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(71) Applicant: **NUMARES AG** [DE/DE]; Am BioPark 9, 93053 Regensburg (DE).

(72) Inventors: **DE JEL, Sebastian**; Eschenweg 2, 86739 Ederheim (DE). **STÄMMLER, Frank**; Wilhelm-Raabe-Straße 9, 93051 Regensburg (DE). **RÖTZER, Sebastian**; Am Klostergrund 16, 93161 Sinzing (DE). **ROBERTSON, Andrew**; Georgenstraße 118, 80798 München (DE). **EIGLSPERGER, Johannes**; Eichenweg 3, 84069 Schierling-Walkenstetten (DE).

(74) Agent: **MAIKOWSKI & NINNEMANN PATENTANWÄLTE PARTNERSCHAFT MBB**; Postfach 150920, 10671 Berlin (DE).

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(54) **Title:** USE OF A MARKER OR A MARKER SET FOR DETERMINING THE RISK OF AN INDIVIDUAL TO HAVE A REDUCED LIVER FUNCTION

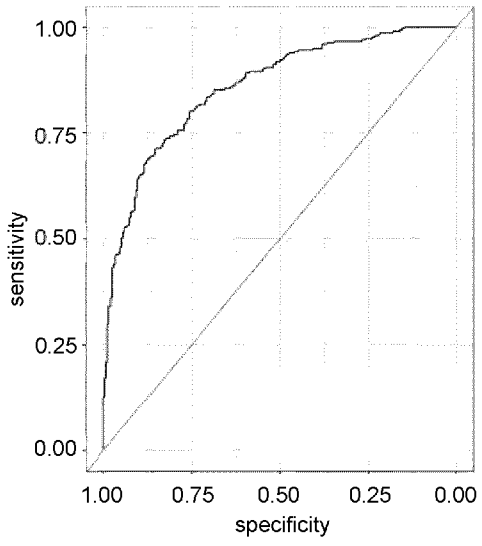

FIG 1A

(57) **Abstract:** The present invention relates, amongst others, to the use of a marker or a marker set in an in vitro method for determining the risk of an individual to have a reduced liver function. The marker is chosen from a first group consisting of high-density lipoprotein, apolipoprotein A1, valine, and lactate; the marker set comprises at least two substances chosen from a second group consisting of high-density lipoprotein, apolipoprotein A1, valine, lactate, pyruvate, and fumarate.

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Use of a marker or a marker set for determining the risk of an individual to have a reduced liver function

Description

The present invention relates to the in-vitro use of a marker or a marker set for determining the risk of an individual to have a reduced liver function according to the preamble of claim 1, to the further medical use of such a marker or a marker set according to the preamble of claim 13, and to an analysis method for determining the risk of an individual to have a reduced liver function according to the preamble of claim 14.

More generally, the present invention relates to biomarkers that are applicable for accurately estimating liver function. The term “liver function” is broad, given the vast array of physiological and biochemical functions the organ carries out, chiefly biliary synthesis for fatty acid digestion, metabolism of carbohydrate, protein and lipids, synthesis of protein and detoxification. Thus, hepatocyte function is paramount to physiological survival.

Given the broad range of functions the liver carries out, it is challenging to accurately define liver function numerically, as opposed to the glomerular filtration rate of the kidney or the ejection fraction of the heart. A number of blood tests are available which reflect the general damage to hepatocytes – mostly products of hepatic metabolic pathways and enzymes – the most common in clinical practice being serum aminotransferases, bilirubin, alkaline phosphatase, albumin, and prothrombin time. These tests are commonly grouped together under the umbrella term ‘Liver Function Tests’ which is misleading, since most are unable to reflect how well the liver is functioning and abnormal values can be due to diseases unrelated to the liver. Moreover, these tests may be normal in patients with advanced liver disease yet abnormal in asymptomatic healthy individuals [1-3]. The combination of detection of serial changes in this test panel interpreted together with patient symptomatology can assist in monitoring progression or remission of disease and can trigger subsequent and more advanced diagnostic testing.

Current specific liver function tests are limited. The dye indocyanine green has been in practice for decades for liver function monitoring. The so-called indocyanine green tracer test (ICG) measures hepatic elimination of the dye and is hence a diagnostic tool of overall liver function and a prognostic predictor of mortality. For example, it has utility in peri-operative liver function

monitoring during liver surgery, an assessment of liver failure acuity, and as a prognostic tool for critically ill patients. Adverse reactions are rare although it is contraindicated in known iodine allergy. Despite its utility, strong levels of evidence are lacking, and it is therefore not recommended for routine liver function assessment [4,5].

Image-based modalities assessing liver function exist but are limited in scope and resource. Nuclear medicine scans metastable technetium-99 (99mTc) galactosyl and mebrofenin using plain scintigraphy and single-photon emission computed tomography (SPECT-CT) have been utilized, however these modalities deliver significant radiation exposure to the patient and user. Gadolinium enhanced MRI [Gd-EOB] can show high spatial and temporal metabolic resolution in the liver, yet is strictly limited by MRI availability and cost [6,7].

A simpler measure of global liver function lies with the Child-Pugh score, particularly in patients having cirrhosis. Originally designed to assess the risk of non-shunt operations in patients with cirrhosis (namely transection of the esophagus for bleeding esophageal varices), it was further validated to stratify the risk of portacaval shunt surgery in patients with cirrhosis. It was also later demonstrated to correlate with survival in patients not undergoing surgery. Additionally, Child-Pugh class is also associated with the likelihood of developing complications of cirrhosis, such as variceal hemorrhage. Components of the modified Child-Pugh classification of the severity of liver disease are degree of ascites, the serum concentrations of bilirubin and albumin, the prothrombin time, and the degree of encephalopathy.

A total Child-Pugh score (sometimes also referred to as Child-Turcotte-Pugh score) of 5 to 6 is considered Child-Pugh class A (well-compensated disease, 10 % postoperative mortality risk), a score of 7 to 9 is class B (significant functional compromise, 30 % postoperative mortality risk), and a score of 10 to 15 is class C (decompensated disease, 82 % postoperative mortality risk).These classes correlate with one- and two-year patient survival: class A: 100 and 85 %; class B: 80 and 60 %; and class C: 45 and 35 % [8-11]. As such, an accurate Child-Pugh score as an estimation of global liver function has significant utility in perioperative planning of patients with cirrhosis, resource management, prioritization of liver treatment, identification of those at risk of decompensated liver failure, and general prognostication. The Child-Pugh score is currently embedded in the treatment algorithm for hepatocellular carcinoma (HCC) [12] and is a feature of liver transplant eligibility [13].

Measurement of an accurate Child-Pugh score however can be challenging, given the difficult nature of ascites and hepatic encephalopathy diagnosis and severity scaling. An accurate diagnosis of ascites is dependent on ultrasound imaging and an invasive paracentesis

procedure [14], while hepatic encephalopathy has no standardized diagnostic method and depends on subjective assessment from a clinician and questionable insensitive psychometric testing [15,16].

The most recent European Association for the Study of the Liver [EASL] management guidelines for HCC, for the purposes of treatment decisions and prognostication, have stratified liver function into "preserved liver function" which refers to Child-Pugh A without any ascites, and hence "reduced liver function" being Child-Pugh A with ascites or Child-Pugh B or C. This "preserved liver function" group is considered a condition required to obtain optimal outcomes, and this prerequisite applies to all treatment options, whereby non-preserved/reduced liver function is deemed terminal stage D disease [17]. This stratification process still requires an accurate measurement of Child-Pugh class with its associated diagnostic difficulties.

In summary, there is a clear need for non-invasive biomarkers enabling a quantitative test which combines a highly accurate estimation of liver function yet as simple as performing a standard liver function panel, in order to stratify patients into preserved vs. reduced liver function, obliviating the need for invasive tracer application, avoiding ionizing radiation and associated labor, and the negating the cost of high-resolution imaging.

US 2020/0378991 A1 describes biomarkers and biomarker panels useful for diagnostic methods evaluating liver disease status in a subject, monitoring liver disease, distinguishing between liver diseases, treating subjects evaluated by diagnostic methods of the invention, providing diagnostic tests for evaluating liver disease status in a subject, and kits therefor. The biomarkers are chosen from bile acids, free fatty acids, amino acids, and carbohydrates listed in Table 1 of this U.S. patent application. A specific example relates to a biomarker panel comprising palmitic acid (C16:0), palmitic acid (C16:0)/palmitoleic acid (C16:1 n7) ratio, tyrosine, fructose, fructose/glucose ratio, glycochenodeoxycholic acid (GCDCA), and glycocholic acid (GCA).

US 2017/0370954 A1 describes biomarkers of nonalcoholic steatohepatitis (NASH), nonalcoholic fatty liver disease (NAFLD), and fibrosis and methods for diagnosis (or aiding in the diagnosis) of NAFLD, NASH and/or fibrosis. Additionally, methods of distinguishing between NAFLD and NASH, methods of classifying the stage of fibrosis, methods of determining the severity of liver disease, methods of determining the severity of liver disease or fibrosis, and methods of monitoring progression/regression of NASH, NAFLD, and/or fibrosis are described. In this context, this U.S. patent application lists scores of various substances. A specific example is a biomarker or biomarker set selected from the group

consisting of 5-methylthioadenosine (5-MTA), glycine, serine, leucine, 4-methyl-2-oxopentanoate, 3-methyl-2-oxovalerate, valine, 3-methyl-2-oxobutyrate, 2-hydroxybutyrate, prolylproline, lanosterol, tauro-beta-muricholate, and deoxycholate.

It is an object of the present invention to provide novel methods and biomarkers for the diagnosis of liver function as well as for determining the risk of an individual to have a reduced liver function.

