might be reason for no cross reactivity in cirrhotic liver and healthy subjects. There will be reduced expression of serpin proteins into blood stream due to decreased synthetic function of liver in cirrhotic liver subjects.

Molecular basis of polymerization is induced by mutations or mild denaturation which is common for all serpins. The conformational change in the serpin structure is crucial for functions and which also is susceptible reason for mutations (203). Mutations which bring about polymerization can also occur anywhere in the serpin and leads to formation and accumulation of stable polymers with similar properties (213, 214). Serpin polymerization can also occur through domain swapping as recorded in antithrombin, α-1 antitrypsin and neuroserpin, which needs further studies to evaluate domain swapping polymerization of entire serpin family proteins (215, 216, 217). Polymerization leads to reduction in serpin secretion with qualitative changes in protein structure (203). The etiological factors of cirrhosis of liver may not induce polymerization which directs to share identical epitope of serpin family proteins. This may be the reason why no cross reactivity was observed in cirrhotic liver subjects in our study.

Even though, incidence of diseases caused by serpin polymerization is rare, homozygous mutations in SERPINA1 gene (α1 antitrypsin) is associated with liver disease including cirrhosis. Human variants of serpin genes has been found in large number as a resultant

of mutations which are associated with many diseases **(Table 6.2)** (206, 218). SERPINA1 alone has 1411 SNPs; SNPs for SERPINA4 are 906 in NCBI's dbSNP database. Mutational studies in terms of cross reactivity, for identification of identical epitope, might be difficult at this point because of huge diversity of serpins.

Table 6.2: Classification of Serpin clade A, chromosomal location, polymerization associated diseases

S.No	Name	Symbols	Synonyms	Chromosome	Associated Diseases
1	SERPINA1	PI	α-1-antitrypsin, AAT	14q32.1	Emphyesma, Chronic Liver Disease, Vasculitis
2	SERPINA2	PIL	ATR, ARGS	14q32.1	----------
3	SERPINA3	AACT	ACT	14q32.1	Emphyesma
4	**SERPINA4**	**PI4**	**KST, KAL, KLST, Kallistatin**	**14q32.1**	**Renal and Cardiovascular Injury**
5	SERPINA5	PLANH3	PA13, PROCI	14q32.1	Angiodema, Papillary thyroid cancer
6	SERPINA6	CBG	-	14q32.1	Chronic fatigue syndrome
7	SERPINA7	TBG	-	Xq22.3	Deficiency results in hypothyroidism
8	SERPINA8	AGT	-	1q42.2	Certain varients linked to essential hypertension
9	SERPINA9	-	CENTERIN	14q32.1	-
10	SERPINA10	-	PZI	14q32.1	Risk of venous thromboembolism, Pregnancy Complications
11	SERPINA11	-	-	14q32.1	-
12	SERPINA12	-	Vaspin, OL-64	14q32.1	Associated with Insulin resistance

Concentration of SERPINA4/Kallistatin is less in cirrhotic liver as well as in healthy subjects and it depends on the degree of severity of different chronic liver diseases (fibrosis, cirrhosis and hepatocellular carcinoma) (22). Hence, the sensitivity of monoclonal antibodies (5ng/lane, by manufacturer's instructions) might not detect SERPINA4/Kallistatin. In case of any cross reactivity, these antibodies may detect other serpins whose concentrations are in nanograms/mL in serum. Use of more sensitive antibodies might detect SERPINA4/Kallistatin in cirrhotic liver as well as in healthy subjects and enhance successful immunological interactions of other serpins. For separation of proteins, 2-DE might be better option than single dimensional SDS-PAGE.

Although, monoclonal antibodies are more specific towards SERPINA4/Kallistatin, they failed to capture protein of interest in natural samples due to less sensitivity. Polyclonal antibodies are more sensitive towards SERPINA4/Kallistatin; even failed to capture protein of interest in natural samples. Well characterized antibodies are needed for quantitative protein studies especially in clinical diagnosis with more sensitivity and specificity especially if concentrations of protein of interest are in pg/mL in circulation (219). Linear epitope specific antibodies are needed for quantification of protein of interest. Monospecific (monoclonal antibody alternative) antibodies are based on epitope specific affinity purification from polyclonal antibodies (199). These antibodies have high specificity and sensitivity towards SERPINA4/Kallistatin and did not show any cross reactivity with other SERPINs even could be able to capture protein of interest in natural samples. Due to this, monospecific (monoclonal antibody alternative) antibodies were selected for development of quantitative ELISA which is having greater sensitivity and specificity.

Concentration of SERPINA4/Kallistatin was reduced significantly in cirrhosis of liver compared to healthy subject as liver is the most important site of SERPINA4/Kallistatin synthesis and expression. The reduction of SERPINA4/Kallistatin levels was high in alcoholic liver cirrhosis which showed that degree of necroinflammation will be varied in different etiologies and is high in alcohol induced cirrhosis of liver. There is a close correlation between the reduction in serum SERPINA4/Kallistatin levels and severity of hepato cellular injury. The magnitude of reduction of SERPINA4/Kallistatin levels appeared to be correlated with degree of cirrhosis of liver and disruption of normal liver function. There is an inverse relationship between oxidative stress and SERPINA4/Kallistatin levels in cirrhotic liver subjects. Chronic oxidative organ damage can markedly reduce circulating SERPINA4/Kallistatin levels. Serum SERPINA4/Kallistatin level predicted the presence of cirrhosis of liver with AUC of 0.969 in 96 subjects.

Thus, serum SERPINA4/Kallistatin level might be a good biomarker for different etiologies of cirrhosis of liver.

Afzali *et al* reported novel association of hyperuricemia and the incidence of cirrhosis of liver development with elevated activities of ALT and γGT in US population (220). Shih *et al* observed significant association between serum uric acid levels and NAFLD among US population; independent of multiple metabolic risk factors (221). Xie *et al* documented crucial role of hyperuricemia is an important independent risk factor for NAFLD in Chinese population (222). In Indian population, study conducted by Paul *et al*, hyperuricemia reflects oxidative stress and is associated with disease progression and can serve as surrogate marker for CLD with varied etiology (223).

Significant elevation of uric acid, an end product of purine metabolism by cellular destruction is a mediator of inflammation and tissue damage (224). Hepatic depletion of adenosine triphosphate (ATP) increases uric acid production leading to hepatocellular injury; reduction in protein synthesis, induces inflammation and pro-oxidative changes which probably elevates activity of liver enzymes (221). Hyperuricemia results in oxidative stress mediated metabolic syndrome which is associated with progression of chronic liver disease. In chronic liver injury, uric acid activates inflammosome (inflammatory cytokines and Nuclear Factor-κB [NF-κB]) and promotes surrounding liver parenchymal damage which in turn leads to hepatic dysfunction (225).

In present study, significant elevation of uric acid in cirrhotic liver subjects was observed when compared with healthy subjects. Despite, no alteration in blood glucose levels indirectly expressing ATP utilization in glycolysis, may be due to altered nucleotide metabolism or oxidative stress associated with disease progression.

Resino *et al* documented the increase in accuracy of serum hyaluronic acid with hepatic fibrotic stages in HIV-HCV coinfected patients (226). Peters *et al,* in their large cohort study, demonstrated that baseline hyaluronic acid is a strong predictor of liver related deaths in HIV-1 patients coinfected with HBV and/or HCV (227). Gudowska *et al* concluded that concentrations of serum hyaluronic acid are elevated in liver diseases and should be considered as a good noninvasive biomarker for diagnosis of liver damage (228). In our study, there was significant elevation of serum hyaluronic acid in cirrhotic liver subjects with varied etiology compared to healthy subjects. However we excluded HIV infected samples and analysed 20 HCV with cirrhosis of liver and our findings correlated well with the findings of Resino *et al,* Peters *et al* and Gudowska *et al.*

It has been documented that, hyaluronic acid is produced from activated HSCs after transdifferentiate into myofibroblasts due to inflammation mediated by complex cytokine network (229). Elevated levels of hyaluronic acid in cirrhotic liver subjects are due to liver damage by chronic liver insults; originating from fibrogenesis (228). Among cirrhotic liver subjects, high levels of hyaluronic acid in ALD suggest that the degree of necroinflammation varies in different etiologies and is high in alcohol induced hepatlcellular injury. Due to decrease in fibrolysis during the disease progression, dysfunction of sinusoidal endothelial cells leads to reduction in degradation of hyaluronic acid resulting in elevated levels in circulation (230).

In chronic hepatic injury, different types of cells (resident innate inflammatory cells, hepatocytes, liver sinusoidal endothelial cells and Kupffer cells) play a key role in liver fibrogenesis. Activation of HSCs is a crucial step in inter-linked process of tissue injury and regeneration. Chief mitogen of HSCs activation is PDGF produced by Kupffer cells. Autocrine and paracrine secretions of cytokines activate and trans-differentiate HSCs into myofibroblasts. This mechanism is responsible for ECM production and accumulation which includes different types of collagen, glycoproteins, proteoglycans and glycosaminoglycans in injured liver (25).

Damaged hepatocytes release ROS and fibrogenic mediators which stimulate inflammatory cells leading to cell damage (25). Decreased hepatic ATP and cell damage result in increased uric acid production leading to histological liver injury (231). Elevated uric acid inhibits endothelial nitric oxide synthase (eNOS) expression which results in reduction of nitric oxide (NO) release and bioavailability in endothelial cells (232). Hyperuricemia induced down regulation of eNOS results in oxidative stress and activates

inflammatory cytokines IL-6 and TNF-α by stimulating HMGB1/RAGE (High Mobility Group Box chromosomal protein1/Receptor for Advanced Glycation Endproducts) signaling pathway results in endothelial dysfunction (233). Accumulation of hyaluronic acid due to increased synthesis and decreased degradation by sinusoidal endothelial cells may lead to significant elevation of the molecule which acts as an indicator of severity of the disease. Our study findings also documented elevated total oxidants and reduced antioxidant capacity and elevated hyaluronic acid in cirrhosis of liver depicting hyaluronic acid having positive correlation with total oxidative stress.

Study conducted by Saitou *et al* demonstrated that serum YKL-40 measurements reflect liver fibrogenesis in HCV patients and may serve as serological marker for evaluating the efficacy of therapies for CLD (234). Kumugai *et al* showed that YKL-40 secretion by macrophages was upregulated by TNF-α and IL-1β, proinflammatory cytokines which are involved in the pathogenesis of NAFLD (235). In large cohort study, Kjaergaard *et al* documented increased expression of YKL-40 in chronic alcoholic liver subjects compared to healthy subjects (236). In present study, we observed serum YKL-40 significantly elevated in cirrhotic liver compared to healthy subjects. Proving YKL-40 might be useful noninvasive tool to diagnose CLD.

YKL-40 is expressed by different cells *viz.*, macrophages, chondrocytes, synovial cells, vascular smooth muscle cells and HSCs (237). Non-enzymatic heparin binding glycoprotein, YKL-40 involves in cell proliferation, inflammation and remodeling of ECM (235). In liver, it modulates angiogenesis by acting as chemo-attractant for endothelial cells. YKL-40 is a growth factor for fibroblasts (238). During disease progression, macrophages will increase which in turn secrete YKL-40 regardless of

etiology of the disease. Enhanced expression of YKL-40 by activated macrophages is mediated by proinflammatory cytokines TNF-α and IL-1β (235). Inflammatory YKL-40 plays a key role in fibrogenesis of liver; elevated levels of YKL-40 indicate disease severity (235, 239). Our findings with respect to YKL-40; elevated in cirrhosis of liver and positively correlated with hyaluronic acid and total oxidative stress; negatively corrleted with total antioxidant capacity and SERPINA4/Kallistatin.

NEW KNOWLEDGE GENERATED

Discovery of protein biomarker candidates

- Two dimensional electrophoresis after depletion of albumin and comparative gel image analysis followed by In-Gel trypsin digestion and LC-MS protein characterization revealed 28 protein biomarker candidates for cirrhosis of liver based on clinical significance and protein scores

- Comparative differential protein expressional analysis by In-Solution trypsin digestion followed by characterization of proteins using LC-MS revealed 14 protein biomarker candidates for cirrhosis of liver based on clinical significance and protein scores

Analytical validation

- SERPINs, a group of protease inhibitors, having structural similarities and expressed from same chromosome 14q32.1; do not have any cross reactivity with SERPINA4/Kallistatin for monoclonal, polyclonal as well as monospecific (monoclonal alternative) antibodies

- Monospecific (monoclonal alternative) antibodies are well characterized antibodies with single linear specific epitope for quantitative protein studies especially in clinical diagnosis which are capable to capture protein of interest whose concentrations are in pg/mL; high sensitivity and high specificity

- Development of quantitative ELISA for quantification of SERPINA4/Kallistatin with accuracy and precision is having a linearity range of 65 pg/mL to 24,000 pg/mL with 95% recovery of protein of interest in natural samples

Clinical validation

- Concentrations of serum SERPINA4/Kallistatin was reduced in cirrhosis of liver compared to healthy subjects; expressed from liver cell (Hep G2 and Hep 3B) which can give a potential insight for diagnosis of cirrhosis of liver

- Concentrations of serum hyaluronic acid, a major proteoglycan of ECM was increased in cirrhosis of liver compared to healthy subjects; hyperuricemia induced endothelial cell dysfunction causes elevation of hyaluronic acid; endothelial cells and are major site for hyaluronic acid degradation

- Concentrations of serum YKL-40, non-enzymatic glycoprotein of ECM was increased in cirrhosis of liver compared to healthy subjects; during disease progression, increased activated macrophages in turn enhance expression of YKL-40 regardless of etiology of the disease which is mediated by proinflammatory cytokines TNF-α and IL-1β

- Among cirrhotic liver subjects, low concentrations of SERPINA4/Kallistatin with high concentrations of hyaluronic acid and YKL-40 in ALD suggest the degree of necroinflammation variation in different etiologies; high in alcohol induced hepato cellular injury when compared to HBV, HCV and NAFLD

CONCLUSION

Technological advancement in –omics approach particularly discovery phase in biomarker pipeline generates numerous biomarker candidates. Prioritization of identified biomarker candidates is a prerequisite for validation in terms of performance characteristics, analytical validation, accuracy, precision and clinical utility. With the help of technological advancement in proteomic approach, we identified 42 protein biomarker candidates for cirrhosis of liver; 28 by In-Gel trypsin digestion after albumin depletion followed by 2DE and LC-MS characterization of proteins and 14 protein biomarker candidates by comparative protein expression analysis using In-Solution trypsin digestion followed by LC-MS characterization of proteins. Despite, numerous biomarker candidates identified, verification may be done only for few qualified candidates which act in cellular pathways and deregulated in cirrhosis of liver.

In this regard, SERPINA4/Kallistatin, a multifunctional protein expressed from liver cells (Hep G2 and Hep 3B) was considered for further analytical and clinical validation. In the development of quantitative diagnostic ELISA, SERPINs do not have any cross reactivity with SERPINA4/Kallistatin due to absence of identical epitope for monoclonal, polyclonal and monospecific (monoclonal alternative) antibodies. Monoclonal and polyclonal antibodies failed to capture protein of interest in natural samples. Monospecific (monoclonal alternative) antibodies with single linear specific epitope are well characterized antibodies and selected for diagnostic quantitative ELISA development; high sensitivity and high specificity. Quantitative diagnostic ELISA (research use) for quantification of SERPINA4/Kallistatin with accuracy and precision with a linearity range of 65 pg/mL to 24,000 pg/mL was developed having 95% recovery of protein of interest in natural samples.

There was significant reduction in SERPINA/Kallistatin levels along with increased levels of hyaluronic acid, YKL-40 and uric acid in cirrhotic liver subjects compared to healthy subjects. Reduction in SERPINA/Kallistatin levels along with elevated activity of liver enzymes and prolonged PT INR lead to poor prognosis of the disease. Thus, serum SERPINA/Kallistatin levels can give potential insights for diagnosis of cirrhosis of liver with varied etiology. Elevated hyaluronic acid levels suggest that there will be increased synthesis and decreased degradation by hyperuricemia induced endothelial cells dysfunction leading to accumulation in ECM. Elevated levels of activated macrophages derived YKL-40 is an indicator of inflammation. Components of ECM turn over *viz.*, hyaluronic acid and YKL-40 expression increased during progression of disease and can serve as direct biomarkers of cirrhosis of liver.

STRENGTHS OF OUR STUDY

- Use of advanced proteomic techniques for discovery of biomarker candidates (depletion of albumin, 2DE, In-Gel trypsin and In-Solution trypsin digestion followed by LC-MS characterization against human data base)
- Identification of 42 specific proteins as biomarker candidates misguiding albumin band in electrophoresis
- Identified 08 new protein biomarker candidates in addition to documented and proved as on date
- Similar protein differential pattern observed with documented studies *viz.*, haptoglobin, Zinc-α2 glycoprotein, CD5 antigen like precursor either with ALD and/or HCV infected haptocellular damage
- Cross reactivity analysis to rule out interference of SERPINs for quantification of SERPINA4
- Use of monospecific (monoclonal alternative) antibodies for development of In-House ELISA quantitative kit
- Development of In-House ELISA quantitative kit (research use) with accuracy and precision with a linearity range of 65 pg/mL to 24,000 pg/mL with 95% recovery of protein of interest from natural samples
- Patent applied for developed ELISA quantitative kit for SERPINA4/Kallistatin

REFERENCES

1. Hernaez R, Sola E, Moreau R, Gines P. Acute on chronic liver failure: an update. Gut. 2017: 66: 541-553.
2. Peng Y, Qi X, Guo X. Child plug verses MELD score for the assessment of prognosis in liver cirrhosis. A systematic review and Meta-analysis of observational studies. Medicine. 2016; 95(8): 1-29.
3. GBD 2013 Mortality and Causes of Death Collaborators. Global, regional, and national age-sex specific all-cause and cause-specific mortality for 240 causes of death, 1990-2013: A systematic analysis for the Global Burden of Disease Study 2013. The Lancet. 2015; 385(9963): 117–171.
4. http//www.worldlifeexpectancy.com
5. Schuppan D, Afdhal NH. Liver Cirrhosis. The Lancet. 2008; 371(9615): 838–851.
6. Kumar M, Sarin SK. Is Cirrhosis of the Liver Reversible? Indian Journal of Pediatrics. 2007; 74: 393-399.
7. Pellicoro A, Prakash R, Iredale JP. Reversibility of liver fibrosis. Fibrogenesis Tissue Repair.2012; 5(Suppl 1): S26.
8. Arroyo V, Moreau R, Kamath PS, Jalan R, Gines P, Nevens F *et al.* Acute on chronic liver failure in cirrhosis. Nature Reviews Disease Primers. 2016; doi: 10.1038/nrdp.2016.41.
9. Benyon RC, Iredale JP. Is liver fibrosis reversible? Gut. 2000; 46:443-446.
10. Gressner OA, Weiskirchen R, Gressner AM. Evolving concepts of liver fibrogenesis provide new diagnostic and therapeutic options. Comparative Hepatology. 2007; 6(7): doi: 10.1186/1476-5926-6-7.

11. Scott LF. Hepatic Fibrosis. In: Schiff's Diseases of the Liver (Edition 11). Eugene R Schiff, Michael F Sorrell, Willis C Maddrey (Ed). Wiley-Blackwell, Singapore 297-305 (2012).
12. Ra BB, Scott LF. Hepatic Fibrogenesis. In: Sherlock's Diseases of the Liver and Biliary System. (Edition 11). James S. Dooley, Anna S.F. Lok, Andrew K. Burroughs, E. Jenny Heathcote (Ed). Wiley Blackwell, Oxford 94-101 (2011).
13. Ekihiro S, David AB. Recent advancement of molecular mechanisms of liver fibrosis. J Hepatobiliary Pancreat. Sci. 2015; 22(7): 512-518.
14. http://www.ezhealthmd.com/medical-condition/cirrhosis/
15. http://www.medicalworld.co.in/2017/08/chronic-liver-failure.html
16. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest. 2005; 115: 209-218.
17. Imbert-Bismut F, Ratziu V, Pieroni L, Charolotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. The Lancet. 2001; 357: 1069-1075.
18. Drucker E, Krapfenbauer K. Pitfalls and limitations in translation from biomarker discovery to clinical utility in predictive and personalized medicine. The EPMA Journal. 2013; 4(1): 7. doi: 10.1186/1878-5085-4-7.
19. Pinzani M, Rombouts K, Colagrande S. Fibrosis in chronic liver disease: diagnostic and management. J Hepatol. 2005; 42: 12-36.
20. Gangadharan B, Antrobus R, Chittenden D, Rossa J, Bapat M, Klenerman P *et al.* New approaches for biomarker discovery: the search for liver fibrosis markers in hepatitis C patients. J Prot Res. 2011; 10: 2643-2650.

21. Parker CE, Borchers CH. Mass spectrometry based biomarker discovery, verification and validation – Quality assurance and control of protein biomarker assays. Molecular Oncology. 2014; 8: 840-858.
22. Cheng Z, Yinghui LV, Suqiu P, Bai R, Wang M, Lin S *et al*. Kallistatin, a new and reliable biomarker for the diagnosis of liver cirrhosis. Acta pharma Sin B. 2015; 5(3): 194-200.
23. Huixiao H, Weida T. Emerging efforts for discovering new biomarkers of liver disease and hepatotoxicity. Biomarkers Med. 2014; 8(2): 143-146.
24. Chin JL, Palvlides M, Moolla A, Ryan JD. Non-invasive markers of liver fibrosis: Adjuncts or alternatives to liver biopsy? Front Pharmacol. 2016; 7: 159; doi: 10.3389/fphar.2016.00159.
25. Sumanth NK, Shashidhar KN, Lakshmaiah V, Muninarayana C. Liver fibrosis: A compilation on the biomarkers status and their significance in disease progress. Future Science OA. 2017; doi: 10.4155/fsoa-2017-0083.
26. Gressner AM, Gao CF, Gressner OA. Non-invasive biomarkers for monitoring the fibrogenic process in liver: A short survey. World J Gastroenterol. 2009; 15(20): 2433-2440.
27. Moran-Salvador E, Mann J. Epigenetics and liver fibrosis. Cell. Mol. Gastroenterol. Hepatol. 2017; 4: 125-134.
28. Sohrabpour AA, Mohamadnejad M, Malekzadeh R. Review article: the reversibility of cirrhosis. Aliment Pharmacol Ther. 2012; 36: 824-832.

29. Bruce R. Bacon. Cirrhosis and its Complications. In: Longo, Fauci, Hauser, Jameson and Loscalzo. Harrison's Principles of Internal Medicine. 18th Edition. India: McGraw Hill 2012. 2592-2602.
30. Poilil SS, Thomas RG, Moon MJ, Jeong YY. Nanoparticles for the treatment of liver fibrosis. International Journal of Nanomedicine. 2017; 12: 6997-7006.
31. Ebrahimi H, Naderian M, Sohrabpour AA. New concepts on pathogenesis and diagnosis of liver fibrosis; A review article. Middle East Journal of Digestive Disease. 2016; 8(3): 166-178.
32. David AB. Reversibility of liver fibrosis. Gastroenterol Hepatol. 2013; 9: 737-739.
33. Sumanth NK, Pradhan R, Shashidhar KN. Molecular and epigenetic mechanisms of bidirectional liver fibrosis. American J of Liver and Clinical Research. 2017; 1(1): 001-006.
34. Ohkoshi S, Hirono H, Watanabe K, Hasegawa K, Kamimura K, Yano M. Natural regression of fibrosis in chronic hepatitis B. World J Gastroenterol. 2016: 22(24): 5459-5466.
35. Seki E, Brenner DA. Recent advancement of molecular mechanisms of liver fibrosis. J Hepatobiliary Pancreat Sci. 2015; 22(7): 512-518.
36. Bedossa P, Dargere D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. Hepatology. 2003; 38: 1449-1457.
37. Carmen FB, Raluca P, Laura T, Anca B. Can We Replace Liver Biopsy with Non-Invasive Procedures? In: Liver Biopsy. Dr Hirokazu Takahashi (Ed.).2011; InTech, doi: 10.5772/19527.

38. Goodman ZD. Grading and staging systems for inflammation and fibrosis in chronic liver diseases. J. Hepatol. 2007; 47(4): 598-607.
39. Sangheun L, Do Young K. Noninvasive diagnosis of hepatitis B virus related cirrhosis. World J Gastroenterol. 2014; 20(2): 445-459.
40. Grigorescu M. Noninvasive biochemical markers of liver fibrosis. J Gastrointestin Liver Dis.2006; 15: 149-159.
41. Afdhal NH, Nunes D. Evaluation of liver fibrosis: A concise review. Am J Gastroenterol.2004; 127: 1704-1713.
42. Nielsen MJ, Kazankov K, Leeming DJ, Karsdal MA, Krag A, Barrera F *et al*. Markers of collagen remodeling detect clinically significant fibrosis in chronic hepatitis C patients. PloS ONE.2010; 10(9): e0137302.
43. Gressner OA, Weiskirchen R, Gressner AM. Biomarkers of liver fibrosis: Clinical translation of molecular pathogenesis or based on liver dependent malfunction tests. Clin. Chim. Acta. 2007; 381: 107-113.
44. Castera L. Noninvasive methods to assess liver disease in patients with hepatitis B or C. Gastroenterology. 2012; 142: 1293-1302.
45. Jarcuska P, Janicko M, Veseliny E, Jarcuka P, Skladany L. Circulating markers of liver fibrosis progression. Clin. Chim. Acta.2010; 411(15, 16): 1009-1017.
46. Cequera A, Garcia MC, Leon M. Biomarkers for liver fibrosis: Advances, advantages and disadvantages. Revista de Gastroenterologia de Mexico. 2014; 79(3): 187-199.

47. Mc Hutchison JG, Blatt LM, de Medina M, Craig JR, Conrad A, Schiff ER *et al*. Measurement of serum hyaluronic acid in patients with chronic hepatitis C and its relationship to liver histology. Consensus Interferon Study Group. J Gastroenterol Hepatol. 2000; 145: 945-951.
48. Orasan OH, Ciulei G, Cozma A, Sava M, Dumitrascu DL. Hyaluronic acid as a biomarker of fibrosis in chronic liver diseases of different etiologies. Clujul. Medical. 2016; 89(1); 24-31.
49. Walsh KM, Fletcher A, MacSween RNM, Morris AJ. Basement membrane peptides as markers of liver diseases in chronic hepatitis C. J Hepatol. 2000; 32: 325-330.
50. Korner T, Kropf J, Gressner AM. Serum laminin and hyaluronan in liver cirrhosis: markers of progression with high prognostic value. J Hepatol. 1996; 25: 684-688.
51. Hu B, Trinh K, Figueira WF, Price PA. Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family. J Biol. Chem. 1996; 271: 19415-19420.
52. Yukiko S, Katsuya S, Yutaka Y, Yamaguchi Y, Kawakita T, Yamamoto N *et al*. Noninvasive estimation of liver fibrosis and response to interferon therapy by a serum fibrogenesis marker, YKL- 40, in patients with HCV- associated liver disease. World J. Gastroenterol. 2005; 11(4): 476-481.
53. Sun J. Matrix metalloproteinases and tissue inhibitor of metalloproteinases are essential for the inflammatory response in cancer cells. Journal of Signal Transduction. 2010; Article ID: 985132.

54. Murawaki Y, Ikuta Y, Idobe Y, Kawasaki H. Serum matrix metalloproteinase -1 in patients with chronic viral hepatitis. Journal of Gastroenterology and Hepatology. 1999; 14(2): 138-145.

55. Boeker KHW, Haberkorn CI, Michels D, Flemming P, Manns MP, Lichtinghagen R. Diagnostic potential of circulating TIMP-1 and MMP-2 as markers of liver fibrosis in patients with chronic hepatitis. Clin. Chim. Acta.2002; 316(1-2): 71–81.

56. Badra G, Lotfy M, El Refaie A, Obada M, Abdelmonem E, Kandeel S *et al*. Significance of serum matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in chronic hepatitis patients. Acta Microbiol Immunol Hung. 2010; 57(1): 29–42.

57. Zhang BB, Min C, Weng HL, Hu ZR, Lu J, Zheng M *et al*. Diagnostic value of platelet derived growth factor-BB, transforming growth factor α1, matrix metalloproteinase-1 in serum and peripheral blood mononuclear cells for hepatic fibrosis. World J Gastroenterol.2003: 2490-2496.

58. Pinzani M, Marra F. Cytokine receptors and signaling in hepatic stellate cells. Semin Liver Dis.2001; 21(3): 397-416.

59. Harada KI, Shiota G, Kawasaki H. Transforming growth factor α and epidermal growth factor receptor in chronic liver disease and hepatocellular carcinoma. Liver. 1999; 19(4): 318-325.

60. Nelson DR, Gonzalez PRP, Qian K, Xu Y, Marousis CG, Davis GL *et al*. Transforming growth factor-β1 in chronic hepatitis C. J Viral Hepat. 1997; 4(1): 29–35.

61. Kanzler S, Baumann M, Schirmacher P, Dries V, Bayer E, Gerken G *et al*. Prediction of progressive liver fibrosis in hepatitis C infection by serum and tissue levels of transforming growth factor-β. J Viral Hepat. 2001; 8(6); 430–437.
62. Zhou J, Deng Y, Yan L, Zhao H, Wang G. Serum platelet derived growth factor BB levels: A potential biomarker for the assessment of liver fibrosis in patients with chronic hepatitis B. International journal of infectious diseases. 2016; 49: 94-99.
63. Pinzami M, Milani S, Herbst H, DeFranco R, Grappone C, Gentilini A *et al*. Expression of platelet-derived growth factor and its receptors in normal human liver and during active hepatic fibrogenesis. Am J Pathol. 1996; 148; 785-800.
64. Ikura Y, Morimoto H, Ogami M, Jomura H, Ikeoka N, Sakurai M. Expression of platelet-derived growth factor and its receptor in livers of patients with chronic liver disease. J Gastroenterol. 1997; 32: 496-501.
65. Yoshida S, Ikenaga N, Liu SB, Peng ZW, Chung J, Sverdlov DY *et al*. Extra hepatic platelet-derived growth factor-beta, delivered by platelets, promotes activation of hepatic stellate cells and biliary fibrosis in mice. Gastroenterology. 2014; 147: 1378-1392.
66. Susanne GS, Belinda M, Peer BC, Lindvig K, Schlusser A, Holst R *et al*. Microfibrillar associated protein 4: A potential biomarker for screening for liver fibrosis in a mixed patient cohort. PLoS One. 2015; 10(10): e0140418.
67. Greenlee KJ, Werb Z, Kheradmand F. Matrix metalloproteinases in lung: Multiple, multifarious and multifaceted. Physiol Rev. 2007; 87: 69-98.

68. Christian M, Barbara S, Corinna H, Gereon P, Bence S, Wiese S *et al*. Detection of novel biomarkers of liver cirrhosis by proteomic analysis. Heptology. 2009; 49: 1257-1266.

69. Chu PG, Weiss LM. Keratin expression in human tissues and neoplasms. Histopathology. 2002; 40: 403-439.

70. Yilmaz Y, Dolar E, Ulukaya E, Akgoz S, Keskin M, Kiyici M *et al*. Soluble forms of extracellular cytokeratin 18 may differentiate simple steatosis from non-alcoholic steatohepatitis. World J Gastroenterol. 2007; 13: 837-844.

71. Yang M, Xu D, Liu Y, Guo X, Li W, Guo C *et al*. Combined serum biomarkers in noninvasive diagnosis of nonalcoholic steatohepatitis. PLoS ONE.2015; 10(6): e0131664.

72. Murawaki Y, Ikuta Y, Okamoto K, Koda M, Kawasaki H. Diagnostic value of serum markers of connective tissue turnover for predicting histological staging and grading in patients with chronic hepatitis C. J Gastroenterol. 2001; 36: 399-406.

73. Rosenberg WMC, Voelker M, Thiel Recka M, Burt A, Schuppan D *et al*. Serum markers detect the presence of liver fibrosis: a cohort study. Gastroenterology. 2004; 127: 1704-1713.

74. Patel K, Gordon SC, Jacobson I, Hezode C, Oh E, Smith KM *et al*. Evaluation of a panel of noninvasive serum markers to differentiate mild from moderate to advanced liver fibrosis in chronic hepatitis C patients. J Hepatol. 2004; 41: 345-350.

75. Hind IF. Noninvasive biomarkers of liver fibrosis: An overview. Advances in Hepatology. 2014; article Id: 357287.

76. Dufour DR, Lott JA, Nolte FS, Gretch DR, Koff RS, Seeff LB. Diagnosis and monitoring of hepatic injury II. Recommendations for use of laboratory tests in screening, diagnosis and monitoring. Clin. Chem. 2000; 46: 2050-2068.
77. Paul YK, Stanley MC, Joseph KL. ACG Practice Guideline: Evalution of abnormal liver chemistries. Am J Gastroenterol. 2016; doi:10.1038/ajg.2016.517.
78. Kim WR, Flamm SL, Di Bisceglie AM, Bodenheimer HC. Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. Hepatology. 2008; 47: 1363-1370.
79. Giannini E, Risso D, Botta F, Chiarbonello B, Fasoli A, Malfatti F *et al*. Validity and clinical utility of the aspartate aminotransferase- alanine aminotransferase ratio in assessing disease severity and prognosis in patients with hepatitis C related chronic liver disease. Arch Intern Med. 2003; 163: 218-224.
80. Carl AB, Edward RA, David EB (Ed). Teitz textbook of clinical chemistry and molecular diagnostics (Edition 15). Elsevier, India. 528, 574, 578, 581, 1681 (2012).
81. Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS *et al*. A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatology. 2003; 38(2): 518-526.
82. Snyder N, Nguyen A, Gajula L, Soloway R, Xiao SY, Lau DT *et al*. The APRI may be enhanced by the use of the FIBROSpect II in the estimation of fibrosis in chronic hepatitis C. Clin. Chim. Acta. 2007; 381(2): 119-123.
83. A Loaeza del C, Paz PF, Oviedo CE, Sanchez-Avila F, Vargas-Vorackova F. AST to platelet ratio index (APRI) for the noninvasive evaluation of liver fibrosis. Annals of Hepatology. 2008; 7(4): 350-357.

84. Lok AS, Marc GG, Goodman ZD, Wright EC, Everson GJ, Sterling RK *et al*. Predicting cirrhosis in patients with hepatitis C based on standard laboratory tests: results of the HALT-C cohort. Hepatology. 2005; 42(2): 282-292.
85. Bonacini M, Hadi G, Govindarajan S, Lindsay KL. Utility of a discriminant score for diagnosing advanced fibrosis or cirrhosis in patients with chronic hepatitis C virus infection. Am J Gastroenterol. 1997; 92: 1302-1304.
86. Vallet PA, Vincent M, Bertrand N, Verkarre V, Nalpas A, Dhalluin-Venier V *et al*. FIB-4: an inexpensive and accurate marker of fibrosis in HCV infection. Comparison with liver biopsy and Fibro Test. Hepatology. 2007; 46(1): 32–36.
87. Mallet V, Dhalluin VV, Roussin C, Bourliere M, Pettinelli ME, Giry C *et al*. The accuracy of the FIB-4 index for the diagnosis of mild fibrosis in chronic hepatitis B. Alimentary Pharmacology & Therapeutics. 2009; 29(4): 409–415.
88. Koda M, Matunaga Y, Kawakami M, Kishimoto Y, Suou T, Murawaki Y. FibroIndex, a practical index for predicting significant fibrosis in patients with chronic hepatitis C. Hepatology. 2007; 45(2): 297–306.
89. Halfon P, Penaranda G, Renou C, Bourliere M. External validation of FibroIndex. Hepatology. 2007; 46(1): 280–281.
90. Imbert BF, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T *et al*. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. The Lancet. 2001; 357(9262): 1069–1075.

91. Poynard T, Imbert BF, Mona M, Messous M, Messous D, Myers RP *et al*. Overview of diagnostic value of biochemical markers of liver fibrosis (Fibro test, HCV Fibro Sure) and necrosis (Acti Test) in patients with chronic hepatitis C. Comparative Hepatology. 2004; 3(8): doi: 10.1186/1476-5926-3-8.
92. Poynard T, Munteanu M, Deckmyn O, Ngo Y, Drane F, Castille JM *et al*. Validation of liver fibrosis biomarker (Fibro Test) for assessing liver fibrosis progression: proof of concept and first application in a large population. Journal of Hepatology. 2012; 57(3): 541–548.
93. Forns X, Ampurdanes S, Josep ML, Aponte J, Quinto L, Martinez-Bauer E *et al*. Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. Hepatology. 2002; 36(4): 986–992.
94. Adams LA, George J, Burgianesi E, Rossi E, De Boer WB, van der Poorten D *et al*. Complex noninvasive fibrosis models are more accurate than simple models in nonalcoholic fatty liver diseases. J Gastroenterol Hepatol.2011; 26: 1536-1543.
95. Poynard T, Aubert A, Bedossa P, Abelle A, Naveau S, Paraf F *et al*. A simple biological index for detection of alcoholic liver disease in drinkers. Gastroenterology.1991; 100(5): 1397–1402.
96. Naveau S, Poynard T, Benattar C, Bedossa P, Chaput JC. Alpha-2-macroglobulin and hepatic fibrosis. Digestive Diseases and Sciences. 1994; 39(11): 2426–2432.
97. Soresi M, Lydia G, Cervello M, Licatc A, Montalto G. Noninvasive tools for the diagnosis of liver cirrhosis. World J Gatroenterol. 2014; 20(48): 18131-18150.

98. WHO library cataloguing in publication data. Guidelines for the prevention, care and treatment of persons with chronic hepatitis B infection. WHO March 2015. https://apps.who.int.
99. Houot M, Ngo Y, Munteanu M, Marque S, Poynard T. Systematic review with meta-analysis: direct comparisons of biomarkers for the diagnosis of fibrosis in chronic hepatitis C and B. Aliment Phormacol Ther.2016; 43: 16-29.
100. Cholongitas E, Papatheodoridis GV, Vangeli M, Terreni N, Patch D, Burroughs AK. Systematic review: the model for end-stage liver disease – should it replace Child-Pugh's classification for assessing prognosis in cirrhosis? Aliment Pharmacol Ther 2005; 22: 1079–1089.
101. Hunter DJ, Losina E, Guermazi A, Burstein D, Lassre MN, Kraus V. A pathway and approach to biomarker validation and qualification for osteoarthritis clinical trials. Curr Drug Targets. 2010; 11(5): 536-545.
102. Drucker E, Krapfenbauer K. Pitfalls and limitations in translation from biomarker discovery to clinical utility in predictive and personalized medicine. The EPMA Journal. 2013; 4: 7.
103. George P. Bring on the biomarkers. Nature. 2011; 469: 156-157.
104. Aebersold R, Anderson L, Caprioli R, Druker B, Hartwell L, Smith R. Perspective: A program to improve protein biomarker discovery for cancer. J Prot Res. 2005; 4: 1104-1109.
105. Anderson NL. The role of multiple proteome platforms in a pipeline for new diagnostics. Mol Cell Proteomics. 2005; 4: 1441-1444.

