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Invited critical review

# Serum proteomics for biomarker discovery in nonalcoholic fatty liver disease

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ABSTRACT

Proteomic platforms have gained increasing attention in the clinical spectrum of nonalcoholic fatty liver disease (NAFLD). This approach allows for the unbiased discovery of circulating biochemical markers, i.e., it is not limited to known molecules of presumed importance. This manuscript provides an overview of proteomic serum biomarker discovery in NAFLD. Hemoglobin is currently the most widely replicated proteomic circulating biomarker of NAFLD; it was identified as a biomarker of fatty liver in two distinct proteomic studies and subsequently validated using distinct analytical methods by independent research groups in large replication cohorts. Given the increasing availability of numerous serum samples and the refinement of the technological platforms available to scrutinize the blood proteome, large collaborative studies between academia and industry are warmly encouraged to identify novel, unbiased circulating biomarkers of NAFLD.



**Contents**



## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease worldwide, particularly in Western countries [1,2]. NAFLD includes a spectrum of liver diseases ranging from simple steatosis, which is usually a benign and non-progressive condition, to nonalcoholic steatohepatitis (NASH), which may progress to liver fibrosis, cirrhosis, and hepatocellular carcinoma [3,4]. NAFLD is closely associated with obesity, hypertension, dyslipidemia, and altered glucose regulation, suggesting that it represents the hepatic expression of metabolic syndrome, which has insulin resistance as a hallmark [5,6]. The progression from simple steatosis to NASH is generally considered a multifactorial process that involves direct lipid toxicity, an increased production of proinflammatory molecules, and the generation of reactive oxygen, which leads to oxidative stress and causes direct toxic damage to the hepatocytes [7,8]. In this context, the widely accepted two-hit hypothesis of NAFLD explains the pathogenesis and progression of this condition via a stepwise mechanism [9]. The first hit is the accumulation of triglycerides in the hepatocytes (simple steatosis), whereas the second hit – the metabolic–oxidative stress and uncontrolled production of cytokines – results from an attempt to compensate for the changes in lipid homeostasis [7,8].

The gold standard for NAFLD diagnosis and staging is the histological examination of the hepatic biopsy [10,11]. Due to the potential

*Abbreviations:* MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; m/z, mass-to-charge ratio; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; SELDI, surfaced-enhanced laser desorption/ionization; TOF, time-of-flight.

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complications of biopsy, including the risk of hemorrhage and patient discomfort, non-invasive imaging techniques have been used to examine the liver for lesions in the spectrum of NAFLD. The most commonly used imaging techniques are ultrasound, computer tomography, and magnetic resonance imaging [12]. In light of the known limitations of liver biopsy and these imaging methods, researchers have recently been searching for noninvasive circulating biomarkers of NAFLD [13–15]. Biomarkers currently hold great promise for detecting, staging, and monitoring patients with NAFLD [16,17]. Biomarkers are cellular, biochemical, and molecular alterations that indicate physiological or pathophysiological status. NAFLD biomarkers are molecules or proteomic patterns that highlight the presence of fatty liver infiltration or steatohepatitis [18]. They can be molecules secreted by the liver itself or as a result of a specific response of the body to the presence of NAFLD. To minimize trauma and the cost of screening while maximizing utility, NAFLD biomarkers should ideally be measurable in serum or plasma [16–18]. A plethora of potential circulating candidate NAFLD biomarkers has been identified so far [13–18], but their clinical utility remains questionable in the absence of replication and validation in large clinical samples. The ideal NAFLD biomarker should be highly sensitive and specific for a particular stage of NAFLD [15,16]. For example, patients with NASH who are at risk for progressive liver disease should test positive for the biomarker, and people with simple steatosis should test negative. Unfortunately, only a few such biomarkers have been identified [14–18]. A panel of biomarkers would offer better sensitivity and specificity compared with a single biomarker [19,20], but there is still a need for biomarkers that are highly sensitive, specific, and easily accessible for analysis from blood samples. The main limitation of the current approaches to biomarker research in NAFLD is the focus on the investigation of candidate molecules, which are presumed to be involved in the pathophysiology and have biological functions that are, at least in part, understood [18]. As a consequence, some molecules are investigated extensively, while others have not been measured at all. An unbiased biomarker discovery strategy, which is not limited to candidate molecules, will be invaluable for the identification of novel, previously unknown biochemical markers of NAFLD. Among the emerging platforms for biomarker detection in NAFLD, proteomics has recently drawn great attention [21–28]. Despite the potential advantages of an unbiased proteomic approach, the large dynamic range of blood protein concentrations allows the investigation of only a limited fraction of protein changes that are potentially relevant for biomarker applications [29,30]. However, advances in analytical instrumentation (particularly in the field of mass spectrometry [MS]) and bioinformatics may now allow the accurate identification of protein biomarkers from serum samples [30]. Although still descriptive, the examination of circulating samples from patients with NAFLD using advanced proteomic techniques can generate novel pathophysiological insights and form the basis for the identification of novel noninvasive biochemical markers.