This object is achieved with the in-vitro use of a marker or a marker set having the claim elements of claim 1. The marker is chosen from a first group consisting of high-density lipoprotein, apolipoprotein A1, valine, and lactate. The marker set comprises or consists of at least two (e.g., 2, 3, 4, 5, 6) substances that are chosen from a second group consisting of high-density lipoprotein (HDL), apolipoprotein A1, valine, pyruvate, lactate, myo-inositol, and anhydrosorbitol.

For this purpose, the concentration of the marker or of the substances contained in the marker set is determined in a body fluid obtained from a patient. This concentration determination can be carried out by any appropriate measuring or analysis method, such as nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, high-performance liquid chromatography (HPLC), infrared spectroscopy such as Fourier-transform infrared (FT-IR) spectroscopy, clinical chemistry, and immunodiagnostics.

An alteration of concentration of the individual marker or of at least two substances of the marker set with respect to the concentration in a control group or an alteration of a concentration ratio between at least two substances with respect to the concentration ratio of the same substances in a control group was correlated in a statistically significant way with a reduced liver function at the time of analysis (i.e., enabling a distinction between individuals having a preserved liver function (Child-Pugh class A without any ascites) and individuals having a reduced liver function (Child-Pugh class A with ascites or Child-Pugh class B or C). Expressed in other words, the use of the biomarkers can also be described as use for classifying an individual into a group of preserved liver function (healthy group; control group) or a group of reduced liver function (diseased group, patient group or case group). I.e., the use is directed to diagnose in a patient or individual a reduced liver function according to the above-given definition.

Already upon testing individual biomarkers, significant results could be obtained for distinguishing the two groups (individuals having a preserved liver function versus individuals

having a reduced liver function), provided that high-density lipoprotein, apolipoprotein A1, valine, or lactate was used as biomarker. The area under the curve (AUC) values of receiver operating characteristic (ROC) plots showed values lying at or above 0.75 or even above 0.80. The AUC value of ROC plots is an aggregated metric that evaluates how well a logistic regression model classifies positive and negative outcomes at all possible cut-offs. It can range from 0 to 1.0. An AUC value of 0 represents a prediction of the opposite of the trained correlation. An AUC value of 0.5 represents a random prediction. An AUC value of higher than 0.5 represents a classification of an event as fulfilling the trained correlation, wherein higher values represent better classification.

Upon testing a combination of at least two biomarkers, the resulting AUC values were significantly above 0.75, in most cases 0.8 or higher or even 0.85 or higher.

The individual biomarkers and the marker sets were tested against a training dataset and a test dataset. After all training and test processes, high-density lipoprotein, apolipoprotein A1, valine, and lactate turned out to be valid biomarkers for the underlying question (i.e., distinguishing individuals having a preserved liver function versus individuals having a reduced liver function). In this context, these biomarkers were very well appropriate biomarkers if used individually. In addition, high-density lipoprotein, apolipoprotein A1, valine, lactate, pyruvate, and fumarate turned out to be particularly valid biomarkers for the underlying question, provided that the concentration of at least two of these biomarkers was determined at the same time (i.e., in one or more body fluid samples from the same patient obtained at the same time point).

The concentration determination can be made with a method being able to determine the concentration of the substances by a single measurement or by a method requiring more than one measurement for such determination. NMR spectroscopy is particularly appropriate for such a concentration determination since it enables a highly accurate concentration determination of multiple substances in a body fluid by a single measurement in a very short measuring time.

In an embodiment, the concentration of the substances is standardized to the concentration of another substance that is naturally present in the sample. This other substance may also be listed in the first group or the second group of substances. In an embodiment, this other substance does not belong to the first or the second group as defined above.

In an embodiment, the marker set comprises at least one substance chosen from the second group and being different from high-density lipoprotein and apolipoprotein A1 if the marker set comprises any of high-density lipoprotein and apolipoprotein A1 as a first of the at least two substances. The concentrations of HDL and apolipoprotein A1 in a body fluid are typically closely interrelated with each other so that both substances can be exchanged against each other for many applications. Therefore, the marker set is particularly appropriate for determining the risk of an individual of having a reduced liver function if HDL and apolipoprotein A1 are not used as the only substances in the marker set.

In an embodiment, the marker is HDL or apolipoprotein A1. HDL as biomarker showed an AUC value of 0.86 in the training dataset (confer Figure 1A) and of 0.84 in the test dataset (confer Figure 1B). Thus, HDL and consequently the closely related apolipoprotein A1 are a particularly appropriate single biomarker for determining the risk of an individual of having a reduced liver function. The accuracy of such risk determination can even be increased if HDL is combined with at least one other biomarker, as will be explained in later sections of the present disclosure.

In an embodiment, the marker is valine. Such a biomarker showed an AUC value of 0.79 in the training dataset (confer Figure 2A) and of 0.75 in the test dataset (confer Figure 2B). Thus, also valine is a particularly appropriate single biomarker for determining the risk of an individual of having a reduced liver function. The accuracy of such risk determination can even be increased if valine is combined with at least one other biomarker, as will be explained in later sections of the present disclosure.

In an embodiment, the marker is lactate. Such a biomarker showed an AUC value of 0.78 in the training dataset (confer Figure 3A) and of 0.76 in the test dataset (confer Figure 3B). Thus, also pyruvate is a particularly appropriate single biomarker for determining the risk of an individual of having a reduced liver function. The accuracy of such risk determination can even be increased if pyruvate is combined with at least one other biomarker, as will be explained in later sections of the present disclosure.

In an embodiment, the marker set comprises or consists of HDL or apolipoprotein A1 as a first of the at least two substances and valine as a second of the at least two substances. Such a biomarker set showed an AUC value of 0.87 in the training dataset (confer Figure 4A) and of 0.85 in the test dataset (confer Figure 4B). Thus, combining HDL and valine as biomarkers in the marker set significantly increases the sensitivity of determination of the risk of an individual

to have a reduced liver function with respect to using any of these substances as individual biomarkers.

In an embodiment, the marker set comprises or consists of HDL or apolipoprotein A1 as a first of the at least two substances and lactate as a second of the at least two substances. Such a biomarker set showed an AUC value of 0.87 in the training dataset (confer Figure 5A) and of 0.85 in the test dataset (confer Figure 5B). Thus, combining HDL and lactate as biomarkers in the marker set significantly increases the sensitivity of determination of the risk of an individual to have a reduced liver function with respect to using any of these substances as individual biomarkers.

In an embodiment, the marker set comprises or consists of HDL or apolipoprotein A1 as a first of the at least two substances and pyruvate as a second of the at least two substances. Such a biomarker set showed an AUC value of 0.88 in the training dataset (confer Figure 6A) and of 0.85 in the test dataset (confer Figure 6B). Thus, supplementing HDL with pyruvate as further biomarker in the marker set significantly increases the sensitivity of determination of the risk of an individual to have a reduced liver function with respect to using HDL as individual biomarker.

In an embodiment, the marker set comprises or consists of HDL or apolipoprotein A1 as a first of the at least two substances and fumarate as a second of the at least two substances. Such a biomarker set showed an AUC value of 0.88 in the training dataset (confer Figure 7A) and of 0.84 in the test dataset (confer Figure 7B). Thus, supplementing HDL with fumarate as further biomarker significantly increases the sensitivity of determination of the risk of an individual to have a reduced liver function with respect to using HDL as individual biomarker.

In an embodiment, the marker set comprises or consists of valine as a first of the at least two substances and lactate as a second of the at least two substances. Such a biomarker set showed an AUC value of 0.84 in the training dataset (confer Figure 8A) and of 0.81 in the test dataset (confer Figure 8B). Thus, combining valine and lactate as biomarkers in the marker set significantly increases the sensitivity of determination of the risk of an individual to have a reduced liver function with respect to using any of these substances as individual biomarkers.

In an embodiment, the marker set comprises or consists of valine as a first of the at least two substances and pyruvate as a second of the at least two substances. Such a biomarker set showed an AUC value of 0.83 in the training dataset (confer Figure 9A) and of 0.81 in the test dataset (confer Figure 9B). Thus, combining pyruvate and valine as biomarkers in the marker

set significantly increases the sensitivity of determination of the risk of an individual to have a reduced liver function with respect to using any of these substances as individual biomarkers.

In an embodiment, the marker set comprises or consists of lactate as a first of the at least two substances and pyruvate as a second of the at least two substances. Such a biomarker set showed an AUC value of 0.80 in the training dataset (confer Figure 10A) and of 0.78 in the test dataset (confer Figure 10B). Thus, supplementing lactate with pyruvate as further biomarker in the marker set significantly increases the sensitivity of determination of the risk of an individual to have a reduced liver function with respect to using lactate as individual biomarker.

In an embodiment, the marker set comprises or consists of HDL or apolipoprotein A1 as a first of the at least two substances and valine and pyruvate as further substances of the at least two substances. Such a biomarker set showed an AUC value of 0.89 in the training dataset (confer Figure 11A) and of 0.86 in the test dataset (confer Figure 11B). Thus, using a combination of the three substances HDL, valine, and pyruvate still slightly increases the sensitivity of determination of the risk of an individual to have a reduced liver function with respect to using only two of these substances.

In an embodiment, the marker set comprises or consists of HDL or apolipoprotein A1 as a first of the at least two substances and valine and lactate as further substances of the at least two substances. Such a biomarker set showed an AUC value of 0.89 in the training dataset (confer Figure 12A) and of 0.85 in the test dataset (confer Figure 12B). Thus, using a combination of the three substances HDL, valine, and lactate still slightly increases the sensitivity of determination of the risk of an individual to have a reduced liver function with respect to using only two of these substances.