106. Nies KM, Ivy DD, Everett DA. The untapped potential of proteomic analysis in pediatric pulmonary hypertension. Proteomics Clin Appl. 2014; 8: 862-874.
107. Rifai N, Gillette AM, Carr AS. Protein biomarker discovery and validation: The long and uncertain path to clinical utility. Nature Biotechnology. 2006; 24(8): 971-983.
108. Parker EC, Borchers HC. Mass spectrometry based biomarker discovery, verification and validation – Quality assurance and control of protein biomarker assays. Molecular Oncology. 2014; 8: 840-858.
109. Farrah T, Deutsch EW, Omenn GS, Campbell DS, Sun Z, Bletz JA *et al*. A high confidence human plasma proteome reference set with estimated concentrations in peptide atlas. Mol Cell Proteomics. 2011; 10: M110.006353.
110. Goossens N, Nakagawa S, Sun X, Hoshida Y. Cancer biomarker discovery and validation. Transl Cancer Res. 2015; 4(3): 256-269.
111. Anderson NL, Anderson NG. The human plasma proteome: history, character and diagnostic prospects. Mol Cell Proteomics. 2002; 1: 845-867.
112. Apple FS, Alan HB, Johanes M, Ravkilde J, Panteghini M, Tate J *et al*. Future biomarkers for detection of ischemia and risk stratification in acute coronary syndrome. Clin Chem. 2005; 51(5): 810-824.
113. Paulovich AG, Whiteaker JR, Hoofnagle AN, Wang P. The interface between biomarker discovery and clinical validation: the tar pit of the protein biomarker pipeline. Proteomics Clin Appl. 2008; 2: 1386-1402.
114. Anderson L. Candidate based proteomics in the search for biomarkers of cardiovascular disease. J Physiol. 2005; 563(1): 23-60.

115. Fillip S, Vougas K, Zoidakis J, Latosinska A, Mullen W, Spasovski G *et al*. Comparison of depletion strategies for the enrichment of low abundance proteins in urine. PloS ONE. 2015; 10(7): e0133773.

116. Steel LF, Trotter MG, Nakajima PB, Mattu TS, Gonye G, Block T. Efficient and specific removal of albumin from human serum samples. Mol Cell Proteomics. 2003; 2(4): 262-270.

117. Xiao Z, Conrads TP, Lucas DA, Janini GM, Schaefer CF, Buetow KH *et al*. Direct ampholyte-free liquid-phase isoelectric peptide focusing: application to the human serum proteome. Electrophoresis. 2004; 25: 128-133.

118. Hoefkens J. Towards unbiased biomarker discovery. Biomarkers. 2010; 1: 19-24.

119. Adkins JN, Susan MV, Auberry KJ, Moore RJ, Angell NH, Smith RD *et al*. Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry. Mol Cell Proteomics. 2002; 1(12): 947-955.

120. Tirumalai RS, Chan KC, Prieto DA, Issaq HJ, Conrads TP, Veenstra TD. Characterization of the low molecular weight human serum proteome. Mol Cell Proteomics. 2003; 2(10): 1096-1103.

121. Krastins B, Prakash A, Sarracino DA, Nedelkov D, Niederkufler EE, Kiernan UA *et al*. Rapid development of sensitive, high throughput, quantitative and highly selective mass spectrometric targeted immunoassays for clinically important proteins in human plasma and serum. Clin Biochem. 2013; 46: 399-410.

122. Marrer E, Dieterle F. Promises of biomarkers in drug development-A reality check. Chem Biol Drug Des. 2007; 69: 381-394.

123. Gillette MA, Mani DR, Carr SA. Place of pattern in proteomic biomarker discovery. J Proteome Res. 2005; 4(4): 1143-1154.

124. Silberring J, Ciborowski P. Biomarker discovery and clinical proteomics. Trends Analyt Chem. 2010; 29(2): doi:10.1016/j.trac.2009.11.007.

125. Vitzthum F, Behrens F, Anderson NL, Shaw JH. Proteomics: from basic research to diagnostic application. A review of requirements & needs. J Proteome Res. 2005; 4: 1086-1097.

126. Doug B, Steve F. Immunoassay development in the *In Vitro* diagnostic industry. In: The immunoassay handbook. (Edition 3). Wild D (Ed). Elsevier, Amsterdam 136 – 143 (2005).

127. Dybkaer R. Vocabulary for use in measurement procedures and description of reference materials in laboratory medicine. Eur J Clin Chem Biochem. 1997; 35: 141-173.

128. Sumanth NK, Shashidhar KN, Lakshmaiah V, Muninarayana C. Cirrhosis of liver: Interference of serpins in quantification of serpina4 - A preliminary study. Practical Laboratory Medicine. 2017; doi.org/10.1016/j.plabm.2017.10.002.

129. NCCLS. Evaluation of the linearity of quantitative measurement procedures: a statistical approach; approved guideline. NCCLS Document EP6-A (NCCLS. Wayne PA) 2003.

130. Lesko LJ, Atkinson AJ. Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation strategies. Annu Rev Pharmacol Toxicol. 2001; 41: 347-366.

131. Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM *et al*. The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. Clin Chem. 2003; 49(1): 7-18.

132. Obuchowski NA, Lreber ML, Wians FH. ROC curves in clinical chemistry: uses, misuses and possible solutions. Clin Chem. 2004; 50: 1118-1125.

133. Sackett DL, Haynes RB. The architecture of diagnostic research. Br Med J. 2002; 324: 539-541.

134. Gangadharan B, Bapat M, Rossa J, Antrobus R, Chittenden D, Kampa B *et al*. Discovery of novel biomarker candidates for liver fibrosis in hepatitis C patients: A preliminary study. PloS One. 2012; 7(6): e39603. doi: 10.1371/journal.pone.0039603.

135. Chai KX, Chen VC, Ni A, Lindpainter K, Rubattu S, Chao L *et al*. Molecular cloning and expression of rat Kallistatin. Bio chim Bio physics Acta. 1997; 1353(3): 277-286.

136. Chao J, Chao L. Biochemistry, regulation and potential function of Kallistatin. Biol Chem Hoppe Seyler. 1995; 376 (12): 705-713.

137. Zhou GX, Chao L, Chao J. Kallistatin: a novel human tissue kallikrein inhibitor. Purification, characterization, and reactive center sequence. J Biol Chem. 1992; 267(36): 25873-25880.

138. Chao J, Schmaier A, Chen LM, Yang Z, Chao L. Kallistatin, a novel human tissue kallikrein inhibitor: levels in body fluids, blood cells and tissues in health and diseases. J Lab Clin Med. 1996; 127(6): 612-620.

139. Chao J, Chai KX, Chao L. Tissue kallikrenin inhibitors in mammals. Immunopharmcology. 1996; 32(1-3): 94-95.

140. Chai KX, Chen LM, Chao J, Chao L. Kallistatin: a novel human serine proteinase inhibitor. Molecular cloning, tissue distribution and expression in Escherichia coli. J Biol Chem. 1993; 268(32): 24498-24505.

141. Bhoola KD, Misso NL, Naran A, Thompson PJ. Current status of tissue kallikrenin inhibitors: importance in cancer. Curr Opin Investig Drugs. 2007; 8(6): 462-468.

142. Fang L, Aiwu Z, Zhenquan W. Crystallization and crystallographic studies of Kallistatin. Acta Crystallogr F Struct Biol Commun. 2015; 71: 1135-1138.

143. Chieh WL, Ling SL, Feng CL, Chen CW, Chao L, Chao J *et al*. Plasma Kallistatin levels in patients with severe community acquired pneumonia. Critical Care 2013; 17(1): R27.

144. Wang CR, Chen SY, Wu CL, Liu MF, Jin YT, Chao L *et al*. Prophylactic adenovirus-mediated human Kallistatin gene therapy suppress rat arthritis by inhibiting angiogenesis and inflammation. Arthritis Rhem. 2005; 52(4): 1319-1324.

145. Guo Y, Li P, Bledsoe G, Yang ZR, Chao L, Chao J. Kallistatin inhibits TGF-β-induced endothelial-mesenchymal transition by differential regulation of microRNA-21 and eNOS expression. Exp Cell Res. 2015; 337(1) 103-110.

146. Miao RQ, Agata J, Chao L, Chao J. Kallistatin is a new inhibitor of angiogenesis and tumor growth. Blood. 2002; 100(9): 3245-3252.

147. Xiaoping H, Xiao Wa, Yinghui LV, Lin J, Diao Y. Protection effects of Kallistatin on carbon tetrachloride-induced Liver fibrosis in rats via Antioxidative stress. PLoS One. 2014; 9(2).

148. Lobo V, Patik A, Phatak A, Chandra N. Free radical, antioxidants and functional foods: impact on human health. Pharmacognosy Reviews. 2010; 4(8): 118-126.

149. Halliwell B. How to characterize an antioxidant - An update. Biochem Soc Symp. 1995; 61: 73–101.

150. Mc Cord JM. The evolution of free radicals and oxidative stress. Am J Med. 2000; 108: 652–659.

151. Elisangela C, Farias JG, Farias ILG, Reetz LGB, OliveiraL, De Carli DM *et al*. Effect of antioxidant potential on severity of cirrhosis in humans. Nutr Hosp. 2015; 32(5): 2294-2300.

152. http://assets.thermofisher.com/MAN0011862_Pierce_Albumin_Depletion_UG.pdf

153. Garfin DE. One dimensional gel electrophoresis, Methods Enzymol. 2009; 463: 497-513.

154. Hjemel LM, Chrambach A. Electrophoresis and electrofocusing in detergent containing media: A discussion of basic concepts, Electrophoresis. 1981; 2: 1–11.

155. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem. 1996; 68: 850-858.

156. https://tools.thermofisher.com/content/sfs/brochures/TR0049-Acetone-precipitation.pdf.

157. http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110009A.pdf

158. Shevchenko A, Henrik T, Jan H, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nature protocols. 2007; 1: 2856-2860.

159. Wiese S, Gronemeyer T, Ofman R, Kunze M, Grou CP, Almeida JA *et al*. Proteomics characterization of mouse kidney peroxisomes by tandem mass spectrometry and protein correlation profiling. Mol Cell Proteomics. 2007; 6(12): 2024-2057.

160. Junqueira M, Spirin V, Balbuena TS, Thomas H, Adzhubei I, Sunyaev S *et al*. Protein identification pipeline for the homology-driven proteomics. J Proteomics. 2008; 71(3): 346-356.
161. http://www.afcs.org/reports/v1/BC0001/Protocols/Protein/Transfer/BIORAD.pdf
162. Bio-Rad Criterion™ Blotter assembly Instruction Manuals.
163. N.H. Horowitz, M. Fling, A method for concentrating dilute protein solutions. Fungal Genetics Report. 1962; 2(18): https://dx.doi.org/10.4148/1941-4765.1057.
164. https://resources.rndsystems.com/pdfs/datasheets/dy1669.pdf
165. https://advansta.com/wikis/setting-up-a-standard-curve-for-an-elisa-assay
166. http://www.elisa-antibody.com/ELISA-test
167. https://resources.rndsystems.com/images/site/rnd-systems-elisa-guide-br3.pdf
168. https://resources.rndsystems.com/pdfs/datasheets/dhyal0.pdf
169. https://resources.rndsystems.com/pdfs/datasheets/dc3l10.pdf
170. https://www.biovision.com/documentation/datasheets/K274.pdf
171. http://www.relassay.com/files/dosya/Package/20Insert/TOS/Assay/Kit.pdf
172. David B, Sacks MB, Path FRC. Carbohydrates. In: Burtis CA, Ashwood ER, Bruns DE. Teitz text book of Clinical Chemistry and Molecular Diagnostics. 5th edition. India: Elsevier; 2012. 709-730.
173. Edmund J. Lamb, F.R.C.Path, Christopher P. Price. Kidney Function Tests. In: Burtis CA, Ashwood ER, Bruns DE. Teitz textbook of Clinical Chemistry and Molecular Diagnostics. 5th edition. India: Elsevier; 2006. 669-707.

174. Matthew R. Pincus, Philip M. Tierno Jr. Maly Fenelus, Wilbur B. Bowne, Martin H. Bluth. Evalution of Liver Function. In: Mcpherson, Pincus. Henry's Clinical Diagnostics and Management by Laboratory Methods. 22nd edition. India; Elsevier 2012. 296-311.

175. Glen L. Hortin. Amino Acids, Peptides and Proteins. In: Carl A. Burtis, Edward R. Ashwood, David E. Bruns. Teitz textbook of Clinical Chemistry and Molecular Diagnostics. 5th edition. India: Elsevier; 2012. 509-563.

176. White IR, Patel K, Symonds WT, Dev A, Griffin P, Tsokanas N *et al*. Serum proteomic analysis focused on fibrosis in patients with hepatitis C virus infection. Journal of Translational Medicine. 2007: 5(33): doi: 10.1186/1479-5876-5-33.

177. Bevin G, Antrobus R, Dwek RA, Nicole Z. Novel serum biomarker candidates for liver fibrosis in hepatitis C patients. Clin Chem. 2007; 53(10): 1792-1799.

178. Katrinli S, Ozdil K, Sahin A, Ozturk O, Kir G, Baykal AT *et al*. Proteomic profiling of HBV infected liver biopsies with different fibrotic stages. Proteome Science. 2017: 15(7): doi: 10.1186/s12953-017-0114-4.

179. Bragulla HH, Homberger DG. Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. J Anat. 2009; 214(4): 516-559.

180. Nikitovic D, Katonis P, Tsatsakis A, Karamanos NK, Tzanakakis GN. Lumican, a Small Leucine-rich Proteoglycan. Life. 2008; 60(12): 818-823.

181. Vijayaraghavan B, Jafferali MH, Figueroa RA, Hallberg E. Samp1, a RanGTP binding transmembrane protein in the inner nuclear membrane. Nucleus. 2016; 7(4): 415-423.

182. Asano M, Komiyama K. Polymeric immunoglobulin receptor. Journal of Oral Science. 2011; 53(2): 147-156.

183. Norman AW. From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. American Journal of Clinical Nutrition. 2008; 88(2): 491S–499S.

184. Wang Y, Kinzie E, Berger FG, LimS-K, Baumann H. Haptoglobin, an inflammation-inducible plasma protein. Redox Report. 2001; 6(6): 379-385. doi: 10.1179/135100001101536580.

185. Trayhurn P, Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. British Journal of Nutrition. 2004; 92: 347-355.

186. http://www.uniprot.org/uniprot/Q5SNT2

187. Janciauskiene SM, Bals R, Koczulla R, Vogelmeier C, Kohntein T, Welte T. The discovery of a 1-antitrypsin and its role in health and disease. Respiratory Medicine. 2011; 105: 1129-1139.

188. Tyagi E, Fiorelli T, Norden M, Padmanabhan J. Alpha 1-antichymotrypsin, an inflammatory protein overexpressed in the brains of patients with alzheimer's disease, induces Tau hyperphosphorylation through c-Jun N-Terminal Kinase Activation. International Journal of Alzheimer's Disease. 2013 (2013); Article ID 606083: doi.org/10.1155/2013/606083.

189. Yoon D, Kueppers F, Genta RM, Klintmalm GB, Khaoustov VI, Yoffe B. Role of alpha-1-antichymotrypsin deficiency in promoting cirrhosis in two siblings with heterozygous alpha-1-antitrypsin deficiency phenotype SZ. Gut. 2002; 50: 730-732.

190. Smith A, McCulloh RJ. Hemopexin and haptoglobin: allies against heme toxicity from hemoglobin not contenders. Front Physiol. 2015; 6:187. doi: 10.3389/fphys.2015.00187.

191. Bergstrom J, Murphy CL, Weiss DT, Solomon A, Sletten K, Hellman U *et al*. Two different types of amyloid deposits— apolipoprotein A-IV and transthyretin in a patient with systemic amyloidosis. Laboratory Investigation. 2004; 84: 981-988.

192. Lei Lu, Li K, Li L, Fang X, Zhou T, Zhang C *et al*. Circulating zinc-α2-glycoprotein levels are low in newly diagnosed patients with metabolic syndrome and correlate with adiponectin. Nutr Metab. 2017; 14: 53. doi: 10.1186/s12986-017-0210-6.

193. Sindhu S, Akhter N, Shenouda S, Wilson A, Ahmdd R *et al*. Plasma fetuin-A/α2-HS-glycoprotein correlates negatively with inflammatory cytokines, chemokines and activation biomarkers in individuals with type-2 diabetes. BMC Immunol. 2016; 17: 33. doi: 10.1186/s12865-016-0171-y.

194. Colomba S, Buclin T, Decosterd LA, Telenti A, Furrer H, Lee BL *et al*. Orosomucoid (α_1-acid glycoprotein) plasma concentration and genetic variants: Effects on human immunodeficiency virus protease inhibitor clearance and cellular accumulation. Clinical Pharmacology & Therapeutics. 2006; 80: 307–318. doi: 10.1016/j.clpt.2006.06.006.

195. Carpenter SL, Mathew P. α2-Antiplasmin and its deficiency: fibrinolysis out of balance. Haemophilia. 2008: 14: 1250–1254.

196. Pereyra D, Offensperger F, Klinglmueller F, Haegle S, Oehlberger L, Gruenberger T *et al*. Early prediction of postoperative liver dysfunction and clinical outcome using antithrombin III-activity. PLoS One. 2017: doi.org/10.1371/journal.pone.0175359.

197. Edwards AM, Isserlin R, Bader GD, Frye SV, Wilson TM, Yu FH. Too many roads not taken. Nature. 2011; 470: 163-165.

198. Uhlen M. Affinity as a tool in life sciences. BioTechniques. 2008; 44: 649-654.

199. Hjelm B, Forsstrom B, Igel U, Johannesson H, Stadler C, Lundberg E, Ponten F *et al*. Generation of monospecific antibodies based on affinity capture of polyclonal antibodies. Protein Science. 2011; 20: 1824-1835.

200. Van Regemortel MH. From absolute to exquisite specificity. Reflections on the fuzzy nature of species, specificity and antigenic sites. J Immunol Methods. 1998; 216(1-2): 37-48.

201. Irving JA, Pike RN, Lesk AM, Whisstock JC. Phylogeny of the serpin super family: implications of patterns of amino acid conservations for structure and function, Genome Res. 2000; 10: 1845-1864.

202. LawRHP, Zhang Q, McGowan S, Buckle AM, Silverman GA, Wong W *et al*. An overview of the serpin super family, Genome Biology. 2006; 7(216): doi: 10.1186/gb-2006-7-5-216.

203. Huntington JA. Serpin structure, function and dysfunction, J Thromb Haemost. 2011; 9: (Suppl 1) 26-34.

204. Huntington JA, Read RJ, Carrell RW. Structure of a serpin protease complex shows inhibition by deformation. Nature. 2000; 407: 923-926.

205. Seixas S, Suriano G, Carvalho F, Seruca R, Rocha J, Di Rienzo A. Sequence diversity at the proximal 14q32.1 SERPIN subcluster: evidence for natural selection favoring the pseudogenization of SERPINA2. Mol Biol Evol. 2007; 24(2): 587-598.

206. Heit C, Jackson BC, McAndrews M, Wright MW, Thompson DC, Silverman GA *et al*. Update of the human and mouse SERPIN gene super family, Human Genomics. 2013; 7(22): doi: 10.1186/1479-7364-7-22.

207. Irving JA, Pike RN, Lesk AM, Whisstock JC. Phylogeny of the serpin super family: implications of patterns of amino acid conservations for structure and function, Genome Res. 2000; 10: 1845-1864.

208. Heiker JT, Kloting N, Kovacs P, Kuettner EB, Strater N, Schultz S *et al*. Vaspin inhibits kallikrein 7 by serpin mechanism, Cell Mol Life Sci. 2013; 70: 2569-2683.

209. Rein CM, Desar UR, Church FC. Serpin-glycosaminoglycan interactions. Methods Enzymol. 2011; 501: 105-137.

210. Billingsley GD, Walter MA, Hammond GL, Cox DW. Physical mapping of four serpin genes: alpha 1-antitrypsin, alpha 1-antichymotrpsin, corticosteroid-binding globulin and protein C inhibitor, within a 280-kb region on chromosome 14q32.1. Am J Hum Genet. 1995; 52: 343-353.

211. Rolini P, Fournier RE. A 370-kb cosmid contig of the serpin gene cluster on human chromosome 14q32.1: molecular linkage of genes encoding alpha 1-antichymotrpsin, protein C inhibitor, kallistatin, alpha 1-antitrypsin and corticosteroid-binding globuli. Genomics. 1997; 46: 409-415.

212. Frank SA. Immunology and Evolution of Infectious Disease. New Jersey. Princeton University Press. 2002.

213. Belorgey D, Hagglof P, Karlsson-Li S, Lomas DA. Protein misfolding and the serpinopathies. Prion. 2007; 1: 15-20.

214. Knaupp AS, Bottomley SP. Serpin polymerization and its role in disease- The molecular basis of α1-antitrypsin deficiency. IUBMB Life. 2009; 61(1): 1-5.

215. Yamasaki M, Li W, Johnson DJ, Huntington JA. Crystal structure of a stable dimer reveals the molecular basis of serpin polymerization. Nature. 2008; 455(7217): 1255-1258.

216. Takehara S, Zhang J, Yang X, Takahashi N, Mikami B, Onda M. Refolding and Polymerization Pathways of Neuroserpin. J Mol Biol. 2010; 403(5): 751-762.

217. Ordonez A, Martinez-Martinez I, Corrales F, Miqueo C, Minano A, Vicente V *et al*. Effect of citrullination on the function and conformation of antithrombin. FEBS Journal. 2009; 276(22): 6763-6772.

218. Carrell RW, Lomas DA. Conformational disease. The Lancet. 1997; 350(9071): 134-138.

219. Gunneras SA, Agaton C, Djerbi S, Hansson M. Prestige antibodies^{TM} – Monospecific antibodies designed for immunohistochemical analysis. BioTechiques. 2008; 44(3): 427-430.

220. Afzali A, Weiss NS, Boyko EJ, Ioannou GN. Association between serum uric acid level and chronic liver disease in the United States. Hepatology. 2010; 52: 578-589.

221. Shih MH, Lazo M, Liu SH, Bonekamp S, Hernaez R, Clark JM. Association between serum uric acid and non-alcoholic fatty liver disease in the US population. Journal of the Formosan Medical Association. 2015; 114: 314-320.

222. Xie Y, Wang M, Zhang Y, Zhang S, Tan A, Gao Y *et al*. Serum uric acid and non-alcoholic fatty liver disease in non-diabetic Chinese men. PloS ONE. 2013; 8(7): doi: 10.1371/journal.pone.0067152.

223. Paul R, Chakravati HN, Mandal SK, Chatterje S, Choudhury PS. Study of serum uric acid in chronic liver disease and its relation with other parameters. Int Res J Pharm. 2013; 4(7): 162-164.

224. Kono H, Chun-Jen CC, Ontiveros F, Rock KL. Uric acid promotes an acute inflammatory response to sterile cell death in mice. J Clin Invest. 2010; 120(6): 1939-1949.

225. Gasse P, Riteau N, Charron S, Girre S, Fick L, Petrilli V *et al*. Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis. Am J Respir Crit Care Med. 2009; 179: 903-913.

226. Resino S, Bellon JM, Aseniso C, Micheloud D, Miralles P, Vargas A *et al*. Can serum hyaluronic acid replace simple noninvasive indexes to predict liver fibrosis in HIV/Hepatitis C coinfected patients? BMC Infectious Diseases. 2010; 10: 244. doi: 10.1186/1471-2334/10/244.

227. Peters L, Mocroft A, Soriano V, Rockstroh J, Rauch A, Karlsson A *et al*. Hyaluronic acid levels predict risk of hepatic encephalopathy and liver-related death in HIV/viral hepatitis coinfected patients. PloS ONE. 2013; 8(5): doi: 10.1371/ journal.pone. 0064283.

228. Gudowska M, Gruszewska E, Panasiuk A, Cylwil B, Flisiak R, Swiderska M *et al*. Hyaluronic acid concentration in liver diseases. Clin Exp Med. 2016; 16: 523-528.

229. Puddu P, Puddu GM, Cravero E, Vizioli C, Muscari A. The relationships among hyperuricemia, endothelial dysfunction and cardiovascular diseases: Molecular mechanisms and clinical implications. Journal of Cardiology. 2012; 59: 235-242.

230. Rocky DC, Montgomery B. Noninvasive measures of liver fibrosis. Hepatology. 2006; 43: S113-120.

231. Wei TY, Wang CC. Uncovered issues in the association between uric acid and nonalcoholic fatty liver disease. Journal of the Formosan Medical Association. 2016; 115: 1022-1023.

232. Huang X, Cai X, Zheng W, Shen Y, Xie L. Relationship between uric acid and endothelial function in hypertensive patients with metabolic syndrome. J Clin Exp Cardiolog. 2016: 7(2): doi: 10.4172/2155-9880.1000416.

233. Cai W, Duan XM, Liu Y, Yu J, Tang YL, Lin ZL *et al*. Uric acid induces endothelial dysfunction by activating HMGB1/RAGE signaling pathway. BioMed Research International article. 2017: id: 4391920.

234. Saitou Y, Shiraki K, Yamanaka Y, Yamaguchi Y, Kawakita T, Yamamoto N *et al*. Noninvasive estimations of liver fibrosis and response to interferon therapy by a serum fibrogenesis marker, YKL-40, in patients with HCV-associated liver disease. World J Gastroenterol. 2005; 11(4): 476-481.

235. Kumagai E, Mano Y, Yoshio S, Shoji H, Sugiyama M, Korenga M *et al*. Serum YKL-40 as a marker of liver fibrosis in patients with non-alcoholic fatty liver disease. Scientific Reports. 2016; 6: 35282. doi: 10.1038/srep35282.

236. Kjaergaard AD, Bojesen SE, Nordestgaard BG, Johansen JS. YKL-40 and alcoholic liver and pancreas damage and disease in 86258 individuals from the general population: cohort and Mendelian randomization studies. Clin Chem. 2014: doi: 10. 1373/clinchem.2014.229096.

237. Johansen JS. Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodeling fibrosis and cancer. Dan Med Bull. 2006; 53: 172-209.

238. Lee CG, Da Silva CA, Dela Cruz CS, Ahangari F, Ma B, Kang MJ *et al*. Role of chitin and chitinase/chitinase- like proteins in inflammation, tissue remodeling and injury. Annu Rev Physiol. 2011; 73: 479-501.

239. Mygind ND, Axelsson A, Ruwald MH, Dalsgaard M, Steffesen R, Iversen K *et al*. The inflammatory biomarker YKL-40 decreases stepwise after exercise stress test. Cardiovascular Endocrinology. 2016; 5: 21-27.

ANNEXURE - I

Review

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Liver fibrosis: a compilation on the biomarkers status and their significance during disease progression

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Liver fibrosis occurs in response to different etiologies of chronic liver injury. Diagnosing degree of liver fibrosis is a crucial step in evaluation of severity of the disease. An invasive liver biopsy is the gold standard method associated with pain and complications. Biomarkers to detect liver fibrosis include direct markers of extracellular matrix turnover and indirect markers as a reflection of liver dysfunction. Although a single marker may not be useful for successful management, a mathematical equation combining tests might be effective. The main purpose of this review is to understand the diagnostic accuracy of biomarkers and scoring systems for liver fibrosis. Advances in -omics approach have generated clinically significant biomarker candidates for liver fibrosis that need further evaluation.

Lay abstract: Liver fibrosis is a global health issue caused by various factors. Early diagnosis of the disease is important for better patient care. Liver biopsy is one of the diagnostic tools but comes with complications. Direct (involved in disease progression) and indirect markers are indicators for liver dysfunction that have easy applicability with less diagnostic value. Combination of these markers may give significant diagnosis but early detection is uncertain. Hence, the present review explains existing biomarkers and their relevance for the generation of ideal biomarkers for effective disease management by using advanced technology.

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Keywords: biomarker • biomarker discovery • genetic markers • hepatic regeneration • liver fibrosis • -omics • sensitivity and specificity • validation strategies

Liver fibrosis is a natural wound healing response which results in the formation of abnormal continuation of connective tissue production and deposition in response to chronic liver injury [1]. Causes of liver fibrosis are multifactorial and include congenital, metabolic, inflammation and toxins. In all these circumstances, replacement of parenchyma by fibrotic tissue, regenerative nodule and loss of liver functions are common [2]. Recent studies to understand the process of hepatic fibrogenesis show that treatment aimed at the underlying cause especially in earlier stage of the disease may improve or even reverse fibrosis. Reasons for resolution may be due to increase in collagenolytic activity and/or increased matrix metalloproteinase (MMP) activity due to decrease in expression of tissue inhibitor of metalloproteinase I (TIMP-I) [3]. Studies have reported that cytokine mobilization of bone marrow derived stem cells will restore neutrophil function and promote hepatic regeneration [4].

In normal liver, extracellular matrix (ECM) is present in space of Disse in direct contact with low-density basal lamina with glycoproteins, proteoglycans and glycosaminoglycans. After an acute liver injury, necrotic or apoptotic cells will be replaced by regenerated parenchymal cells. If the hepatic injury is chronic, there will be failure of regeneration and substitution of hepatocytes with abundant ECM and fibrillar collagen (Figure 1) [5]. Liver fibrosis is associated with major alterations in both quantity and composition of ECM. In advanced stage, fibrotic liver

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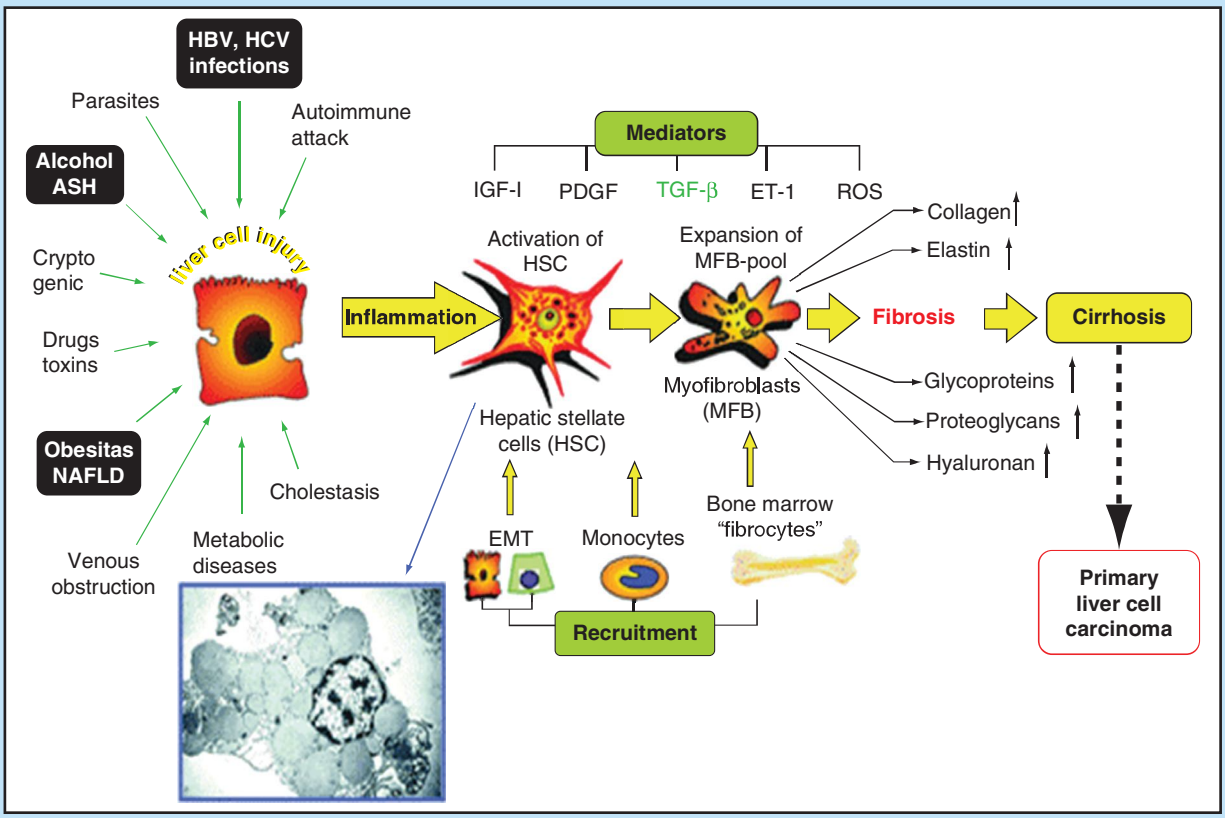

Figure 1. Pathophysiology of liver fibrosis.
After chronic liver injury, necrotic or apoptotic cells will be replaced by regenerated parenchymal cells. Inflammation-connected activation of hepatic stellate cells takes place and transdifferentiation into myofibroblast-like cells which attains contractile, proinflammatory and fibrogenic property.
ASH: Alcoholic steatohepatitis; EMT: Epithelial mesenchymal transition; ET-1: Endothelin-1; HSC: Hepatic stellate cell; NAFLD: Nonalcoholic fatty liver disease; ROS: Reactive oxygen species.
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contains three- to ten-times more ECM than normal liver which includes collagens (I, III and IV), fibronectin, elastin, laminin, hyaluronic acid (HA) and proteoglycans [6].

ECM-producing cells in the injured liver are hepatic stellate cells (HSC) which dwell in the space of Disse and are the major storage cells of vitamin A [7]. Due to chronic liver injury, activation of HSCs takes place and transdifferentiate into myofibroblast-like cells and attains contractile, proinflammatory and fibrogenic property. Chief mitogen for activation of HSCs is PDGF which is produced by Kupffer cells. Activated HSCs migrate and accumulate at tissue repair sites and secrete large amounts of ECM and regulates ECM degradation. HSCs collagen synthesis is regulated at transcription and post-transcriptional levels [7]. Replacement of normal low-density matrix by high-density interstitial matrix disturbs the hepatocyte synthetic and metabolic function and impairs solute transport from sinusoid to hepatocyte. Cellular behavior alterations are mediated by cell membrane receptors termed as integrins [6,8].

The activation of HSCs takes place in two phases (initiation and perpetuation). Initiation includes early changes in HSCs resulting from paracrine stimuli by neighboring cells viz., sinusoidal endothelium, kupffer cells, hepatocytes and platelets. Inflammatory marker cells stimulate matrix synthesis, cell proliferation and release of vitamin A by HSC through the action of cytokine TGF-β, reactive oxygen intermediates and lipid peroxides. Perpetuation involves seven discrete changes in cell behavior; proliferation, chemotaxis, fibrogenesis, contractility, matrix degradation,

Box 1. Advantages and disadvantages of biomarkers of liver fibrosis.

Advantages:
- Minimal invasive
- Not associated with morbidity and mortality
- Easy to apply with great availability and easier reproducibility
- Validated biomarkers with scores may be useful for monitoring therapy
- Biomarkers are less expensive

Disadvantages:
- Direct markers are not organ specific, influenced by unrelated sites of inflammation
- Not sensitive enough to discriminate intermediate stages
- Dependent on clearance rate and influenced by impaired biliary function and renal excretion
- None of the biomarkers have a high degree of accuracy
- Most of the biomarkers need further validation

retinoid loss and inflammatory signaling, and WBC chemoattraction with cytokine release. Among the discrete changes in cell behavior following the perpetuation of HSC activation, fibrogenetic factors play a vital role in fibrogenesis [6,8].

Though the liver biopsy is considered the gold standard method, it is an invasive procedure associated with pain and complications. Scoring system for diagnosis and prognosis of fibrosis include routine laboratory tests viz., serum proteins, liver enzymes, bilirubin, prothrombin time (PT) and direct markers of ECM turn over. Liver fibrosis can be diagnosed by imaging techniques such as ultrasonography, computed tomography and MRI [7]. These diagnostic modalities can detect parenchymal changes but required skills and costs are exorbitant. In the present review, an attempt has been made to understand the diagnostic accuracy, advantages and disadvantages (Box 1) for existing biomarkers and different scores for liver fibrosis.

Diagnosis & assessment of liver fibrosis

Accurate assessment of the extent of liver fibrosis is essential for clinical management so as to predict prognosis and therapeutic decision in patients with liver fibrosis (Figure 2).

Liver biopsy

Despite development of potential diagnostic tests for the past 50 years, liver biopsy is considered as gold standard method to classify liver fibrosis and provides useful information about diagnosis and also other damaging process viz., necrosis, inflammation and steatosis [9]. Three of the widely used methods to assess histological fibrosis are: Ishak score, Metavir score and Desmet/Scheuer staging system (Figure 3) [10]. Each scoring system relies on progressive development of periportal fibrosis followed by septal fibrosis and finally nodule formation [11].

Limitation of liver biopsy is highly invasive. Moreover, poor sample quality and tissue size make biopsy nonreproducible and depend on the experience of pathologist which leads to interobserver variations. Risk allied for liver biopsy range from pain (84%) and hypertension, bleeding (0.5%) and damage to biliary system with approximately 0.01% mortality rate [12]. These limitations of liver biopsy have given urgency for development of noninvasive diagnostic procedure for liver fibrosis. An ideal biomarker should be organ specific, sensitive to indicate active damage, easily accessible in peripheral tissue and cost effective [13]. Advantages of biomarkers over liver biopsy are that their estimations in serum are by minimal invasive procedure. Further advantages are easy applicability, interlaboratory reproducibility and broad availability.