## 2. Proteomic approaches in NAFLD: general issues

Traditionally, the identification of clinically useful disease markers is a stepwise process comprising discovery and validation [31,32]. In the field of proteomics, the term discovery refers to the unbiased and semiquantitative identification of significant differences in protein expression between patients and healthy subjects [30]. The outcome of the discovery phase is therefore a list of proteins that are differentially expressed (generally in terms of their relative abundances) between NAFLD patients and controls [21–28]. In general, tens to hundreds of potential biomarkers are identified during the discovery phase. Because several candidate biomarkers identified during this phase may actually be false positives, replication and validation in different clinical cohorts and using different analytical methods is a fundamental prerequisite for the translational application of NAFLD proteomics from bench to bedside. To date, different proteomic platforms have been used for biomarker discovery in NAFLD [21–28]. Published studies have been chiefly based on the use of MS, an analytical method that enables the precise measurement of molecular weights of distinct proteins in a biological sample [33,34]. A mass spectrometer comprises an ion source, an analyzer, and a detector. In a classic MS experiment, sample molecules are initially converted into a gaseous phase, ionized at the ion source, separated with respect to their mass-to-charge ratios (m/z) in the analyzer, and subsequently detected [33]. The most commonly used ion sources include matrix-assisted laser desorption/ionization (MALDI) and surfaced-enhanced laser desorption/ionization (SELDI) [33,34]. MALDI and SELDI sources are based on pulsed laser beams that (1) sublimate analyte molecules from the solid to the gaseous state and (2) promote ionization. The most commonly used mass analyzer used for NAFLD proteomics is the time-of-flight (TOF), which measures the velocity of the ions, from which the m/z ratio can be determined. Over the last 10 years, few proteomic studies have been performed on the serum samples of patients with NAFLD [21–28]. In general, the direct MS examination of a sample may offer a fast and comprehension identification of its protein profile through the acquisition of a vast range of m/z values. However, several MS-based proteomic approaches do not allow rigorous protein identification because the information is restricted to the m/z value [25]. Protein characterization by means of the m/z values still remains of limited clinical value because the unknown molecular identity of the identified proteins does not allow validation in different clinical cohorts using a variety of analytical methods. Despite these shortcomings, proteomic approaches have been successfully applied for the identification of potential circulating biomarkers of NAFLD. I will now review the human clinical studies that have used proteomic methods for the purpose of biomarker discovery.

## 3. Circulating proteomic biomarkers of NAFLD: clinical studies

The first study of the serum protein profiles in patients with NAFLD was published by Younossi et al. [28] using SELDI-TOF MS. The authors investigated 98 obese patients, 91 with a diagnosis of NAFLD, and 7 without NAFLD (who served as obese controls). Proteomic analyses revealed twelve significantly different protein peaks between the two groups [28]. Given the inherent limitation of the SELDI approach [32], however, the authors were unable to identify these proteins. Using MALDI TOF-MS, our research group sought to investigate a serum proteomic pattern analysis that could distinguish simple steatosis from NASH [25]. A total of 80 patients with biopsy-proven NAFLD (48 definite NASH, 22 borderline NASH, and 10 simple fatty liver) and 19 healthy comparison subjects were investigated. The mass spectra of serum samples were obtained using an Ultraflex II mass spectrometer. The highest accuracy for NASH diagnostics was reached using 15 peaks, with a sensitivity of 73.95% and a specificity of 88.71%. However, the mass spectra did not discriminate between NASH and simple steatosis. Taken together, these results suggested that the proteomic analysis of serum samples from NAFLD patients does not seem to have a major clinical value for identifying patients with NASH at risk of progressive liver disease [25]. Bell et al. [24] used a label-free, MS-based approach to describe changes in the serum proteome and identify biomarker candidates in serum samples from 69 obese patients with varying stages of NAFLD and 16 obese controls without evidence of liver fat infiltration. The authors showed that the expression levels of 55 and 15 proteins changed significantly between the simple steatosis and NASH plus fibrosis group and the NASH without fibrosis and NASH plus fibrosis group, respectively. The functional classification of proteins that showed significant changes identified their involvement in several distinct pathways, including immune system regulation and inflammation, coagulation, cellular and extracellular matrix structure and function, and roles as carrier proteins in the blood [24]. The study