In an embodiment, the marker set comprises or consists of valine, pyruvate, and lactate. Such a biomarker set showed an AUC value of 0.86 in the training dataset (confer Figure 13A) and of 0.83 in the test dataset (confer Figure 13B). Thus, using a combination of the three substances valine, pyruvate, and lactate still slightly increases the sensitivity of determination of the risk of an individual to have a reduced liver function with respect to using only two of these substances.

In an aspect, the present invention relates to the further medical use of a marker or a defined marker set for in-vivo diagnostics of having a reduced liver function. The marker is chosen from a first group consisting of high-density lipoprotein, apolipoprotein A1, valine, and lactate. The marker set comprises or consists of at least two (e.g., 2, 3, 4, 5, 6) substances that are chosen

from a second group consisting of high-density lipoprotein, apolipoprotein A1, valine, lactate, pyruvate, and fumarate.

In an aspect, the present invention relates to a method for analyzing an isolated body fluid sample in vitro, comprising the steps explained in the following. This method is carried out on an isolated body fluid sample originating from an individual.

In a first step, the concentration of a single substance or of at least two substances is determined by analyzing the body fluid sample with a suited measuring technique. The single substance is chosen from a first group consisting of high-density lipoprotein, apolipoprotein A1, valine, and lactate. The at least two (e.g., 2, 3, 4, 5, 6) substances are chosen from a second group consisting of high-density lipoprotein, apolipoprotein A1, valine, lactate, pyruvate, and fumarate.

Afterwards, a score is calculated from the determined concentrations, wherein the score is indicative for the risk of the individual to have a reduced liver function.

The score can be calculated by taking into consideration the concentrations measured or expected in a body fluid sample from a control group. To give a simple example, the score can be the median of the concentration ratios of the at least two substances between the body fluid test sample of the patient and corresponding control values of a body fluid control sample that have been measured in the past. If the score is above or below a predetermined threshold value, a significant increase or decrease of the marker substances is present in the body fluid test sample that is indicative for having a reduced liver function. It should be noted that other calculation methods as well as a weighting of individual marker concentrations with respect to other marker concentrations can also be performed in an embodiment.

A suited way to calculate the score is disclosed on pages 25 to 27 of WO 2012/045773 A9. Another suited way to calculate the score is the following:

$$score = \frac{1}{1+e^{-\omega}},$$

wherein

$$\omega = a + \sum_{x=1}^{n} b_x \cdot I_x$$

$a = const.$

$b_x = substance\ specific\ coefficient$

$I = parameter\ being\ indicative\ for\ the\ concentration\ of\ substance\ x$

Thereby, the individual factors a, b need to be adjusted according to the underlying model and can vary in dependence on the specific substances considered in the marker set. Parameter "I" can be, e.g., the signal intensity or signal integral of an according signal observed in the evaluated measuring result. To give an example, "I" can be the signal intensity or signal integral of an NMR signal in an NMR spectrum if NMR spectroscopy is used as measuring technique.

In an embodiment, "I" is a ratio between two signal intensities or two signal integrals. In such a case, it is, e.g., possible to standardize the concentration of a first substance (or a plurality of substances) by the concentration of a second substance.

The score is a (semi-)quantitative measure for the likelihood that the individual has a reduced liver function. Thus, the score serves for (semi-)quantitatively determining the risk of a reduced liver function.

Calculating the score comprises multiplying each of the concentrations of the substances by a substance-specific weighting factor to provide a plurality of weighted values and combining the weighted values into a risk equation. Afterwards, an output of the risk equation is compared to a predefined threshold. If the score is above the threshold, there is a likelihood that the individual has a reduced liver function. In an embodiment, the likelihood and/or the risk is higher, the higher the score is (i.e., the likelihood and/or the risk increases with increasing distance of the score from the threshold).

In an embodiment, the calculated score is output and presented to the individual and/or to a third person such as a physician or medical staff. The output can be performed on a display (i.e., in an electronic way) or in printed form. Thereby, it is also possible to generate a report indicating the score, optionally in combination with a comparative scale of possible scores and their meaning with respect to the risk of having a reduced liver function.

In an embodiment, the method is a computer-implemented method. In particular, all steps of spectral analysis and concentration determination as well as of score calculation are performed on a computer. Such steps are far too complex to be done in a manual way. The computer-implemented concentration determination is, in an embodiment, based on a spectral analysis, such as an analysis of NMR spectra. The spectral analysis and the further required steps until the score can be output can be done on the same computer that is used for controlling a spectrometer performing the spectral analysis or on a different computer.

In an embodiment, the body fluid sample is a urine sample or a blood sample. In an embodiment, the blood sample is a whole blood sample, a blood serum sample, a blood plasma sample, or any other blood preparation derivable from whole blood or from other blood preparations. Blood serum is a particularly appropriate body fluid for carrying out the method or for the above-mentioned in vitro or in vivo uses of specific biomarkers contained in the marker set. Blood plasma is also an appropriate body fluid. In an embodiment, lactate is present in the marker set if blood plasma is used as body fluid to be analyzed.

In an embodiment, the body fluid sample (and therewith the patient from whom the body fluid sample originates) is grouped into one of at least two predefined groups based on the calculated score. Typically, one group encompasses patients having a reduced liver function, wherein the other group encompasses individuals having a preserved liver function. The terms "reduced liver function" and "preserved liver function" are defined above in detail. The resulting grouping can also be indicated on an according report. In an embodiment, the grouping encompasses more than two groups (yes/no), namely information on the grade of severity of the reduced liver function.

In an embodiment, the individual of whom the body fluid is analyzed, is a healthy individual. In an embodiment, the individual is a risk patient, i.e., an individual belonging to a group that has an increased risk of having a reduced liver function with respect to the risk of a healthy standard population. In an embodiment, the individual suffers from (etiology-independent) cirrhosis. In an embodiment, the individual is a patient having cirrhosis but being asymptomatic. In an embodiment, the individual suffers from a (in particular chronic) viral liver infection and has a cirrhosis. In an embodiment, the individual suffers from chronic hepatitis B and has a cirrhosis. In an embodiment, the individual suffers from chronic hepatitis C and has a cirrhosis. In an embodiment, the individual suffers from non-alcoholic fatty liver disease (NAFLD), in particular from non-alcoholic steatohepatitis (NASH), and has a cirrhosis. In an embodiment, the individual suffers from alcoholic liver disease and has a cirrhosis. In an embodiment, the individual has a cryptogenic cirrhosis. In an embodiment, the individual suffers from a (in particular chronic) viral liver infection without having a cirrhosis. In an embodiment, the individual suffers from chronic hepatitis B without having a cirrhosis. In an embodiment, the individual suffers from grade IV fibrosis (i.e., a fibrosis that led to cirrhosis). All of the precedingly mentioned embodiments can be particularly well combined so that the individual may be chosen from any of the beforementioned groups of patients/healthy individuals.

In an embodiment, the score is calculated by not only considering the concentration of the at least two marker substances but also includes the age and/or the gender of the individual who donated the body fluid sample.

In an embodiment, calculating the score involves calculating a ratio between at least two concentration values. To give an example, a ratio between HDL and valine or between pyruvate and lactate is calculated in an embodiment. Generally, individual ratios of pairs of two of all marker substances the concentrations of which have been determined upon carrying out the method can be calculated for calculating the score.

In an aspect, the present invention relates to a medical method for diagnosing a reduced liver function in an individual. The method comprises the steps explained in the following.

In a first step, a body fluid sample is gathered from an individual. In a second step, the concentration of a single substance or of at least two substances is determined by analyzing the body fluid sample with a suited measuring technique. The single substance is chosen from a first group consisting of high-density lipoprotein, apolipoprotein A1, valine, and lactate. The at least two (e.g., 2, 3, 4, 5, 6) substances are chosen from a second group consisting of high-density lipoprotein, apolipoprotein A1, valine, lactate, pyruvate, and fumarate.

Afterwards, a score is calculated from the determined concentrations, wherein the score is indicative for determining the risk of the individual to have a reduced liver function.

In a further aspect, the present invention relates to a decision support system for analyzing an isolated body fluid sample in vitro, the decision support system comprising:

a) a unit for providing a body fluid sample from an individual;

b) a unit for determining the concentration of a single substance or of at least two substances by analyzing the body fluid sample with a suited measuring technique. The single substance is chosen from a first group consisting of high-density lipoprotein, apolipoprotein A1, valine, and lactate. The at least two (e.g., 2, 3, 4, 5, 6) substances are chosen from a second group consisting of high-density lipoprotein, apolipoprotein A1, valine, lactate, pyruvate, and fumarate; and

c) a unit for calculating a score from the determined concentrations, the score being indicative for determining the risk of the individual to have a reduced liver function.

In an embodiment, the unit for determining the concentration of the single substance or of the at least two substances is configured to determine the concentration of any exemplary substance or substance combination of the embodiments explained above.

While some of the explained uses and methods are described as in vitro uses and methods and some of the explained uses and methods are described as in vivo uses and methods, it should be noted that each in vitro use or method can also be carried out as in vivo use or method, and vice versa.

All embodiments of the use of the marker or the marker set can be combined in any desired way and can be transferred either individually or in any arbitrary combination to the further medical use of the marker or the marker set as well as to the different methods and to the decision support system. Likewise, all embodiments of the further medical use of the marker or the marker set can be combined in any desired way and can be transferred either individually or in any arbitrary combination to the use of the marker or the marker set, to the different methods, and to the decision support system. Finally, all embodiments of the different methods can be combined in any desired way and can be transferred either individually or in any arbitrary combination to the use of the marker or the marker set, to the further medical use of the marker or the marker set, to any other of the described methods, and to the decision support system.