Serum biomarkers for liver fibrosis are classified into two categories [14]:

- Direct markers: which reflects ECM turnover
- Indirect markers: molecules released into blood which reflect alterations of hepatic function

Direct markers of liver fibrosis

Direct markers are directly involved in deposition and removal of ECM produced by HSC and other hepatic cells. Serum levels of these markers are elevated with progressing fibrosis and have a tendency to decrease with response to treatment [15]. Assessment of these markers may be useful for bringing about effective treatment, but they are

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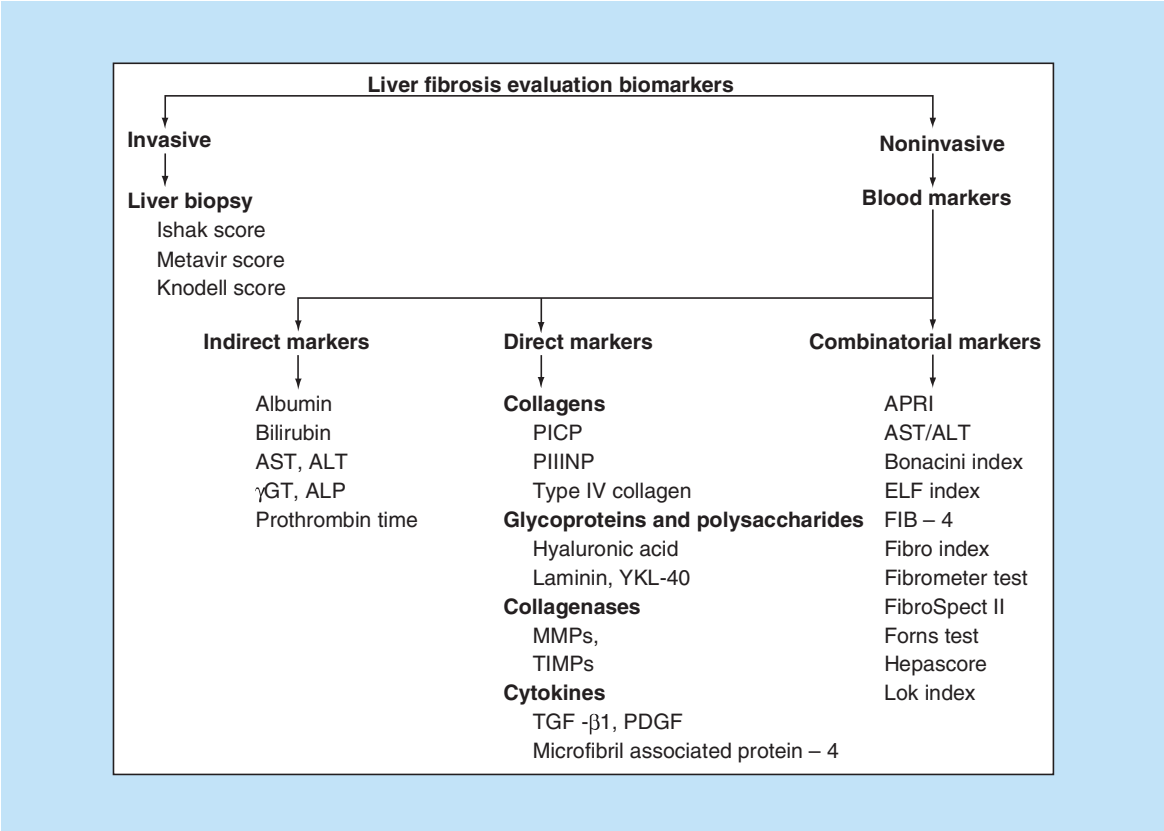


Figure 2. Algorithm of liver fibrosis markers.
γGT: Gamma glutamyl transferase; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; APRI: AST to platelet ratio index; AST: Aspartate aminotransferase; ELF: Enhanced liver fibrosis; FIB-4: Fibrosis-4; MMP: Matrix metalloproteinase; PICP: Procollagen I carboxy peptide; PIIICP: Procollagen III amino peptide; TIMP: Tissue inhibitor of metalloproteinase.

Table 1. Classification of direct markers for liver fibrosis according to structure.

Collagens – PICP – PIIINP – Type IV collagen	**Collagenases and their inhibitors** – MMPs – TIMPs
Glycoproteins and polysaccharides – Hyaluronic acid – Laminin – YKL-40	**Cytokines and proteomic markers** – TGF-β1 – PDGF – Microfibril associated protein-4

MMP: Matrix metalloproteinase; PICP: Procollagen I carboxy peptide; PIIINP: Procollagen III amino peptide; TIMP: Tissue inhibitors of metalloproteinase.
Data taken from [16].

neither organ specific nor readily available. Direct markers of liver fibrosis are classified according to their molecular structure (Table 1) [16].

Collagens

Procollagen I carboxy peptide & procollagen III amino peptide

During synthesis of collagen, procollagen undergoes enzymatic cleavage at carboxy and amino terminal ends by procollagen C-peptidase and procollagen N-peptidase and peptides are released into serum whose estimations can be used to assess matrix deposition [17]. Fibril-forming type I collagen is profuse in healthy liver. During fibrogenesis, type I collagen will be increased up to eightfold [18]. Serum estimations can give an indication regarding the severity of disease. Type III collagen, a fibril-forming collagen is an important component of connective tissue. Concentrations of procollagen III amino peptide (PIIINP) in basal membrane are greater during hepatic fibrosis

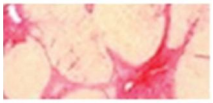
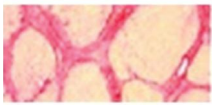
Appearance	Ishak stage: categorical description	Ishak	Metavir
	No fibrosis (normal)	0	F0
	Fibrosis expansion of some portal areas ± short fibrous septa	1	F1
	Fibrosis expansion of portal areas ± short fibrous septa	2	F2
	Fibrosis expansion of most portal areas with occasional portal to portal (P-P) bridging	3	
	Fibrosis expansion of portal areas with marked portal to portal (P-P) bridging as well as portal to central (P-C)	4	F3
	Marked bridging (P-P and / or P-C) with occasional nodules (incomplete cirrhosis)	5	
	Cirrhosis, probable or definite	6	F4

Figure 3. Histological scoring system for liver fibrosis.
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due to chronic liver injury. PIIINP will be correlated with aminotransferase levels in acute hepatitis which reflects degree of fibrosis [18,19].

Relatively low sensitivity and specificity (78 and 81%) of these markers have limited their clinical use. There is no correlation between procollagen I carboxy peptide and PIIINP serum levels with histological grading of liver fibrosis. Hence, these are not reliable to establish fibrosis grading [19,20].

Type IV collagen

Type IV collagen is a crucial component of hepatic ECM which is deposited integrally in matrix. Serum estimation of type IV collagen is a sign of direct degradation and has positive correlation with grade of liver fibrosis. Combinatorial use of type IV collagen with PIIINP has a sensitivity and specificity of 88% [19,21].

Glycoproteins & polysaccharides

Hyaluronic acid

HA is a glycosaminoglycan synthesized by HSCs and is the main component of ECM. In normal liver, HA uptake and degradation take place in hepato sinusoidal endothelial cells. Increased concentrations in serum are attributable to increased production and decreased hepatic elimination or both [22]. Serum HA levels are related to stage of fibrosis and degree of necroinflammation. High levels have been detected in liver fibrosis with varied etiology [23]. HA has sensitivity and specificity of 88–95% and 86–100%, respectively, in liver fibrosis especially nonalcoholic fatty liver diseases, but positive and negative predictive value of HA has been reported as 61% and 98–100%, respectively [18].

Laminin

Laminin is noncollagenous glycoprotein deposited in basal membrane of liver, synthesized by HSCs. In liver fibrosis, laminin increases around the vessels, in perisinusoidal space and portal triad. Serum laminin levels are elevated in liver fibrosis irrespective of etiology and have a correlation with severity of fibrosis and liver inflammation [24]. Laminin cut-off concentration at 1.45 U/ml has sensitivity and specificity of 87 and 74%, respectively, with positive-predictive value of 77% and negative-predictive value of 85%. Estimations of serum HA and laminin have good prognostic value for liver fibrosis complications [25].

YKL-40

YKL-40 (chondrex, human cartilage glycoprotein-39) is a glycoprotein. YKL-40 mRNA is strongly expressed by liver [26]. It can be used as a marker to assess liver fibrosis and helps distinguish between mild stage and extensive stage of liver fibrosis and has positive-predictive value of 80%. Between HA and YKL-40, HA is a better predictive marker for liver fibrosis [27].

Collagenases & their inhibitors

MMPs & TIMPs

Degradation of ECM of liver is due to activity of MMP. Three MMPs are expressed in humans viz., MMP-1 (collagenases), MMP-2 (gelatinase A) and MMP-9 (gelatinase B) [28]. These enzymes are synthesized intracellularly and secreted as zymogens. MMPs are activated by membrane-type MMP and inhibited by tissue inhibitors of metalloproteinases (TIMPs) [15]. In liver fibrosis, there will be inverse correlation between levels of MMP-1 and histological severity [29]. MMP-2 secreted from hepatic stellate cells in liver disease has high diagnostic accuracy of 92% to detect liver fibrosis. There will be a 2.4-fold increase in the levels of MMP-2 in fibrotic patients when compared with controls [30]. MMP-9 from Kupffer cells has negative correlation with histological severity [31].

ECM degradation by MMPs is inhibited by TIMPs, which affect MMPs function. TIMP-1 will interact with almost all the 3MMPs where as TIMP-2 specifically interacts with MMP-2. With progression of liver disease, serum levels of TIMPs will increase. MMP-1/TIMP-1 ratio is useful for the diagnosis of hepatic fibrosis and correlates with degree of portal inflammation [32].

Cytokines & proteomic markers

TGF-α & TGF-β1

In liver fibrosis, TGF-α enhances proliferation of HSCs and correlates well with progression of the disease [33,34]. Homodimetric polypeptide, TGF-β1, secreted in an inactive form, has pleiotropic effect through membrane receptors. TGF-β1 stimulates production of ECM by HSCs and inhibits hepatocyte growth and proliferation in liver fibrosis [35]. High levels of TGF-β1 correlate with progression of hepatic fibrosis. TGF-β1 cut-off value of less than 75 ng/ml is an indicator of stable disease. Limitation of levels of TGF-β1 is due to contamination of sample by platelet TGF-β [36].

PDGF-BB

PDGF-BB is expressed by platelets, fibroblasts, endothelial cells, mast cells and macrophages [37]. It is the main subunit which stimulates HSC proliferation and migration. Serum levels of PDGF-BB have correlation with severity of hepatic fibrosis. In early studies by Pinzani *et al.* and Ikura *et al.*, PDGF-BB mRNA expression was found to be markedly elevated in chronic liver disease [38,39]. Recent studies by Yoshida *et al.* and Jiyuan *et al.* showed decreased serum levels of PDGF-BB in liver fibrosis [37,40].

Microfibrillar-associated protein 4

Microfibrillar-associated protein 4 present in ECM including elastin and collagen is a disulfide-linked dimer that forms higher oligomeric structure [41]. In its C-terminal end, MFAP4 has fibrinogen like domain and in the N-terminal end an integrin binding motif is present [42]. Recent studies suggest that MPAF4 has a sensitivity of 91.6% and a specificity of 95.6%. MPAF4 is an ideal serum marker among liver-specific proteins [43].

Cytokeratin-18 fragments

The major intermediate filament present in hepatocyte are cytokeratin-18 fragments (CK18). Caspase-induced apoptosis takes place by cleavage of CK18 in different positions and results in the formation of CK18 fragments [44].

Table 2. Area under receiver's operating curve for direct markers in various etiology of liver fibrosis.

Marker	Liver disease evaluated by biochemical marker				AUROC for advanced fibrosis
	CHC	*CHB*	*NAFLD*	*ALD*	
PICP	NA	–	–	NA	NA
PIIINP	0.69–0.78	–	NA	0.67–0.87	0.67–0.87
Type IV collagen	0.73–0.83	–	0.82	NA	0.58–0.83
HA	0.82–0.92	0.98	0.97	0.69–0.93	0.69–0.98
Laminin	0.54–0.82	–	NA	NA	0.46–0.82
YKL-40	0.7–0.81	–	NA	NA	0.7–0.81
MMP-2	0.59	–	–	–	0.59

ALD: Alcoholic liver disease; AUROC: Area under receiver's operating curve; CHB: Chronic hepatitis B; CHC: Chronic hepatitis C; HA: Hyaluronic acid; MMP: Matrix metallo proteinase; NA: AUROC is not available; NAFLD: Nonalcoholic fatty liver disease; PICP: Procollagen I carboxy peptide; PIIINP: Procollagen III amino peptide. Data taken from [50].

According to Yilmaz *et al.*, levels of M30 antigen (a neoepitope in CK18) and M65 (cytosolic pool of CK18) can distinguish between advanced fibrosis and early-stage fibrosis [45,46].

Although a single direct marker may serve as an indicator of disease severity, there is growing consensus that combination of multiple markers as an integrated panel will enhance the performance characteristics in terms of specificity and sensitivity.

According to Oberti *et al.*, best diagnostic accuracy was found for HA (86%), laminin (81%), PIIINP (74%) and TGF-β (67%) [15]. But in this study, the diagnostic advantages over nonspecific markers like prothrombin index, gamma glutamyl transferase (γGT), and α2 macroglobulin were not reported [15]. Murawaki *et al.* inferred that HA and MMP-2 are useful for diagnosing stages of fibrosis, but cannot replace liver biopsy as there is an overlap among stages and grades in liver fibrosis [47]. The European Liver Fibrosis study compared the diagnostic performance of HA, PIIINP and TIMP-1 with liver biopsy with threshold sensitivity greater than 90% and specificity greater than 90% can detect liver fibrosis [48]. Patel *et al.* observed that diagnostic value of HA, TIMP-1 and α2-macroglobulin can differentiate chronic hepatitis C patients with moderate/severe fibrosis from those with no or mild fibrosis (Table 2) [49,50].

Indirect markers of liver fibrosis

Indirect markers reflect alteration in hepatic function. These markers are useful in diagnosing, evaluating severity, monitoring therapy and also assessing the prognosis of liver diseases. These include measurement of activity of enzymes viz., aminotransferases, alkaline phosphatase (ALP) and γ-glutamyl transferase (γGT), and estimations of bilirubin and albumin in blood [51]. These are the markers for liver injury, not for liver function and should be referred as liver chemistries or liver tests [52].

Aminotransferases

Liver disease is most important cause of increased transaminase activity in serum. Serum activities of aspartate aminotransferase (AST; EC 2.6.1.1) and alanine aminotransferase (ALT; EC 2.6.1.2) are elevated when disease processes affect liver cell integrity. Between these two, ALT is more specific enzyme for liver insult. Alterations of ALT activity persist longer than AST activity. Activities of both enzymes may reach as high as 100-times upper reference limit in liver diseases. Peak activities bear no relationship to prognosis and may fall with worsening of patient's condition [53]. AST/ALT ratio >1 is a prediction of cirrhosis, and has sensitivity and specificity of 81.3 and 55.3%, respectively. In some etiologies of chronic hepatitis, the ratio is ≤1, whereas ratio >2 suggests alcoholic hepatitis [54].

Alkaline phosphatase (EC 3.1.3.1)

Zinc metalloproteinase enzyme, ALP, catalyzes the hydrolysis of phosphate esters at an alkaline pH. The response of liver to any form of biliary tree obstruction induces the synthesis of ALP from canalicular membrane of hepatocytes [52]. Thus newly formed enzyme enters the circulation to increase the enzyme activity in serum. Elevation tends to be more notable in extra hepatic obstruction than in intrahepatic obstruction. Serum enzyme activities may reach 10- to 12-times the upper reference limit. Liver diseases that principally affect parenchymal

cells such as infectious hepatitis typically show only moderate increase or even normal serum ALP activity. Increase may also be seen as a consequence of response to drug therapy [55].

γGT (EC 2.3.2.2)

Elevated activities of γGT are found in serum of alcoholic hepatitis patients. Moderate elevations occur in infectious hepatitis. Increased concentrations of enzyme are also found in serum of subjects receiving anticonvulsant drugs (phenytoin and phenobarbital). γGT is a sensitive indicator and elevated in most of the subjects with liver disease regardless of cause, but its efficacy is limited due to lack of specificity [52,55].

Albumin

Liver has synthesizing capacity to maintain albumin concentrations until parenchymal damage is more than 50%. Plasma albumin measurements are useful in assessing chronicity and severity of the disease. However, its utility for this purpose is limited, as plasma albumin concentration is also decreased in acute kidney disease [55].

Bilirubin

Sequential measurement of bilirubin is supportive in assessing the severity of liver damage due to different etiology. In acute hepatitis, serum bilirubin peaks later than enzymes but remains elevated for longer time [52,55].

Prothrombin time

Serial PT measurements can be used to differentiate between cholestasis and severe hepatocellular diseases. In severe hepatocellular damage, PT remains elevated for a longer time. Cholestasis will cause a decrease in PT as a result of malabsorption of vitamin K [15,55].

Combinatorial use of biomarkers

Combination of different markers can improve sensitivity and specificity of these tests (Table 3) [50].

AST/platelet ratio (APRI)

$$\text{APRI} = \frac{\text{AST(upper normal value)}}{\text{Platelet count } (10^9 / \text{L})} \times 100$$

Wai *et al.* developed 'AST to platelet ratio index' (APRI). APRI more than 1.5 has area under receiver's operating curve (AUROC) of 80% and 89% for advanced fibrosis F3–F4 and cirrhosis, respectively [56]. According to Snyder *et al.*, APRI cut-off of 0.42 or less has high diagnostic accuracy with a negative predictive value (NPV) of 95% [57]. In autoimmune hepatitis, Loaeza Del Castillo *et al.* showed that APRI does not have any diagnostic value in assessing fibrosis [58]. Lok *et al.* enhanced diagnostic accuracy of APRI in incorporating ALT and international normalized ratio in assessing the progression of fibrosis in postliver transplant patients [59].

Table 3. Area under receiver's operating curve for indirect marker panel in various etiology of liver fibrosis.

Marker	Liver disease evaluated by biochemical marker				AUROC for advanced fibrosis
	CHC	*CHB*	*NAFLD*	*ALD*	
AST/ALT ratio	0.54–0.71	NA	0.74–0.83	NA	0.54–0.83
APRI	0.65–0.87	0.67–0.72	0.56–0.86	–	0.56–0.87
FibroTest	0.72–0.87	0.76–0.85	0.82–0.89	0.83–0.91	0.72–0.87
Fibro index	0.8–0.83	NA	–	–	0.82
Frons index	0.78–0.86	NA	–	–	0.78–0.86

ALD: Alcoholic liver disease; ALT: Alanine aminotransferase; APRI: AST to platelet ratio index; AST: Aspartate aminotransferase; AUROC: Area under receiver's operating curve; CHB: Chronic hepatitis B; CHC: Chronic hepatitis C; NA: AUROC is not available; NAFLD: Nonalcoholic fatty liver disease.
Data taken from [50].

Table 4. Bonacini cirrhosis discriminant parameters score.

Score	Platelets ($10^3/\mu l$)	ALT:AST ratio	INR
0	>340	>1.7	<1.1
1	280–340	1.2–1.7	1.1–1.4
2	220–279	0.6–1.19	>1.4
3	160–219	<0.9	–
4	100–159	–	–
5	40–99	–	–
6	<40	–	–

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; INR: International normalized ratio.
Data taken from [60].

Bonacini index

$$\text{Bonacini cirrhosis discriminant Score} = \text{Platelet score} + \text{ALT : AST ratio score} + \text{INR score}$$

Bonacini *et al.* developed a discriminant score (Table 4) [60] for diagnosis of advanced fibrosis and cirrhosis by taking three parameters: platelets, ALT/AST ratio and PT which have positive correlation with histological scores and have 98% specificity but 46% sensitivity [60].

FIB-4 score

$$\text{FIB}-4 = \frac{\text{Age (years)} \times \text{AST(U/L)}}{\text{Platelet count } (10^9/\text{L})} \times \sqrt{\text{ALT(U/L)}}$$

Sterling *et al.* developed a score to assess fibrosis in HIV/HCV coinfected patients and successfully classified 87% of patients at a cut-off of 3.25 with an AUROC of 76% [61]. Further validation of this score showed AUROCs of 85 and 81% for monoinfected HCV and HBV patients, respectively [62,63].

Fibro index

$$\text{Fibro index} = 1.738 - 0.064 \times \text{platelet count } (10^4/\text{mm}^3) + 0.005 \times \text{AST (IU/L)} + 0.463 \text{ gamma globulin (g/dl)}$$

Koda *et al.* developed score from platelet count, AST and γGT to assess fibrosis [63]. A cut-off of 2.25, was correlated with F2–F3 fibrosis and has 90% NPV [64]. However, further validation showed this score has less diagnostic accuracy [65].

FibroTest

$$z = 4.467 \times \log_{10}[\text{alpha2 macroglobulin (g/l)}] - 1.357 \times \log_{10}[\text{haptoglobin (g/l)}] + 1.017 \times \log_{10}[\gamma\text{GT (IU/L)}] + 0.0281 \times [\text{age (years)}] + 1.737 \times \log_{10}[\text{bilirubin } (\mu \text{ mol/L})] - 1.184 \times [\text{apolipoprotein A1 (g/l)}] + 0.301 \times \text{sex (female} = 0, \text{male} = 1) - 5.54$$

FibroTest (Fibro Sure in USA) was patented since 2001 by APHP (Assistance publique - Hopitaux de Paris), the Parisian public hospital system. It is the most validated test and is based on age, gender, serum haptoglobin, α2 macroglobulin, apolipoprotein A1, γGT and bilirubin. However, it is less significant in detection of intermediate

Table 5. Conversion between FibroTest and fibrosis stages.

FibroTest	METAVIR score	Knodell score	Ishak score
0.75–1.00	F4	F4	F6
0.73–0.74	F3–F4	F3–F4	F5
0.59–0.72	F3	F3	F4
0.49–0.58	F2	F1–F3	F3
0.32–0.48	F1–F2	F1	F2–F3
0.28–0.31	F1	F1	F2
0.22–0.27	F0–F1	F0–F1	F1
0.00–0.21	F0	F0	F0

Conversion between FibroTest and fibrosis stages using METAVIR, Knodell and Ishak fibrosis scoring systems.
Data taken from [66].

stages of fibrosis (Table 5) [66]. Poynard *et al.* established high accuracy of FibroTest in steatohepatitis with AUC of 85% [67].

Forns index

$$\text{Forns Index} = 7.811 - 3.131 \times \ln(\text{platelet count } [10^9/\text{L}]) + 0.781 \times \ln(\gamma\text{GT } [\text{IU/L}]) + 3.467 \times \ln(\text{age}) - 0.014 \times \text{cholesterol (mg/dl)}$$

In 2002, Forns *et al.* developed this score by calculating age, platelet count, serum cholesterol and γGT which can differentiate mild fibrosis with advanced fibrosis at a cut-off value of 6.9 [68]. Further validation of this index showed sensitivity of 94%, specificity of 51% with AUROC ranging from 81 to 86% [69].

PGA index

- PT (% of control): ≥80 = 0; 70–79 = 1; 60–69 = 2; 50–59 = 3; <50 = 4
- γGT (IU/l): <20 = 0; 20–49 = 1; 50–99 = 2; 100–199 = 3; ≥200 = 4
- Apolipoprotein A1 (mg/dl): ≥200 = 0; 175–199 = 1; 150–174 = 2; 125–149 = 3; <125 = 4
- α2 macroglobulin (g/l): <1.25 = 0; 1.25–1.74 = 1; 1.75–2.24 = 2; 2.25–2.74 = 3; ≥2.75 = 4

PGAA index is the sum of the above.

Poynard *et al.* anticipated PGA index in combination with γGT, prothrombin index and apolipoprotein A to assess alcoholic liver disease [70]. The accuracy of this index has been increased from 65 to 70% by addition of α2 macroglobulin (PGAA) [71].

Calculating such a score greatly improves sensitivity and specificity and can avoid limitations of individual markers. Combinations of direct and indirect markers may increase diagnostic accuracy, but has not been implemented in clinical practice (Table 6) [72]. Scores may give clear positive or negative prediction only at early stages of fibrosis. In acute hepatic injury, there will be false positive results in scores such as APRI, Forns index and FIB-4. In hemolytic and hyper bilirubinemia, false positive results may be possible for FibroTest [21]. According to WHO 2015 report, APRI and FibroTest are preferred noninvasive tests to assess the presence of cirrhosis caused by hepatitis B [73]. APRI has low performance when compared with FIB-4 and FibroTest in liver disease caused by hepatitis B and hepatitis C [74]. FIB-4 cut-offs were initially validated only for F3 and F4, and need specific validation before comparing with FibroTest and APRI [61].

Evolving biomarker candidates for liver fibrosis

α-smooth muscle actin is an isoform of actin expressed from myofibroblasts which plays an important role in fibrogenesis. Active myofibroblasts proliferate and synthesize large amounts of extracellular components. Expression of α-smooth muscle actin correlates with activation of myofibroblasts, and is a reliable marker for HSCs activation [75–77]. Maieron *et al.* identified Von Willebrand factor as a new biomarker for chronic liver diseases; further established VITRO score (Von Willebrand factor-Ag/PLT) to evaluate stages of liver fibrosis in chronic hepatitis C patients [78].

Table 6. Main scoring system for liver fibrosis with sensitivity and specificity.

Test	Parameters	Sensitivity (%)	Specificity (%)
APRI	AST/platelet count	57	93
AST/ALT	AST/ALT	51	71
Bonacini index	ALT/AST, INR, platelet count	46	98
ELF index	Age, HA, PIIINP and TIMP-1	90	69
FIB-4	Platelet count, AST, ALT and age	65	97
Fibro index	Platelet count, AST and γ-globulin	35	97
Fibrometer test	Platelet count, INR, AST, α2 macroglobulin, HA, urea and age	80	84
FibroSpect II	HA, TIMP-II and α2 macroglobulin	76	73
Forns test	Age, platelet count, γGT and cholesterol	30	95
Globulin–albumin ratio	Globulin and albumin	43	98
GUCI	Platelet count, AST and INR	80	78
Hepascore	Age, gender, bilirubin, γGT, HA and α2 macroglobulin	84	71
Lok index	Platelet count, AST, ALT and INR	68	72

γGT: Gamma glutamyl transferase; ALT: Alanine aminotransferase; APRI: AST to platelet ratio index; AST: Aspartate aminotransferase; ELF: Enhanced liver fibrosis; FIB-4: Fibrosis-4; GUCI: Goteborg University cirrhosis index; HA: Hyaluronic acid; INR: International normalized ratio; PIIINP: Procollagen III amino peptide; TIMP-1: Tissue inhibitor of metalloproteinase I; TIMP-II: Tissue inhibitor of metalloproteinase II.
Data taken from [72].

White *et al.* and Gangadharan *et al.* discovered protein marker candidates: apolipoprotein AIV, lipid transfer inhibitory protein, complement C3, apolipoprotein L1, apolipoprotein J and corticosteroid-binding protein for liver fibrosis by proteomic approach in various studies [79]. Zhiyun *et al.* observed the alterations in concentrations of Kallistatin depending on the degree of severity of disease and also have documented that Kallistatin levels in serum vary in different liver diseases (fibrosis, cirrhosis and hepatocellular carcinoma) [80]. Irvine *et al.* identified 17 analytes with differential expression between patients with no advanced fibrosis and patients with advanced fibrosis which have the potential to enhance the diagnostic accuracy. Data suggest that MMP7 is a valuable indicator of advanced fibrosis [81].

Hu *et al.* discovered eight marker candidates viz., malic acid, oxidized glutathione, γ-glutamyl-cysteinyl-glycine, ATP, phenylalanine, AMP, nitrotyrosine and tryptophan by metabolomic approach [82]. Zeng *et al.* showed the use of ceruloplasmin to identify various liver fibrosis stages via AUROC values [83]. They further developed a model in combination with ceruloplasmin and γGT, which has sensitivity and specificity of 84 and 83.1%, respectively [83]. Newly discovered candidate markers may have vital responsibility for assessment of chronic liver injury which needs further evaluation. Statistical comparison should be made with established biomarkers and panels.

Genetic markers for liver fibrosis

Genetics of progression in liver fibrosis is multifactorial (genes, environmental factors and cell types) and highly complex. Hall *et al.* identified seven genomic loci on chromosomes 4, 5, 7, 12 and 17 which influences fibrosis phenotypes based on quantitative trait locus analysis [84]. Aravinthan *et al.* investigated the relationship between the variants of *CDKN1A* in different population and concluded that *CDKN1A* variant rs762623 related to the development but not the progression of liver disease in nonalcoholic fatty liver disease [85]. Lopez-Rodriguez *et al.* identified seven single nucleotide polymorphisms located in *IL-28B* (rs12979860), *JAK1* (rs11576173 and rs1497056), *TYK2* (rs280519), *OAS1* (rs2057778), *SOCS1* (rs33932899) and *RNASEL* (rs3738579) genes in severe necroinflammatory activity grade of chronic hepatitis C patients [86]. The genotypes of IL-10–1082G/A and TNF-α 308G/A expressed elevated levels of inflammatory cytokines in nonalcoholic steatohepatitis' patients can signify changes in liver functions, disease severity and to forecast the risk for progression [87].

Epigenetic mechanisms (DNA methylation, histone modification and noncoding RNA mediated gene silencing) regulate chromatin structure, modification and initiation of transcription are involved in fibrogenesis of liver. Epigenome is influenced by age, gender, environment and underlying genome through presence of single nucleotide polymorphisms [88,89]. Abnormal DNA methylation patterns are associated with inappropriate gene repression in liver fibrosis. Differential DNA methylation at peroxisome proliferator-activated receptor-γ promoter in cell free DNA may distinguish mild with severe liver fibrosis [90].

Table 7. Circulating miRNA signatures in liver disease.

Etiology	miRNA signature	Expression
ALD	miR-122 (acute alcohol, microsteatosis) miR-122 and miR-155 (chronic alcohol, macrosteatosis)	Increases Increases
NAFLD/NASH	miR-122, miR34a and miR-192	Increases
HCV	miR-122, miR-34a, miR-155, miR-125b, miR-146a and miR-21	Increases
HBV	miR-192 and miR-122	Increases
Liver fibrosis/ cirrhosis	miR-29 and miR-652 miR-513-3p and miR-571	Decreases Increases
HCC	miR-21, miR-16, miR-199a, miR-122, miR-223 and miR-885-5p	Increases
Drug overdose	miR-122 and miR-192	Increases

ALD: Alcoholic liver disease; HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; HCV: Hepatitis C virus; NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.
Reproduced with permission from [94].

Small, noncoding micro RNAs (miRNAs) regulate gene expression by binding to mRNA and control diverse biological functions viz., apoptosis, cell proliferation and differentiation [91,92]. Alterations of intracellular miRNAs play an important role in pathophysiology of chronic liver disease with different etiology. Normal liver homoeostasis requires miR-122 which regulates genes that are involved in hepatic cholesterol and lipid metabolism [93]. After chronic liver injury, HSCs' proliferation and differentiation into myofibroblast-like cells are regulated by mi-R221. miR-9, miR-21 and miR-188 regulate activation of myofibroblast, synthesis of extracellular proteins and collagen deposition. The broad variety of miRNAs which are involved in liver fibrosis and enters into systemic circulation can serve as potential biomarkers. Studies show that identification of circulating miRNA expression profiles are distinct between liver diseases with varied etiology (Table 7) [94].

Future perspective

Existing biomarkers for liver fibrosis in clinical practice have narrow applicability due to lack of specificity (predict etiology) and lack of sensitivity (distinguish intermediate stages). An ideal biomarker would give insights for diagnosis, monitor the activity of disease and assess therapeutic response. The determination of biomarkers could be an easy, noninvasive and inexpensive method to monitor the progression of liver fibrosis. This leads to urgency in the progression of biomarker discovery for liver fibrosis and hepatotoxicity with the help of advances in -omics approach. Discovery of biomarker candidates should be a simplified, unbiased, semi-quantitative binary comparison between diseased and normal. During discovery phase, the variables (study design, preanalytical and analytical) which affect precision should be minimized. Newly identified biomarker candidates need validation in terms of performance characteristics. Biomarker validation (analytical validation, clinical validation and clinical utility) links biomarker with biological process and clinical end point and is necessary for fit-for-purpose which helps research data for better patient care.

Analytical validation is to develop optimized assay which has consistency to measure the specific biomarker. Considerable care should be taken during the process in terms of preanalytical variation, interfering substance, indicators of accuracy, precision, analytical measurement range and proficiency testing procedures. Clinical validation gives diagnostic accuracy and discriminates between those with and without disease in terms of sensitivity, specificity, likelihood ratio and receiver operating characteristics curve. Likelihood ratio and receiver operating characteristics curve are derived from sensitivity and specificity values of a biomarker. Clinical utility of a biomarker is evaluated in a series of human population in terms of performance characteristics which needs stratified and sub population studies across geographical setting. To overcome the pitfalls in translation from biomarker discovery to clinical utility, there is a need for definite study design in selection of patients, proper biomarker validation and robustness in analytical techniques.

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Executive summary

Liver fibrosis

- Development of liver fibrosis is a pathological condition caused by varied etiology and is associated with significant morbidity and mortality.

Liver biopsy

- An invasive liver biopsy is still considered as a gold standard diagnostic tool for liver fibrosis associated with pain and complications.

Direct & indirect biochemical markers

- No doubt extracellular matrix markers and cytokines have diagnostic value, but they do not have much significance compared with routine biomarkers.
- Moreover, they are not organ specific and may also correlate with diseases in other organs.

Combinatorial biochemical markers

- Scoring system for diagnosis and prognosis of liver fibrosis plays a role only after pathological outcome.

Future perspectives for liver fibrosis markers

- Considering these limitations, successful management of liver fibrosis needs more reliable biomarkers which are specific to liver.
- In this regard, newly discovered biomarkers may have vital responsibility for assessment of chronic liver injury which needs further evaluation and statistical comparison with established biomarkers and panels.

Conclusion

- In present scenario, there is a need for extensive research to establish an accurate, precise, organ specific and sensitive noninvasive biomarker for early diagnosis, management and therapeutic monitoring of liver fibrosis.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1. Ebrahimi H, Naderian M, Sohrabpour AA. New concepts on pathogenesis and diagnosis of liver fibrosis; A review article. *Middle East J. Did. Dis.* 8, 166–178 (2016).
2. Wen-Ce Z, Quan-Bao Z, Liang Q. Pathogenesis of liver cirrhosis. *World J. Gastroenterol.* 20(23), 7312–7324 (2014).
3. Pellicoro A, Prakash R, Iredale JP. Reversibility of liver fibrosis. *Fibrogenesis Tissue Repair.* 5(Suppl. 1), S26 (2012).

• **Reversibility of liver fibrosis.**

4. Vicente A, Richard M, Patrik Sk *et al.* Acute on chronic liver failure in cirrhosis. *Nat. Rev. Dis. Primers* 2, 16041 (2016).
5. Gressner OA, Weiskirchen R, Gressner AM. Evolving concepts of liver fibrogenesis provide new diagnostic and therapeutic options. *Comp. Hepatol.* 6(7), doi:10.1186/1476–5926–6–7 (2007) (Epub ahead of print).
6. Scott LF. Hepatic Fibrosis. In: *Schiff's Diseases of the Liver*(11th Edition). Eugene R Schiff, Michael F Sorrell, Willis C Maddrey (Eds). Wiley-Blackwell, Singapore, 297–305 (2012).
7. Meena BB, Scott LF. Hepatic Fibrogenesis. In: *Sherlock's Diseases of the Liver and Biliary System*(11th Edition). James S Dooley, Anna SF Lok, Andrew K Burroughs, E Jenny Heathcote (Eds). Wiley Blackwell, Oxford, United Kingdom, 94–101 (2011).
8. Ekihiro S, David AB. Recent advancement of molecular mechanisms of liver fibrosis. *J. Hepatobiliary Pancreat. Sci.* 22(7), 512–518 (2015).
9. Bedossa P, Dargere D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. *Hepatology* 38, 1449–1457 (2003).
10. Carmen FB, Raluca P, Laura T, Anca B. Can we replace liver biopsy with non-invasive procedures? In: Liver Biopsy, Dr Hirokazu Takahashi (Ed.). InTech, doi 10.5772/19527 (2011).
11. Goodman ZD. Grading and staging systems for inflammation and fibrosis in chronic liver diseases. *J. Hepatol.* 47(4), 598–607 (2007).
12. Sangheun L, Do Young K. Non-invasive diagnosis of hepatitis B virus related cirrhosis. *World J. Gastroenterol.* 20(2), 445–459 (2014).
13. Pinzani M, Rombouts K, Colagrande S. Fibrosis in chronic liver disease: diagnostic and management. *J. Hepatol.* 42, 12–36 (2005).

14. Gressner AM, Gao CF, Gressner OA. Non invasive biomarkers for monitoring the fibrogenic process in liver: A short survey. *World J. Gastroenterol.* 15, 2433–2440 (2009).
15. Grigorescu M. Noninvasive biochemical markers of liver fibrosis. *J. Gastrointestin. Liver Dis.* 15, 149–159 (2006).
16. Afdhal NH, Nunes D. Evaluation of liver fibrosis: a concise review. *Am. J. Gastroenterol.* 127, 1704–1713 (2004).
17. Nielsen MJ, Kazankov K, Leeming DJ *et al.* Markers of collagen remodeling detect clinically significant fibrosis in chronic hepatitis C patients. *PLoS ONE* 10(9), e0137302 (2010).
18. Gressner OA, Weiskirchen R, Gressner AM. Biomarkers of liver fibrosis: clinical translation of molecular pathogenesis or based on liver dependent malfunction tests. *Clin. Chim. Acta* 381, 107–113 (2007).
19. Castera L. Noninvasive methods to assess liver disease in patients with hepatitis B or C. *Gastroenterology* 142, 1293–1302 (2012).
20. Jarcuska P, Janicko M, Veseliny E, Jarcuka P, Skladany L. Circulating markers of liver fibrosis progression. *Clin. Chim. Acta* 411(15,16), 1009–1017 (2010).
21. Cequera A, Garcia MC, Leon M. Biomarkers for liver fibrosis: advances, advantages and disadvantages. *Revista de Gastroenterologia de Mexico.* 79(3), 187–199 (2014).
22. Mc Hutchison JG, Blau LM, de Medina M *et al.* Measurement of serum hyaluronic acid in patients with chronic hepatitis C and its relationship to liver histology. Consensus Interferon Study Group. *J. Gastroenterol. Hepatol.* 145, 945–951 (2000).
23. Orasan OH, Ciulei G, Cozma A, Sava M, Dumitrascu DL. Hyaluronic acid as a biomarker of fibrosis in chronic liver diseases of different etiologies. *Clujul. Medical* 89(1), 24–31 (2016).
24. Walsh KM, Fletcher A, MacSween RNM, Morris AJ. Basement membrane peptides as markers of liver diseases in chronic hepatitis C. *J. Hepatol.* 32, 325–330 (2000).
25. Korner T, Kropf J, Gressner AM. Serum laminin and hyaluronan in liver cirrhosis: markers of progression with high prognostic value. *J. Hepatol.* 25, 684–688 (1996).
26. Hu B, Trinh K, Figueira WF, Price PA. Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family. *J. Biol. Chem.* 271, 19415–19420, (1996).
27. Yukiko S, Katsuya S, Yutaka Y *et al.* Noninvasive estimation of liver fibrosis and response to interferon therapy by a serum fibrogenesis marker, YKL- 40, in patients with HCV- associated liver disease. *World J. Gastroenterol.* 11(4), 476–481 (2005).
28. Sun J. Matrix metalloproteinases and tissue inhibitor of metalloproteinases are essential for the inflammatory response in cancer cells. *J. Signal. Transduct.*2010, 985132 (2010).
29. Murawaki Y, Ikuta Y, Idobe Y, Kawasaki H. Serum matrix metalloproteinase -1 in patients with chronic viral hepatitis. *J. Gastroenterol. Hepatol.* 14(2), 138–145 (1999).
30. Boeker KHW, Haberkorn CI, Michels D, Flemming P, Manns MP, Lichtinghagen R. Diagnostic potential of circulating TIMP-1 and MMP-2 as markers of liver fibrosis in patients with chronic hepatitis. *Clin. Chim. Acta* 316(1–2), 71–81 (2002).
31. Badra G, Lotfy M, El Refaie A *et al.* Significance of serum matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in chronic hepatitis patients. *Acta Microbiol. Immunol. Hung.* 57(1), 29–42 (2010).
32. Zhang BB, Min C, Weng HL *et al.* Diagnostic value of platelet derived growth factor-BB, transforming growth factor α1, matrix metalloproteinase -1 in serum and peripheral blood mononuclear cells for hepatic fibrosis. *World J. Gastroenterol.* 9(11), 2490–2496 (2003).
33. Pinzani M, Marra F. Cytokine receptors and signalling in hepatic stellate cells. *Semin Liver Dis.* 21(3), 397–416 (2001).
34. Harada KI, Shiota G, Kawasaki H. Transforming growth factor α and epidermal growth factor receptor in chronic liver disease and hepatocellular carcinoma. *Liver*19(4), 318–325 (1999).
35. Nelson DR, Gonzalez PRP, Qian K *et al.* Transforming growth factor-β1 in chronic hepatitis C. *J. Viral Hepat.* 4(1), 29–35 (1997).
36. Kanzler S, Baumann M, Schirmacher P *et al.* Prediction of progressive liver fibrosis in hepatitis C infection by serum and tissue levels of transforming growth factor-β. *J. Viral Hepat.* 8(6), 430–437 (2001).
37. Zhou J, Deng Y, Yan L, Zhao H, Wang G. Serum platelet derived growth factor BB levels: a potential biomarker for the assessment of liver fibrosis in patients with chronic hepatitis B. *Int. J. Infect. Dis.* 49, 94–99 (2016).
38. Pinzami M, Milani S, Herbst H *et al.* Expression of platelet-derived growth factor and its receptors in normal human liver and during active hepatic fibrogenesis. *Am. J. Pathol.* 148, 785–800 (1996).
39. Ikura Y, Morimoto H, Ogami M *et al.* Expression of platelet-derived growth factor and its receptor in livers of patients with chronic liver disease. *J. Gastroenterol.* 32, 496–501 (1997).
40. Yoshida S, Ikenaga N, Liu SB *et al.* Extra hepatic platelet-derived growth factor-beta, delivered by platelets, promotes activation of hepatic stellate cells and biliary fibrosis in mice. *Gastroenterology* 147, 1378–1392 (2014).
41. Susanne GS, Belinda M, Peer BC *et al.* Microfibrillar associated protein 4: a potential biomarker for screening for liver fibrosis in a mixed patient cohort. *PLoS ONE* 10(10), e0140418 (2015).