Further details of aspects of the present invention will be explained in the following making reference to exemplary embodiments and accompanying Figures. In the Figures:

Figures 1A to 13B	show ROC plots illustrating the ability of different markers or marker sets for determining the risk of an individual to have a reduced liver function upon evaluating a training dataset (always in Figure A) or upon evaluating a test dataset (always in Figure B); and
Figures 1C to 13D	show confusion matrices illustrating the distributions of calculated scores from the determined concentrations of the individual biomarkers or the biomarkers contained in the respective marker set upon evaluating a training dataset (always in Figure C) or upon evaluating a test dataset (always in Figure D).

All ROC plots shown in Figures 1A to 13A (in the A Figures) and the corresponding confusion matrices shown in Figures 1C to 13C (in the C Figures) as well as all ROC plots shown in Figures 1B to 13B (in the B Figures) and the corresponding confusion matrices shown in Figures 1D to 13D (in the D Figures) were obtained by analyzing blood serum samples of individuals having a reduced liver function (cases) or having a preserved liver function (control). The recruiting of patients for the analyzed samples as well as the sample preparation and measuring will be explained in the following in more detail.

Study design

A retrospective case-control design using serum samples from individuals having a reduced liver function or having a preserved liver function was chosen. The samples were either banked or retained samples from routine clinical care collected for other research studies or non-research purposes, or samples collected as part of a clinical study. Serum cohorts were obtained from four different study sites, namely three in Europe and one in the U.S. Thus, this study was a retrospective, multi-centric, cross-sectional case-control study.

Group assignment and reference standard

The case group consisted of patients classified as having a reduced liver function (i.e., being classified into Child-Pugh class A and having ascites; or being classified into Child-Pugh classes B or C (irrespective of having ascites)). The control group consisted of patients classified into Child-Pugh class A without having ascites

Cohort details

Details of the tested patient cohort are listed in the following Table A:

Table A: Details of the tested patient cohort.

Characteristic	N	Training, N = 563	Test, N = 238
Center, n / N (%)	801		
Center 1A		214 / 563 (38%)	109 / 238 (46%)
Center 1B		40 / 563 (7.1%)	17 / 238 (7.1%)
Center 2		235 / 563 (42%)	84 / 238 (35%)
Center 4		74 / 563 (13%)	28 / 238 (12%)
Age, Mean (SD)	801	61.17 (10.94)	61.52 (10.26)
Age Group, n / N (%)	801		
<63		293 / 563 (52%)	117 / 238 (49%)
>=63		270 / 563 (48%)	121 / 238 (51%)

Characteristic	N	Training, N = 563	Test, N = 238
Sex, n / N (%)	801		
female		161 / 563 (29%)	67 / 238 (28%)
male		402 / 563 (71%)	171 / 238 (72%)
HCC Stage (Milan), n / N (%)**	801		
negative		360 / 563 (64%)	146 / 238 (61%)
early		110 / 563 (20%)	52 / 238 (22%)
late		93 / 563 (17%)	40 / 238 (17%)
Ascites: yes, n / N (%)	801	206 / 563 (37%)	94 / 238 (39%)
Reduced Liver Function*: yes, n / N (%)	801	295 / 563 (52%)	125 / 238 (53%)
Child-Pugh Class, n / N (%)	801		
Class A		294 / 563 (52%)	128 / 238 (54%)
Class B		200 / 563 (36%)	89 / 238 (37%)
Class C		69 / 563 (12%)	21 / 238 (8.8%)

* The target variable used in modelling for this use case.

** Milan criteria stratifies HCC into early-stage disease (transplant-appropriate, curative intent) and late-stage (transplant inappropriate) based on lesion size (one lesion smaller than 5 cm; alternatively, up to three lesions, each smaller than 3 cm), no extrahepatic manifestations and no gross vascular invasion.

Sample preparation

Samples were prepared with reagents of the AXINON® serum kit 2.0 offered by numares AG. During sample preparation, all reagents were used at ambient temperature (15-30°C).

a) Calibration samples

The AXINON® serum calibrator 2.0 was filled into an NMR tube. A cap for NMR tubes was placed onto the NMR tube. Subsequently, the calibration sample was placed in a defined position of an NMR rack.

b) Control samples

The AXINON® serum control 2.0 was filled into an NMR tube. A cap for NMR tubes was placed onto the NMR tube. Subsequently, two control samples were placed in defined positions of the same NMR rack.

c) Analytical samples

The AXINON® serum additives solution 2.0 was combined with the blood serum to be analyzed in a ratio of 1:10 in a suitable reagent container. The liquid was gently mixed, wherein foaming was avoided. The volume of the mixture required for the NMR measurement was transferred into an NMR tube. A cap for NMR tubes was placed onto the NMR tube. Each analytical sample was placed at a defined position into the same NMR rack according to a rack list (as defined by the AXINON® Sample Wizard, see AXINON® Sample Wizard User manual). Attention was paid to ensure proper positioning of the samples. The analysis results of samples that were not clearly assignable were discarded.

Measurement

All measurements were carried out on a Bruker Avance II+ 600MHz NMR spectrometer with an UltraShield 600 Plus NMR Magnet System, or a Bruker Avance III HD 600MHz NMR spectrometer with an UltraShield 600 Plus NMR Magnet System, or a Bruker Avance III HD 600MHz NMR spectrometer with an Ascend NMR Magnet System using a PATXI 1H/D-13C/15N Z-GRD probe. All samples were kept at 5-7°C in the SampleJet and brought to the target temperature in the integrated preheating block before measurement.

Two measurement sequences were used for all samples:

- on the one hand, a standard pulse program with 30-degree excitation pulse and pre-saturation for water suppression was used (zgpr30);
- on the other hand, a pulse program to measure T2-weighted spectra without J modulation using refocusing pulses between double spin echoes (project = Periodic Refocusing Of J Evolution by Coherence Transfer) was used.

Samples were measured in batches of up to 93 analytical samples per run. In addition to the analytical samples, each run included one AXINON® blood serum calibrator sample and two AXINON® blood serum control samples (before and after the analytical blood serum samples, respectively) to assure ideal measurement conditions throughout the run.

Signal analysis

a) Spectrum Qualification (Quality Control for measurement)

NMR spectra underwent automatic referencing, phase correction and baseline correction before further analysis.

Subsequently, the NMR spectra underwent an automatic standardization and calibration procedure to minimize between-device, between-day and between-run effects. The quality of each of these spectra was assessed by a custom spectrum qualification algorithm that

analyzes general spectral properties, e.g., offset and tilt of the baseline in selected spectral regions, and properties of selected indicator signals, e.g., signal position, shape and width. Spectra that did not meet the predefined quality criteria were excluded from further analysis.

b) Bins

Successfully qualified spectra (typically covering a chemical shift from -5 to 14 ppm) were subjected to further modifications. In particular, broad background signals were separated with a suitable algorithm, e.g., background intensities (such as generated from proteins) were subtracted from the spectra, resulting in spectral intensities devoid of such background signals.

The cohort (i.e., the plurality) of modified spectra was checked for regions in which the cohort did not show a significant number of signals. These regions – like the region of the water signal and the regions in which signals are contained that originate from substances contained in AXINON® serum additives – were ignored in the steps explained in the following.

The remaining spectral regions were subject to an adaptive binning, which divides the spectrum in bins of differing size or extent (typically covering 0.01 to 0.05 ppm, but in extreme cases also covering 0.005 to 0.5 ppm). The resulting boundaries for the bins are tailored to represent signals and/or signal structures in the spectrum as good as possible.

Depending on the cohort of modified spectra, the size and thus the number of bins varies. Typical numbers of bins lie in a range of from 100 to 400.

c) Quantifier

Quantification of substances was done by fitting a predefined, characteristic set of Pseudo-Voigt functions, which represent a linear combination of a Gaussian and a Lorentzian function, to the substance specific signal structure(s). The resulting signal fits were checked for goodness of fit and physical plausibility of properties related to fit parameters in order to reject results of insufficient fit quality.

Alternatively, quantification models making use of the previously assigned bins were applied. After substance identification, substance labels have been assigned to these bins. The quantification was then determined by the bin value, which calculates as [(sum of intensities in bin)/(number of data points in bin)]. The standardization by data points is used to compensate for a varying number of data points in the bins. The number of data points in a bin may vary by one data point due to shifts of the applied discretization grid.

In either case, the resulting integral of the quantification is then translated into a substance concentration by applying a conversion factor which has been determined experimentally for each of the substances.

Test of identified marker substances

The identified marker substances were tested in different combinations to assess their suitability for determining the risk of an individual of having a reduced liver function. In doing so, the result of the determination based on the marker substances (predicted risk) has been checked against clinical signs of a reduced liver function in the patient who donated the serum sample, as already explained above.

The results are summarized in receiver operating characteristic (ROC) plots. In these plots, the area under the curve (AUC) indicates the fitness of the prediction. If the AUC is 0.5, the prediction is to be considered random and thus not well suited. The higher the AUC, the better is the prediction model.

The obtained results will be explained in the following in more detail making reference to Figures 1A to 13D.

Figures 1A, 2A, 3A, 4A, 5A, 6A, 7A, 8A, 9A, 10A, 11A, 12A, and 13A show ROC plots of different individual markers or marker combinations upon evaluation of a training dataset. Figures 1C, 2C, 3C, 4C, 5C, 6C, 7C, 8C, 9C, 10C, 11C, 12C, and 13C show the corresponding confusion matrices based on the scores assigned to the patient's health status (i.e., having a preserved liver function or a reduced liver function) confirmed by other tests.