42. Greenlee KJ, Werb Z, Kheradmand F. Matrix metalloproteinases in lung: multipe, multifarious and multifaceted. *Physiol. Rev.* 87, 69–98 (2007).
43. Christian M, Barbara S, Corinna H *et al.* Detection of novel biomarkers of liver cirrhosis by proteomic analysis. *Heptology* 49, 1257–1266 (2009).
44. Chu PG, Weiss LM. Keratin expression in human tissues and neoplasms. *Histopathology* 40, 403–439 (2002).
45. Yilmaz Y, Dolar E, Ulukaya E *et al.* Soluble forms of extracellular cytokeratin 18 may differentiate simple steatosis from non-alcoholic steatohepatits. *World J. Gastroenterol.* 13, 837–844 (2007).
46. Yang M, Xu D, Liu Y *et al.* Combined serum biomarkers in non-invasive diagnosis of non-alcoholic steatohepatitis. *PLoS ONE* 10(6), e0131664 (2015).
47. Murawaki Y, Ikuta Y, Okamoto K *et al.* Diagnostic value of serum markers of connective tissue turnover for predicting histological staginig and grading in patients with chronic hepatitis C. *J. Gastroenterol.* 36, 399–406 (2001).
48. Rosenberg WMC, Voelker M, Thiel R *et al.* Serum markers detect the presence of liver fibrosis: a cohort study. *Gastroenterology* 127, 1704–1713 (2004).
49. Patel K, Gordon SC, Jacobson I *et al.* Evaluation of a panel of non-invasive serum markers to differentiate mild from moderate to advanced liver fibrosis in chronic hepatitis C patients. *J. Hepatol.* 41, 345–350 (2004).
50. Hind IF. Noninvasive biomarkers of liver fibrosis: an overview. *Adv. Hepatol.* 2014, 357287 (2014).
51. Dufour DR, Lott JA, Nolte FS *et al.* Diagnosis and monitoring of hepatic injury II. Recommendations for use of laboratory tests in screening, diagnosis and monitoring. *Clin. Chem.* 46, 2050–2068 (2000).
52. Paul YK, Stanley MC, Joseph KL. ACG Practice Guideline: evalution of abnormal liver chemistries. *Am. J. Gastroenterol.* 112(1), 18–35 (2016).
53. Kim WR, Flamm SL, Di Bisceglie AM *et al.* Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. *Hepatology* 47, 1363–1370 (2008).
54. Giannini E, Risso D, Botta F *et al.* Validity and clinical utility of the aspartate aminotransferase- alanine aminotransferase ratio in assessing disease severity and prognosis in patients with hepatitis C related chronic liver disease. *Arch. Intern. Med.* 163, 218–224 (2003).
55. Carl AB, Edward RA, David EB. *Teitz textbook of clinical chemistry and molecular diagnostics (15th edition).* Elsevier, India. 528, 574, 578, 581, 1681 (2012).
56. Wai CT, Greenson JK, Fontana RJ *et al.* A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. *Hepatology.* 38(2), 518–526 (2003).
57. Snyder N, Nguyen A, Gajula L *et al.* The APRI may be enhanced by the use of the FIBROSpect II in the estimation of fibrosis in chronic hepatitis C. *Clin. Chim. Acta* 381(2), 119–123 (2007).
58. Loaeza-del-Castillo A, Paz PF, Oviedo CE *et al.* AST to platelet ratio index (APRI) for the noninvasive evaluation of liver fibrosis. *Ann. Hepatol.* 7(4), 350–357 (2008).
59. Lok AS, Marc GG, Goodman ZD *et al.* Predicting cirrhosis in patients with hepatitis C based on standard laboratory tests: results of the HALT-C cohort. *Hepatology* 42(2), 282–292 (2005).
60. Parsian H, Alizadeh M, Yahyapour Y. Clinical application of non-invasive markers of liver fibrosis. In: *Practical Management Of Chronic Viral Hepatitis.* Servidido G (Ed.). InTech, Croatia (2013).
61. Sterling RK, Lissen E, Clumeck N *et al.* Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. *Hepatology* 43(6), 1317–1325 (2006).
62. Mallet V, Dhalluin VV, Roussin C *et al.* The accuracy of the FIB-4 index for the diagnosis of mild fibrosis in chronic hepatitis B. *Aliment. Pharmacol. Ther.* 29(4), 409–415 (2009).
63. Koda M, Matunaga Y, Kawakami M *et al.* FibroIndex, a practical index for predicting significant fibrosis in patients with chronic hepatitis C. *Hepatology* 45(2), 297–306 (2007).
64. Halfon P, Penaranda G, Renou C *et al.* External validation of FibroIndex. *Hepatology* 46(1), 280–281 (2007).
65. Imbert BF, Ratziu V, Pieroni L *et al.* Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* 357(9262), 1069–1075 (2001).
66. Poynard T, Imbert BF, Mona M *et al.* Overview of diagnostic value of biochemical markers of liver fibrosis (Fibro test, HCV Fibro Sure) and necrosis (Acti Test) in patients with chronic hepatitis C. *Comp. Hepatol.* 3(1), 8 (2004).
67. Poynard T, Munteanu M, Deckmyn O *et al.* Validation of liver fibrosis biomarker (Fibro Test) for assessing liver fibrosis progression: proof of concept and first application in a large population. *J. Hepatol.* 57(3), 541–548 (2012).
68. Forns X, Ampurdanes S, Josep ML *et al.* Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. *Hepatology* 36(4), 986–992 (2002).
69. Adams LA, George J, Burgianesi E *et al.* Complex noninvasive fibrosis models are more accurate than simple models in non-alcoholic fatty liver diseases. *J. Gastroenterol. Hepatol.* 26, 1536–1543 (2011).

70. Poynard T, Aubert A, Bedossa P *et al.* A simple biological index for detection of alcoholic liver disease in drinkers. *Gastroenterology* 100(5), 1397–1402 (1991).
71. Naveau S, Poynard T, Benattar C *et al.* Alpha-2-macroglobulin and hepatic fibrosis. *Digest. Dis. Sci.* 39(11), 2426–2432 (1994).
72. Soresi M, Lydia G, Cervello M *et al.* Non invasive tools for the diagnosis of liver cirrhosis. *World J. Gatroenterol.* 20(48), 18131–18150 (2014).
73. WHO library cataloguing in publication data. Guidelines for the prevention, care and treatment of persons with chronic hepatitis B infection. WHO March 2015. http://apps.who.int/iris/bitstream/10665/154590/1/9789241549059_eng.pdf
74. Houot M, Ngo Y, Munteanu M *et al.* Systematic review with meta analysis: direct comparisons of biomarkers for the diagnosis of fibrosis in chronic hepatitis C and B. *Aliment. Pharmacol. Ther.* 43, 16–29 (2016).
75. Cherng S, Young J, Hongbao Ma. Alpha-smooth muscle actin (α-SMA). *J. Am. Sci.* 4(4), 7–9 (2008).
76. Carpino G, Morini S, Merlik M *et al.* Alpha-SMA expression in hepatic stellate cells and quatative analysis of hepatic fibrosis in cirrhosis and in recurrent chronic hepatitis after liver transplantation. *Digest. Liver Dis.* 37(5), 349–356 (2005).
77. Rockey DC, Weymouth N, Shi Z. Smooth muscle α actin (Acta2) and myofibroblast function during hepatic wound healing. *PLoS ONE* 8(10), e77166 (2013).
78. Maieron A, Salzl P, Peck-Radosavljevic M *et al.* Von Willebrand Factor as a new marker for non-invasive assessment of liver fibrosis & cirhhosis in patients with chronic hepatitis C. *Aliment. Pharmacol. Ther.* 39, 331–338 (2014).

•• **New biomarker and score establishment.**

79. Gangadharan B, Manisha B, Rossa J *et al.* Discovery of novel biomarker candidates for liver fibrosis in hepatitis C patients: a preliminary study. *PLoS ONE* 7(6), e39603 (2012).

• **Proteomic approach for biomarker candidate discovery.**

80. Zhiyun C, Yinghui LV, Suqiu P *et al.* Kallistatin, a new and reliable biomarker for the diagnosis of liver cirrhosis. *Acta Pharma. Sin. B* 5(3), 194–200 (2015).

• **Plasma protein as new biomarker for chronic liver injury.**

81. Irvine KM, Wockner LF, Hoffmann I *et al.* Multiplex serum protein analysis identifies novel biomarkers of advanced fibrosis in patients with chronic liver disease with the potential to improve diagnostic accuracy of established biomarkers. *PLoS ONE* 11(11), e0167001 (2016).
82. Hu Q, Wei J, Liu Y *et al.* Discovery and identification of potential biomarkers for alcohol-induced oxidative stress based on cellular metabolomics. *Biomed. Chromatogr.* 31(7), doi:10.1002/bmc.3907 (2016) (Epub ahead of print).
83. Zeng DW, Dong J, Jiang JJ *et al.* Ceruloplasmin, a reliable marker of fibrosis in chronic hepatitis B virus patients with normal or minimally raised alanine aminotransferase. *World J. Gastroenterol.* 22(43), 9586–9594 (2016).
84. Hall RA, Liebe R, Hochrath K *et al.* Systems genetics of liver fibrosis: identification of fibrogenic and expression quantitative trail loci in the BXD murine reference population. *PLoS ONE* 9(2), e89279 (2014).
85. Aravinthan A, Mells G, Allison M *et al.* Gene polymorphisms of cellular senescence marker p21 and disease progression in non-alcohol-related fatty liver disease. *Cell Cycle* 13(9), 1489–1494 (2014).
86. Lopez-Rodriguez R, Hernandez-Bartolome A, Borque MJ *et al.* Interferon-related genetic markers of necroinflammatory activity in chronic hepatitis C. *PLoS ONE* 12(7), e0180927 (2017).

• **Single nucleotide polymorphisms as biomarkers for liver fibrosis.**

87. Bocsan IC, Milaciu MV, Pop RM *et al.* Cytokines genotype-phenotype correlation in nonalcoholic steatohepatitis. *Oxid. Med. Cell. Longev.*2017, 4297206 (2017).

•• **Genotype of proinflammatory cytokine genes to assess liver function.**

88. Bartke T, Vermeulen M, Xhemalce B, Robson SC, Mann M, Kouzarides T. Nucleosome-interacting proteins regulated by DNA and histone methylation. *Cell* 143, 470–484 (2010).
89. Dongen JV, Nivard MG, Willemsen G *et al.* Genetic and environmental influences interact with age and sex in shaping the human methylome. *Nat. Commun.* 7, 11115 (2016).
90. Moran-Salvador E, Mann J. Epigenetics and liver fibrosis. *Cell. Mol. Gastroenterol. Hepatol.* 4, 125–134 (2017).

•• **Role of epigenetics in liver fibrosis.**

91. Ambros V. The functions of animal microRNAs. *Nature*431, 350–355 (2004).
92. Kren BT, Wong PY, Sarver A, Zhang X, Zeng Y, Steer CJ. Micro RNAs identified in highly purified liver-derived mitochondria may play a role in apoptosis. *RNA Biol.* 20, 65–72 (2009).
93. Lanford RE, Hildebrandt-Eriksen ES, Petri A *et al.* Therapeutic silencing of microRNA-122 in patients with chronic hepatitis C virus infection. *Science* 327, 198–201 (2010).
94. Szabo G, Shashi B. MicroRNAs in liver disease. *Nat. Rev. Gastroenterol. Hepatol.* 10(9), 542–552 (2013).

•• **Circulating micro-RNAs as biomarkers for liver fibrosis.**

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Cirrhosis of liver: Interference of serpins in quantification of SERPINA4 – A preliminary study

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A B S T R A C T

Background: Cirrhosis of liver is a pathological condition, wherein functions of liver are impaired by chronic liver exploitations. Due to decrease in synthetic capacity, expressions of plasma proteins tend to decrease in blood stream. Serpins (Serine protease inhibitors) are class of plasma proteins expressed from liver with structural similarities and diverse functions. SERPINA4 (Kallistatin) is a multifunctional serpin clade A protein expressed from liver and concentration in serum is the reflection of extent of liver dysfunction.

Objective: To identify interference of other serpins by immunological cross reactivity with SERPINA4 in cirrhotic liver and healthy subjects.

Materials and methods: Blood samples were collected from 20 subjects (10 cirrhotic liver, 10 healthy) from R.L. Jalappa Hospital and Research Centre, Kolar, Karnataka, India. Separation of proteins was carried out by SDS-PAGE. Cross reactivity study was analyzed using western blot.

Results: Proteins present in cirrhotic liver and healthy subject's serum were separated by SDS PAGE. There was no band detection on both (cirrhotic liver and healthy) PVDF (polyvinylidene diflouride) membranes. However, a significant band was observed with recombinant kallistatin.

Conclusion: Structurally similar serpins with minor amino acid sequence similarities did not show any immunological cross reactivity with SERPINA4 due to non identical epitope in cirrhotic liver and healthy subjects. Present study revealed that there is no interference of serpins for immunological reactions in quantitative estimation of kallistatin which needs further validation.

1. Introduction

Cirrhosis of liver is a pathological condition characterized by diffuse fibrosis, severe disruption of intra hepatic arterial and venous flow, portal hypertension and finally liver failure resulting from varied etiologies of chronic liver diseases [1]. Despite different etiological factors, pathological characteristics, degeneration, necrosis of hepatocytes, replacement of parenchyma by fibrotic tissue, regenerative nodules; loss of liver functions are common [2]. Liver is a major organ with synthetic capacity to produce plasma proteins. Reduction in concentration of plasma proteins is reflected as decreased hepatic synthesis [3].

Serpins (Serine Protease Inhibitors) are class of plasma proteins that have similar structure and diverse functions. Serpins are divided into clades based on sequence similarities. In humans (clades A to I), 36 serpin coding genes and 5 pseudogenes are identified based on phylogenetic relationship [4]. Extracellular clade A molecules are localized on chromosomes 1, 14 and X. Intracellular clade B serpins are localized on chromosome 6 and 18 [5]. Serpins are interrelated due to highly conserved core structure [6]. Majority of clade A serpins are localized on chromosome 14 which are expressed from liver.

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SERPINA1, (α1-antitrypsin) is an inhibitor of neutrophil elastase [5]. Pseudogene SERPINA2 indicates an ongoing process of pseudogenization [7]. Antichymotrypsin, SERPINA3 is an inhibitor of chymotrypsin and cathepsin G found in blood, liver, kidney and lungs [5]. SERPINA5 inhibits active C protein and are expressed from liver [4]. Non inhibitory hormone binding protein, SERPINA6 is a cortisol transporter [8]. SERPINA9 which is expressed from liver plays an important role in maintaining native B cell [4]. The inhibitory protein of activated coagulation factors Z and XI is SERPINA10 [5]. SERPINA11 is a pseudogene and uncharacterized [4]. SERPINA12 is an inhibitory protein of kallikrein and plays a role in insulin sensitivity [9].

Kallistatin (SERPINA4, serpin family A member 4, tissue kallikrein inhibitor), belongs to clade A serpins encoded by the SERPINA4 gene with 5 exons and 4 introns mapped to chromosome 14q31-32.1 in humans and expressed from liver cell lines (Hep G2 and Hep 3B). It is an acidic glycoprotein with a molecular weight of 58kD and isoelectric pH ranges from 4.6 to 5.2 [10,11]. Apart from inhibitory action on human tissue kallikrein, it is a potent vasodilatory protein [12]. Kallistatin is involved in prevention of cancer, cardiovascular disease and arthritis through the effects of antiangiogenic, anti-inflammatory, antiapoptotic and antioxidative properties [13].

Kallistatin concentration in serum depends on the degree of severity of different chronic liver diseases (fibrosis, cirrhosis and hepatocellular carcinoma) [13]. Interference of other serpins with antibodies may give a significant false positive/negative value in quantitative estimations of kallistatin, which may mislead in assessment of extent of the disease. Hence, in the present study, an attempt has been made to identify immunological cross reactivity between kallistatin and other serpins in cirrhotic liver and compared with healthy subjects.

2. Materials and methods

2.1. Samples

Blood samples were collected from 20 subjects: 10 clinically and diagnostically proven cirrhotic liver subjects with varying degree, age and gender matched 10 healthy subjects **(**Table 1**)** from R. L. Jalappa Hospital and Research Centre, Kolar, Karnataka, India. Collection of blood samples from cirrhotic liver and healthy subjects was carried out after obtaining informed consent and study is approved by Institutional Ethical Committee (DMC/KLR/IEC/61/2016-17).

2.2. Serum preparation

Serum was collected from clotted blood using serum separator tubes centrifuged at 4000 rpm for 10 min. Serum was stored at − 20 °C for further analysis. All the samples were used to find cross reactivity of other serpins with kallistatin by western blot after protein segregation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. Reagents

Primary monoclonal antibodies specific for kallistatin along with secondary antibodies and recombinant kallistatin were procured from R & D systems, USA. Other chemicals of analytical grade were procured from Bio-Rad and Sigma Aldrich, USA.

2.4. SDS-PAGE analysis

SDS gels were prepared as per standard protocol. Cirrhotic liver and healthy subject's serum samples were loaded in different gels and SDS-PAGE was carried with duplication at 25 mA (2 gels run @ 50 mA) in 1X SDS running buffer. After electrophoresis, gels were incubated in fixing solution (7% acetic acid, 10% methanol) at room temperature for 20 min. At this point, the gels were transferred onto a PVDF (polyvinylidene diflouride) membrane for western blot and duplicate gels were subjected for staining with colloidal Coomassie brilliant blue in a shaker at room temperature for 2 h. Excess staining solution was removed and the gels were washed

Table 1
Details of 20 blood samples (10 liver cirrhotic subjects, age and gender matched 10 healthy subjects) used for SDS PAGE and Western blot.

Sample ID	Gender	Age	Etiology	Sample ID	Gender	Age	Etiology
C1	M	36	NA	D1	M	36	ALD
C2	M	28	NA	D2	M	28	ALD
C3	M	62	NA	D3	M	62	ALD
C4	F	26	NA	D4	F	26	NAFLD
C5	M	35	NA	D5	M	35	ALD
C6	F	26	NA	D6	F	26	NAFLD
C7	M	70	NA	D7	M	70	ALD
C8	M	30	NA	D8	M	30	ALD
C9	M	62	NA	D9	M	62	ALD
C10	M	30	NA	D10	M	30	ALD

Abbreviations: C: Control; D: Diseased (Cirrhosis of liver); M: Male; F: Female; NA: Not Applicable; ALD: Alcoholic Liver Disease; NAFLD: Non Alcoholic Fatty Liver Disease.

with 10% acetic acid and placed in deionized water for destaining till the appearance of bands [14,15].

2.5. Western blot analysis

Proteins separated by SDS-PAGE were transferred onto PVDF membranes using a Transblot-Blot SD semi dry transfer cell (Bio-Rad) at 15 V for 2 h (1X transfer buffer: Tris/Glycine with 20% Methanol). After transfer, PVDF membranes were kept for blocking using blocking buffer (5% skimmed milk powder in 1X PBST) and incubated over night at 4 °C. After overnight blocking, PVDF membranes were washed with 1X PBST thrice for 3 min each. Primary antibodies were diluted (1:100) and PVDF membranes were incubated in diluted primary antibody solution at room temperature with slow shaking on rocker for 2–3 h. PVDF membranes were washed with 1X PBST thrice for 3 min each.

Secondary antibody was diluted (1:5000) and PVDF membranes were incubated in diluted secondary antibody solution at room temperature with slow shaking on rocker for 2–3 h. After incubation, PVDF membranes were washed with 1X PBST thrice for 3 min each. 12.5 mL Tris buffer (pH 7.35), 30 µl of 30% H_2O_2, a pinch of DAB were added into detection tray, mixed well and PVDF membranes were kept into the tray. The tray was gently shaken for a period of 10 min until the colour developed in the control lane [16,17]. SDS-PAGE and western blot were repeated with pooled and concentrated cirrhotic liver and healthy serum samples along with recombinant kallistatin.

2.6. Concentration of serum proteins by dialysis using solid sucrose

Dialyzing tube containing serum to be concentrated is coiled up in a beaker and covered with commercial sucrose for 4 h. The liquid accumulated outside the dialyzing bag was poured off. Tubing was removed from the sugar at the end of 4 h and is tied off above the solution placed in water to dialyze away the sugar [18].

3. Results

3.1. Separation of proteins

Since SDS-PAGE is an efficient tool for separation of proteins based on molecular weight, proteins in serum were separated in both diseased (D1–D10) and healthy (C1–C10) gels along with corresponding molecular weight marker. Recombinant kallistatin was spotted on another SDS-PAGE with pooled and concentrated samples of cirrhotic liver and healthy subjects.

3.2. Immunological cross reactivity analysis

Western blot analysis allowed identification of cross reactivity of serpins in diseased and healthy samples using monoclonal antibodies specific for kallistatin followed by secondary antibodies conjugated with HRP. No bands were observed on PVDF membranes of diseased **(**Fig. 1**a)** as well as healthy **(**Fig. 1**b)** samples. However, a significant band was observed with recombinant kallistatin. There was no band detection with pooled and concentrated samples of diseased and healthy **(**Fig. 1**c)** indicating that there is no cross reactivity of other serpins with kallistatin.

4. Discussion

Serpins are broadly distributed family of protease inhibitors which circulates in blood and are mainly expressed from liver [19]. Highly conserved similar structure (native, monomeric, active, latent, cleaved, delta and polymeric proteins) of serpins are crucial for their inhibitory function and play an important role in haemostasis and fibrinolysis [4,6]. These proteins are suicide or single use inhibitors that use conformational changes to inhibit target enzymes [20]. Inhibitor binds tightly to a protease by incorporating

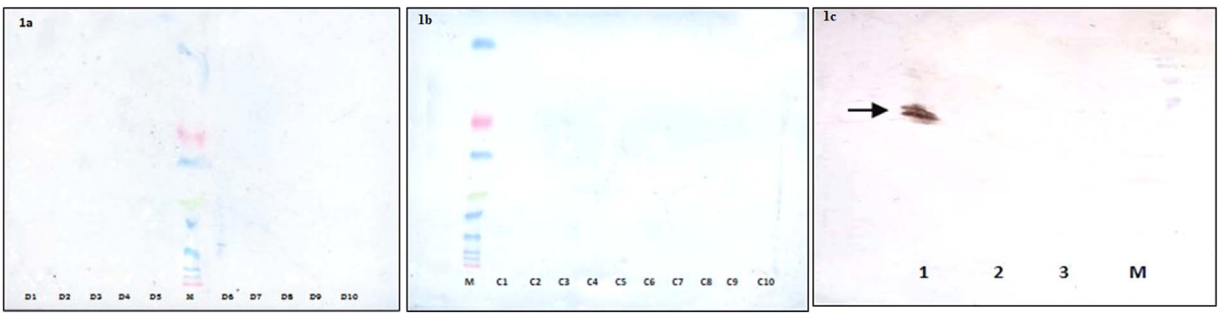

Fig. 1. a: Western blot with diseased serum; M: Marker; D: Diseased subjects (Cirrhosis of Liver); **b:** Western blot with control serum; M: Marker; C: Healthy subjects; **c:** Western blot with Recombinant kallistatin; 1: Recombinant kallistatin; 2: Pooled and concentrated diseased serum; 3: Pooled and concentrated control serum; 4: Marker; **Arrow: Detection of band with recombinant kallistatin**.

reactive centre loop of inhibitor into β sheet of the enzyme by forming SDS and heat stable complex [6].

A highly conserved secondary and tertiary structure is the main criteria for the classification with modest amino acid similarities [4]. Despite chromosomal proximity, these genes have divergent function [21]. Serpin genes are present in clusters on same chromosome with common precursor. The human genes encoding α1-antitrypsin, corticosteroid-binding globulin, α1-antichymotrypsin and protein C inhibitor are mapped to the chromosome 14q32.1. Kallistatin is also mapped within the region on the same chromosome [22,23].

In spite of similarity in chemical properties having minor amino acids sequence resemblance and mapped on same gene, our study did not show any cross reactivity between serpin class proteins in cirrhotic liver and healthy subjects which may be attributed due to absence of identical epitope among serpins. Cross reactivity occurs when two different serpins share an identical epitope. Epitope comprises approximately 15 amino acids of which 5 amino acids influence strongly for binding to definite paratope of Fab region on variable domain of antibody [24]. Due to the absence of identical epitope among serpins might be reason for no cross reactivity in cirrhotic liver and healthy subjects. There will be reduced expression of serpin proteins into blood stream due to decreased synthetic function of liver in cirrhotic liver subjects.

Molecular basis of polymerization is induced by mutations or mild denaturation which is common for all serpins. The conformational change in the serpin structure is crucial for functions and which also is susceptible reason for mutations [6]. Mutations which bring about polymerization can also occur anywhere in the serpin and leads to formation and accumulation of stable polymers with similar properties [25,26]. Serpin polymerization can also occur through domain swapping as recorded in antithrombin, α-1 antitrypsin and neuroserpin, which needs further studies to evaluate domain swapping polymerization of entire serpin family proteins, [27–29]. Polymerization leads to reduction in serpin secretion with qualitative changes in protein structure [6]. The etiological factors of cirrhosis of liver may not induce polymerization which directs to share identical epitope of serpin family proteins. This may be the reason why no cross reactivity was observed in cirrhotic liver subjects in our study.

Even though, incidence of diseases caused by serpin polymerization is rare, homozygous mutations in SERPINA1 gene (α1 antitrypsin) is associated with liver disease including cirrhosis. Human variants of serpin genes has been found in large number as a resultant of mutations which are associated with many diseases **(**Table 2**)** [4,30]. SERPINA1 alone has 1411 SNPs; SNPs for SERPINA4 are 906 in NCBI's dbSNP database (Accessed: July 2017). Mutational studies in terms of cross reactivity, for identification of identical epitope, might be difficult at this point because of huge diversity of serpins.

Concentration of kallistatin is less in cirrhotic liver as well as in healthy subjects. Hence, the sensitivity of monoclonal antibodies (5 ng/lane, by manufacturer's instructions) might not detect kallistatin. In case of any cross reactivity, these antibodies may detect other serpins whose concentrations are in nanograms in serum. Use of more sensitive antibodies might detect kallistatin in cirrhotic liver as well as in healthy subjects and enhance successful immunological interactions of other serpins. For separation of proteins, 2 dimensional electrophoresis (2-DE) might be better option than single dimensional SDS-PAGE.

5. Conclusion

In the present study, no immunological cross reactivity was observed between serpins and SERPINA4 (kallistatin) due to the absence of identical epitope in cirrhotic liver and healthy subjects. Because of enormous diversity of serpins, validation of quantitative ELISA should be carried out to check interference of other factors (buffer components, sample matrix, compliment and rheumatoid factor) along with cross reactivity by using different types of antibodies. Further quantitative studies of Kallistatin may provide insights into potential diagnostic options for chronic liver diseases.

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Table 2
Classification of serpin clade A, chromosomal location, polymerization associated diseases.

Sl. no.	Name	Symbols	Synonyms	Chromosome	Associated diseases
1	SERPINA1	PI	α-1-antitrypsin, AAT	14q32.13	Emphyesma, Chronic Liver Disease, Vasculitis
2	SERPINA2	PIL	ATR, ARGS	14q32.13	–
3	SERPINA3	AACT	ACT	14q32.13	Emphyesma
4	SERPINA4	PI4	KST, KAL, KLST, Kallistatin	14q32.13	Renal and Cardiovasular Injury
5	SERPINA5	PLANH3	PA13, PROCI	14q32.13	Angiodema, Papillary thyroid cancer
6	SERPINA6	CBG	–	14q32.13	Chronic fatigue syndrome
7	SERPINA7	TBG	–	Xq22.3	Deficiency results in hypothyroidism
8	SERPINA8	AGT	–	1q42.2	Certain varients linked to essential hypertension
9	SERPINA9	–	CENTERIN	14q32.13	–
10	SERPINA10	–	PZI	14q32.13	Risk of venous thromboembolism, Pregnancy Complications
11	SERPINA11	–	–	14q32.13	–
12	SERPINA12	–	Vaspin, OL-64	14q32.13	Associated with Insulin resistance

References

[1] N. Salman, S.K. Muhammad, F. Javid, F.M. Mohammad, Cirrhosis and its complications: evidence based treatment, World J. Gastroenterol. 20 (18) (2014) 5442–5460.
[2] Z. Wen-Ce, Z. Quan-Bao, Q. Liang, Pathogenesis of liver cirrhosis, World J. Gastroenterol. 20 (23) (2014) 7312–7324.
[3] M. Sandeep, L.G. John, Assessment of liver function, in: James S. Dooley, Anna S.F. Lok, Andrew K. Burroughs, E. Jenny Heathcote (Eds.), Sherlock's Diseases of the Liver and Biliary System, 12th edition, Wiley Blackwell, Oxford, 2011, pp. 30–31.
[4] H. Claire, C.J. Brain, M. McAndrews, W.W. Mathew, C.T. David, A.S. Gary, et al., Update of the human and mouse SERPIN gene super family, Hum. Genom. 7 (22) (2013).
[5] H.P.L. Ruby, Z. Qingwei, S. McGowan, M.B. Ashley, A.S. Gary, W. Wilson, et al., An overview of the serpin super family, Genom. Biol. 7 (216) (2006), http://dx.doi.org/10.1186/gb-2006-7-5-216.
[6] J.A. Huntington, Serpin structure, function and dysfunction, J. Thromb. Haemost. 9 (Suppl. 1) (2011) S26–S34.
[7] S. Seixas, G. Suriano, F. Carvalho, R. Seruca, J. Rocha, A. Di Rienzo, A sequence diversity at the proximal 14q32.1 SERPIN subcluster: evidence for natural selection favouring the pseudogenization of SERPINA2, Mol. Biol. Evol. 24 (2007) 587–598.
[8] J.A. Irving, R.N. Pike, A.M. Lesk, J.C. Whisstock, Phylogeny of the serpin super family: implications of patterns of amino acid conservations for structure and function, Genom. Res. 10 (2000) 1845–1864.
[9] J.T. Heiker, N. Kloting, P. Kovacs, E.B. Kuettner, N. Strater, S. Schulyz, et al., Vaspin inhibits kallikrein 7 by serpin mechanism, Cell. Mol. Life Sci. 70 (2013) 2569–2683.
[10] J. Chao, K.X. Chai, L. Chao, Tissue kallikrein inhibitors in mammals, Immunopharmacology 32 (1–3) (1996) 67–72.
[11] X.Z. Gray, L. Chao, J. Chao, Kallistatin: a novel human tissue kallikrein inhibitor, J. Biol. Chem. 267 (36) (1992) 25873–25880.
[12] C.W. William, A.H. Russell, S. Dan, L. Chao, J. Chao, Localization and expression of tissue kallikrein and kallistatin in human blood vessels, J. Histochem. Cytochem. 47 (2) (1999) 221–228.
[13] C. Zhiyum, L.V. Yinghui, P. Suqiu, B. Ruyu, W. Minggxi, L. Shuyu, et al., Kallistatin, a new and reliable biomarker for the diagnosis of liver cirrhosis, Acta Pharma. Sin. B 5 (3) (2015) 194–200.
[14] D.E. Garfin, One dimensional gel electrophoresis, Methods Enzymol. 463 (2009) 497–513.
[15] L.M. Hjemel, A. Chrambach, Electrophoresis and electrofocusing in detergent containing media: a discussion of basic concepts, Electrophoresis 2 (1981) 1–11, http://dx.doi.org/10.1002/elps.1150020102.
[16] 〈http://www.afcs.org/reports/v1/BC0001/Protocols/Protein%20Transfer%20BIORAD.pdf〉.
[17] Bio-Rad Criterion™ Blotter assembly Instruction Manuals.
[18] N.H. Horowitz, M. Fling, A method for concentrating dilute protein solutions, Fungal Genet. Rep. 2 (18) (1962), http://dx.doi.org/10.4148/1941-4765.1057.
[19] J.A. Irving, R.N. Pike, A.M. Lesk, J.C. Whisstock, Phylogeny of the serpin super family: implications of patterns of amino acid conservations for structure and function, Genom. Res. 10 (2000) 1845–1864.
[20] J.A. Huntington, R.J. Read, R.W. Carrell, Structure of a serpin protease complex shows inhibition by deformation, Nature 407 (2000) 923–926.
[21] C.M. Rein, U.R. Desar, F.C. Church, Serpin-glycosaminoglycan interactions, Methods Enzymol. 501 (2011) 105–137.
[22] G.D. Billingsley, M.A. Walter, G.L. Hammond, D.W. Cox, Physical mapping of four serpin genes: alpha 1-antitrypsin, alpha 1-antichymotrpsin, corticosteroid-binding globulin and protein C inhibitor, within a 280-kb region on chromosome 14q32.1, Am. J. Hum. Genet. 52 (1995) 343–353.
[23] P. Rolini, R.E. Fournier, A 370-kb cosmid contig of the serpin gene cluster on human chromosome 14q32.1: molecular linkage of genes encoding alpha 1-antichymotrpsin, protein C inhibitor, kallistatin, alpha 1-antitrypsin and corticosteroid-binding globulin, Genomics 46 (1997) 409–415.
[24] S.A. Frank, Immunology and Evolution of Infectious Disease, Princeton University Press, New Jersey, 2002.
[25] D. Belorgey, P. Hagglof, S. Karlsson-Li, D.A. Lomas, Protein misfolding and the serpinopathies, Prion 1 (2007) 15–20.
[26] A.S. Knaupp, S.P. Bottomley, Serpin polymerization and its role in disease – the molecular basis of α1-antitrypsin deficiency, IUBMB Life 61 (1) (2009) 1–5.
[27] M. Yamasaki, W. Li, D.J. Johnson, J.A. Huntington, Crystal structure of a stable dimer reveals the molecular basis of serpin polymerization, Nature 455 (7217) (2008) 1255–1258.
[28] S. Takehara, J. Zhang, X. Yang, N. Takahashi, B. Mikami, M. Onda, Refolding and polymerization pathways of neuroserpin, J. Mol. Biol. 403 (5) (2010) 751–762.
[29] A. Ordonez, I. Martinez-Martinez, F. Corrales, C. Miqueo, A. Minano, V. Vicente, et al., Effect of citrullination on the function and conformation of antithrombin, FEBS J. 276 (22) (2009) 6763–6772.
[30] R.W. Carrell, D.A. Lomas, Conformational disease, Lancet 350 (9071) (1997) 134–138.

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Mini Review

Molecular and Epigenetic Mechanisms of Bidirectional Liver Fibrosis -

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ABSTRACT

Liver fibrosis is natural wound healing response to different etiologies of chronic liver insults leading to accumulation of Extra Cellular Matrix (ECM) due to imbalance between synthesis and degradation. Fibrogenesis is consequence of multicellular response; activation of Hepatic Stellate Cells (HSCs) and transdifferentiation into myofibroblasts are crucial for development of hepatic scar. Recent studies evidenced that liver fibrosis is potentially bidirectional regulated by complex cytokines, growth factors, genetic and epigenetic mechanisms (DNA methylation, histone modifications and miRNAs mediated gene silencing). Regression of liver fibrosis is due to increase in collagenolytic activity and increased Metalloproteinase (MMPs) activity with decreased expression and activity of Tissue Inhibitors of Metalloproteinases (TIMPs). Reversible epigenetic mechanisms, pro-fibrotic and anti-fibrotic miRNAs regulate progression and regression of liver fibrosis which initiates to discover diagnostic, prognostic and therapeutic should be comprehensively defined. Hence, in this review we made an attempt to understand molecular, genetic and epigenetic mechanisms of bidirectional liver fibrosis.

Keywords: Liver fibrosis; Hepatic regeneration; Cytokines; Epigenetics

ABBREVIATIONS

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GLDH: Glutamate Dehydrogenase; γGT: Gamma Glutamyl Transferase; LDH: Lactate Dehydrogenase; CRP: C-Reactive Protein; α2M: α 2 Macroglobulin; CTGF: Connective Tissue Growth Factor; PIIINP: Procollagen III amino peptide; MMPs: Matrix Metallo Proteinases; TIMPs: Tissue Inhibitors of Metalloproteinases; HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; ASH: Alcoholic Steatohepatitis; NAFLD: Non Alcoholic Fatty Liver Diseases; IGF-1: Insulin like Growth Factor 1; EMT: Epithelial Mesenchymal Transition; TGF-β: Transforming Growth Factor β; PDGF: Platelet Derived Growth Factor; ET-1: Endothelin-1; ROS: Reactive Oxygen Species

INTRODUCTION

Liver, a vital organ performs several crucial functions; substrate metabolism, detoxification, protein and digestive enzyme synthesis and immune response for human survival [1]. Being highly vascular organ, it is continuously exposed to injury and damage by hepatotoxins *viz* viruses, drugs, alcohol, excess fat etc., leading to inflammation and fibrosis [2]. Liver fibrosis is natural wound healing response to chronic liver insults which involves deposition of Extra Cellular Matrix (ECM). Accumulated ECM destroys liver by forming fibrotic scar and subsequent nodular development ultimately leading to liver cirrhosis. Fibrotic liver contains three to ten times more ECM which in turn distorts liver parenchyma and vascular architecture and results in liver dysfunction. Hepatic Stellate Cells (HSCs) are ECM producing cells in fibrotic liver effective after activation and trans-differentiation into myofibroblasts which attains contractile, inflammatory and fibrogenic properties (Figure 1). HSCs activation results from interactions with damaged hepatocytes, Kupffer cells, disintegrated platelets and sub-fractions of leucocytes [3,4]. Progression of inflammatory and fibrogenic pathways are mediated by cytokines, genetic and epigenetic mechanisms. After chronic liver injury, fibrogenesis starts with necrosis or apoptosis of hepatocytes and inflammation connected activation of hepatic stellate cells, their trans-differentiation to myofibroblasts with enhanced expression and secretion of extracellular matrix and deposition which attains contractile, proinflammatory and fibrogenic property. In pathophysiology of liver fibrogenesis, Transforming Growth Factor-β (TGF-β), Platelet Derived Growth Factor (PDGF), endothelin-1 and Vascular Endothelial Growth Factor (VEGF) play a dominant role [4]. Genes regulating hepatocellular damage, inflammatory response to injury and Reactive Oxygen Species (ROS) generation regulates extent of hepatic damage, inflammation and ECM deposition [5]. Epigenetic mechanisms [DNA methylation, histone modifications and noncoding micro RNA (miRNA)] have been shown to orchestrate many aspects of fibrogenesis of liver [6]. Recent studies have shown that liver fibrosis is dynamic and potentially bidirectional process. Treatment aimed at underlying cause especially at early stage of the disease can reverse fibrosis to normal liver architecture by spontaneous resolution of hepatic scar. Reasons for resolution may be due to increase in collagenolytic activity, increased Matrix Metalloproteinase (MMPs) activity and decreased expression of Tissue Inhibitors of Metalloproteinase (TIMPs). Cytokine mobilization of bone marrow derived stem cells restores neutrophil function and promotes hepatic regeneration [3]. The stage at which disease become irreversible is not well established but it is believed that irreversibility attains once septal neovascularisation happens and portal pressure increases significantly [7]. Hence, we have made an attempt in this review to understand the molecular and epigenetic mechanisms involved in bidirectional liver fibrogenesis.

PATHOPHYSIOLOGY OF BIDIRECTIONAL LIVER FIBROSIS

Natural wound healing response of liver for chronic liver injury results in the formation of hepatic scar leading to fibrosis of liver. After an acute injury, liver parenchymal cells regenerate and replace the necrotic and apoptotic cells. If hepatic injury persists, there will be failure in hepatic regeneration and substitution of hepatocytes with abundant ECM having contractile, inflammatory and fibrogenic properties [8]. Different types of cells (resident innate inflammatory cells, hepatocytes, liver sinusoidal endothelial cells and Kupffer cells) play a role in liver fibrogenesis. Activation of HSCs is a crucial step in inter-linked process of tissue injury and regeneration [9]. Quiescent HSCs present in space of Disse will be activated and trans-differentiate into myofibroblasts like cells which are responsible for ECM production and accumulation in injured liver [10]. Accumulation of ECM is due to increased synthesis and decreased degradation by over expression of TIMPs which inhibits MMPs [9]. Fibrotic liver contains three to ten times more ECM compared to normal liver includes collagen types, glycoproteins, proteoglycans and glycosaminoglycans [3]. Chief mitogen of HSCs activation is PDGF produced by Kupffer cells; macrophages are source of pro-fibrotic chemokines [11,12]. Activated HSCs activate immune response by secretion of cytokines, chemokines and interacting with immune cells. Complex network of cytokines (Table 1) modify activities of circulating immune cells, HSCs, hepatocytes, liver sinusoidal endothelial cells and Kupffer cells (Figure 2). Autocrine and paracrine secretions of cytokines activate and trans-differentiate HSCs into myofibroblasts [9]. Activated HSCs migrates to tissue repair site and secrete ECM; collagen synthesis is

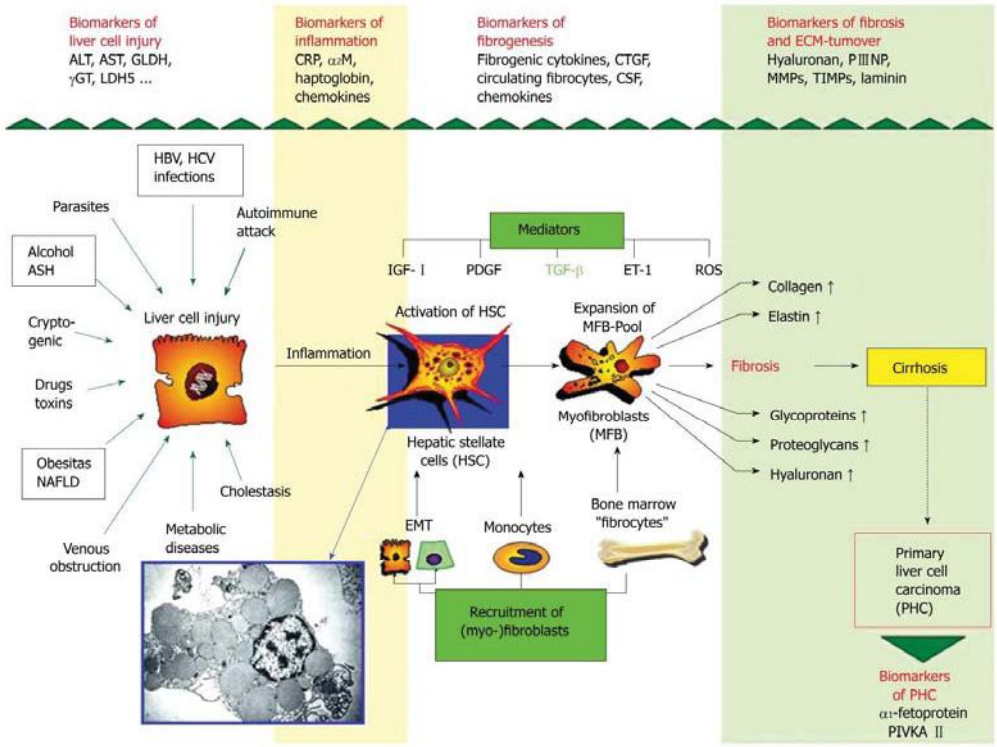

Figure 1: Pathophysiology of liver fibrosis [4]. Reproduced with permission from Gressner, et al.

Table 1: Role of cytokines involved in regulation of liver fibrosis [9].

Mediators	Mechanism of action
Growth Factors	
Platelet-derived growth factor (PDGF)	Stimulates activation and proliferation of HSCs. Upregulates expression of TIMP-1 Inhibits collagenase activity
Transforming growth factor-β (TGF-β)	Stimulates activation of HSCs. Upregulates type I collagen and α-SMA synthesis. Promotes MFs survival through activation of FAK. Inhibits DNA synthesis and induces hepatocytes apoptosis Upregulates expression of TIMPs
Interleukins	
Interleukin-1 (IL-1)	Activates HSCs and promotes HSCs survival through NF-κB. Promotes lipid accumulation in NAFLD Promotes type I collagen synthesis, Upregulates TIMP-1
Tumor necrosis factor-α (TNF-α)	Promotes activation of HSCs and reduces apoptosis of activated HSCs Upregulates TIMP-1, Stimulates hepatocyte apoptosis
Interleukin-17 (IL-17)	Upregulates type I collagen, TGF-β, and TNF-α through STAT3 pathway, Promotes activation of HSCs
Interleukin-10 (IL-10)	Inhibits HSCs activity. Inhibits expression of TIMP-1 and TGF-β
Interleukin-22 (IL-22)	Promotes HSCs senescence
Interleukin-6 (IL-6)	Attenuates hepatocytes apoptosis and induce regeneration of hepatocytes through NF-κB Induces insulin resistance
Interferon	
Interferon-α (IFN-α)	Has an anti-apoptotic effect on activated HSCs
Interferon-β (IFN-β)	Inactivates HSCs and decrease production of type I collagen and α-SMA through inhibition of PDGF and TGF-β
Interferon-γ (IFN-γ)	Inhibits activation of HSCs and reduce type I collagen deposition. Induces hepatic HSCs apoptosis and cell cycle arrest
Chemokine	
CCL2	Promotes migration of bone marrow-derived monocyte to liver. Activates HSCs. Induces insulin resistance in NAFLD
CXCL10	Promotes hepatocytes apoptosis. Inhibits NK cells-mediated HSCs inactivation. Stimulates T-cell chemotaxis to the liver
CXCL16	Promotes intrahepatic accumulation of NKT cells

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HSC: Hepatic Stellate Cell; NF-κB: Nuclear Factor-κB; NAFLD: Non-Alcoholic Fatty Liver Disease; TIMP: Tissue Inhibitors of Metalloproteinase; STAT: Signal Transducer and Activator of Transcription; SMA: Smooth Muscle Actin; NKT: Natural Killer T cell; FAK: Focal Adhesion Kinase; CXCL: Chemokine (C-X-C motif) Ligand; CCL: Chemokine (C-C motif) Ligand.

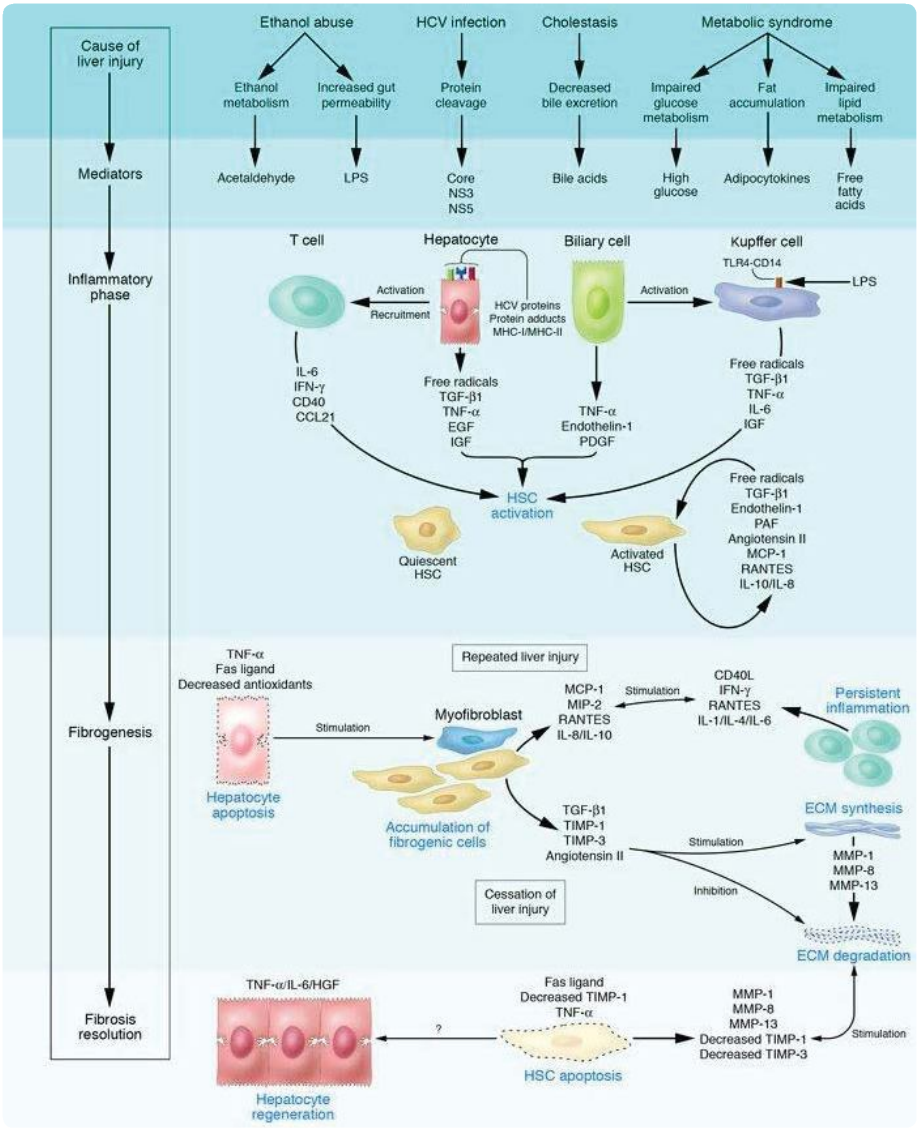

Figure 2: Cellular mechanisms of Liver fibrosis [5]. Reproduced with permission from Bataller, et al.

regulated by transcription and post-transcription. Collagen fibrils can be cross-linked by tissue transglutaminase and lysyl oxidase pathways which make collagen susceptible for collagenase activity [13]. Low density matrix is replaced by high density interstitial matrix which disturbs metabolic functions and impairs solute transport; altered cellular behavior is mediated by Integrins [11]. Damaged hepatocytes release ROS and fibrogenic mediators which stimulate inflammatory cells and fibrogenic action of activated HSCs. Activated HSCs stimulate lymphocytes by secreting inflammatory chemokines. It is a cyclic stimulation process of inflammatory and fibrogenic cells vice versa [5]. Damaged hepatocytes release inflammatory cytokines which activate Kupffer cells and stimulate the recruitment of activated T cells. This activates quiescent HSCs into fibrogenic myofibroblasts secrete cytokines. Due to chronic liver injury, activated HSCs express and deposits ECM leads fibrosis of liver. ECM degradation is inhibited by the actions of TIMPs. When etiology of liver fibrosis removed, there will be spontaneous resolution of fibrosis by apoptosis of activated HSCs and regeneration of hepatocytes. Accumulated collagen is degraded by increased activity of MMPs. Spontaneous resolution of liver fibrosis is possible after successful treatment of causative agent and may take several years depending on cause and severity of the disease [5,12]. Characteristic features of liver fibrosis reversal are decreased inflammation and decreased fibrogenic cytokines, increased collagenase activity and disappearance of myofibroblast and fibrotic scar [14]. Regression of liver fibrosis consists of thinning of fibrous septa, regeneration of hepatocytes and recovery of acinal structure [15]. Reversal of liver fibrosis can be achieved by inhibition of HSCs activation, neutralization of proliferative, fibrogenic, contractile and proinflammatory response of HSCs, stimulation of HSCs apoptosis or senescence and degradation of scar matrix. Inhibition of HSCs activation and trans-differentiation into myofibroblasts can be attained by reducing oxidant stress [10]. Interferon-β (IFN-β) inactivates HSCs and decrease production of collagen I and α Smooth Muscle Actin (α-SMA) by inhibiting PDGF and TGF-β; Interferon-γ (IFN-γ) has inhibitory action on activation of HSCs [9]. Fibrillar collagens are degraded by interstitial MMPs (MMP-1, -8 and -13) which are released in pro-enzyme form and activated by cleavage of inhibitory N-terminal peptide by plasmin. Plasmin synthesis

in fibrotic liver is inhibited by synthesis of plasminogen activator inhibitor-1 expressed from activated HSCs [13]. During resolution of fibrosis, MMPs activity is increased due to decreased expression of TIMPs; monocyte/macrophage lineage expresses MMPs [16]. After removal of inflammation, macrophages are differentiated into $Ly6c^{low}$ phenotype which produces MMP9 and MMP12 capable of matrix degradation [14]. Altered interactions between activated HSCs and ECM favor apoptosis [5,13]. Myofibroblast apoptosis is contributed by activation of death receptor mediated pathway, increased expression of pro-apoptotic proteins (p53, Bax and Bcl-2) and decreased expression of pro-survival proteins [14]. After successful removal of causative agent, HSCs undergo caspase-8/caspase-3 dependent apoptosis. Over expression of pro-apoptotic proteins leads to caspase-9 mediated programmed cell death. Over expression of CXCL9 by macrophages and VEGF expression accelerate fibrosis resolution by angiogenesis [16].

GENETICS OF BIDIRECTIONAL LIVER FIBROSIS

Genetics of liver fibrosis progression are highly complex and influenced by multiple factors. Hepatocellular apoptosis/ necrosis genes *viz* Bcl-xL, Fas influence the extent of hepatic damage and fibrinogenesis. Inflammatory genes *viz* IL-1 β, IL-6, IL-10, IFN-γ, SOCS-1 and osteopontin determines the fibrogenic response to injury. Genes regulating ROS generation (NADPH oxidase) regulate inflammation and ECM deposition [5]. Trans-differentiation of activated HSCs into myofibroblasts is mediated by down regulation of lipogenic genes like peroxisome proliferator-activated receptor gamma (PPARγ) and up-regulation of fibrogenic genes. Activated HSCs and myofibroblasts migrate to site of injury and express fibrogenic genes *viz* vimentin, collagen α1 (Colla1) and α-SMA stimulated by increased levels of PDGF and TGF-β [17,18]. Fibrogenic growth factors, vasoactive substances and adipokines are required for fibrogenesis (Table 2). In fibrosis resolution, expression of fibrogenic genes is decreased by inactivated HSCs in association with increased expression of genes like PPAR-γ. Genes *viz GFAP, Adiporl, Adpf* and *Dbp* are not expressed by inactive HSCs shows the difference between quiescent HSCs and inactive HSCs [19]. Gene polymorphisms play a major role in progression of liver fibrosis due to chronic liver diseases. In Alcoholic Liver Disease (ALD), polymorphisms of genes encoding alcohol metabolizing enzymes and proteins (alcohol dehydrogenase, aldehyde dehydrogenase and P450), genes encoding inflammatory mediators and antioxidants influence fibrogenesis [5]. In nonalcoholic steatohepatitis, genotypes of IL-10-1082G/ A and TNF-α 308G/ A express elevated levels of inflammatory cytokines [20]. In non-alcoholic fatty liver disease, *CDKN1A* variant rs762623 is related to development of liver fibrosis [21]. In primary biliary cholangitis, polymorphisms of genes encoding IL-1β, IL-1 and TNF-α are responsible for diseases progression. In Hepatitis C Virus (HCV) infection, polymorphisms of genes involved in immune response (transporter associated with antigen processing 2, specific HLA-II alleles), fibrogenic agonists (angiotensinogen and TGF-β) enhances fibrosis [5]

EPIGENETICS OF BIDIRECTIONAL LIVER FIBROSIS

Cellular composition and phenotype changes in chronic liver diseases are under the control of chromatin configuration of regulatory genes directed by epigenetic mechanisms [6]. Multifactorial causes influence the epigenetic mechanisms through SNPs [3]. Epigenetics are reversible changes in gene expression which are inherited through cell division without altering underlying DNA sequence; DNA methylation, post transcriptional modifications of amino acid tails of histones and non-coding RNA mediated gene silencing. These mechanisms organize many aspects of liver fibrosis by regulating chromatin structure, modifications and initiation of transcription that alters the accessibility of genes [22]. Diverse biological functions of liver are regulated by noncoding small microRNAs and play role in pathophysiology of liver [23]. Epigenetic mechanisms are determinants of gene expression during HSCs activation and deactivation by controlling transcription activity during fibrosis progression and regression. Unlike genetic mutations, epigenetic changes undergo reversion with the resolution of liver fibrosis and can be modulated pharmacologically [6,22].

Table 2: Genetic and non-genetic factors of liver fibrosis [5].

Type of Liver Disease	Genes	Non-genetic factors
HCV	Hereditary hemochromatosis Angiotensinogen Transforming growth factor β1 Tumor necrosis factor α Apolipoprotein E Microsomal epoxide hydroxylase Monocyte chemotactic protein type 1 Monocyte chemotactic protein type 2 Factor V (Leiden)	Alcohol intake Co-infection with HBV/ HIV Age at time of acute infection Diabetes mellitus
Alcohol-induced	Interleukin 10 Interleukin 1β Alcohol dehydrogenase Aldehyde dehydrogenase Cytochrome P450, family 2, subfamily e, polypeptide 1 Tumor necrosis factor α Cytotoxic T lymphocyte antigen type 4 Transporter-associated antigen-processing type 2 Manganese superoxide dismutase	Episodes of alcoholic hepatitis
NASH	Hereditary hemochromatosis gene Angiotensinogen Transforming growth factor β1	Age, Severity of obesity Diabetes mellitus Hypertriglyceridemia
PBC	Interleukin 1β Tumor necrosis factor α Apolipoprotein E	
Autoimmune hepatitis	Human leukocyte antigen type II haplotypes	Type II Autoimmune hepatitis No response to therapy

Modified from Bataller *et al*
HCV: Hepatitis C Virus; HBV: Hepatitis B Virus; HIV: Human Immuno deficiency Virus; NASH: Non Alcoholic Steatohepatitis; PBC: Primary Biliary Cholangitis

DNA METHYLATION

Development of liver fibrosis is associated with aberrant DNA methylation patterns which lead to inappropriate gene expression. DNA methylation is regulated by DNA methyltransferases (DNMT1, DNMT3a and DNMT3b), hydroxymethylases which increases in fibrotic liver while hepatic expression of Ten Eleven Translocation (TET) demethylase is down regulated [6]. Hypermethylation of cell cycle genes (p15 and p16), tumor suppressor genes (RASSF1A and E-cadherin) and anti-fibrotic gene PPARγ is associated with liver fibrosis progression [22]. Transdifferentiation of HSCs expresses methyl-CpG-binding domain nuclear proteins (MeCP2, MBD1, MBD2 and MBD3) which are transcriptional repressors of epigenetic

silencing of PPARγ gene. MeCP2 has positive regulation on expression of histone methyltransferase ASH1 which is required for expression of pro-fibrogenic genes collagen1, TIMP1 and TGF-β1. In hepatic myofibroblasts, MeCP2 regulate gene expression by direct methyl-CpG-dependent transcription and indirect post-transcriptional mechanisms [6]. Transdifferentiation of HSCs to pro-fibrogenic myofibroblast phenotype is suppressed by DNMT inhibitor 5'-aza-deoxycytidine [24].

HISTONE MODIFICATIONS

Liver damaging agents dysregulate chromatin structure by epigenetic mechanisms which involve action of ROS on histone modification. In post-translational modifications of histone proteins, lysine methylation or acetylation regulates liver fibrosis by perspective activation of HSCs. In activated HSCs, histone methyltransferase (H3K4 methyltransferase), ASH1 is up-regulated during progression of fibrosis and binds to promoters and 5' end of α-SMA, collagen1, TIMP-1 and TGF-β1 which results in transcriptional activation of gene expression. H3K27 methylation leads to repression of PPARγ gene; H3K9 dimethylation results in repression of inhibitory protein IκBα leads to up-regulation of transcription factor NFκB which has an important role in liver fibrosis. HSCs transdifferentiation requires chromatin signature H3K27me3 by recruited PPARγ gene. Lysine acetylation of histone proteins can up-regulate expression of collagen1, TIMP-1 and TGF-β1. Histone acetylation can be inhibited which can reverse myofibroblast differentiation by Histone Acetylation (HDAC) inhibitors [6,22,25].

SMALL NON CODING RNAS (MIRNAS)

miRNAs are essential for cellular process by regulating mRNA transcripts and are involved in activation of HSCs and liver fibrosis through regulation of proliferation and apoptosis (**Table 3**). Liver homeostasis is regulated by miRNA-122 affects on various genes involved in metabolism. miRNA-155 involved in innate and adaptive immune response by targeting TNF and promotes liver inflammation; miRNA-146a is a negative regulator of Toll Like Receptor (TLR) signaling proinflammatory response. Hepatocyte proliferation is regulated by miRNA-21 gene mediated cell cycle and DNA synthesis. Cytokines and growth factors of liver fibrosis regulate expression of pro-fibrogenic and anti-fibrogenic miRNAs (**Figure 3**). Key factors of fibrogenesis *viz* Col1α1, TGF-β receptor II, hepatocyte nuclear factor 4 α (HNF4 α) are regulated by miRNAs effect on mRNA 3'-UTR [6,22,23].

LONG NON-CODING RNAS (LNCRNAS) AND CIRCULAR RNAS (CIRCRNAS)

lncRNAs (exonic, intronic, overlapping and intergenic) effect gene expression by modulation of chromatin remodeling, control of gene transcription, post-transcriptional mRNA processing, protein function or localization and intracellular signaling; H19 and XIST were first identified lncRNAs for liver diseases [26]. lncRNA maternally expressed gene3 (lncRNA MEG3) located on chromosome 14q32.3 is a tumor suppressor gene which is down regulated due to TGF-β mediated methylation in disease progression of liver fibrosis [27]. Epigenetic regulation of MEG3 regulates fibrosis by inducing apoptosis by Bax/ Bcl-2 and cytoplasmic cytochrome C expressed p53 mediation. ECM synthesis will be reduced by over expression of MEG3 by suppressing cell proliferation [26]. LALR1 (human ortholog hLALR1) enhances hepatocyte proliferation through activation of Wnt/ β-catenin signaling and suppressing Axin1 [28]. Circular RNAs (circRNAs) a class of endogenous RNAs regulate gene expression at transcriptional or post-transcriptional level by acting as miRNAs sponges [29]. In bidirectional liver fibrosis, has_circ-0004018 transcribed by *SMYD4* has lower expression in disease progression [30]. circHIPK3 derived from Exon2 of *HIPK3* gene is a modulator of cell proliferation and significantly up-regulated in liver cancer [29].

Table 3: Role of miRNAs in liver diseases [23].

miRNAs	Functions
Mitochondrial miRNAs	
Rno miR-21, miR-130a, miR-130b, miR-140, miR-320, miR-494 and miR-671	Potential role in apoptosis, cell proliferation and Differentiation
miR-705, miR-494, miR-202-5p miR-451-7b, miR-26a, miR-122, miR805, miR-690, miR-155 and miR-134	Mitochondrial dysfunctions
pre-miR-302a, pre-let-7b, miR-365, miR-720, miR-133b, miR-1974, miR-24, miR-133a, miR-125a-5p and let-7 family members	Potential role in cell proliferation and differentiation
has-miR-494, miR-1275 and miR-1974	A possible role in regulating translation in mitochondria
miR-181c	Mitochondrial dysfunctions
Nuclear miRNAs	
miR-29b	Might regulate transcription or splicing of target Transcripts
miR-320	Transcriptional gene silencing
miR-671	Gene silencing of noncoding antisense transcripts
Circulating miRNAs	
Exosomal miRNAs Epstein–Barr virus-encoded miRNAs	Cell–cell communication
miR-126	Atherosclerosis
miR-150	Cell migration
miR-146a	Cell growth inhibition
miR-29a	HIV Tat and morphine-mediated neuronal dysfunction
miR-133b	Neurite outgrowth

Modified from Szabo, *et al.*

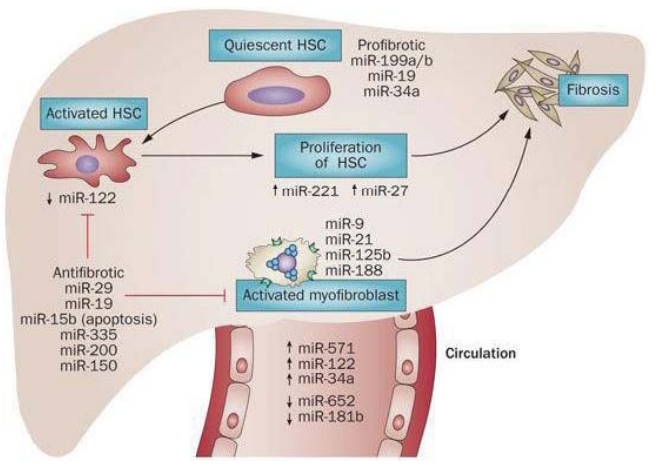

Figure 3: miRNA regulation of liver fibrosis [23]. Reproduced with permission from Szabo, et al.

CONCLUSION

Natural wound healing response to chronic liver insults results in the formation of liver fibrosis which is mediated by complex network of cytokines, growth factors, genetic and epigenetic mechanisms. Recent studies have shown that liver fibrosis is potentially bidirectional. Molecular mechanisms for liver regression in humans need to be more comprehensively defined. At which point, liver fibrosis will become irreversible is not well established, early stages may give witness for reversibility. Even though reversible epigenetic mechanisms of liver fibrosis can be modulated pharmacologically, it needs extensive research to improve anti-fibrotic drug therapies.

REFERENCE

1. Hong H, Tong W. Emerging efforts for discovering new biomarkers of liver disease and hepatotoxicity. Biomark Med. 2014; 8: 143-146. https://goo.gl/2Ya7ZT
2. Jun LC, Michael P, Ahmad M, John DR. Non-invasive markers of liver fibrosis: Adjuncts or alternatives to liver biopsy? Front Pharmacol. 2016; 7: 159. https://goo.gl/ns4em2
3. Krishna SN, Shashidhar KN, Lakshmaiah V, Muninarayana C. Liver fibrosis: A compilation on the biomarkers status and their significance in disease progress. Future Sci OA. 2017; 4: https://goo.gl/bfrr2x
4. Axel MG, Chun-Fang G, and Olav AG. Non-invasive biomarkers for monitoring the fibrogenic process in liver: A short survey. World J Gastroenterol. 2009; 15: 2433-2440. https://goo.gl/6HyasN
5. Ramón B, David AB. Liver fibrosis. J Clin Invest. 2005; 115: 209-218. https://goo.gl/1kRus8
6. Eva Moran-Salvador, Jelena M. Epigenetics and liver fibrosis. Cell Mol Gastroenterol Hepatol. 2017; 4: 125-134. https://goo.gl/LxzrDf
7. Sohrabpour AA, Mohamadnejad M, Malekzadeh R. Review article: the reversibility of cirrhosis. Aliment Pharmacol Ther. 2012; 36: 824-832. https://goo.gl/4W6i63
8. Gressner OA, Weiskirchen R, Gressner AM. Evolving concepts of liver fibrogenesis provide new diagnostic and therapeutic options. Comp Hepatol. 2007; 6: https://goo.gl/A2Epjo
9. Ebrahimi H, Naderian M, Sohrabpour AA. New concepts on pathogenesis and diagnosis of liver fibrosis; A review artcle. Middle East J Dig Dis. 2016; 8: 166-178. https://goo.gl/yXUAT5
10. Scott LF. Hepatic Fibrosis. In: Schiff's Diseases of the Liver (Edition 11). Eugene R Schiff, Michael F Sorrell, Willis C Maddrey (Ed). Wiley-Blackwell, Singapore. 2012: 297-305.
11. Meena BB, Scott LF. Hepatic Fibrogenesis. In: Sherlock's Diseases of the Liver and Biliary System. (Edition 11). James S. Dooley, Anna S.F. Lok, Andrew K. Burroughs, E. Jenny Heathcote (Ed). Wiley Blackwell, Oxford. 2011: 94-101.
12. David AB. Reversibility of liver fibrosis. Gastroenterol Hepatol (N Y). 2013; 9: 737-739. https://goo.gl/WzsfK8
13. Manoj KS, Sarin K. Is cirrhosis of the liver reversible? The Indian Journal of Pediatrics. 2007; 74: 393-399. https://goo.gl/M1nFNf
14. Antonella P, Prakash R, and John P I. Reversibility of liver fibrosis. Fibrogenesis Tissue Repair. 2012; 5: 26. https://goo.gl/MjwccN
15. Shogo O, Haruka H, Kazuhiko W, Katsuhiko H, Kenya K, and Masahiko Y. Natural regression of fibrosis in chronic hepatitis B. World J Gastroenterol. 2016; 22: 5459-5466. https://goo.gl/p5nSy5
16. Seki E, Brenner DA. Recent advancement of molecular mechanisms of liver fibrosis. J Hepatobiliary Pancreat Sci. 2015; 22: 512-518. https://goo.gl/WPCYs4
17. Krizhanovsky V, Yon M, Dickins RA, Hearn S, Simon J, Miething C, et al. Senescence of activated stellate cells limits liver fibrosis. Cell. 2008; 134: 657-667. https://goo.gl/RTVEee
18. Hernández-Gea V, Ghiassi-Nejad Z, Rozenfeld R, Gordon R, Fiel M, Yue Z, et al. Autophagy releases lipid that promotes fibrogenesis by activated hepatic stellate cells in mice and in human tissues. Gastroenterology. 2012; 142: 938-946. https://goo.gl/GEG5Lh
19. Kisseleva T, Cong M, Paik Y, Scholten D, Jiang C, Benner C, et al. Myofibroblasts revert to an inactive phenotype during regression of liver fibrosis. Proc Natl Acad Sci USA. 2012; 109: 9448-9453. https://goo.gl/5JhkU3
20. Ioana CB, Mircea VM, Raluca MP, Stefan CV, Lorena C, Daniela MM, et al. Cytokines genotype-phenotype correlation in nonalcoolic steatohepatitis. Oxidative Medicine and Cellular Longevity. 2017; 7. https://goo.gl/ZP2Cqy
21. Aravinthan A, Mells G, Allison M, Leathart J, Kotronen A, Yki-Jarvinen H, et al. Gene polymorphisms of cellular senescence marker p21 and disease progression in non-alcohol-related fatty liver disease. Cell Cycle. 2014; 13: 1489-1494. https://goo.gl/na9YBE
22. Atta HM. Reversibility and heritability of liver fibrosis: Implications for research and therapy. World J Gastroenterol. 2015; 21: 5138-5148. https://goo.gl/ECGvgM
23. Szabo G, Bala S. MicroRNAs in liver disease. Nat Rev Gastroenterol Hepatol. 2013; 10: 542-552. https://goo.gl/9MFQNU
24. Mann J, Oakley F, Akiboye F, Elsharkawy A, Thorne AW, Mann DA. Regulation of myofibroblast transdifferentiation by DNA methylation and MeCp2: implications for wound healing and fibrogenesis. Cell Death Differ. 2007; 14: 275-285. https://goo.gl/QiycQd
25. Perugorria MJ, Wilson CL, Zeybel M, Walsh M, Amin S, Robinson S, et al. Histone methyltransferase ASH1 orchestrates fibrogenic gene transcription during myofibroblast transdifferentiation. Hepatology. 2012; 56: 1129-1139. https://goo.gl/bkNhpz
26. Takahashi K, Yan I, Haga H, Patel T. Long non-coding RNA in liver diseases. Hepatology. 2014; 60: 744-753. https://goo.gl/Aj1wcu
27. Braconi C, Kogure T, Valeri N, Huang N, Nuovo G, Costinean S et al. MicroRNA-29 can regulate expression of the long non-coding RNA gene MEG3 in hepatocellular cancer. Oncogene. 2011; 30: 4750-4756. https://goo.gl/6y4akq
28. Xu D, Yang F, Yuan JH, Zhang L, Bi HS, Zhou CC et al. Long noncoding RNAs associated with liver regeneration 1 accelerates hepatocyte proliferation during liver regeneration by activating wnt/beta-catenin signaling. Hepatology. 2013; 58: 739-751. https://goo.gl/zsuiBR
29. Zheng Q, Bao C, Guo W, Li S, Chen J, Chen B et al. Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs. Nat Commun. 2016; 7: 11215. https://goo.gl/uVCK6c
30. Liyun Fu, Ting Yao, Qingqing Chen, Xiaoyan Mo, Yaoren Hu and Junming Guo. Screening differential circular RNA expression profiles reveals hsa_circ_0004018 is associated with hepatocellular carcinoma. Oncotarget. 2017; 8: 58405-58416. https://goo.gl/6NhnjA

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Research Article

Correlative Study of Hyaluronic Acid and YKL-40 with Conventional Markers for Cirrhosis of Liver

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Abstract

Background: Multifactorial liver cirrhosis leads to liver dysfunction and portal hypertension. Diagnosing the degree of disease is crucial for successful management; early diagnosis may reverse cirrhotic liver to normal architecture. Highly invasive liver biopsy is gold standard diagnostic tool with pain and complications. Components of Extra Cellular Matrix (ECM) may serve as indicator of severity of disease. Hence, the present study aimed to correlate serum levels of Hyaluronic Acid (HA), YKL-40 and uric acid with conventional markers of liver cirrhosis.

Methods: Blood samples were collected from cirrhotic liver (n=96) and age and gender matched healthy subjects (n=96) from Department of Medicine, RL Jalappa Hospital & Research Centre, Kolar, Karnataka, India. Serum was used to estimate HA, YKL-40, serum transaminases (Aspartate transaminase [AST] & Alanine Transaminase [ALT]), γ-Glutamyl Transferase (γGT), total protein, albumin, total bilirubin, uric acid, total antioxidant capacity and total oxidative stress. Plasma was used to estimate Prothrombin Time (PT) and International Normalized Ratio (INR) was calculated.

Results: Hyperuricemia with significant elevation of serum HA and YKL-40 levels in cirrhotic liver compared to healthy subjects were observed. There was an increase in total oxidative stress with decreased antioxidant capacity in liver cirrhotic compared to healthy subjects. Serum levels of HA and YKL-40 were positively correlated with activities of AST, ALT and γGT with concentrations of total bilirubin, total oxidative stress and PT INR. Negative correlation was observed between serum levels of uric acid, HA and YKL-40 to serum levels of total protein, albumin and total antioxidant capacity.

Conclusion: Components of ECM turn over viz., HA and YKL-40 expression increased during disease progression and can serve as direct biomarkers of liver cirrhosis which needs to be validated in diverse population. Hyperuricemia with elevated liver enzymes activity lead to poor prognosis.

Keywords: Endothelial dysfunction; HMGB1/RAGE signaling pathway; Hyaluronic acid; Hyperuricemia; Inflammatory cytokines; YKL-40

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Introduction

Liver cirrhosis is a natural wound healing response to chronic liver injury which leads to development of fibrotic scar surrounded by regenerative nodules results in portal hypertension and hepatic encephalopathy; end stage liver disease [1]. Despite multifactorial; the pathological characteristics which include degeneration, necrosis of hepatocytes, replacement of liver parenchyma by fibrotic tissue and regenerative nodular formation are common in cirrhosis. Defenestration and capillarization of liver sinusoidal endothelial cells are also major contributing factors for hepatic dysfunction [2]. In cirrhotic liver, the Extra Cellular Matrix (ECM) produced by myofibroblasts which are trans-differentiated from activated Hepatic Stellate Cells (HSCs) differs qualitatively and quantitatively compared to normal liver. An increase in fibril and non fibril forming collagens along with glycoproteins, proteoglycans and glycosaminoglycans reflects ECM turnover and serve as direct biomarkers for severity of cirrhosis [3].

Inflammatory factors [Tumor Necrosis Factor α (TNF- α) and interleukins] induced oxidative and apoptotic stress are important features for liver fibrosis/cirrhosis progression which results in uric acid production, an end product of purine metabolism [4]. Hyperuricemia, increased degradation of nuclear material cause endothelial dysfunction, insulin resistance, oxidative stress and systemic inflammation (metabolic syndrome) which are risk factors for hepatic damage [5]. High levels of uric acid lead to disease progression and elevation of liver enzymes activity in circulation resulting in poor prognosis of the disease [6].

Hyaluronic Acid (HA), a high molecular non-sulfated glycosaminoglycan is synthesized by synovial cells and HSCs by enzyme hyaluronic acid synthase and degraded by sinusoidal endothelial cells of liver [7]. HA plays a vital role in the formation of ECM during progression of cirrhosis. Inflammatory mediated cytokine transdifferentiation of HSCs into myofibroblasts during disease progression may alter serum HA levels which indicates severity of the disease [8].

YKL-40 (Chitinase-3-like protein 1, chondrex, breast regression protein-39 and human cartilage glycoprotein-39) is a secreted heparin binding glycoprotein [9]. It is one of the 18 glycosyl hydrolases, with no enzymatic activity due to mutations within active site. YKL-40 biological role still remains unclear but may play an important role in inflammation, proliferation and angiogenesis [10,11]. Levels of YKL-40 are enhanced in inflammation, increased extracellular remodeling,

fibrosis, cancer, heart failure and ischemic cerebrovascular disease [9]. YKL-40 acts as a chemoattractant for endothelial cells which can modulate angiogenesis during tissue repair. It is secreted by activated macrophages mediated by proinflammatory cytokines and expressed in human liver [12].