Figures 1B, 2B, 3B, 4B, 5B, 6B, 7B, 8B, 9B, 10B, 11B, 12B, and 13B show ROC plots of different individual markers or marker combinations upon evaluation of a test dataset. Figures 1D, 2D, 3D, 4D, 5D, 6D, 7D, 8D, 9D, 10D, 11D, 12D, and 13D show the corresponding confusion matrices based on the scores assigned to the patient's health status (i.e., having a preserved liver function or a reduced liver function) confirmed by other tests.

The following Table 1 summarizes the results depicted in the Figures.

Table 1: Summary of biomarkers/biomarker set composition and corresponding AUC values depicted in the Figures.

Figure	Biomarker	AUC value in training dataset (268 controls; 295 cases)	AUC value in test dataset (113 controls; 125 cases)
1A and 1B	HDL	0.86	0.84
2A and 2B	Valine	0.79	0.75
3A and 3B	Lactate	0.78	0.76
4A and 4B	HDL and valine	0.87	0.85
5A and 5B	HDL and lactate	0.87	0.85
6A and 6B	HDL and pyruvate	0.88	0.85
7A and 7B	HDL and fumarate	0.88	0.84
8A and 8B	Valine and lactate	0.84	0.81
9A and 9B	Valine and pyruvate	0.83	0.81
10A and 10B	Lactate and pyruvate	0.80	0.78
11A and 11B	HDL, valine, and pyruvate	0.89	0.86
12A and 12B	HDL, valine, and lactate	0.89	0.85
13A and 13B	Valine, pyruvate, and lactate	0.86	0.83

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Claims

1. Use of a marker or of a marker set in an in vitro method for determining the risk of an individual to have a reduced liver function,

characterized

in that the marker is chosen from a first group consisting of high-density lipoprotein, apolipoprotein A1, valine, and lactate and in that the marker set comprises at least two substances chosen from a second group consisting of high-density lipoprotein, apolipoprotein A1, valine, lactate, pyruvate, and fumarate.

2. Use according to claim 1, **characterized** in that the marker set comprises at least one substance chosen from the second group and being different from high-density lipoprotein and apolipoprotein A1 if the marker set comprises any of high-density lipoprotein and apolipoprotein A1 as a first of the at least two substances.

3. Use according to claim 1 or 2, **characterized** in that the marker set comprises high-density lipoprotein or apolipoprotein A1 as a first of the at least two substances and valine as a second of the at least two substances.

4. Use according to claim 1 or 2, **characterized** in that the marker set comprises high-density lipoprotein or apolipoprotein A1 as a first of the at least two substances and lactate as a second of the at least two substances.

5. Use according to claim 1 or 2, **characterized** in that the marker set comprises high-density lipoprotein or apolipoprotein A1 as a first of the at least two substances and pyruvate as a second of the at least two substances.

6. Use according to claim 1 or 2, **characterized** in that the marker set comprises high-density lipoprotein or apolipoprotein A1 as a first of the at least two substances and fumarate as a second of the at least two substances.

7. Use according to claim 1 or 2, **characterized** in that the marker set comprises valine and lactate.

8. Use according to claim 1 or 2, **characterized** in that the marker set comprises valine and pyruvate.

9. Use according to claim 1 or 2, **characterized** in that the marker set comprises lactate and pyruvate.

10. Use according to claim 1 or 2, **characterized** in that the marker set comprises high-density lipoprotein or apolipoprotein A1 as a first of the at least two substances as well as valine and pyruvate as further substances.

11. Use according to claim 1 or 2, **characterized** in that the marker set comprises high-density lipoprotein or apolipoprotein A1 as a first of the at least two substances as well as valine and lactate as further substances.

12. Use according to claim 1 or 2, **characterized** in that the marker set comprises valine, pyruvate, and lactate.

13. Marker or marker set for use in in-vivo diagnostics of a reduced liver function,

characterized

in that the marker is chosen from a first group consisting of high-density lipoprotein, apolipoprotein A1, valine, and lactate and in that the marker set comprises at least two substances chosen from a second group consisting of high-density lipoprotein, apolipoprotein A1, valine, lactate, pyruvate, and fumarate.

14. Method for analyzing an isolated body fluid sample in vitro, comprising the following steps:

a) determining the concentration of a substance or of at least two substances, wherein the substance is chosen from a first group consisting of high-density lipoprotein, apolipoprotein A1, valine, and lactate and wherein the at least two substances are chosen from a second group consisting of high-density lipoprotein, apolipoprotein A1, valine, lactate, pyruvate, and fumarate in an isolated body fluid sample from an individual by analyzing the body fluid sample with a suited measuring technique,

b) calculating a score from the determined concentrations, the score being indicative for determining the risk of the individual to have a reduced liver function.

15. Method according to claim 14, **characterized** in that calculating the score involves calculating a ratio between at least two concentration values.