Diagnosing the degree of hepatotoxicity is crucial for therapeutic management of cirrhosis; early diagnosis may help reversing cirrhotic liver to normal liver architecture. Liver biopsy is considered the gold standard method; it is an invasive procedure associated with pain and complications. Existing biomarkers for cirrhosis in clinical practice have narrow applicability; unable to predict etiology (specificity) and distinguish intermediate stages (sensitivity). Ideal biomarker should be organ specific, sensitive to indicate active damage, easily accessible in peripheral tissue, cost effective; should give insights for tailor made therapy for effective clinical management of the disease [3]. Hence, the present study aimed to correlate serum levels of uric acid, HA and YKL-40 with conventional markers of liver cirrhosis.

Materials and Methods

Samples

Blood samples were collected from clinically and diagnostically proven cirrhotic liver subjects with varying degree from the subjects registered in the Department of Medicine, RL Jalappa Hospital & Research Centre attached to Sri Devaraj Urs Medical College, A constituent of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka, India. Individuals diagnosed with cirrhosis caused by different etiologies *viz.,* Alcoholic Liver Diseases (ALD), viral hepatitis caused by Hepatitis C and B (HCV & HBV) and Non-Alcoholic Fatty Liver Disease (NAFLD) based on clinical symptoms *viz.,* ascites, encephalopathy, jaundice and altered biochemical parameters were included. Individuals with diabetes and/or its complications, myocardial infarction, acute and chronic renal failure, pneumonia and cancer and individuals on thrombolytic therapy were excluded from the study.

Collection of blood samples from cirrhotic liver (n=96) and age and gender matched healthy subjects (n=96) were carried out after obtaining informed consent and the study is approved by institutional ethical committee (DMC/KLR/IEC/61/2016-17). Venous blood, 5ml from antecubital vein in comfortable position of subject (cirrhotic and healthy) was collected; 2.0 ml of blood was transferred into sodium citrate tube and 3 ml into serum separator tube.

Serum separation

Serum was collected from clotted blood using serum separator tubes centrifuged at 4000 rpm for 10 min. Serum and citrate blood were stored at -20°C for further analysis. Serum was used to estimate biochemical parameters; serum transaminases [Aspartate Transaminases (AST) and Alanine Transaminases (ALT)], γ-Glutamyl Transferase (γGT), total protein, albumin, total bilirubin, uric acid, total antioxidant capacity and total oxidative stress. Plasma was used to estimate Prothrombin Time (PT) and International Normalized Ratio (INR) was calculated.

Determination of proteins concentration and enzyme activities

Hyaluronic acid and YKL-40 were estimated by ELISA quantikine kits procured from R&D Systems, USA. Activity of liver enzymes *viz.,* AST, ALT and γGT were estimated by IFCC (International Federation of Clinical Chemistry) kinetic method. Concentrations of total protein and albumin were estimated by biuret and bromocresol green method respectively. Total bilirubin was estimated by diazo method and uric acid was estimated by uricase enzymatic end point method. Total oxidative status and total antioxidant capacity were estimated by colorimetric method. PT was estimated by coagulation method and INR was calculated.

Statistical analysis

Statistical analysis was performed by using institutional licensed version of IBM SPSS 20. Descriptive statistics were represented in terms of mean and standard deviation. Comparison for quantitative variables between groups for significance was done by using Student t-test. Pearson correlation was used to assess the relation between quantitative measures. One way ANOVA test was performed to find out difference of variables (HA and YKL-40) between cirrhotic liver subjects with varied etiology. Area Under Receiver's operating Characteristics (AUC) curve was analyzed to find out the diagnostic accuracy of HA and YKL-40.

Results

In the present study, cirrhotic liver subjects were in the age group of 25 to 65 years with a mean age of 43.27 ± 8.56; 78% (n=75) were males while 22% were females (n=21) with varying degree and different etiological factors (Figure 1).

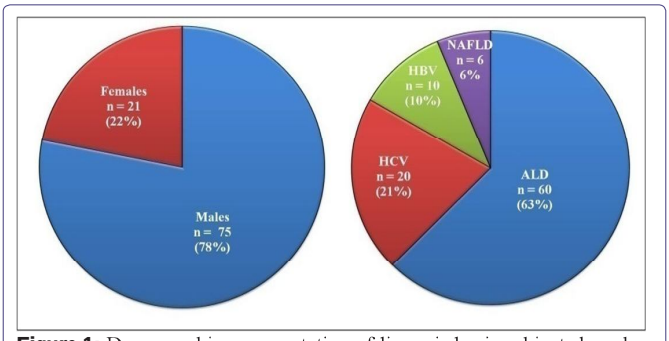

Figure 1: Demographic representation of liver cirrhosis subjects based on gender & etiology.

ALD: Alcoholic Liver Disease; HCV: Hepatitis C Virus; HBV: Hepatitis B Virus; NAFLD: Non-Alcoholic Fatty Liver Disease.

Significant elevation was observed for the variables serum HA (22.84 ± 6.11 Vs 7.99 ± 3.02), YKL-40 (140.72 ± 23.93 Vs 29.93 ± 6.64) and uric acid (6.11 ± 0.67 Vs 3.56 ± 0.50) in cirrhotic liver compared to healthy subjects (Figure 2).

Significant increase in activity of AST, ALT and γGT with increased serum total bilirubin and prolonged PT INR were observed in cirrhotic liver than healthy subjects. Serum total protein and albumin were reduced significantly in cirrhotic liver compared to healthy subjects. In cirrhotic liver there was an increase in total oxidative status (33.27 ± 6.05 Vs 12.18 ± 3.59) with decreased antioxidant capacity (20.75 ± 4.97 Vs 33.37 ± 5.95) compared to healthy subjects (Table 1).

Pearson correlation analysis showed positive correlation between serum levels of uric acid, HA and YKL-40 with activities of AST, ALT and γGT with serum levels of total bilirubin, PT INR and total oxidative status. A negative correlation was observed between serum levels of uric acid, HA and YKL-40 with total protein, albumin and total antioxidant capacity (Tables 2A, 2B and 2C).

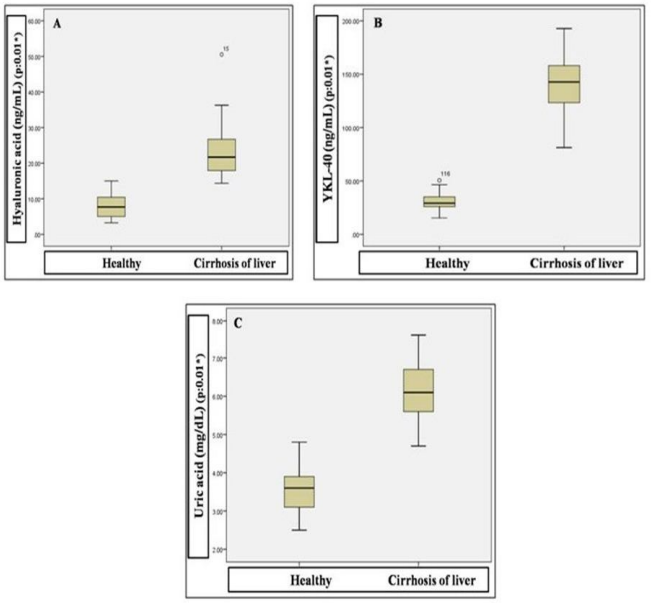

Figure 2: Comparison of serum hyaluronic acid (A), YKL-40 (B) and uric acid (C) concentrations between healthy and cirrhosis of liver subjects.

Among cirrhotic liver subjects, one way ANOVA analysis showed high levels of serum HA (24.41 ± 6.46) in ALD subjects compared to HCV (21.26 ± 5.00), HBV (18.00 ± 2.84) and NAFLD (20.49 ± 3.94) subjects. YKL-40 was expressed high in ALD (149.80 ± 20.53) compared to HCV (129.71 ± 19.59), HBV (115.41 ± 22.19) and NAFLD (128.79 ± 25.21) subjects (Table 3).

ROC analysis was carried out to define the diagnostic accuracy for serum HA and YKL-40 in cirrhotic liver subjects. Serum HA with an AUC of 0.961 (95% CI) with sensitivity 95.05% and specificity 97.80% and YKL-40 with an AUC of 0.973 (95% CI) with sensitivity 95% and specificity 99% compared with that of ALT with an AUC of 0.999 were observed (Figure 3).

Discussion

Till date, liver biopsy is the gold standard tool for diagnosis and distinguishes between intermediate stages of liver fibrosis and cirrhosis [13]. Highly invasive liver biopsy involves pain and complications with approximately 0.01% mortality rate [14]. Indirect biomarkers which are reflection of liver dysfunction have narrow applicability in clinical practice due to lack of sensitivity and specificity. Direct biomarkers which reflect ECM turnover needs validation in large population studies in diverse geographical settings [3]. Serum levels of HA and YKL-40 which are crucial components of ECM in disease progression may reflect severity of the disease [12]. In present study, we examined diagnostic value of serum HA and YKL-40 by correlating with conventional markers of liver cirrhosis.

Variable	Groups	Varied Etiology				Total (n=96)
		ALD (n=60)	HCV (n=20)	HBV (n=10)	NAFLD (n=6)	
Hyaluronic acid (ng/mL) 0.001*	II	24.41 ± 6.46	21.26 ± 5.00	18.00 ± 2.84	20.49 ± 3.94	22.84 ± 6.11
	I	8.17 ± 3.08	7.57 ± 3.00	7.89 ± 2.92	7.67 ± 3.21	7.99 ± 3.02
YKL-40 (ng/mL) 0.001*	II	149.80 ± 20.53	129.70 ± 19.59	115.40 ± 22.19	128.79 ± 25.21	140.7 ± 23.93
	I	29.20 ± 6.42	31.25 ± 6.11	29.53 ± 7.85	33.43 ± 8.50	29.93 ± 6.64
AST (U/L)	II	216.13 ± 60.53	188.75 ± 41.09	205.10 ± 29.94	243.33 ± 51.50	209.94 ± 55.70
	I	38.90 ± 15.39	35.75 ± 15.59	35.60 ± 7.22	39.50 ± 9.35	37.93 ± 14.41
ALT (U/L)	II	268.25 ± 61.38	260.90 ± 38.79	268.50 ± 23.32	284.83 ± 53.46	267.78 ± 53.58
	I	33.25 ± 13.50	36.65 ± 23.59	33.40 ± 16.64	30.83 ± 5.70	33.82 ± 15.96
γGT (U/L)	II	259.03 ± 66.67	272.05 ± 60.94	293.10 ± 90.19	327.00 ± 54.75	269.54 ± 69.06
	I	37.10 ± 14.12	42.00 ± 17.14	36.20 ± 6.35	31.66 ± 7.78	37.68 ± 14.01
Total Protein (g/dL)	II	4.83 ± 0.47	5.10 ± 0.72	5.46 ± 0.95	5.18 ± 0.59	4.97 ± 0.62
	I	6.97 ± 0.34	6.91 ± 0.29	6.78 ± 0.37	6.90 ± 0.42	6.93 ± 0.34
Albumin (g/dL)	II	2.47 ± 0.35	2.63 ± 0.40	2.89 ± 0.63	2.65 ± 0.33	2.56 ± 0.41
	I	3.94 ± 0.35	4.01 ± 0.34	3.76 ± 0.32	4.10 ± 0.44	3.94 ± 0.35
Total Bilirubin (mg/dL)	II	5.88 ± 1.32	4.50 ± 1.29	4.24 ± 1.09	5.58 ± 2.09	5.40 ± 1.49
	I	0.80 ± 0.25	0.71 ± 0.19	0.63 ± 0.18	0.81 ± 0.24	0.77 ± 0.24
Uric acid (mg/dL) 0.001*	II	6.30 ± 0.59	5.86 ± 0.75	5.77 ± 0.62	5.56 ± 0.57	6.11 ± 0.67
	I	3.66 ± 0.49	3.32 ± 0.42	3.33 ± 0.39	3.68 ± 0.76	3.56 ± 0.50
PT INR	II	2.63 ± 0.54	2.55 ± 0.42	2.50 ± 0.36	2.26 ± 0.45	2.57 ± 0.50
	I	1.09 ± 0.13	1.03 ± 0.15	1.06 ± 0.17	1.13 ± 0.10	1.08 ± 0.14
TAC (nmol/μL) 0.001*	II	20.15 ± 4.43	20.81 ± 6.59	22.28 ± 4.60	24.02 ± 3.52	20.75 ± 4.97
	I	33.70 ± 6.21	32.83 ± 5.32	32.93 ± 6.22	32.60 ± 5.95	33.37 ± 5.95
TOS (μmolH_2O_2 Equiv/L) 0.001*	II	32.82 ± 4.94	31.83 ± 6.17	37.90 ± 10.34	34.82 ± 3.91	33.27 ± 6.05
	I	12.48 ± 3.55	12.36 ± 4.05	9.93 ± 3.33	12.30 ± 1.65	12.18 ± 3.59

Table 1: Statistical analysis of biochemical parameters (liver cirrhosis with varied etiology vs. healthy subjects)
*Sig (2-tailed) $p < 0.05$: Significant.
Data were presented as numbers and mean ± SD; Group II: Clinically and diagnostically proven liver cirrhotic subjects; Group I: Healthy subjects; SD: Standard Deviation; ALD: Alcoholic Liver Disease; HCV: Hepatitis C Virus; HBV: Hepatitis B Virus; NAFLD: Non Alcoholic Fatty Liver Disease; AST: Aspartate Transaminase; ALT: Alanine Transaminase; γGT: Gamma Glutamyl Transferase; TAC: Total Antioxidant Capacity; TOS: Total Oxidative Status; PT INR: Prothrombin Time International Normalized Ratio.

Afzali et al., in their large cohort study reported novel association of hyperuricemia and the incidence of cirrhosis development and risk of cirrhosis related hospitalization or death with elevated activities of ALT and γGT which are markers of hepatic necroinflammation in US population [6]. In Indian population, Paul et al., in their study found that hyperuricemia reflects oxidative stress and is associated with disease progression which can serve as surrogate marker for CLD with varied etiology [5]. Xie et al., documented crucial role of hyperuricemia is an important independent risk factor for NAFLD in Chinese population [4]. Shih et al., observed significant association between serum uric acid levels and NAFLD among US population; independent of multiple metabolic risk factors [15].

In the present study, significant elevation of uric acid in cirrhotic liver subjects was observed compared to healthy subjects. Significant elevation of uric acid, an end product of purine metabolism by cellular destruction is a mediator of inflammation and tissue damage [16]. In chronic liver injury, uric acid activates inflammosome (inflammatory cytokines and Nuclear Factor-κB [NF-κB]) and promotes surrounding liver parenchymal damage which in turn leads to hepatic dysfunction [17]. Hepatic depletion of Adenosine Triphosphate (ATP) increases uric acid production leading to hepatocellular injury; reduction in protein synthesis, induces inflammation and pro-oxidative changes which elevates liver enzymes activity in circulation [15]. Hyperuricemia results in oxidative stress mediated metabolic syndrome which are associated with progression of chronic liver disease. Uric acid levels can enhance the inflammatory cytokines *viz.,* interleukin-1 (IL-1), IL-6, IL-10, IL-18 and TNF-α resulting in pro-inflammation for disease progression. Hyperuricemia may alter expression of endothelin-1 which promotes disease and stimulates innate immune response [18].

Resino et al., documented the increase in accuracy of serum hyaluronic acid with hepatic fibrotic stages in HIV-HCV coinfected patients [19]. Peters et al., in their large cohort study, demonstrated that baseline hyaluronic acid is a strong predictor of liver related deaths in HIV-1 patients coinfected with HBV and/or HCV [20]. Gudowska et al., concluded that concentrations of serum hyaluronic acid are elevated in liver diseases and should be considered as a good noninvasive biomarker for diagnosis of liver damage [8].

In our study, there was a significant elevation of serum hyaluronic acid in cirrhotic liver subjects with varied etiology compared to healthy subjects. Among cirrhotic liver subjects, high levels of HA in ALD suggests that the degree of necroinflammation will be varied in different etiologies and it is high in alcohol induced liver injury. HA is produced from activated HSCs after transdifferentiate into myofibroblasts due to inflammation mediated by complex cytokine network [21]. Elevated levels of HA in cirrhotic liver subjects is due to imbalance between synthesis from fibrogenesis and decrease in fibrolysis during the disease progression, dysfunction of sinusoidal endothelial cells leads to reduction in degradation of HA resulting in elevated levels in circulation [8,22].

Variable	Correlation	AST	ALT	γGT	TP	ALB	TB	UA	PT INR	TAC	TOS
Hyaluronic acid (ng/mL)	Pearson Correlation	0.74**	0.77**	0.78**	-0.79**	-0.78**	0.81**	0.79**	0.75**	-0.67**	0.77**
	Sig (2-tailed)	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	n	192	192	192	192	192	192	192	192	192	192

** Correlation is significant at the 0.01 level (2-tailed).

Table 2A: Correlation of hyaluronic acid with other biochemical parameters.

Variable	Correlation	AST	ALT	γGT	TP	ALB	TB	UA	PT INR	TAC	TOS
YKL40 (ng/mL)	Pearson Correlation	0.87**	0.89**	0.87**	-0.88**	-0.86**	0.93**	0.92**	0.86**	-0.73**	0.86**
	Sig (2-tailed)	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.01	0.001	0.001
	n	192	192	192	192	192	192	192	192	192	192

** Correlation is significant at the 0.01 level (2-tailed).

Table 2B: Correlation of YKL-40 with other biochemical parameters.

Variable	Correlations	AST	ALT	γGT	TP	ALB	TB	PT INR	TAS	TOS
Uric acid (mg/dL)	Pearson Correlation	0.84**	0.86**	0.83**	-0.82**	-0.79**	0.90**	0.85**	-0.70**	0.84**
	Sig. (2-tailed)	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	N	192	192	192	192	192	192	192	192	192

** Correlation is significant at the 0.01 level (2-tailed)

Table 2C: Correlation of uric acid with other biochemical parameters.

AST: Aspartate Transaminase; ALT: Alanine Transaminase; γGT: Gamma Glutamyl Transferase; TP: Total Protein; ALB: Albumin; TB: Total Bilirubin; UA: Uric Acid; PT INR: Prothrombin Time International Normalized Ratio; TAC: Total Antioxidant Capacity; TOS: Total Oxidative Status.

Variables	Groups	Mean ± SD	Std. Error	Sig
Hyaluronic acid (ng/mL)	1 (n=60)	24.41 ± 6.46	0.83	0.005*
	2 (n=20)	21.26 ± 5.00	1.12	
	3 (n=10)	18.00 ± 2.84	0.89	
	4 (n=6)	20.49 ± 3.94	1.61	
YKL-40 (ng/mL)	1 (n=60)	149.80 ± 20.53	2.65	0.001*
	2 (n=20)	129.71 ± 19.59	4.38	
	3 (n=10)	115.41 ± 22.19	7.01	
	4 (n=6)	128.79 ± 25.21	10.29	

Table 3: Post Hoc and Bonferroni test for ANOVA analysis; comparison of hyaluronic acid and YKL-40 between cirrhotic liver groups with varied etiology.

Group 1: Alcoholic Liver Disease; Group 2: Hepatitis C; Group 3: Hepatitis B; Group 4: Non Alcoholic Fatty Liver Disease; n: Sample number; SD: Standard Deviation.

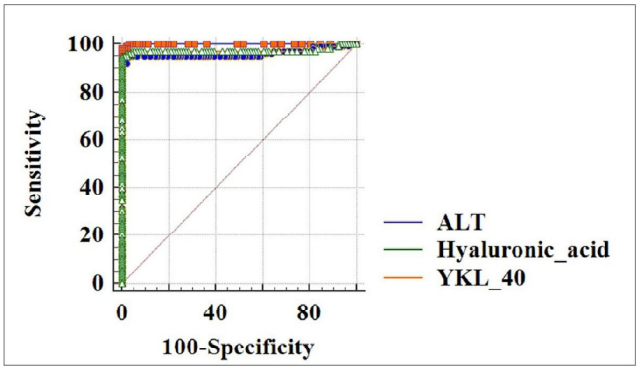

Figure 3: ROC analysis; comparison of AUC curve for Hyaluronic acid (0.961) and YKL-40 (0.973) with ALT.

Damaged hepatocytes release Reactive Oxygen Species (ROS) and fibrogenic mediators which in turn stimulate inflammatory cells leads to cell damage [3]. Decreased hepatic ATP and cell damage result in increased uric acid production which lead to histological liver injury [23]. Elevated uric acid inhibits endothelial Nitric Oxide Synthase (eNOS) expression which results in reduction of Nitric Oxide (NO) release and bioavailability in endothelial cells [24]. Hyperuricemia induced down regulation of eNOS results in oxidative stress and activates inflammatory cytokines IL-6 and TNF-α by stimulating HMGB1/RAGE (High Mobility Group Box chromosomal protein1/ Receptor for Advanced Glycation End products) signaling pathway results in endothelial dysfunction [25]. Accumulation of HA due to increased synthesis and decreased degradation leads to significant elevation which acts as an indicator of severity of the disease.

Study conducted by Saitou et al., demonstrated that serum YKL-40 measurements reflects liver fibrogenesis in HCV patients; serve as serological marker for evaluating the effectiveness of therapy [12]. Kumugai et al., showed that YKL-40 secretion by macrophages was upregulated by TNF-α and IL-1β, proinflammatory cytokines which are involved in the pathogenesis of NAFLD [26]. In large cohort study, Kjaergaard et al., documented increased expression of YKL-40 in chronic alcoholic liver subjects compared to healthy subjects [27].

In the present study, we observed serum YKL-40 significantly elevated in cirrhotic liver compared to healthy subjects; concentrations may be useful noninvasive tool for diagnosis of liver cirrhosis. YKL-40 is expressed from macrophages, chondrocytes, synovial cells, vascular smooth muscle cells and HSCs [28]. Non-enzymatic heparin binding glycoprotein, YKL-40 involves in cell proliferation, inflammation and remodeling of ECM [26]. In liver, it modulates angiogenesis by acting as chemo-attractant for endothelial cells. YKL-40 is a growth factor for fibroblasts [29]. During disease progression, macrophages will increase which in turn secrete YKL-40 regardless of etiology of the disease. Enhanced expression of YKL-40 by activated macrophages is mediated by proinflammatory cytokines TNF-α and IL-1β [26]. Inflammatory YKL-40 plays a key role in fibrogenesis of liver; elevated levels of YKL-40 indicate the disease severity [9,26].

Limitations

Different etiologies of liver cirrhosis were considered in the present study; sample size for HBV and NAFLD was less and needs to be validated with large sample number. Etiologies *viz.,* biliary cirrhosis, auto immune cholangiopathy, inherited metabolic liver disease (haemochromatosis, Wilson's disease and α-1antitrypsin deficiency), autoimmune hepatitis and cryptogenic cirrhosis need to be considered. Interference of confounding factors for the quantification of proteomic biomarkers *viz.*, hyaluronic acid and YKL-40 which may give false values and misleads diagnosis should be ruled out.

Future Directions

Possibilities of combinatorial use of biomarkers and to define cut off values and new scores with the help of biomarkers validated in this study may distinguish different stages of liver cirrhosis. Variations in concentrations of biomarkers required to be validated in different (sub and stratified) population; gender variation needs to be checked. Physiological, pathological and habitual factors which may affect the concentrations of hyaluronic acid and YKL-40 have to be ruled out.

Conclusion

Elevated activity of liver enzymes and prolonged PT INR along with hyperuricemia lead to poor prognosis. Elevated HA levels suggest that there will be increased synthesis and decreased degradation which leads to accumulation of HA in ECM. An elevated level of YKL-40 is an indicator of inflammation. Components of ECM turn over *viz.,* HA and YKL-40 expression increased during progression of disease and can serve as direct biomarkers for liver cirrhosis which needs to be validated in different population.

Conflict of Interest:

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

References

1. Detlef S, Nezam HA (2008) Liver Cirrhosis. The Lancet 371: 838-851.
2. Zhou W-C, Zhang Q-B, Qiao L (2014) Pathogenesis of liver cirrhosis. World J Gastroenterol 20: 7312-7324.

3. Nallagangula KS, Nagaraj SK, Venkataswamy L, Chandrappa M (2017) Liver fibrosis: a compilation on the biomarkers status and their significance during disease progression. Future Sci OA 4.

4. Xie Y, Wang M, Zhang Y, Zhang S, Tan A, et al. (2013) Serum uric acid and non-alcoholic fatty liver disease in non-diabetic Chinese men. PLoS One 8: 67152.

5. Paul R, Chakravarti HN, Mandal SK, Chatterjee S, Choudhury PS (2013) Study of serum uric acid in chronic liver disease and its relation with other parameters. Int Res J Pharm 4: 162-165.

6. Afzali A, Weiss NS, Boyko EJ, Ioannou GN (2010) Association between serum uric acid level and chronic liver disease in the United States. Hepatology 52: 578-589.

7. Rostami S, Parsian H (2013) Hyaluronic Acid: From Biochemical Characteristics to its Clinical Translation in Assessment of Liver Fibrosis. Hepat Mon 13: 13787.

8. Gudowska M, Gruszewska E, Panasiuk A, Cylwili B, Flisiak R, et al. (2016) Hyaluronic acid concentration in liver diseases. Clin Exp Med 16: 523-528.

9. Dam Mygind N, Axelsson A, Ruwald MH, Dalsgaard M, Steffensen R, et al. (2016) The inflammatory biomarker YKL-40 decreases stepwise after exercise stress test. Cardiovasc Endocrinol 5: 21-27.

10. Bussink AP, Speijer D, Aerts JMFG, Boot RG (2007) Evolution of Mammalian Chitinase(-Like) Members of Family 18 Glycosyl Hydrolases. Genetics 177: 959-970.

11. Kzhyshkowska J, Mamidi S, Gratchev A, Kremmer E, Schmuttermaier C, et al. (2006) Novel stabilin-1 interacting chitinase-like protein (SI-CLP) is up-regulated in alternatively activated macrophages and secreted via lysosomal pathway. Blood 107: 3221-3228.

12. Saitou Y, Shiraki K, Yamanaka Y, Yamaguchi Y, Kawakita T, et al. (2005) Noninvasive estimation of liver fibrosis and response to interferon therapy by a serum fibrogenesis marker, YKL-40, in patients with HCV-associated liver disease. World J Gastroenterol 11: 476-481.

13. Bedossa P, Dargere D, Paradis V (2003) Sampling variability of liver fibrosis in chronic hepatitis C. Hepatology 38: 1449-1457.

14. Lee S, Kim DY (2014) Non-invasive diagnosis of hepatitis B virus-related cirrhosis. World J Gastroenterol 20: 445-459.

15. Shih MH, Lazo M, Liu SH, Bonekamp S, Hernaez R, et al. (2015) Association between serum uric acid and nonalcoholic fatty liver disease in the US population. J Formos Med Assoc 114: 314-320.

16. Kono H, Chen CJ, Ontiveros F, Rock KL (2010) Uric acid promotes an acute inflammatory response to sterile cell death in mice. J Clin Invest 120: 1939-1949.

17. Gasse P, Riteau N, Charron S, Girre S, Fick L, et al. (2009) Uric acid is a danger signal activating NALP3 inflammasome in lung injury inflammation and fibrosis. Am J Respir Crit Care Med 179: 903-913.

18. Puddu P, Puddu GM, Cravero E, Vizioli L, Muscari A (2012) Relationships among hyperuricemia, endothelial dysfunction and cardiovascular disease: molecular mechanisms and clinical implications. J Cardiol 59: 235-242.

19. Resino S, Bellón JM, Asensio C, Micheloud D, Miralles P, et al. (2010) Can serum hyaluronic acid replace simple non-invasive indexes to predict liver fibrosis in HIV/Hepatitis C coinfected patients? BMC Infect Dis 10: 244.

20. Peters L, Mocroft A, Soriano V, Rockstroh J, Rauch A, et al. (2013) Hyaluronic acid levels predict risk of hepatic encephalopathy and liver-related death in HIV/viral hepatitis coinfected patients. PLoS One 8: 64283.

21. Orasan OH, Ciulei G, Cozma A, Sava M, Dumitrascu DL (2016) Hyaluronic acid as a biomarker of fibrosis in chronic liver diseases of different etiologies. Clujul Med 89: 24-31.

22. Rockey DC, Bissell DM (2006) Noninvasive measures of liver fibrosis. Hepatology 43: 113-120.

23. Wei TY, Wang CC (2016) Uncovered issues in the association between uric acid and nonalcoholic fatty liver disease. J Formos Med Assoc 115: 1022-1023.

24. Huang X, Cai X, Zheng W, Shen Y, Xie L (2016) Relationship between Uric Acid and Endothelial Function in Hypertensive Patients with Metabolic Syndrome. J Clin Exp Cardiolog 7: 416.

25. Cai W, Duan XM, Liu Y, Yu J, Tang YL, et al. (2017) Uric Acid Induces Endothelial Dysfunction by Activating the HMGB1/RAGE Signaling Pathway. BioMed Research International 4391920.

26. Kumagai E, Mano Y, Yoshio S, Shoji H, Sugiyama M, et al. (2016) Serum YKL-40 as a marker of liver fibrosis in patients with non-alcoholic fatty liver disease. Sci Rep 6: 35282.

27. Kjaergaard AD, Bojesen SE, Nordestgaard BG, Johansen JS (2014) YKL-40 and alcoholic liver and pancreas damage and disease in 86,258 individuals from the general population: cohort and mendelian randomization studies. Clin Chem 60: 1429-1440.

28. Johansen JS (2006) Studies on serum YKL-40 as a biomarker in diseases with inflammation, tissue remodelling, fibroses and cancer. Dan Med Bull 53: 172-209.

29. Lee CG, Da Silva CA, Dela Cruz CS, Ahangari F, Ma B, et al. (2011) Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. Annu Rev Physiol 73: 479-501.

Review

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Evolution of proteomic biomarker for chronic liver disease: Promise into reality

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Abstract

Liver is the vital organ for synthesis of proteins whose concentration in blood reflects liver dysfunction. Variations in protein domain can generate clinically significant biomarkers. Biomarker pipeline includes discovery of candidates, qualification, verification, assay optimization, and validation. Advances in proteomic approach can discover protein biomarker candidates based on "up-or-down" regulation or fold change in expression which is correlated with disease state. Despite numerous biomarker candidates been discovered, only few are useful in clinical practice which indicates the need for well-established validation regimen. Hence, the main purpose of this review is to understand the protein biomarker development and pitfalls. Companion diagnostics provide insights into potential cost-effective diagnosis for chronic liver disease.

Keywords

Chronic liver disease, proteomics, biomarker discovery, assay optimization, specificity and sensitivity, validation strategies, pitfalls, companion diagnostics

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Introduction

Human liver is a vital organ for health and survival, performing biochemical functions, namely, protein synthesis, production of digestive enzymes, and detoxification.[1]Liver fibrosis/cirrhosis is a pathological condition in which functions of liver are impaired by chronic liver insult. Cirrhosis of liver is the histological development of regenerative nodules surrounded by fibrous bands in response to chronic liver injury which leads to portal hypertension and end-stage liver disease. Fibrosis is a reversible natural wound healing response to chronic liver injury resulting in accumulation of extra cellular matrix (ECM); precursor of cirrhosis. Despite varied etiology, the pathological characteristics which include degeneration, necrosis of hepatocytes, and replacement of liver parenchyma by fibrotic tissues and regenerative nodules are common and ultimately result in liver dysfunction.[2]

After an acute injury, there will be regeneration and replacement of liver parenchymal cells to necrotic and apoptotic cells. If injury persists, there will be substitution of hepatocytes with abundant ECM having contractile, inflammatory, and fibrogenic properties.[3] Activation of hepatic stellate cells (HSCs) is a crucial step of tissue injury and regeneration.[4] Quiescent HSCs present in space of Disse will be activated and trans-differentiate into myofibroblasts like cells which are responsible for ECM production and accumulation in injured liver.[5] Accumulation of

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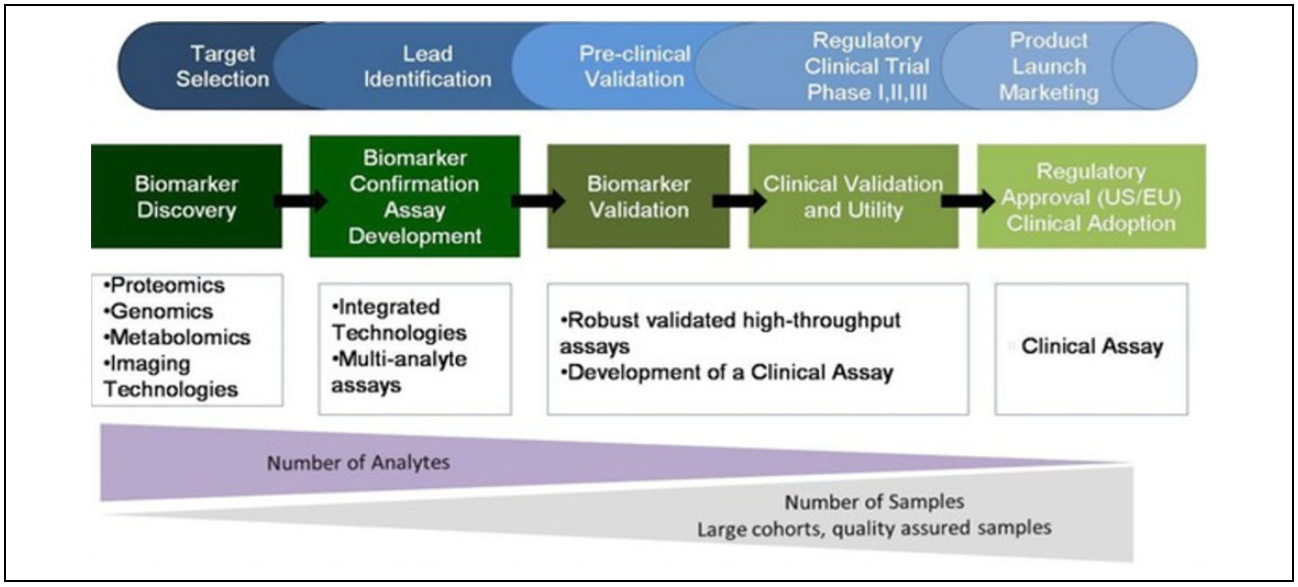

Figure 1. Biomarker discovery and validation; advances in omics will generate clinically significant biomarker candidates which need validation through verification studies and clinical assay development. *Source*: Adapted from the study by Nies et al.[12]

ECM is due to increased synthesis and decreased degradation by over expression of tissue inhibitors of metallo proteinases which inhibits matrix metallo proteinases. Fibrotic liver contains 3–10 times more ECM compared to normal liver which includes collagen types, glycoproteins, proteoglycans, and glycosaminoglycans.[2]

Accurate assessment of degree of hepatotoxicity especially at early stage is crucial for clinical management to predict prognosis and therapeutic decision even to reverse liver fibrosis/cirrhosis to normal architecture of liver. Despite development of potential diagnostic tests, for the past 50 years, liver biopsy is still considered as gold standard for diagnosis of chronic liver diseases (CLDs), which is associated with pain and complications. Biomarkers are used to diagnose or monitor the activity of disease and to assess therapeutic response for CLD.[2]

A biomarker is a molecule that is analytically measured with well-established performance characteristics in an established scientific frame work of evidence that elucidates physiological, toxicological, pharmacological, or clinical outcome.[6] Biomarkers can be gene variants, single nucleotide polymorphisms, gene expression products, metabolites, polysaccharides, circulating nucleotides, and proteins.[7]

Validation of a biomarker includes assessing the biomarker, measuring the performance characteristics, and determining the range of conditions for reproducibility and accuracy. Biomarker validation relates biomarker with biological process and clinical end point and is necessary for fit-for-purpose. Validation helps research data for better patient care. An ideal biomarker for CLD should be specific, sensitive to indicate active damage, prior to histological changes, easily accessible in peripheral tissue, and cost-effective.[2,6] A biological marker objectively measures and evaluates normal biological, pathogenic process, or pharmacological response to a drug. Surrogate markers serve as a substitute for a clinically meaningful end point. Prognostic biomarker indicates likely outcome of a disease irrespective of treatment. Predictive biomarker helps to assess response to a particular treatment. Pharmacodynamic biomarker gives interaction between drug and target.[6,7] In this review, an attempt has been made to understand the process of proteomic biomarker development which includes protein biomarker discovery, validation, and pitfalls in biomarker pipeline for CLD.