5

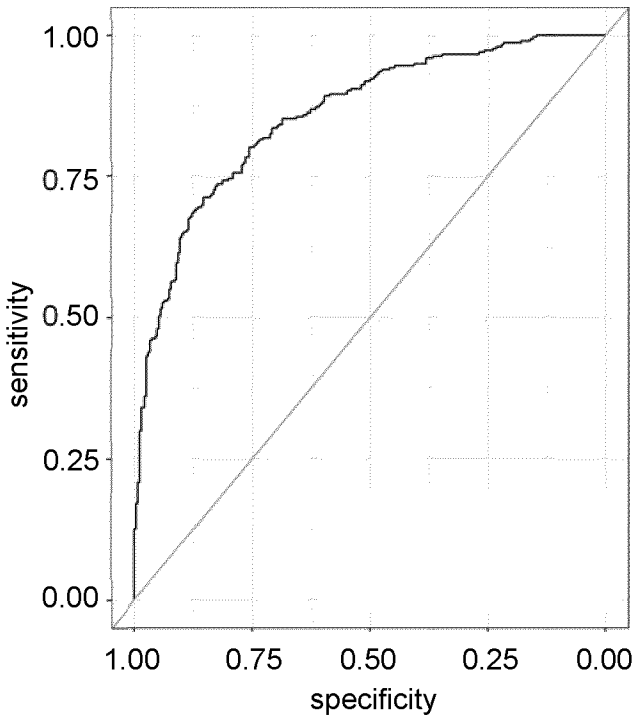

FIG 1A

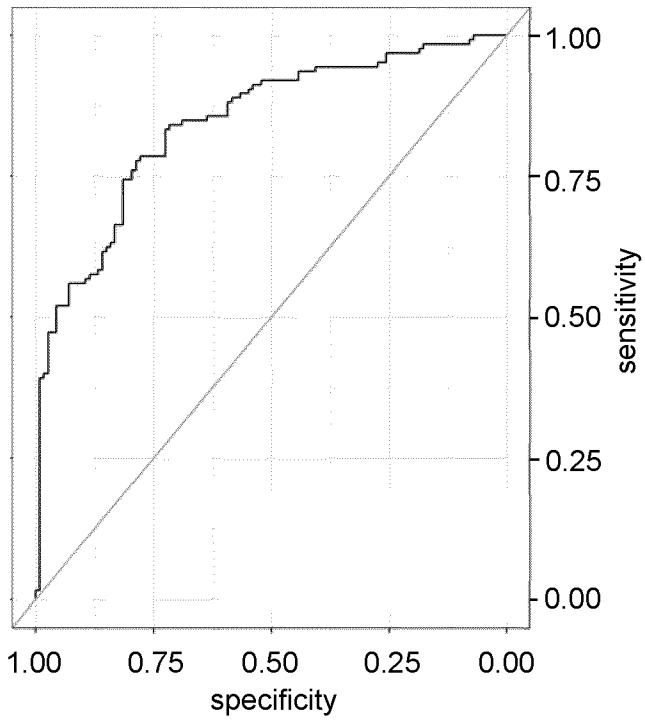

FIG 1B

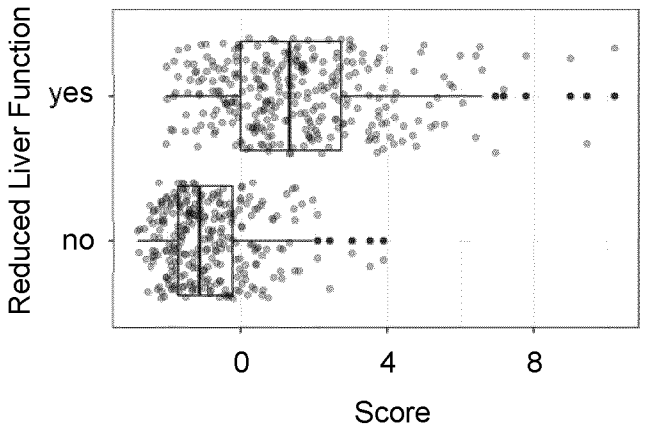

FIG 1C

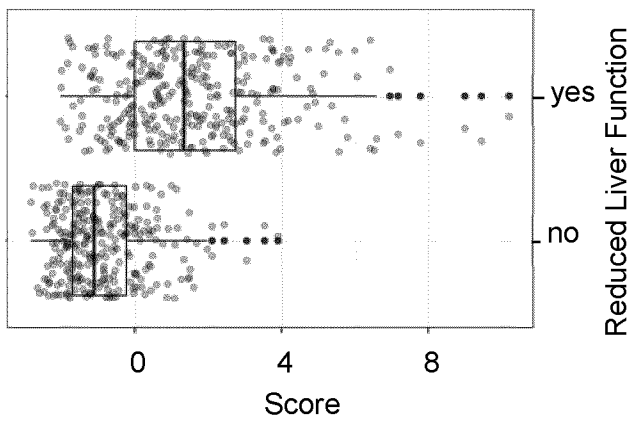

FIG 1D

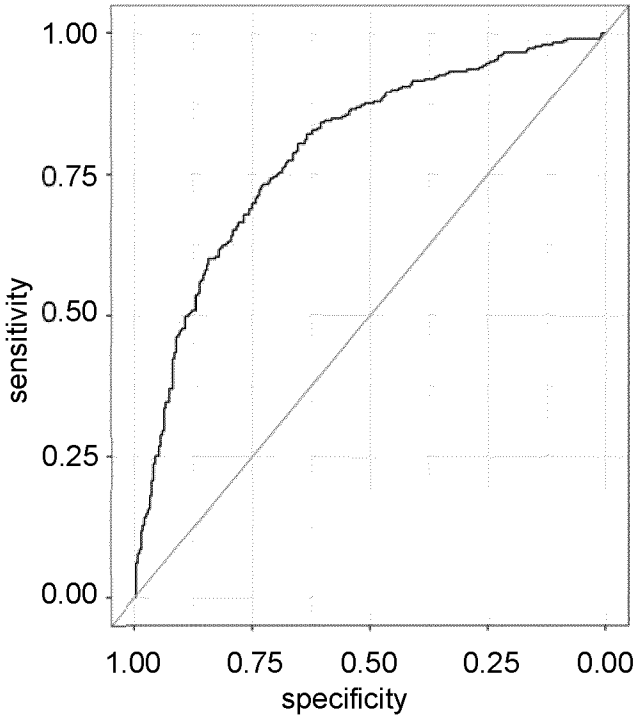

FIG 2A

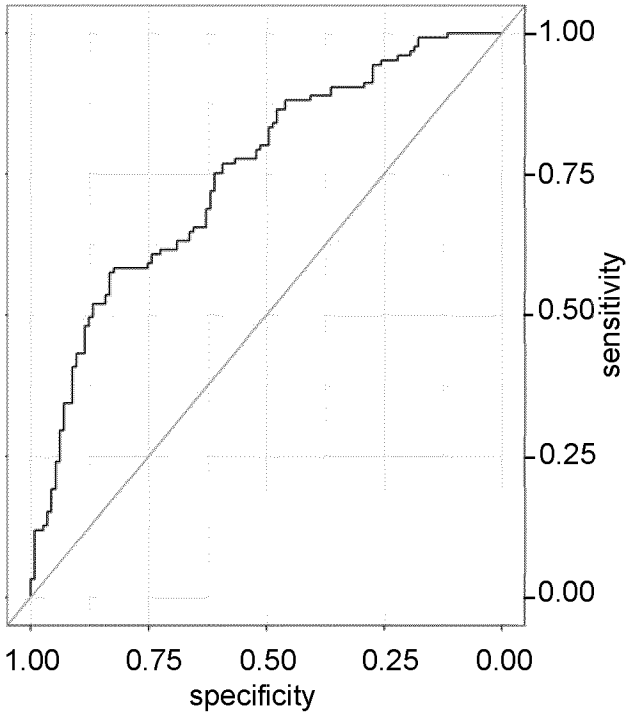

FIG 2B

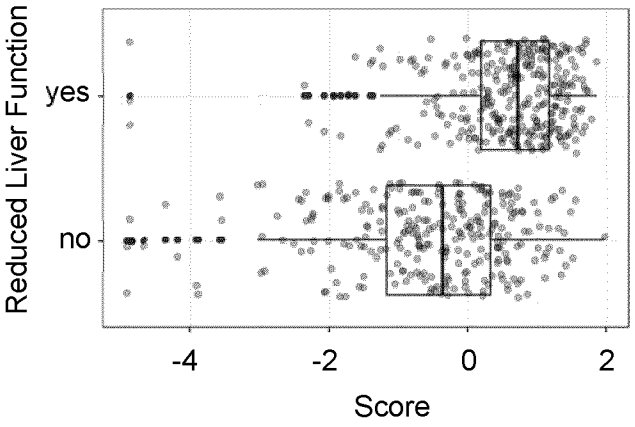

FIG 2C

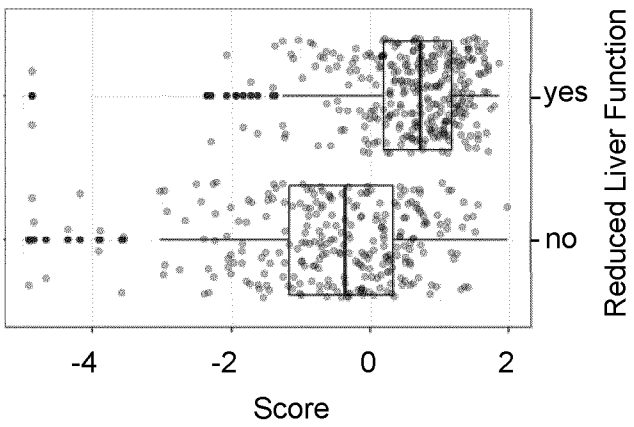

FIG 2D

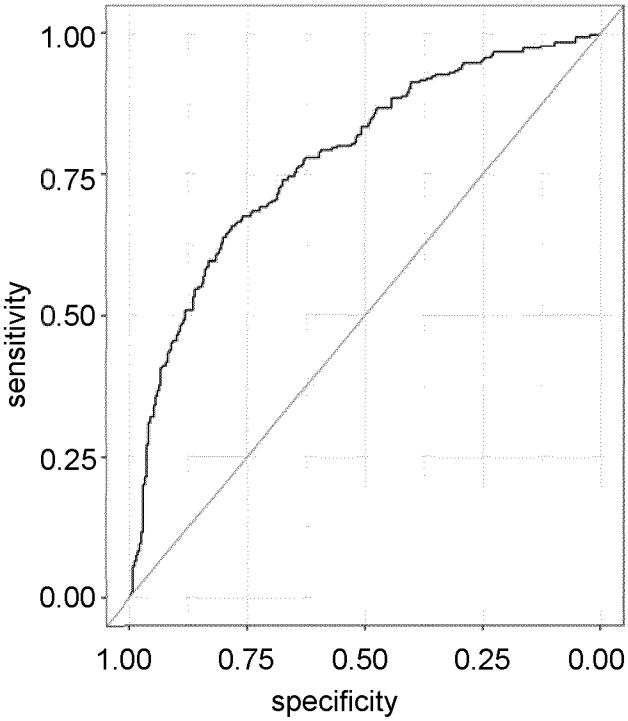

FIG 3A

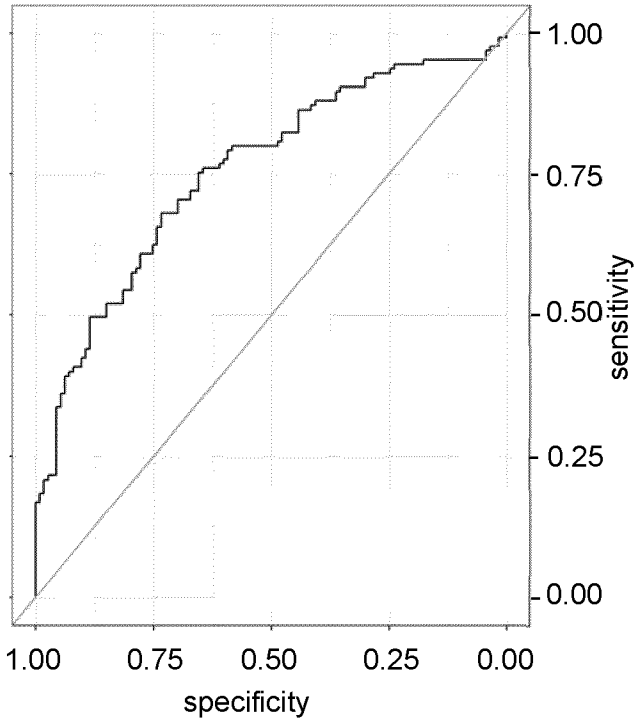

FIG 3B

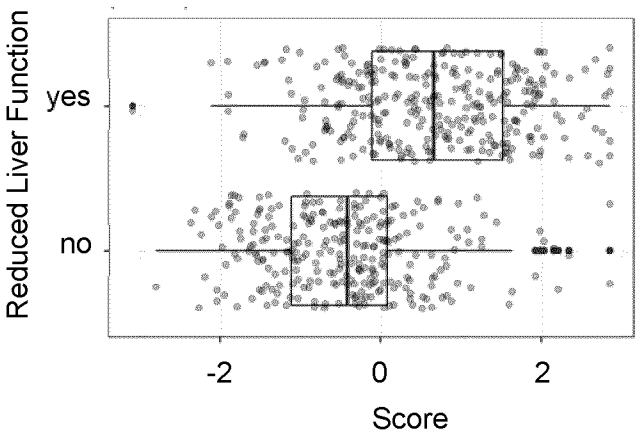

FIG 3C

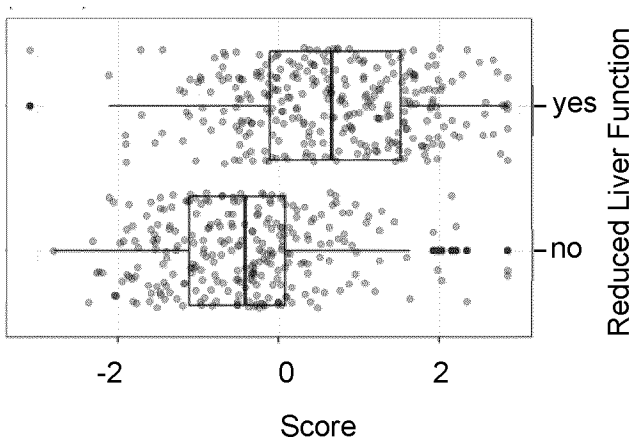

FIG 3D

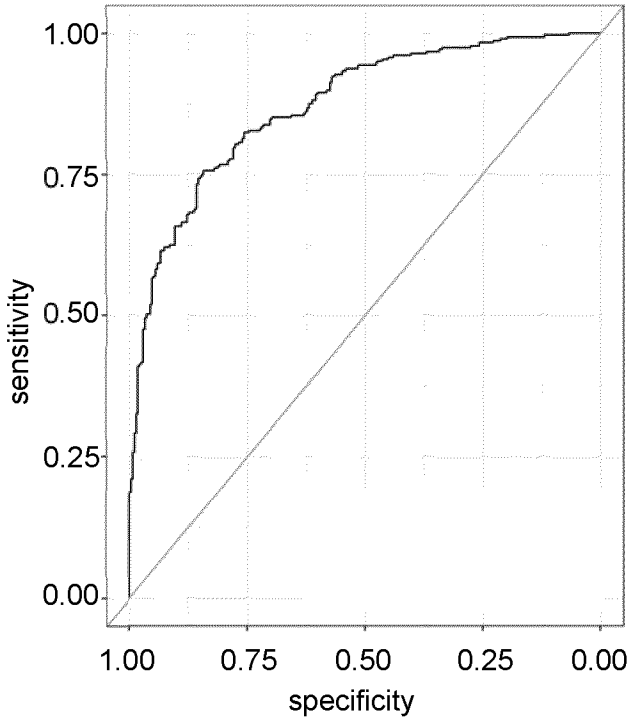

FIG 4A

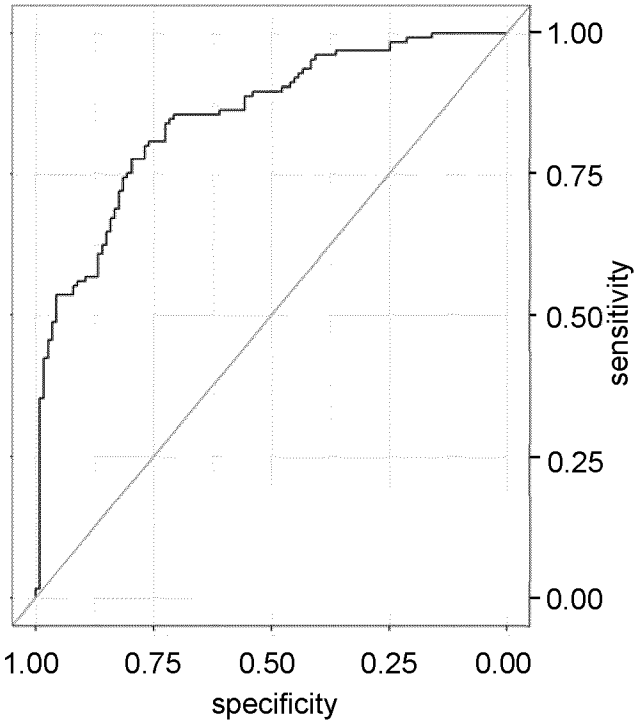

FIG 4B

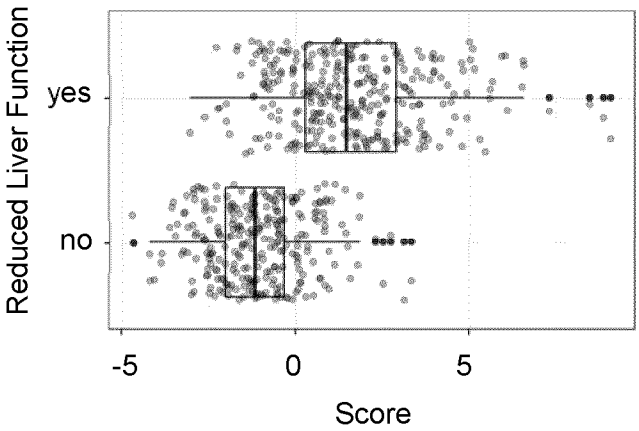

FIG 4C

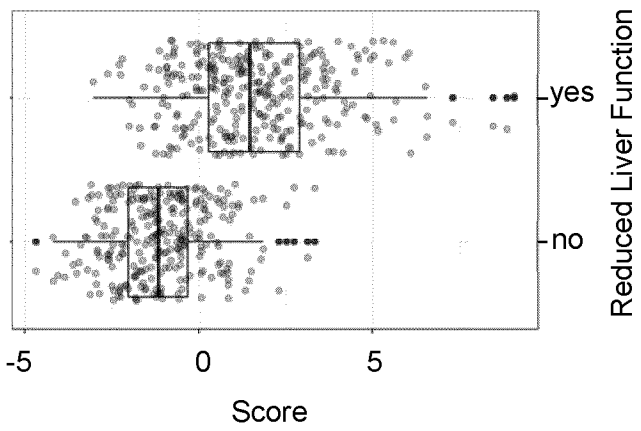

FIG 4D

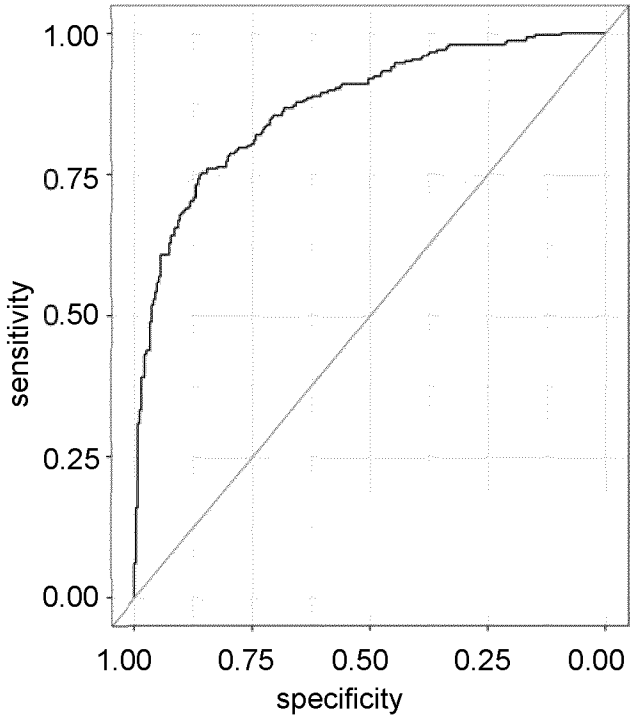

FIG 5A

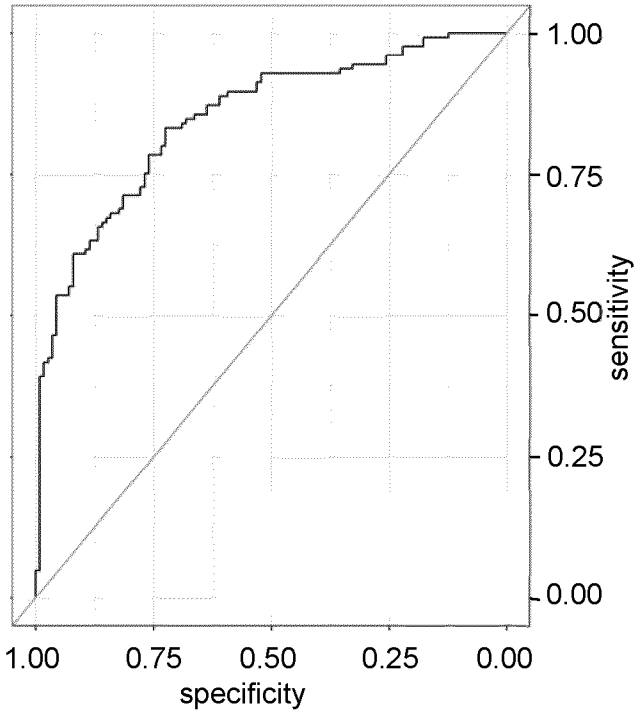

FIG 5B

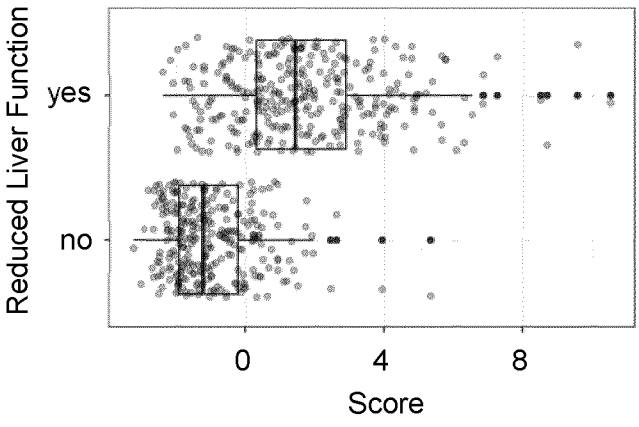