Biomarker development by proteomic approach

Establishment of correlation between disease state and biomarker alterations will help clinician for diagnosis and tailored therapy.[8] In CLD, protein domain will have alterations where the amount of protein from liver enters into circulation and serves as an indicator for degree of liver dysfunction, which holds good for discovery of novel protein biomarkers using proteomics.[9] Proteins have more structural diversity and stability than DNA and RNA and carry more information than nucleic acids which are dynamic and reflection of cellular physiology.[10] Advances in proteomic approach help discover and identify clinically significant protein biomarker candidates for CLD. Protein biomarker pipeline includes a series of essential components such as discovery, research assay optimization, analytical and clinical validation, and clinical utility (Figure 1).[11,12]

Protein biomarker candidate discovery

Protein biomarker discovery is a simplified, semiquantitative, unbiased binary comparison between diseased and

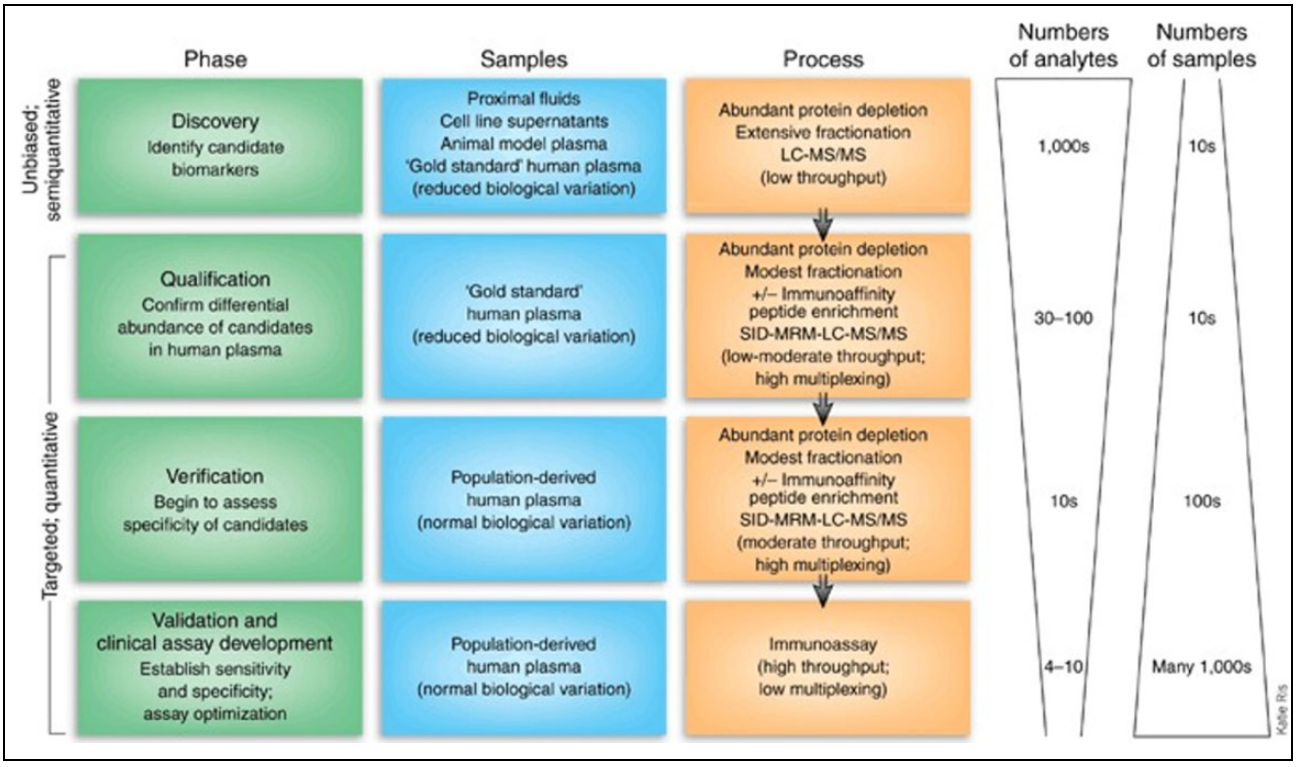

Figure 2. Discovery of protein biomarker; protocol for the development of protein biomarker candidate. *Source*: Adapted from the study by Rifai et al.[13]

normal using biological samples for maximal detection of significant protein expression differences. It needs avoiding contamination of other diseases and confounding factors.[2,13] Proteins that are differentially expressed between CLD and normal are due to changes in translation, posttranslational modifications, and degradation or that are involved in pathophysiological changes which are good sources of biomarker candidates.[10] Comparative analysis between diseased and healthy generates hundreds of protein biomarker candidates that are differentially expressed. There is an inverse relationship between number of samples analyzed and number of proteins quantified (Figure 2).[13,14]

Discovery of protein biomarker candidates for CLD, plasma/serum is the best choice among other body fluids, represents physiological and pathological process.[15] During discovery phase, the variables (study design, preanalytical, and analytical) which affect precision should be minimized. Study population should be selected from a well-defined study design with definite inclusion and exclusion criteria to minimize bias. Case control study or cohort study usually considered as a better study design for discovery phase of biomarker. Multiple sources of bias could be seen in retrospective and observational studies.[16] Preanalytical variables such as type and manufacture of collection tubes, phlebotomy device, patient's posture, time of sample collection, type of sample to be collected, storage conditions, and sample preparation should be controlled in order to get significant observation. Analytical variables such as mass resolution and collision energy need to be controlled to minimize the source of variations.[13,14]

In candidate discovery for CLD, to obtain significant protein expressional difference, use of gold standard sample is recommended. Plasma is the biofluid of choice (Human Proteomic Organization), and contains proteins that reflect a variety of human diseases.[17] Anticoagulants (EDTA or citrate) cause osmotic shifting of fluid from cell to plasma, which gives 10% less values when compared to serum and are known to chelate cations, and give negative results in case protein of interest has cations in its structure. Antigenic epitope mask might happen which reduces immunoreactivity because of heparin.[13,18] Compared to plasma, proteins are more stable in serum. For large studies, serum is the preferred sample by clinicians since it is the most simple matrix.[14] Although individual sample analysis is recommended, pooling strategy with definite criteria from multiple individuals reduces sample number and cost.[19]

Protein biomarker discovery in plasma/serum is complicated. Around 99% of protein content is comprised of 20 abundant proteins which interfere in identification and characterization of low abundant proteins by mass spectral and electrophoretic analysis.[20–23] Depletion of abundant proteins allows detection of low abundant proteins. But there is a chance for further removal of low abundant proteins that are bound to high abundant proteins.[14] For

accurate protein biomarker candidate discovery for CLD, depletion of albumin (55% of total protein) and immunoglobulins may be achieved using high affinity columns.[21,22] In two-dimensional gel electrophoresis (2-DE), depletion dilemma can be rectified using narrow pH (3–5.6) range which avoids interference of abundant proteins (albumin, transferrin, and immunoglobulins).[9,14]

After depletion, discovery may be carried out by fractionation and purification using different analytical methods: 2-DE for separation of proteins followed by identification of significant protein spots using software tools. Identified spots are subjected for in-gel digestion to identify peptides and proteins either by surface-enhanced laser desorption-ionization (SELDI) or matrix-assisted laser desorption-ionization (MALDI) or liquid chromatography-mass spectrometry (LC-MS) and proteomics search engines, that is, Mascot or SEQUEST.[24–26] Unlike gel-based discovery, LC-MS carried out before or after enrichment of proteins by trypsin digestion, splitting long proteins into short peptides followed by chromatographic separation in addition to mass to charge ratio.[14,27,28]

2-DE has limited sensitivity and reproducibility compared to LC-MS. The main disadvantage of SELDI/MALDI is difficulty in detection of differential pattern and identification of peaks. Automated LC-MS is suitable for protein biomarker discovery. Secondary ions collected from chromatographic profiles from MS spectra are subjected to proteomic search engines.[29,30] Identified peptides are used to determine differential expression between CLD and normal. Use of parametric statistical tools prior to peptide identification is recommended. Biomarker candidates reported and identified for CLD by one group of researchers are not identified by another group (Table 1) because of lack of standardization of multistep procedures. Selection of specific criteria during LC-MS gives complexity and errors for reproducibility between laboratories. Biomarker discovery and validation should be performed in a blinded fashion, free from bias, and performed in a similar fashion that remove all confounding factors and generate significant biomarker candidates.[30]

Biomarker validation

Biomarker validation is necessary to deliver high-quality research data for effective use of biomarker for better patient care. Great interest and technological advancement in biomarker discovery results in identification of protein biomarker candidates for CLD. Biomarker candidates require verification that demonstrates the differential expression which remains detectable by assay to be used for validation.[13] Despite numerous biomarker candidates identification, verification may be done only for few qualified candidates in terms of marker performance and reagent availability.[10] Proteins that act in cellular pathways and deregulated in CLD should be considered for further validation.[19] Validation of biomarker and clinical assay optimization requires measurement of thousands of patient samples with narrow measurement coefficient of variation values.[13]

Assay optimization

As MS is unable to achieve high measurement accuracy and precision, it is necessary to develop antibodies for quantification of biomarker candidates. Concentration of protein in serum or plasma ranges from picograms to nanograms per milliliter; highly sensitive immunotechniques are required for quantification. Enzyme-linked immunosorbent assay (ELISA) is the best alternative for quantification of these proteins compared to sophisticated nonimmune-based techniques.[35] Capture and detection antibodies (monoclonal or polyclonal), which detect distinct epitope of the protein, are needed to form sandwich reaction. Specificity of antibodies is established using Western blot or immunostaining. During development of ELISA, care should be taken to minimize the effect of variables such as avidity, concentration of antibodies (monoclonal capture/detection 0.5–4/0.25–2 μg/ml and polyclonal capture/detection 0.2–0.8/0.05–0.4 μg/ml), incubation time and temperature, sample volume, dilution of sample, pH, composition and concentration of diluents, enzyme, substrate, and quality of detector which affect performance characteristics. Fluorescent or chemiluminescent are other alternatives for better sensitivity.[13,36]

Analytical evaluation

Newly developed assay requires analytical validation before evaluating clinical utility in terms of performance characteristics such as outcome studies, clinical requirement, proficiency testing, and goals set by regulatory agencies.[35] Preanalytical variables should be characterized and controlled in various physiological and pathological conditions. Time of collection of sample (fasting or fed state) should be defined. In fed state, chylomicrons do not affect ELISA. Selection of appropriate sample (plasma or serum) and use of anticoagulants should be determined. Storage conditions and duration of storage should be examined. Physiological factors such as age, gender, and ethnicity significantly affect protein concentrations along with lifestyle factors. Pathological conditions and drugs which influence protein concentrations should be examined before estimation.[13]

Indicators of accuracy, precision, analytical measurement range, and reference intervals should be defined. Trueness is the closeness of agreement between average measured values of different samples which reflect bias (systemic error). Accuracy is the closeness of agreement between the values measured and true concentration of analyte.[37] Newly discovered methods usually do not have reference materials and methods and should use alternative

Table 1. Protein biomarker candidates identified by proteomic analysis for liver fibrosis.

Authors	Etiology of liver fibrosis	Type of sample	Proteomic techniques	Protein biomarker candidates identified
White et al.[31]	HCV	Serum	2-DE and LC-MS	α2 macroglobulin Haptoglobin Complement C4 Serum retinol binding protein Apolipoprotein A1 Apolipoprotein A-IV
Gangadharan et al.[32]	HCV	Serum	2-DE and LC-MS	α2 macroglobulin Inter-α-trypsin inhibitor heavy chain H4 α1 antichymotrypsin Apolipoprotein L1 Paraoxonase/aryleserase 1 Zinc-α2-glycoprotein CD5 antigen-like protein β2 glycoprotein 1
Gangadharan et al.[9]	HCV	Serum	2-DE, LC-MS, and in-solution isoelectric focusing	Beta chains of C3 and C4
Gangadharan et al.[33]	HCV	Serum	2-DE and LC-MS	Adiponectin Sex hormone binding protein 14-3-3 protein zeta/delta Complement C3dg Immunoglobulin J chain Apolipoprotein CIII Corticosteroid binding globulin α2 HS glycoprotein Lipid transfer inhibitor protein Haptoglobin-related protein
Katrinli et al.[34]	HBV	Liver tissue	2-DE and LC-MS	Apolipoprotein A1 Pyruvate kinase Glyceraldehyde 3-phosphate dehydrogenase Glutamate dehydrogenase Alcohol dehydrogenase Transferrin, peroxiredoxin 3 Keratin 5, annexin
Nallagangula et al. (2017) (unpublished data)	ALD	Serum	2-DE and LC-MS	Serotransferrin Keratin isoforms Vitamin D binding protein isoform 3 Angiotensinogen preproprotein CD5 antigen-like protein Hemopexin precursor α1 antichymotrypsin Glycerol kinase isoform X1 Sex hormone binding protein

HCV: hepatitis C virus; HBV: hepatitis B virus; ALD: alcoholic liver disease; 2-DE: two-dimensional electrophoresis; LC-MS: liquid chromatography-mass spectrometry.

protocols such as spike, recovery, and linearity. Use of specific antibodies should be necessary to have no cross reactivity with other proteins. Care should be taken during ELISA development to minimize the errors because of exogenous and endogenous substances. The factors (buffer components, sample matrix, compliment, and rheumatoid factor) can impact antibody binding in natural samples and therefore influence the accuracy of results should be ruled out.[38] Repeatability is the measurement performed in the same condition, and reproducibility is the measurement performed in different conditions. To assess precision, two replicates per sample per run, and two runs per day for least 20 days are recommended.[39]Reference intervals must be defined for protein of interest and new methodology by comparing healthy individual values similar to those of patient values.[39] Reference values should be subdivided into groups based on age, gender, race, and physiological states. Normal distribution of reference intervals for protein of interest for parametric analysis is presented as mean $\pm$ 2SD and for nonparametric analysis will be presented as percentiles. Limits of detection and quantification must be defined with acceptable accuracy and precision. Limit of

detection is the lowest value that exceeds the measurand value against blank sample which does not have protein of interest. Linearity gives the relation between observed value and expected value which is above the range of measurand values.[13,39]

Clinical validation

After analytical validation of new methodology for protein of interest, biomarker candidate should confirm the performance characteristics in terms of consistency and accuracy in clinical evaluation to diagnose or predict the clinical outcome of CLD. The newly identified biomarker candidate should satisfy the following criteria.[40]

Sensitivity of biomarker

The ability of a biomarker or change in magnitude of a biomarker with precision which is sensitive enough to reflect a meaningful change in clinical end point of CLD.

Specificity of biomarker

The ability of a biomarker or change in magnitude of a biomarker which distinguish patients who are responders and nonresponders in terms of change in clinical end point of CLD.

Probability of false positive

Desired change of biomarker is not reflected by positive change in clinical end point or even worse is associated with negative change in a clinical end point of CLD.

Probability of false negative

No change or small change is observed in magnitude of biomarker which fails to signal positive and meaningful change in a clinical end point of CLD.

Pharmacokinetic/pharmacodynamic model

Correlation between changes in biomarker and drug exposure, to predict future outcome or standardization of dose adjustments based on biomarker measurements.

Likelihood ratio of biomarker indicates certainty of the diagnosis of disease prevalence and calculates posttest odds of having a disease as the prevalence changes. Receiver operating characteristic (ROC) curve is the comparison of diagnostic accuracy of two or more tests and to define appropriate cutoff for clinical utility of test. Likelihood ratio and ROC curve are derived from sensitivity and specificity values.[2,41]

Clinical utility

Clinical utility predicts positive outcome of drug in selected and unselected groups. Novel biomarker candidate needs to be evaluated in a series of human population (sub and stratified). In phase I (exploratory phase), test results should be different from patients with confirmed CLD and those of control population without CLD. Area under ROC curve should be >0.5 for newly identified biomarker candidate to proceed further. In phase II (challenge phase), different cutoff values for sensitivity and specificity should be defined with diagnostic accuracy to predict the presence or the absence of CLD. Phase III (advanced clinical phase) is to establish diagnostic accuracy of biomarker in target population in different geographical regions independently. Phase IV (outcome phase) gives the positive influence of test to get healthy outcome of CLD by evaluating both tested and untested patients with respect to diagnostic and therapeutic intervention.[13,42,43]

Pitfalls and limitations

In current medical research, novel biomarker should have the ability to improve treatment which is cost-effective.[7] Newly identified biomarkers for CLD are unable to replace the existing conventional markers in clinical practice due to errors in study design or experimental execution. Despite numerous biomarker candidates identified for CLD, few biomarkers only validated successfully. Pitfalls in biomarker pipeline are because of no proper relation between discovery, verification, and clinical validation (due to lack of definite selection criteria in discovery phase, biomarker verification (sensitivity and specificity), and less robustness in analytical validation) as well as less structure and scientific factors which fail to give strong evidence for better patient care (Figure 3).[7,19]

During discovery phase, appropriate and well characterized clinical specimen has impact on outcome of identification process.[44] Selection of patients for biomarker studies should be done by specialist to ensure the presence or absence of the disease. Randomization and optimal selection of patients with single etiology are necessary and are well matched with age and gender with the same ethnicity. Other lifestyle factors play an important role in selection criteria, namely, body mass index, habits, physical activity as well as metabolic syndromes, and use of drugs.[7] Small sample size and lack of information about history may give false negative values in discovery phase.[13,45] Proper procedures for samples collection, handling, and storage to avoid denaturation of proteins should be followed. Systematic monitoring of quality of sample over a time period is necessary.[7] Suitable semiquantitative methods and sophisticated technologies like LC-MS along with proper analysis and data interpretation can improve biomarker candidate quality and yield.

Validation of biomarker is expensive and time-consuming. Protein biomarker quantification is essential to have specific detection and capture antibodies with high sensitivity to form sandwich method with low concentrated protein in biological specimens. Analysis of protein of

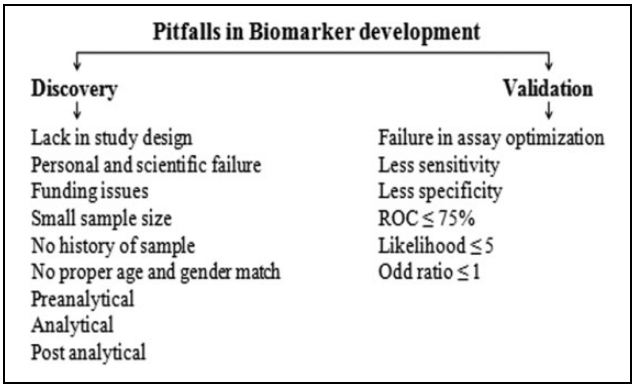

Figure 3. Pitfalls and limitations in biomarker development.

interest should be carried in triplicates and reported in mean and standard deviation.[7,13] In research laboratory, quality control procedures are less compared to clinical laboratories, and basic steps should be implemented to get accuracy and precision.[46] Difficulties in validation strategies, which need well-defined sub and stratified population matched with pathological and physiological factors of early disease state, are main reasons for pitfalls. Lack of sensitivity, specificity of biomarker for disease progression and regression with ROC $\leq$ 75%, and likelihood ratio $\leq$ 5 with odd ratio $\leq$ 1 are limitations in biomarker pipeline.[7]

Transition from research to routine

Newly identified biomarker needs to satisfy and fulfill the need of application which can be able to separate patients into groups that clinicians would treat differently and could be able to give reliable outcome of the treatment and it should be evidence based.[46] New test should add or replace the information provided by existing biomarkers for CLD and cost-effective for better patient care.[47] Introduction of new biomarker from research laboratory into clinical laboratory is three-way collaboration involving research laboratory, diagnostic industry, and clinical laboratory.[48] Care should be taken in research laboratory about selection of novel biomarker evaluation at early stage to minimize the methodological bias (preanalytical, analytical, and postanalytical) which may affect results.[49,50] Validated biomarker in research laboratory will be transferred to specialist referral laboratory to confirm the assessment in clinical setting.[47]

Once new test has beneficial effect on patient outcome which is evidence based and cost-effective, it will be introduced into funded health care system. Biomarker has to meet analytical validation, quality control, external validation, personal qualification, training, and documentation for approval (510(k)/premarket approval (PMA)/in vitro diagnostic (IVD) Directive 98/97/EC).[13,47] The test developed should be suitable for clinical laboratory and capable to meet basic requirements (robustness, stability of reagents, acceptable turnaround time, adaptability for automation, and low cost). If biomarker is ideally measured in serum which does not require special handling, storage with robust analytical procedure, rejection of samples will be minimized which decreases turnaround time. Internal quality control should be robust as external quality control may not be available for newly discovered and validated biomarkers. Interlaboratory comparison can provide information about accuracy and precision at early stages. Definite reference intervals and linearity range will help for best practice of biomarker.[47]

Regulatory requirements

IVD device to enter into market must meet the rules and regulations of 510(k) premarketing clearance or PMA by the food and drug administration (FDA) in the United States, the Pharmaceutical Affairs Law (PAL) and Market Authorization Holder by Pharmaceutical and Medical Devices Agency of Japan, and IVD Directive 98/97/EC by member states of European Union. According to FDA, 510(k) process is that new test should measure existing FDA classified analyte I or II where there is predicate cleared test which are commercially available. Information about new test should include classification, performance characteristics, and analytical capability (accuracy, precision, linearity, specificity, and sensitivity) comparison with that of existing predicate test. Class III, which is associated with high risk or clinical utility of biomarker or novel technological measurements or no predicate device, needs PMA process.[13,51,52]

Companion diagnostics

Companion diagnostics (CDx) is the central part of personalized medicine. CDx is simultaneous development of drug and diagnostic test: in vitro diagnostic device which provides information about safe and effective use of corresponding therapeutic product. CDx includes screening and detection, prognosis, monitoring, and theranostics. The key indicator for CDx is robustness of financing environment for drug and diagnostic companies which minimizes costs from selection of patient population till clinical trials. CDx improves chances for approval and increases market uptake. There is a need for CDx which can be able to provide diagnostic test specific for therapeutic drug for cost-effective and successful management of CLD.[7,53]

Conclusion

Early diagnosis of CLD is essential for disease management and even reversibility of liver fibrosis/cirrhosis. Concentration of proteins expressed from liver into circulation serves as an indicator for liver dysfunction and good source of biomarker development based on proteomic approach. Technological advancement generates biomarker candidates which is a prerequisite for validation in terms

of performance characteristics, analytical validation, accuracy, precision, and clinical utility. Factors that affect discovery and validation should be controlled to overcome pitfalls in biomarker pipeline. Evidence-based biomarker which fulfills regulatory requirements should be introduced into clinical practice by collaboration with research laboratory, diagnostic industry, and clinical laboratory. Rapid development in CDx would provide a cost-effective best practice for the management of CLD.

Declaration of Conflicting Interests

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References

1. Hong H and Tong W. Emerging efforts for discovering new biomarkers of liver disease and hepatotoxicity. *Biomark Med* 2014; 8(2): 143–146.
2. Nallagangula KS, Nagaraj SK, Venkataswamy L, et al. Liver fibrosis: a compilation on the biomarkers status and their significance in disease progress. *Future Sci OA* 2017; 4(1): FSO250. DOI: 10.4155/fsoa-2017-0083.
3. Gressner OA, Weiskirchen R, and Gressner AM. Evolving concepts of liver fibrogenesis provide new diagnostic and therapeutic options. *Comp Hepatol* 2007; 6: 7. DOI: 10.1186/1476-5926-6-7.
4. Ebrahimi H, Naderian M, and Sohrabpour AA. New concepts on pathogenesis and diagnosis of liver fibrosis; a review article. *Middle East J Dig Dis* 2016; 8(3): 166–178.
5. Scott LF. Hepatic fibrosis. In: Schiff ER, Sorrell MF and Maddrey WC (eds) *Schiff's diseases of the liver*, 11th ed. Singapore: Wiley-Blackwell, 2012, pp. 297–305.
6. Hunter DJ, Losina E, Guermazi A, et al. A pathway and approach to biomarker validation and qualification for osteoarthritis clinical trials. *Curr Drug Targets* 2010; 11(5): 536–545.
7. Drucker E and Krapfenbauer K. Pitfalls and limitations in translation from biomarker discovery to clinical utility in predictive and personalized medicine. *EPMA J* 2013; 4: 7.
8. George P. Bring on the biomarkers. *Nature* 2011; 469: 156–157.
9. Gangadharan B, Antrobus R, Chittenden D, et al. New approaches for biomarker discovery: the search for liver fibrosis markers in hepatitis C patients. *J Proteome Res* 2011; 10: 2643–2650.
10. Aebersold R, Anderson L, Caprioli R, et al. Perspective: a program to improve protein biomarker discovery for cancer. *J Proteome Res* 2005; 4: 1104–1109.
11. Anderson NL. The role of multiple proteome platforms in a pipeline for new diagnostics. *Mol Cell Proteomics* 2005; 4: 1441–1444.
12. Nies KM, Ivy DD, and Everett DA. The untapped potential of proteomic analysis in pediatric pulmonary hypertension. *Proteomics Clin Appl* 2014; 8: 862–874.
13. Rifai N, Gillette AM, and Carr AS. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat Biotechnol* 2006; 24(8): 971–983.
14. Parker CE and Borchers CH. Mass spectrometry based biomarker discovery, verification and validation—quality assurance and control of protein biomarker assays. *Mol Oncol* 2014; 8: 840–858.
15. Farrah T, Deutsch EW, Omenn GS, et al. A high confidence human plasma proteome reference set with estimated concentrations in peptide atlas. *Mol Cell Proteomics* 2011; 10: M110.006353.
16. Goossens N, Nakagawa S, Sun X, et al. Cancer biomarker discovery and validation. *Trans Cancer Res* 2015; 4(3): 256–269.
17. Anderson NL and Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics* 2002; 1: 845–867.
18. Apple FS, Alan HB, Johanes M, et al. Future biomarkers for detection of ischemia and risk stratification in acute coronary syndrome. *Clin Chem* 2005; 51(5): 810–824.
19. Paulovich AG, Whiteaker JR, Hoofnagle AN, et al. The interface between biomarker discovery and clinical validation: the tar pit of the protein biomarker pipeline. *Proteomics Clin Appl* 2008; 2: 1386–1402.
20. Anderson L. Candidate based proteomics in the search for biomarkers of cardiovascular disease. *J Physiol* 2005; 563(1): 23–60.
21. Fillip S, Vougas K, Zoidakis J, et al. Comparison of depletion strategies for the enrichment of low-abundance proteins in urine. *PLoS One* 2015; 10(7): e0133773.
22. Steel LF, Trotter MG, Nakajima PB, et al. Efficient and specific removal of albumin from human serum samples. *Mol Cell Proteomics* 2003; 2(4): 262–270.
23. Xiao Z, Conrads TP, Lucas DA, et al. Direct ampholyte-free liquid-phase isoelectric peptide focusing: application to the human serum proteome. *Electrophoresis* 2004; 25: 128–133.
24. Hoefkens J. Towards unbiased biomarker discovery. *Biomarkers* 2010; 1: 19–24.
25. Adkins JN, Susan MV, Auberry KJ, et al. Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry. *Mol Cell Proteomics* 2002; 1(12): 947–955.
26. Tirumalai RS, Chan KC, Prieto DA, et al. Characterization of the low molecular weight human serum proteome. *Mol Cell Proteomics* 2003; 2(10): 1096–1103.
27. Krastins B, Prakash A, Sarracino DA, et al. Rapid development of sensitive, high-throughput, quantitative and highly selective mass spectrometric targeted immunoassays for clinically important proteins in human plasma and serum. *Clin Biochem* 2013; 46: 399–410.
28. Marrer E and Dieterle F. Promises of biomarkers in drug development–a reality check. *Chem Biol Drug Des* 2007; 69: 381–394.

29. Gillette MA, Mani DR, and Carr SA. Place of pattern in proteomic biomarker discovery. *J Proteome Res* 2005; 4(4): 1143–1154.
30. Silberring J and Ciborowski P. Biomarker discovery and clinical proteomics. *Trends Analyt Chem* 2010; 29(2): 128. DOI: 10.1016/j.trac.2009.11.007.
31. White IR, Patel K, Symonds WT, et al. Serum proteomic analysis focused on fibrosis in patients with hepatitis C virus infection. *J Transl Med* 2007; 5: 33. DOI: 10.1186/1479-5876-5-33.
32. Gangadharan B, Antrobus R, Dwek RA, et al. Novel serum biomarker candidates for liver fibrosis in hepatitis C patients. *Clin Chem* 2007; 53(10): 1792–1799.
33. Gangadharan B, Bapat M, Rossa J, et al. Discovery of novel biomarker candidates for liver fibrosis in hepatitis C patients: a preliminary study. *PLoS One* 2012: 7(6): e39603. DOI: 10.1371/journal.pone.0039603.
34. Katrinli S, Ozdil K, Sahin A, et al. Proteomic profiling of HBV infected liver biopsies with different fibrotic stages. *Proteome Sci* 2017; 15: 7. DOI: 10.1186/s12953-017-0114-4.
35. Vitzthum F, Behrens F, Anderson NL, et al. Proteomics: from basic research to diagnostic application. A review of requirements & needs. *J Proteome Res* 2005; 4: 1086–1097.
36. Wild D. *The immunoassay handbook*. 3rd ed. Amsterdam: Elsevier, 2005.
37. Dybkaer R. Vocabulary for use in measurement procedures and description of reference materials in laboratory medicine. *Eur J Clin Chem Biochem* 1997; 35: 141–173.
38. Nallagangula KS, Shashidhar KN, Lakshmaiah V, et al. Cirrhosis of liver: interference of serpins in quantification of SERPINA4–a preliminary study. *Pract Lab Med* 2017; 9: 53–57. DOI: 10.1016/j.plabm.2017.10.002.
39. NCCLS. *Evaluation of the linearity of quantitative measurement procedures: a statistical approach, approved guideline*. Wayne, PA: NCCLS Document EP6-A, NCCLS, 2003.
40. Lesko LJ and Atkinson AJ. Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation strategies. *Annu Rev Pharm Toxicol* 2001; 41: 347–366.
41. Bossuyt PM, Reitsma JB, Bruns DE, et al. The STARD statement for reporting studies of diagnostic accuracy: explanation and elaboration. *Clin Chem* 2003; 49(1): 7–18.
42. Obuchowski NA, Lreber ML, and Wians FH. ROC curves in clinical chemistry: uses, misuses, and possible solutions. *Clin Chem* 2004; 50: 1118–1125.
43. Sackett DL and Haynes RB. The architecture of diagnostic research. *Br Med J* 2002; 324: 539–541.
44. Zolg W. The proteomic search for diagnostic biomarkers: lost in translation? *Mol Cell Proteomics* 2006; 5(10): 1720–1726.
45. Waerner T, Urthaler J, and Krapfenbauer K. The role of laboratory medicine in healthcare: quality requirements of immunoassays, standardization and data management in prospective medicine. *EPMA J* 2010; 1: 619–626.
46. Sturgeon CM, Hoffman BR, Chan DW, et al. National academy of clinical biochemistry laboratory medicine practice guidelines for use of tumor markers in clinical practice: quality requirements. *Clin Chem* 2008; 54: e1–e10.
47. Sturgeon C, Hill R, Hortin GL, et al. Taking a new biomarker into routine use–a perspective from the routine clinical biochemistry laboratory. *Proteomics Clin Appl* 2010; 4: 892–903.
48. Hortin GL, Jortani SA, Ritchie JC, et al. Proteomics: a new diagnostic frontier. *Clin Chem* 2006; 52: 1218–1222.
49. Ransohoff DF. How to improve reliability and efficiency of research about molecular markers: roles of phases, guidelines, and study design. *J Clin Epidemiol* 2007; 60: 1205–1219.
50. Hemingway H, Riley RD, and Altman DG. Ten steps towards improving prognosis research. *Br Med J* 2009; 339: 410–414.
51. Code Federal Regulations, Vol.21 CFR807. http://frwebgate.access.gpo.gov/cgi-bin/get-cfr.cgi?YEAR=current&TITLE=21&PART=807&SECTION=81&SUBPART=&TYPE=TEXT (accessed August 2017).
52. Code Federal Regulations, Vol.21 CFR814. http://frwebgate.access.gpo.gov/cgi-bin/get-cfr.cgi?YEAR=current&TITLE=21&PART=814&SECTION=1&SUBPART=&TYPE=TEXT (accessed August 2017).
53. Agarwal A, Ressler D, and Snyder G. The current and future state of companion diagnostics. *Pharm Personal Med* 2015; 8: 99–110.

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A proteomic approach of biomarker candidate discovery for alcoholic liver cirrhosis

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Abstract

Alcoholic liver disease (ALD) progresses from steatosis to alcoholic hepatitis to fibrosis and cirrhosis. Liver biopsy is considered as the gold standard method for diagnosis of liver cirrhosis and provides useful information about damaging process which is an invasive procedure with complications. Existing biomarkers in clinical practice have narrow applicability due to lack of specificity and lack of sensitivity. The objective of this article is to identify proteomic biomarker candidates for alcoholic liver cirrhosis by differential expression analysis between alcoholic liver cirrhotic and healthy subjects. Blood samples were collected from 20 subjects (10 alcoholic liver cirrhosis and 10 healthy) from R. L. Jalapa Hospital and Research Centre, Kolar, Karnataka, India. Differential protein analysis was carried out by two-dimensional electrophoresis after albumin depletion, followed by liquid chromatography–mass spectrometry. The image analysis found 46 spots in cirrhotic gel and 69 spots in healthy gel, of which 14 spots were identified with significant altered expression levels. Based on the protein score and clinical significance, among 14 spots, a total of 28 protein biomarker candidates were identified: 13 with increased expression and 15 with decreased expression were categorized in alcoholic liver cirrhosis compared to healthy subjects. Protein biomarker candidates identified by "-omics" approach based on differential expression between alcoholic liver cirrhotic subjects and healthy subjects may give better insights for diagnosis of ALD. Prioritization of candidates identified is a prerequisite for validation regimen. Biomarker candidates require verification that demonstrates the differential expression will remain detectable by assay to be used for validation.

Keywords

Alcoholic liver cirrhosis, protein biomarker candidates, albumin depletion, two-dimensional electrophoresis, liquid chromatography–mass spectrometry

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Introduction

Cirrhosis of the liver is the histological development of regenerative nodules surrounded by fibrous bands in response to chronic liver injury, leading to portal hypertension and end-stage liver disease. Causes of cirrhosis of the liver are multifactorial. Despite varied etiology, the pathological characteristics which result in liver dysfunction are common.[1] In recent years, alcohol consumption has correlated with deaths from asymptomatic and self-limited fatty liver to cirrhosis of the liver. Alcoholic liver disease (ALD) progresses from steatosis to alcoholic hepatitis to fibrosis

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and frank cirrhosis (micronodular, occasionally mixed micronodular, and macronodular) and often occurs acutely against background chronic liver disease.[2] Multifactorial pathogenesis plays a role in progression of the disease. Accumulation of triacylglycerol in liver is an early and reversible effect of alcohol which increases peripheral lipolysis and alters liver redox potential leading to fatty acid synthesis. In ALD, generated prooxidants enhance antioxidant system results in lipid peroxidation.[3]

Acetaldehyde generated from alcohol is highly reactive and toxic; it binds to phospholipids and amino acids, resulting in the formation of abnormal folding of proteins in endoplasmic reticulum (ER) leading to ER stress.[3] Proliferation and activation of hepatic stellate cells (HSCs) in ALD are induced by Kupffer cells and hepatocytes. Chief mitogen for the activation of HSCs is platelet-derived growth factor, which is produced by Kupffer cells. Kupffer cells induce collagen synthesis through the production of transforming growth factor β, tumor necrotic factor α (TNF-α), and reactive oxygen species (ROS). Activated HSCs migrate and accumulate at tissue repair sites and secrete large amounts of extracellular matrix (ECM). HSCs collagen synthesis is regulated at transcription and post-transcriptional levels. Dysregulation of cytokine metabolism and activity is vital for alcohol-induced liver damage. TNF-α, a pro-inflammatory cytokine, is one of the key factors for pathophysiology of ALD. Hepatocytes induce fibrosis through the production of ROS or apoptosis followed by regenerative nodular formation.[3,4]

Diagnosing the degree of disease is a crucial step for successful management of ALD. Despite the development of potential diagnostic tests for the past 50 years, liver biopsy is considered as the gold standard method for diagnosis of liver cirrhosis and provides useful information about damaging process, namely, steatosis, lobular inflammation, periportal fibrosis, Mallory bodies, nuclear vacuolation, bile ductal proliferation, and fibrosis/cirrhosis.[2,5] Limitation of liver biopsy is highly invasive; poor sample quality and tissue size make biopsy nonreproducible, and it depends on the experience of the pathologist leading to interobserver variations. Risk allied for liver biopsy range from pain (84%) and hypertension, bleeding (0.5%), and damage to biliary system with approximately 0.01% mortality rate.[6] No single biomarker can establish alcohol to be the etiology of liver disease; existing biomarkers in clinical practice have narrow applicability due to lack of specificity and lack of sensitivity (distinguish intermediate stages) which prevent reliance on any single biomarker.[2,7]

An ideal biomarker should be organ specific, a sensitive indicator for active damage, easily accessible in peripheral tissue, and cost-effective and should give insights for diagnosis, monitor the activity of disease, and assess therapeutic response. The determination of biomarkers could be an easy, noninvasive, and inexpensive method to monitor the progression of liver disease. This leads to urgency in the progression of biomarker discovery for cirrhosis of the liver with the help of technological advancement in "-omics" approach. Discovery of biomarker candidates should be a simplified, unbiased, semiquantitative binary comparison between diseased and normal.[8] Although individual sample analysis is recommended, pooling strategy with definite selection criteria from multiple individuals reduces sample number and cost.[9] Alterations in protein domain due to ALD which enters into circulation hold good for discovery of biomarker candidates. In the present study, we tried to discover protein biomarker candidates for alcoholic liver cirrhosis whose concentration may be altered due to changes in translation, posttranslational modifications, and/or degradation using two-dimensional electrophoresis (2DE) after albumin depletion, followed by liquid chromatography–mass spectrometry (LC-MS).

Table 1. Details of 20 blood samples (10 alcoholic liver cirrhotic subjects and age- and gender-matched 10 healthy subjects) used for discovery of biomarker candidates by proteomic approach.

Sample ID	Gender	Age	Etiology	Sample ID	Gender	Age	Etiology
C1	M	36	NA	D1	M	36	ALD
C2	M	28	NA	D2	M	28	ALD
C3	M	62	NA	D3	M	62	ALD
C4	M	36	NA	D4	M	36	ALD
C5	M	35	NA	D5	M	35	ALD
C6	M	40	NA	D6	M	40	ALD
C7	M	70	NA	D7	M	70	ALD
C8	M	30	NA	D8	M	30	ALD
C9	M	62	NA	D9	M	62	ALD
C10	M	30	NA	D10	M	30	ALD

C: control; D: diseased (alcoholic liver cirrhosis); M: male; NA: not applicable; ALD: alcoholic liver disease.

Materials and methods

Samples

Blood samples were collected from 20 subjects: 10 clinically and diagnostically proven alcoholic liver cirrhotic subjects with varying degree and age- and gender-matched 10 healthy subjects (Table 1) from R. L. Jalappa Hospital and Research Centre, attached to Sri Devaraj Urs Medical College, a constituent of Sri Devaraj Urs Academy of Higher Education, Kolar, Karnataka, India. Individuals diagnosed with cirrhosis of the liver caused by ALD based on clinical history and symptoms, namely, ascites, encephalopathy, jaundice, and altered biochemical parameters, were included in the study. Individuals with diabetes and/or its complications, myocardial infarction, acute and chronic renal failure, pneumonia, and cancer were excluded from the study. Collection of blood samples from cirrhotic liver subjects and healthy subjects was carried out after obtaining informed consent, and the study is approved by Institutional Ethical Committee (DMC/KLR/IEC/61/2016-17).

Serum separation

Serum was collected from clotted blood using serum separator tubes centrifuged at 4000 r/min for 10 min. Serum was stored at −20°C till further analysis. All samples were used for discovery of protein biomarker candidates after depletion of abundant albumin. Desalting was carried out by acetone precipitation. 2DE was carried out to find differentially expressed proteins between cirrhotic and healthy subjects. Identified spots were characterized by LC-MS after in-gel trypsin digestion.

Reagents

Dye-based (Cibacron blue) prefractionation albumin depletion kit was procured from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Precast gels and other chemicals of analytical grade for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 2DE, in-gel trypsin digestion, and LC-MS were procured from Bio-Rad (Hercules, California, USA) and Sigma Aldrich (St Louis, Missouri, USA).

Depletion of albumin

Resuspended resin (200 µL aliquot of resin) was transferred into spin column (column volume: 900 µL; 10-µm pore-size polyethylene filter). Bottom of the column was twisted off and placed in a 1.5-mL collecting tube. Then, the sample is centrifuged at 12,000 × *g* for 1 min, the flow-through is discarded, and finally the spin column back is placed into the same collection tube. Around 200 µL of binding/wash buffer was added to the spin column. Then, the sample is centrifuged at 12,000 × *g* for 1 min, the flow-through is discarded, and, finally, the spin column back is placed into the new collection tube. About 50 µL of pooled serum sample (cirrhotic and healthy in separate columns) was added into resin and incubated for 2 min at room temperature. Then, the sample is centrifuged at 12,000 × g for 1 min, flow-through is reapplied to spin column, and incubated for 2 min at room temperature. Again, the sample is centrifuged at 12,000 × g for 1 min and flow-through is retained. Spin column was placed in a new collection tube. Resin was washed to release unbound proteins by adding 50 µL of binding/wash buffer for each 200 µL of the resin used. Retained fractions of cirrhotic and healthy samples were run in SDS-PAGE for confirmation of albumin depletion.[10]

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS gel was prepared according to the standard protocol. Samples from cirrhotic liver subjects and healthy subjects after depletion of albumin along with un-depleted samples were loaded in gel, and SDS-PAGE was carried out at 25 mA in 1× SDS running buffer for confirmation of depletion along with molecular weight marker. After electrophoresis, gel was incubated in a fixing solution (40% methanol, 10% acetic acid) at room temperature for 20 min. Gel was subjected for staining with sliver stain (0.1% silver nitrate, 36% formaldehyde) at room temperature for 20 min. Excess staining solution was removed, and the gel was washed with 5% acetic acid.[11–13]

Acetone precipitation

Acetone precipitation was carried out to remove excess salts which interferes electrophoretic run. Protein samples after depletion of albumin (cirrhotic and healthy separately) were placed in acetone-compatible tubes. Four times the sample volume of cold acetone (−20°C) was added into both tubes. The tubes were vortexed and incubated for 60 min at −20°C, followed by centrifugation at 13,000 × *g* for 10 min. Then the supernatant was disposed carefully for the retention of protein pellet.[14]

Two-dimensional polyacrylamide gel electrophoresis

Albumin depleted and desalted protein pellet (200 µg) from cirrhotic and healthy subjects were diluted with rehydration buffer (8 M urea, 2% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate, 50 mM dithiothreitol (DTT), 0.2% w/v Bio-Lyte 3/10 ampholyte, bromophenol blue) and used separately for 2DE with 7 cm (pH 4–7) nonlinear immobilized pH gradient dry strips (Bio-Rad). Samples were left overnight for rehydration on 7 cm (pH 4–7) dry strips. Isoelectric focusing was carried out at 250 V for 20 min, followed by 4000 V for 5 h at 20°C. Proteins were separated by 8–16% precast polyacrylamide gels at 200 V for 40 min. After electrophoresis, gels were subjected to staining with silver stain (0.1% silver nitrate, 36% formaldehyde) at room temperature for 20 min. Excess staining solution was removed, and the gel was washed with 5% acetic acid.[15]

Image analysis

For image analysis, scanned gels were processed using PDQuest 2-D analysis software (Bio-Rad). For differential analysis, the cirrhotic gel was compared with that of the healthy gel. Differential expression of proteins present in both cirrhotic and healthy gels was considered significant when the fold change was least 2 and $p \leq 0.05$ with 95% confidence interval with the application of rank-sum test.

In-gel digestion and peptide extraction

Excised spots were cut into cubes and transferred into a microcentrifuge tube, and 100 µL of destaining solution (100 mM ammonium bicarbonate/acetonitrile (1:1 vol/vol)) was added and incubated for 30 min; 500 µL of neat acetonitrile was added and the tubes were incubated for 10 min until gel pieces shrink; 50 µL of DTT solution (10 mM DTT in 100 mM ammonium bicarbonate buffer)

was added to cover the gel pieces and incubated in 56°C thermostat for 30 min; and 500 µL of acetonitrile was added to the tubes and further incubated for 10 min. All the liquid was removed from the tube. Following DTT treatment, to get reduction and alkylation of cystines and cysteines in the protein, 50 µL of iodoacetamide solution (55 mM iodoacetamide in 100 mM ammonium bicarbonate solution) was added to the tubes and incubated for 20 min at room temperature in dark. The gel pieces were again treated with acetonitrile for 10 min, and the entire liquid was removed from the tube. The gel pieces were saturated with trypsin buffer (13 ng/µL of trypsin in 10 mM ammonium bicarbonate in 10% acetonitrile) for 30 min. Cold trypsin (20 µg of trypsin in 1.5 mL of ice-cold 1 mM hydrochloric acid) was added to the tubes and incubated overnight at 37°C. Tubes were cooled to room temperature, gel pieces were centrifuged at 10,000 r/min for 1 min, and peptides were extracted in 100 µL of extraction buffer (1:2, 5% formic acid/acetonitrile) by incubating for 15 min at 37°C shaker, and the supernatant was withdrawn directly for LC-MS analysis.[16]

Mass spectrometric analysis

Mass spectrometric analysis of the extracted peptides was performed using Nano LCMS-LTQ-Orbitrap Discovery (Thermo Scientific) coupled to Nano LC (Agilent 1200). The samples were reconstituted in 0.1% formic acid prior to injection; 70-min gradient run was set up using acetonitrile and water with formic acid as the mobile phase. LTQ Orbitrap Discovery is a hybrid-type MS system with the ability to determine accurate *m*/*z* of intact precursors. The raw files post-MS run was analyzed using Proteome Discover software and MASCOT as search engine against human database.[17]

Results

SDS-PAGE analysis for depletion of albumin

Immobilized resin form of Cibacron blue was effective in binding abundant albumin from plasma/serum samples for depletion of significant amount. Human serum albumin (HSA) from pooled serum samples of cirrhotic and healthy subjects was depleted using Cibacron blue dye-loaded resin columns. Proteins in the flow-through were analyzed by SDS-PAGE (Figure 1) along with prestained molecular weight marker to investigate efficient depletion of HSA. Sensitive staining, silver stain, was helpful for the detection of low nanogram proteins when compared with Coomassie Brilliant Blue. Silver-stained gel demonstrated significant amount of abundant albumin depletion from serum samples of both cirrhotic and healthy subjects.

Identification of biomarker candidates

Synthetic gel image representative of all features in the differential analysis comparing samples from cirrhotic and healthy is shown in Figure 2. The image analysis software and statistical analysis found 46 spots in cirrhotic gel and 69 spots in control gel, of which 14 spots were identified with significant altered expression levels between cirrhotic and healthy based on quantitative ratio. These spots were

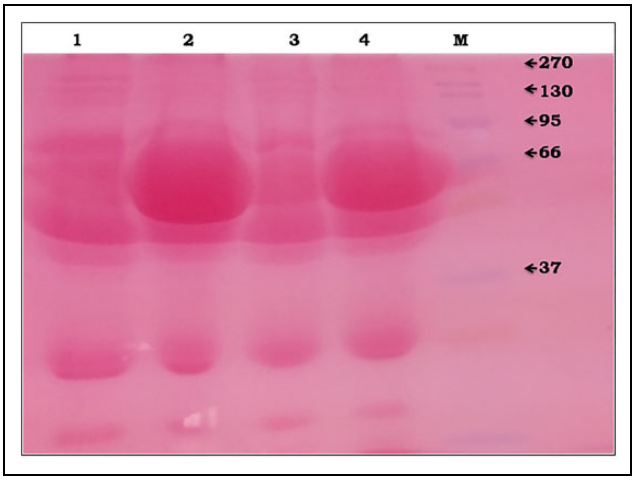

Figure 1. SDS-PAGE analysis for confirmation of albumin depletion (silver-stained gel). 1: Normal pooled albumin depleted serum. 2: Normal pooled serum. 3: Cirrhotic liver pooled albumin depleted serum. 4: Cirrhotic liver pooled serum. M: pre-stained molecular weight marker. SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis.

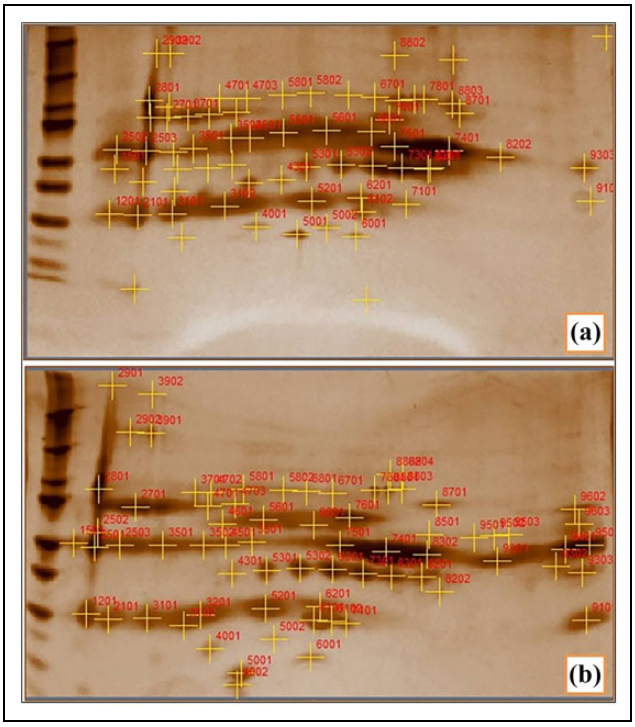

Figure 2. Comparison of 2DE gel images representative of all features in differential analysis from alcoholic cirrhotic and healthy subjects. (a) 2DE gel image of alcoholic liver cirrhotic subjects. (b) 2DE gel image of healthy subjects. 2DE: two-dimensional electrophoresis.

Table 2. Proteomic biomarker candidates identified by 2DE followed by LC-MS for alcoholic liver cirrhosis.

Biomarker candidate	Expression in ALD	Mol. Wt	Cal. pI	Physiological role
Keratin isoforms				Protects epithelial cells from damage or stress
Keratin type II cuticular Hb6 isoform X1	Increases	62	6.37	
Keratin type I cuticular Ha1	Increases	47.2	4.88	
Keratin type II cuticular Hb5 isoform 1	Increases	55.8	6.55	
Keratin type II cytoskeletal 6C	Increases	60	8	
Keratin type II cytoskeletal 2 epidermal	Increases	65.4	8	
Keratin type I cytoskeletal 9	Increases	62	5.24	
Lumican precursor	Increases	38.4	6.61	Collagen binding proteoglycan
pIgR isoform X1	Increases	83.2	5.74	Mediates transcellular transport of Igs
Serotransferrin precursor	Increases	77	7.12	Ferric ion binding protein
Ig lambda like polypeptide 5 isoform 1	Increases	23	8.84	Not known
Vitamin D binding protein isoform 3 precursor	Increases	55	5.63	Vitamin D transport and storage
Haptoglobin isoform 1 preproprotein	Increases	45.2	6.58	Binds free plasma hemoglobin
Transmembrane protein 201 isoform 1	Increases	72.2	9.22	Involved in nuclear movement during fibroblast polarization and migration
α-1-Antitrypsin precursor	Decreases	46.7	5.59	Protease inhibitor
Hemopexin precursor	Decreases	51.6	7.02	Scavenging heme
Apolipoprotein A-IV precursor	Decreases	45.3	5.38	Chylomicrons and VLDL secretion and catabolism
CD5 antigen like isoform X1	Decreases	38.7	5.66	Key regulator of lipid synthesis
Zinc-α2-glycoprotein precursor	Decreases	34.2	6.05	Lipid mobilization and fertilization
Dermcidin isoform 1 preproprotein	Decreases	11.3	6.54	Antimicrobial activity
α1-B-glycoprotein precursor	Decreases	54.2	5.86	Not known
Glycerol kinase isoform X1	Decreases	63.6	6.54	Transfer of phosphate from ATP to glycerol
α2-HS-glycoprotein preproprotein	Decreases	39.3	5.72	Role in endocytosis
Kininogen-1 isoform 1 precursor	Decreases	71.9	6.81	Role in blood coagulation
Sex hormone binding globulin isoform1 precursor	Decreases	43.8	6.71	Androgen transport protein
α1-Acid glycoprotein 1 precursor	Decreases	23.5	5.11	Acute phase protein
Leucine-rich α2-glycoprotein precursor	Decreases	38.2	6.95	Protein-protein interactions, signal transduction and cell adhesion
α2-Antiplasmin isoform X1	Decreases	56.6	6.89	Inactivating plasmin
Antithrombin-III precursor	Decreases	52.6	6.71	Serine protease inhibitor

pIgR: polymeric Ig receptor; 2DE: two-dimensional electrophoresis; LC-MS: liquid chromatography–mass spectrometry; ALD: alcoholic liver disease; Mol. Wt: molecular weight; Calc. pI: calculated isoelectric pH; Igs: immunoglobulins.

excised, digested with trypsin, analyzed by LC-MS, and identified by MASCOT database. These spots contain more than one protein; among 14 spots, a total of 68 proteins were identified. Many of the proteins were identified as the same protein in different locations on the gels and so among 68 proteins, we identified 46 candidate biomarkers for alcoholic liver cirrhosis. Among 46 candidate biomarkers, 28 were identified based on protein score and clinical significance (Table 2).

Among 28 protein biomarker candidates, 13 with increased expression and 15 with decreased expression were identified in alcoholic liver cirrhotic when compared to healthy subjects. Serum concentrations of keratin isoforms were found to increase in alcoholic cirrhosis. Immunoglobulins (Igs), namely, polymeric immunoglobulin receptor isoform X1 and IgGFc-binding protein precursor, were increased in cirrhotic liver when compared to healthy subjects but with low protein scores. Along with 13 features whose expression is increased in cirrhosis of the liver, angiotensinogen preproprotein, α2-macroglobulin isoform X1, were found to increase compared to healthy subjects. Serum albumin preproprotein, α-1-antitrypsin precursor and α-1-antichymotrypsin precursor showed decreased expression in alcoholic liver cirrhosis. Concentrations of glycerol kinase isoform X1 and kininogen-1 isoform 1 precursor were decreased in cirrhotic liver compared to healthy subjects.

Discussion

Invasive liver biopsy is the gold standard diagnostic tool for liver fibrosis/cirrhosis with varied etiology and to distinguish between intermediate stages. Reliable noninvasive biomarker with sensitivity and specificity is needed for diagnosis/prognosis and effective management of the disease.[8] In the present study, we used 2DE followed by LC-MS for identification of biomarker candidates for alcoholic liver cirrhosis. For maximal detection of meaningful protein expression difference, cases and controls should differ absolutely in terms of disease of interest. Simplified, unbiased binary comparison between diseased and healthy avoids contamination by other diseases and confounding

Table 3. Protein biomarker candidates identified by proteomic analysis for liver fibrosis.

Authors	Etiology of liver fibrosis	Type of sample	Proteomic techniques	Protein biomarker candidates identified
White et al.[23]	HCV	Serum	2DE, LC-MS	α2 Macroglobulin Haptoglobin Complement C4 Serum retinol binding protein Apolipoprotein A1 Apolipoprotein A-IV
Bevin et al.[24]	HCV	Serum	2DE, LC-MS	α2 Macroglobulin Inter-α-trypsin inhibitor heavy chain H4 α-1-Antichymotrypsin Apolipoprotein L1 Paraoxonase/aryleserase 1 Zinc-α2-glycoprotein CD5 antigen-like protein β2 Glycoprotein 1
Bevin et al.[25]	HCV	Serum	2DE, LC-MS, in-solution isoelectric focusing	Beta chains of C3 and C4
Bevin et al.[22]	HCV	Serum	2DE, LC-MS	Adiponectin, sex hormone binding protein 14-3-3 protein zeta/delta, complement C3dg Immunoglobulin J chain Apolipoprotein CIII Corticosteroid binding globulin α2-HS-glycoprotein Lipid transfer inhibitor protein Haptoglobin-related protein
Katrinli et al.[26]	HBV	Liver tissue	2DE, LC-MS	Apolipoprotein A1 Pyruvate kinase Glyceraldehyde 3-phosphate dehydrogenase Glutamate dehydrogenase Alcohol dehydrogenase Transferrin, peroxiredoxin 3 Keratin 5, Annexin

HCV: hepatitis C virus; HBV: hepatitis B virus; 2DE: two-dimensional electrophoresis; LC-MS: liquid chromatography–mass spectrometry.

factors which may alter the expression of protein results in false discovery of biomarker candidates.[18]

Discovery of biomarker candidates by proteomic approach is difficult, especially when the pH range is between 3 and 7, as abundant albumin interferes in identification and characterization of low abundant proteins by mass spectral and electrophoretic analysis. Accurate protein biomarker candidate discovery was achieved after depletion of albumin using dye-based affinity columns. Antibody-based immunoprecipitation is more robust for depletion of abundant proteins from plasma/serum and is suitable for identification of novel biomarker candidates.[19–21] Depletion dilemma can be rectified using narrow pH (3–5.6) range and avoids interference of abundant proteins (albumin, transferrin, and Igs) but chance to miss proteins whose isoelectric pH is in the alkaline range.[22]

Technological advancement in biomarker candidate discovery resulted in identification of protein biomarker candidates for chronic liver diseases (CLDs) with varied etiology (Table 3). Biomarker candidates identified require verification which demonstrates that the differential expression should remain detectable by assay to be used for validation.[18] Despite numerous biomarker candidates identified, verification may be done only for few qualified candidates in terms of marker performance and reagent availability.[27] Biomarker candidates that show significant expressional differences between diseased and healthy in discovery phase are prioritized. Proteins that are secreted and/or present on cell surface and that act in cellular pathways and deregulated in ALD should be considered for further validation.[9]

In the present study, keratin isoforms showed upregulation in alcoholic liver cirrhosis. Keratin is a fibrous structural protein that protects epithelial cells from damage and stress and regulates key cellular activities, namely, cell growth and protein synthesis.[28] Lumican, leucine-rich repeat proteoglycan, constitute an important fraction of noncollagenous ECM proteins. It plays a major role in tissue homeostasis and modulates cellular functions, namely, cell proliferation, migration, and differentiation.[29] Polymeric Ig receptor (pIgR) isoform X1 is a type-I transmembrane protein expressed from glandular epithelial cells of liver and breast. It mediates transcellular transport of polymeric Igs. Pro-inflammatory cytokines, namely,

interferon-γ, TNF, and interleukin 1, which are the key regulators of pIgR expression, upregulate in ALD.[8,30,31]

Vitamin D-binding protein, a multifunctional protein that belongs to the albumin gene family can bind various forms of vitamin D (ergocalciferol, cholecalciferol, and calcifediol) for the transport. It is synthesized by hepatic parenchymal cells.[32] Haptoglobin which is included in the existing noninvasive marker panel has showed increased expression in the present study as it is an acute phase protein. Liver is the major site for the synthesis of haptoglobin; hepatic expression will be stimulated by upregulated IL-6 in ALD.[23,33,34] Transmembrane protein 201 is involved in nuclear movement during fibroblast polarization and migration; actin-dependent nuclear movement is through association with transmembrane actin-associated nuclear lines.[35]

Serine protease inhibitors, α-1-antitrypsin (SERPINA1) and α-1-antichymotrypsin (SERPINA3), produced primarily in liver hepatocytes and released directly into the blood stream showed downregulation in alcoholic liver cirrhosis compared to healthy subjects.[36–38] Hemopexin which showed decreased expression in ALD is a single polypeptide chain of 439 amino acid residues with a molecular weight of 63 kDa is expressed from liver, and it acts as a heme-scavenging protein.[39] Apolipoprotein A-IV, even though not evident from liver, its expression, was also decreased in ALD. Downregulation of Apolipoprotein A-IV was reported in hepatic fibrosis in rat models.[23,40] CD5 antigen-like isoform X1, a key regulator of lipid synthesis, was downregulated in alcoholic liver cirrhosis, whereas upregulation was noted in liver cirrhotic patients in hepatitis C virus infection.[24] Zinc-α2-glycoprotein, adipokine, which plays an important role in fat catabolism and which reduces insulin resistance, was downregulated in ALD.[24,41]

Glycerol kinase, a phosphotransferase and a key enzyme in the regulation of glycerol uptake and metabolism, is involved in triglyceride and glycerophospholipid synthesis. Glycerol kinase converts glycerol, a product of lipolysis to glucose in the liver, and shows downregulation in alcoholic liver cirrhosis. In the present study, α2-HS-glycoprotein has shown decreased expression in ALD. It is a secretory protein expressed from liver and key regulator in inhibition of vascular calcification, bone metabolism regulation, control of protease activity, insulin resistance, and breast tumor cell proliferative signaling.[42] α-1-Acid glycoprotein, an acute phase synthesized primarily in hepatocytes, which acts as a carrier of lipophilic compounds was down regulated in ALD.[43] An SERPINF2, α2-antiplasmin, synthesized in the liver as a single-chain glycoprotein with a molecular weight of 51 kDa inhibits plasmin and was decreased in liver cirrhotic patients.[44] Antithrombin III, a member of the serpin family and an inhibitor of proteinases, namely, thrombin and factor Xa, is primarily synthesized by hepatocytes and downregulated in ALD.[45] Our studies are also corroborated with the downregulation of α2-antiplasmin and antithrombin III in ALD.

Newly identified proteomic biomarker candidates for ALD need validation and clinical assay optimization which require measurement of thousands of patient samples with narrow measurement coefficient of variation values.[18] Concentration of proteins in serum/plasma ranges from picograms to nanograms per milliliter. Enzyme-linked immunosorbent assay is the best alternative for quantification of these protein candidates for ALD with high specificity and sensitivity capture and detection antibodies. Newly developed assay requires analytical validation before evaluating clinical utility in terms of performance characteristics, namely, outcome studies, clinical requirement, proficiency testing, and goals set by regulatory agencies.[46] Indicators of accuracy, precision, analytical measurement range, and reference intervals should be defined for newly discovered biomarker candidates.[47] After analytical validation of new methodology for protein of interest, biomarker candidate should confirm the performance characteristics in terms of consistency and accuracy in clinical evaluation to diagnose or predict the clinical outcome of ALD. The newly identified biomarker candidate should satisfy specificity and sensitivity. Evidence-based biomarker should fulfill regulatory requirements before introduced into clinical practice for ALD.[18,48]

Conclusion

With the help of technological advancement in "-omics" approach, we identified 28 protein biomarker candidates (13 with increased expression and 15 with decreased expression) for alcoholic liver cirrhosis. Despite numerous biomarker candidates identified, verification may be done only for few qualified candidates that act in cellular pathways and deregulated in ALD. These differentially expressed proteins between alcoholic cirrhosis and healthy subjects need to be validated to get the same differential expression detectable by assay to be used for validation.

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Declaration of Conflicting Interests

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References

1. Zhou WC, Zhang QB, and Qiao L. Pathogenesis of liver cirrhosis. *World J Gastroenterol* 2014; 20(23): 7312–7324.

2. O'Shea RS, Dasarathy S, and McCullough AJ. Practice guideline committee of the American association for the study of liver diseases; Practice parameters committee of the American college of gastroenterology alcoholic liver disease. *Hepatology* 2010; 51(1): 307–328.
3. Stewart S and Day C. Alcohol and liver. In: Dooley JS, Anna SFL, Andrew KB and Heathcote EJ (eds) *Sherlock's diseases of the liver and biliary system*. 12th Edition. Singapore: Wiley Blackwell, 2011, pp. 507–520.
4. An L, Wang X and Cederbaum AI. Cytokines in alcoholic liver disease. *Arch Toxicol* 2012; 86(9): 1337–1348.
5. Bedossa P, Dargere D, and Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. *Hepatology* 2003; 38(6): 1449–1457.
6. Sangheun L and Do Young K. Non-invasive diagnosis of hepatitis B virus related cirrhosis. *World J Gastroenterol* 2014; 20(2): 445–459.
7. Hong H and Tong W. Emerging efforts for discovering new biomarkers of liver disease and hepatotoxicity. *Biomarkers Med* 2014; 8(2): 143–146.
8. Nallagangula KS, Nagaraj SK, Venkataswamy L, et al. Liver fibrosis: a compilation on the biomarkers status and their significance in disease progress. *Future Sci OA* 2017; 4(1): FSO250. doi: 10.4155/fsoa-2017-0083.
9. Paulovich AG, Whiteaker JR, Hoofnagle AN, et al. The interface between biomarker discovery and clinical validation: the tar pit of the protein biomarker pipeline. *Proteomics Clin Appl* 2008; 2(10–11): 1386–1402.
10. http://assets.thermofisher.com/MAN0011862_Pierce_Albumin_Depletion_UG.pdf.
11. Garfin DE. One dimensional gel electrophoresis. *Methods Enzymol* 2009; 463: 497–513.
12. Hjemel LM and Chrambach A. Electrophoresis and electrofocusing in detergent containing media: a discussion of basic concepts. *Electrophoresis* 1981; 2(1): 1–11.
13. Shevchenko A, Wilm M, Vorm O, et al. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal Chem* 1996; 68(5): 850–858.
14. https://tools.thermofisher.com/content/sfs/brochures/TR0049-Acetone-precipitation.pdf (accessed August 2017).
15. http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110009A.pdf.
16. Shevchenko A, Tomas H, Havlis J, et al. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* 2007; 1(6): 2856–2860.
17. Junqueira M, Spirin V, Balbuena TS, et al. Protein identification pipeline for the homology-driven proteomics. *J Proteomics* 2008; 71(3): 346–356.
18. Rifai N, Gillette AM, and Carr AS. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat Biotechnol* 2006; 24(8): 971–983.
19. Anderson L. Candidate based proteomics in the search for biomarkers of cardiovascular disease. *J Physiol* 2005; 563(pt 1): 23–60.
20. Fillip S, Vougas K, Zoidakis J, et al. Comparison of depletion strategies for the enrichment of low abundance proteins in urine. *PloS One* 2015; 10(7): e0133773.
21. Steel LF, Trotter MG, Nakajima PB, et al. Efficient and specific removal of albumin from human serum samples. *Mol Cell Proteomics* 2003; 2(4): 262–270.
22. Bevin G, Bapat M, Rossa J, et al. Discovery of novel biomarker candidates for liver fibrosis in hepatitis C patients: a preliminary study. *PloS One* 2012: 7(6): e39603. doi:10.1371/journal.pone.0039603.
23. White IR, Patel K, Symonds WT, et al. Serum proteomic analysis focused on fibrosis in patients with hepatitis C virus infection. *J Transl Med* 2007; 5: 33. doi:10.1186/1479-5876-5-33.
24. Bevin G, Antrobus R, Dwek RA, et al. Novel serum biomarker candidates for liver fibrosis in hepatitis C patients. *Clin Chem* 2007; 53(10): 1792–1799.
25. Bevin G, Antrobus R, Chittenden D, et al. New approaches for biomarker discovery: the search for liver fibrosis markers in hepatitis C patients. *J Prot Res* 2011; 10(5): 2643–2650.
26. Katrinli S, Ozdil K, Sahin A, et al. Proteomic profiling of HBV infected liver biopsies with different fibrotic stages. *Proteome Sci* 2017: 15: 7. doi:10.1186/s12953-017-0114-4.
27. Aebersold R, Anderson L, Caprioli R, et al. Perspective: a program to improve protein biomarker discovery for cancer. *J Prot Res* 2005; 4(4): 1104–1109.
28. Bragulla HH and Homberger DG. Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. *J Anat* 2009; 214(4): 516–559.
29. Nikitovic D, Katonis P, Tsatsakis A, et al. Lumican, a small leucine-rich proteoglycan. *Life* 2008; 60(12): 818–823.
30. Balaje V, Jafferali MH, Figueroa RA, et al. Samp1, a RanGTP binding transmembrane protein in the inner nuclear membrane. *Nucleus* 2016; 7(4): 415–423.
31. Asano M and Komiyama K. Polymeric immunoglobulin receptor. *J Oral Sci* 2011; 53(2): 147–156.
32. Norman AW.From vitamin D to hormone D: fundamentals of the vitamin D endocrine system essential for good health. *Am J Clin Nutr* 2008; 88(2): 491S–499S.
33. Wang Y, Kinzie E, Berger FG, et al. Haptoglobin, an inflammation-inducible plasma protein. *Redox Report* 2001; 6(6): 379–385. doi: 10.1179/135100001101536580.
34. Trayhurn P and Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr* 2004; 92(3): 347–355.
35. http://www.uniprot.org/uniprot/Q5SNT2 (accessed August 2017).
36. Janciauskiene SM, Bals R, Koczulla R, et al. The discovery of a 1-antitrypsin and its role in health and disease. *Respir Med* 2011; 105(8): 1129–1139.
37. Tyagi E, Fiorelli T, Norden M, et al. Alpha 1-antichymotrypsin, an inflammatory protein overexpressed in the brains of patients with Alzheimer's disease, induces Tau hyperphosphorylation through c-Jun N-Terminal Kinase

Activation. *International Journal of Alzheimer's Disease* 2013; 2013: 1–11.
38. Yoon D, Kueppers F, Genta RM, et al. Role of alpha-1-antichymotrypsin deficiency in promoting cirrhosis in two siblings with heterozygous alpha-1-antitrypsin deficiency phenotype SZ. *Gut* 2002; 50(5): 730–732.
39. Smith A and McCulloh RJ. Hemopexin and haptoglobin: allies against heme toxicity from hemoglobin not contenders. *Front Physiol* 2015; 6: 187. doi: 10.3389/fphys.2015.00187.
40. Bergstrom J, Murphy CL, Weiss DT, et al. Two different types of amyloid deposits—apolipoprotein A-IV and transthyretin—in a patient with systemic amyloidosis. *Lab Invest* 2004; 84(8): 981–988.
41. Lei Lu, Li K, Li L, et al. Circulating zinc-α2-glycoprotein levels are low in newly diagnosed patients with metabolic syndrome and correlate with adiponectin. *Nutr Metab (Lond)* 2017; 14: 53. doi: 10.1186/s12986-017-0210-6.
42. Sindhu S, Akhter N, Shenouda S, et al. Plasma fetuin-A/α2-HS-glycoprotein correlates negatively with inflammatory cytokines, chemokines and activation biomarkers in individuals with type-2 diabetes. *BMC Immunol* 2016; 17(1): 33. doi: 10.1186/s12865-016-0171-y.
43. Colomba S, Buclin T, Decosterd LA, et al. Orosomucoid (α_1-acid glycoprotein) plasma concentration and genetic variants: effects on human immunodeficiency virus protease inhibitor clearance and cellular accumulation. *Clin Pharmacol Ther* 2006; 80(4): 307–318. doi:10.1016/j.clpt.2006.06.006.
44. Carpenter SL and Mathew P. α2-Antiplasmin and its deficiency: fibrinolysis out of balance. *Haemophilia* 2008; 14(6): 1250–1254.
45. Pereyra D, Offensperger F, Klinglmueller F, et al. Early prediction of postoperative liver dysfunction and clinical outcome using antithrombin III-activity. *PLoS One* 2017; 12(4): e0175359. doi.org/10.1371/journal.pone.0175359.
46. Vitzthum F, Behrens F, Anderson NL, et al. Proteomics: from basic research to diagnostic application. A review of requirements and needs. *J Proteome Res* 2005; 4(4): 1086–1097.
47. Dybkaer R. Vocabulary for use in measurement procedures and description of reference materials in laboratory medicine. *Eur J Clin Chem Biochem* 1997; 35(2): 141–173.
48. Lesko LJ and Atkinson AJJr. Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation strategies. *Annu Rev Pharmacol Toxicol* 2001; 41: 347–366.

ANNEXURE - II

PROFORMA

Date:

Title of the Study: A Correlative Study Of Advanced Biomarker: Kallistatin with Conventional and Oxidative Markers in Cirrhosis of Liver

Case History of the Patient:

Case No:

Name: OP No: IP No:

Age: Gender: M / F / Trans:

DOB: Ward:

Vaccination Status: Yes/ No

Hepatitis B: Yes/ No

Hepatitis C: Yes/ No

Occupation:

Weight: Height:

BMI: Abdominal Girth: Waist: Hip Ratio:

Address:

Contact no.:

E Mail:

Contact person:

Approximate distance from the hospital:

Informant: Patient/ attendant (His relation:)

Chief Complaints:

History of Present Illness and Duration:

Past History:

Surgical History:

Obstetric History:

History of previous hospitalization and details:

History of Deaddiction:

Hypertension	: yes/no	if yes, duration:
Diabetes	: yes/no	if yes, duration:
Tuberculosis	: yes/no	if yes, duration:
Heart diseases	: yes/no	if yes, duration:

Drug history for DM/HTN/ Any Hepatotoxic Drugs: yes/no; if yes, duration & details:

Family History:

Diabetes: yes/no; if yes, duration:

Hypertension: yes/no; if yes, duration:

Tuberculosis: yes/no; if yes, duration:

Personal History:

Economic status:

Diet: vegetarian / Non vegetarian/mixed

Smoking: yes/no; if yes, duration and number, type of smoking

Consumption of Smokeless Tobacco:

Use of Addiction causing substances:

Exposure with STD/ HIV infected individual

Alcohol: yes/no; if yes, duration and quantity in grams:

General Physical Examination:

Built: Average/ below normal / normal / well built / obese

Nourishment: well / poor nourished

Edema :	Ascites:	Encephalopathy:
Blood pressure:	Pulse rate:	

Systemic Examination:

CVS:	RS:
CNS:	Per Abdomen:

Diagnosis:

Biochemical Investigations:

Blood (Serum)

Kallistatin:	pg/mL
Hyaluronic acid:	ng/mL
YKL-40:	ng/mL
Blood sugar:	mg/dL
Blood urea:	mg/dL
Serum creatinine:	mg/dl
Total bilirubin:	mg/dL
Total protein:	g/dL
Albumin:	g/dL
A/G Ratio:	
AST:	U/L
ALT:	U/L
ALP:	U/L
Gamma GT:	U/L
Uric Acid:	mg/dL
Total Antioxidant Capacity:	nmol/µL
Total Oxidant Status	µmol H_2O_2 Equiv/L

Whole Blood (Citrate)

Total leukocyte count:	10^9 / Litre
Platelet count:	10^9/Litre
INR:	

ANNEXURE – III

VOLUNTEER INFORMATION SHEET

Title of the Study: A Correlative Study of Advanced Biomarker: Kallistatin with Conventional and Oxidative Markers in Cirrhosis of Liver

Principal investigator: Mr. N. KRISHNA SUMANTH
Organization: Sri Devaraj Urs Medical College, Tamaka, Kolar.

I, Mr. N. Krishna Sumanth, Ph.D scholar, Dept. of Biochemistry is carrying out a study titled: A Correlative Study of Advanced Biomarker: Kallistatin with Conventional and Oxidative Markers in Cirrhosis of Liver. This study is a case control study where kallistatin an advanced biochemical marker will be compared with proven markers and oxidative stress parameters of cirrhotic patients.

Cirrhosis of liver is a pathological condition which impairs the functions of liver by chronic liver insults. Cirrhosis of liver has emerged as a major global health burden and is commonly caused by Alcohol, Hepatitis B, Hepatitis C and Non-Alcoholic Fatty Liver Disease (NAFLD). The development of fibrosis/cirrhosis is associated with a significant morbidity and mortality.

Recent development in understanding the process of hepatic fibrosis suggests that a capacity for recovery from any degree of fibrosis including those associated with cirrhosis is possible with early diagnosis and management.

Hepatic cirrhosis even though can be approached by imaging techniques these diagnostics modalities require skill, costlier and unaffordable by rural population and has its own limitations.

In this regard the recent marker kallistatin is gaining a momentum as an early diagnostic and/or a prognostic marker. This has created interest in me to do a research on kallistatin.

For my research work, I need 5 ml of venous blood to estimate kallistatin; the newer marker and established markers as well as oxidative stress parameters in the blood sample. Thus, I request your kind self to volunteer and consent for clinical information and permit me to collect 5 ml of venous blood from your antecubital vein in your comfortable position, analyze and store the left out sample if required for further analysis.

There is no compulsion for you to participate in this study. You need to sign and duly fill the prescribed form if you volunteer to participate in this study. Further you are at the liberty to withdraw from the study at any point of time in case you do not wish to continue for what so ever the reason may be. But you need to substantiate the reason and the reason will be between the principal investigator and the volunteer. I assure you that all the results, values and the outcome of the study shall be highly confidential and between the principal investigator and the volunteer, there are no adverse effects from this study, done free of cost, no hidden charges.

Contact information:

Principal investigator:
N.Krishna Sumanth
PhD Scholar, Dept. of Biochemistry
SDUMC, Tamaka, Kolar
Phone Number: +91-0- 8886006888 (M)
Email:sumanthnk@gmail.com

Guide:
Dr. Shashidhar.K.N.
Professor
Dept. of Biochemistry
SDUMC, Tamaka, Kolar.
08152-210604, 210605 ext.: 110

INFORMED CONSENT

I understand that I remain free to withdraw from this study at any time with due substantiation.

I have read or had read to me and understand the purpose of this study and the confidential nature of the information that will be collected & disclosed during the study.

I have had the opportunity to ask questions regarding the various aspects of this study & my questions have been answered to my fullest satisfaction.

I, the undersigned agree to participate in this study, authorize for the collection of blood sample and disclosure of my personal information only after my knowledge as outlined in this consent form.

I am also informed that I will not be charged or make me pay for any of the investigations pertaining to this study.

Participant's Name & Signature/ Thumb Impression

Date

Witness

1. Signature and Name
   Date
   Address:

2. Signature and Name
   Date
   Address:

Signature of the Principal Investigator

Date

NAME: N. Krishna Sumanth

PHONE NUMBER: +91- 0- 8886006888

ANNEXURE – IV

DrillBit Anti-Plagiarism Report

A Correlative Study of Advanced Biomarker Kallistatin with Conventional and Oxidative Markers for

By

Sumanth

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10	16	Ur Inaryneutrophilgelat Inaseassociatedlipocal Inasanearlymarker Ofacutekidney Injury In Therecipientafterl Publication	<1
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14	15	Acharyanagarjuna University Thesis Published in Shodganga www.inflibnet.ac.in **Student Paper**	<1
15	19	Serumlevels Ofosteo Proteger In And R A N K L Inpatients Withrheumatoidarthritis Andtheirrelationtobonem Ineralde **Publication**	<1
16	1	medind.nic.in **Internet Data**	<1
17	27	Elsevier Article **Journal**	<1
18	30	Bharathiar University Article published in - www.inflibnet.com **Publication**	<1
19	13	A Systematic Comparison Identifies an ATP-Based Viability Assay as Most Suitable Read-Out for Drug Screening in Glioma Stem-Like Cells **Student Paper Published in Hindawai**	<1
20	28	Brain Differences in First-Episode Schizophrenia Treated With Quetiapine A	<1

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21	14	Investigation Into Drug Solubilisation Potential Of Sulfonated Calix4resorcinarenes 157 439 000370. Student Paper	<1
22	10	Recovery Components as Determinants of the Health-Related Quality of Life Among Patients With Schizophrenia Structur www.ncbi.nlm.nih.gov	<1
23	20	Article Published in Sensors Journal - MDPI 2016 Student Paper.	<1
24	11	Evaluationofcombinationantiemeticprophylaxis inhighriskemetogenicpatientsundergoingthyroid surgeryAran Journal Elsevier	<1
25	26	Student Thesis Published by Vellore Institute of Technology - www.vit.ac.in Publication	<1
26	9	Evaluating The Frequency Of Vitamin D	

		Deficiency In The Pediatric Age Group And Identifying The Biochemical Predictors Associated 161 665 000289. **Journal**	<1
27	24	Article Published in Journal of Kaohsiung J Med Science **Student Paper**	<1
28	12	Effects Of Noncoherent And Coherent Light On Complete Blood Picture And Osmotic Fragility Of Human Blood 155 864.1000134. **Journal**	<1
29	18	Elsevier Article **Journal**	<1
30	23	Article Published in Hematol Oncol Stem Cell Ther Journals370 **www.sciencedirect.com**	<1

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