FIG 5C

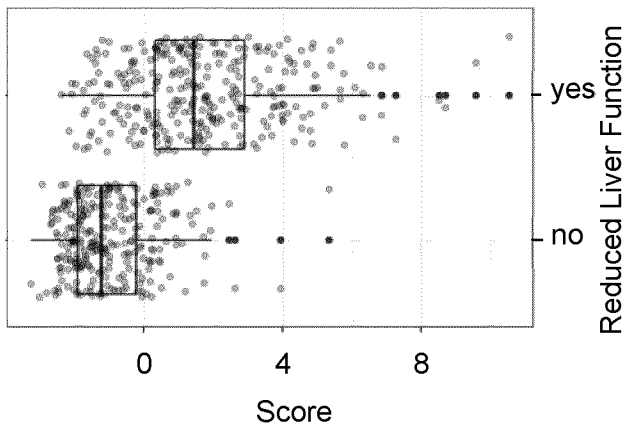

FIG 5D

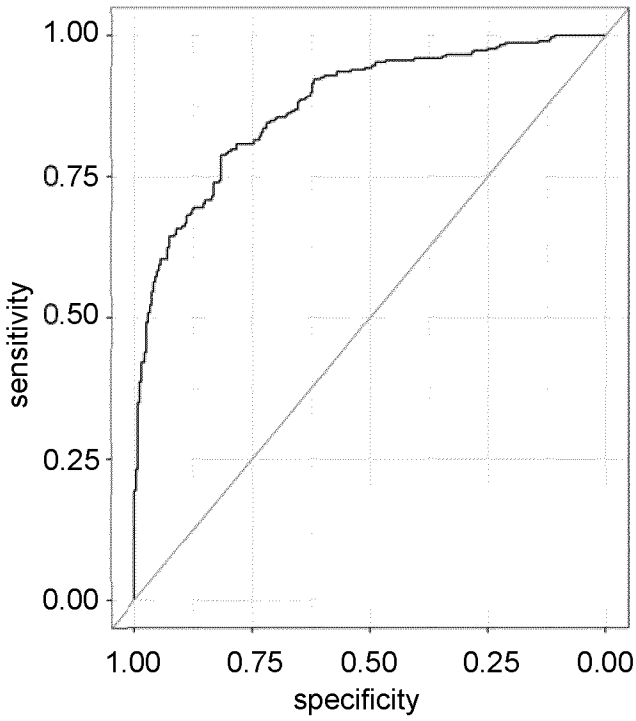

FIG 6A

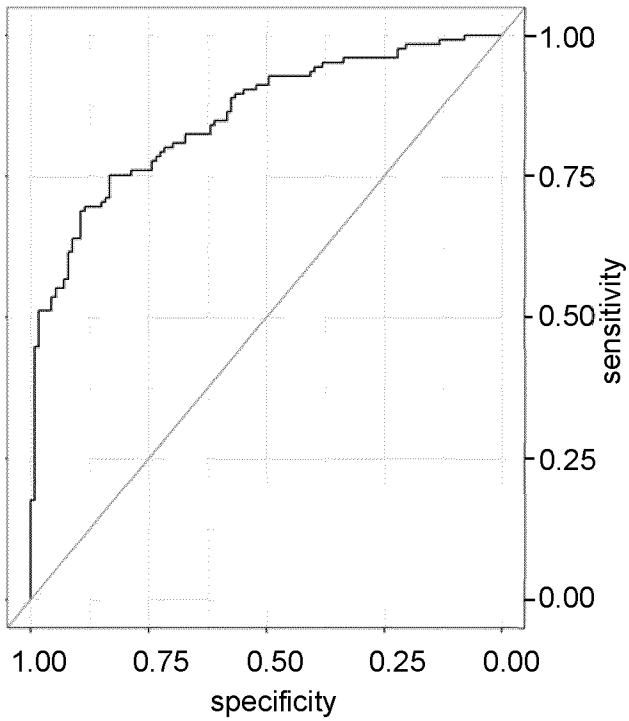

FIG 6B

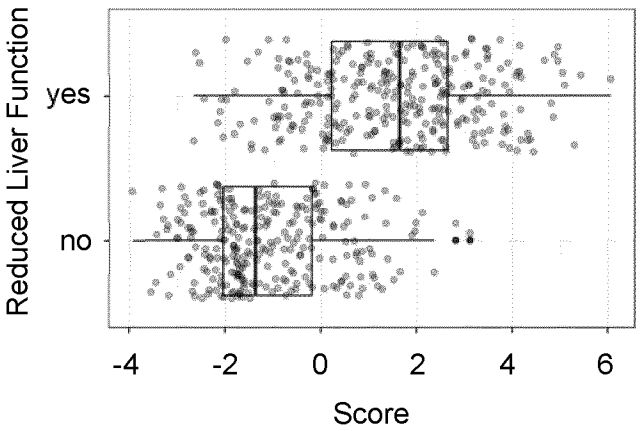

FIG 6C

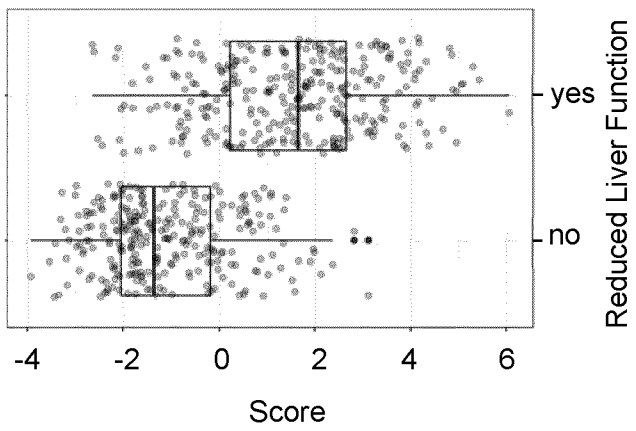

FIG 6D

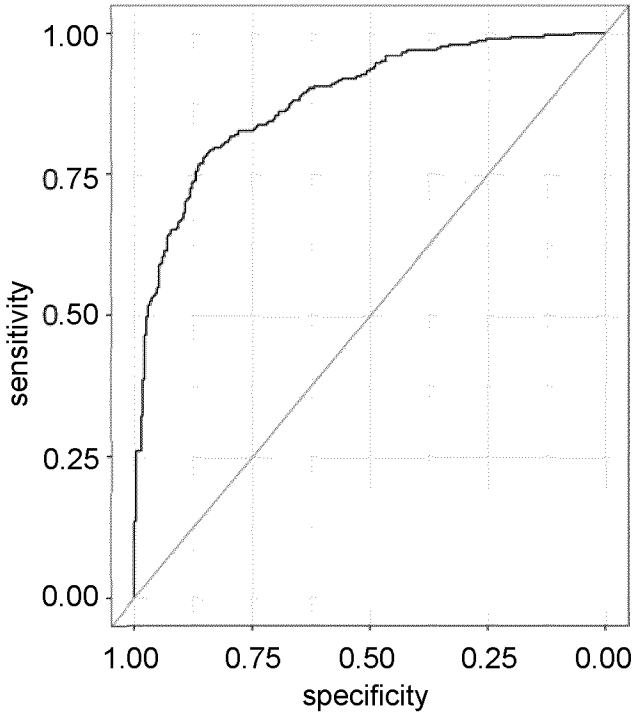

FIG 7A

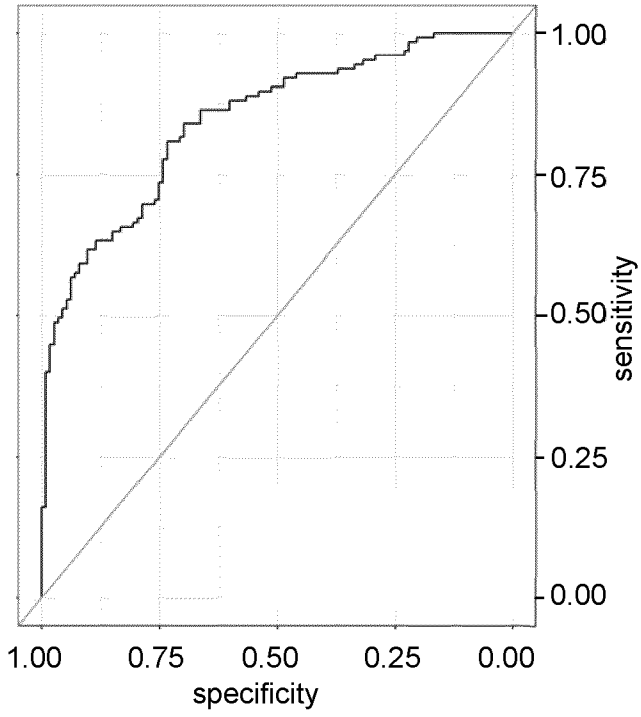

FIG 7B

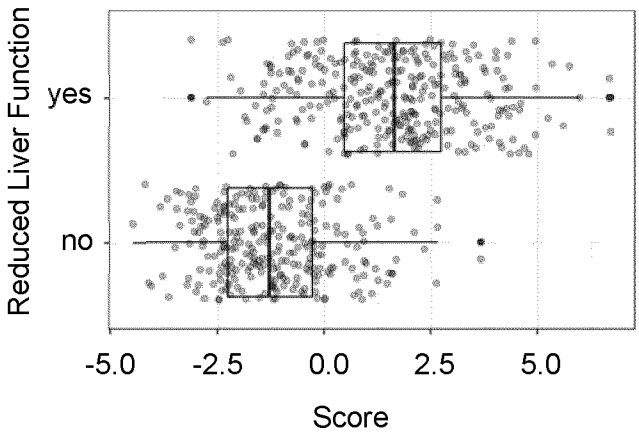

FIG 7C

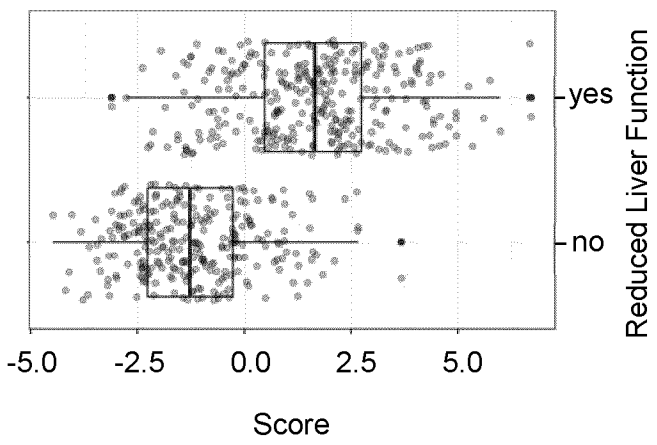

FIG 7D

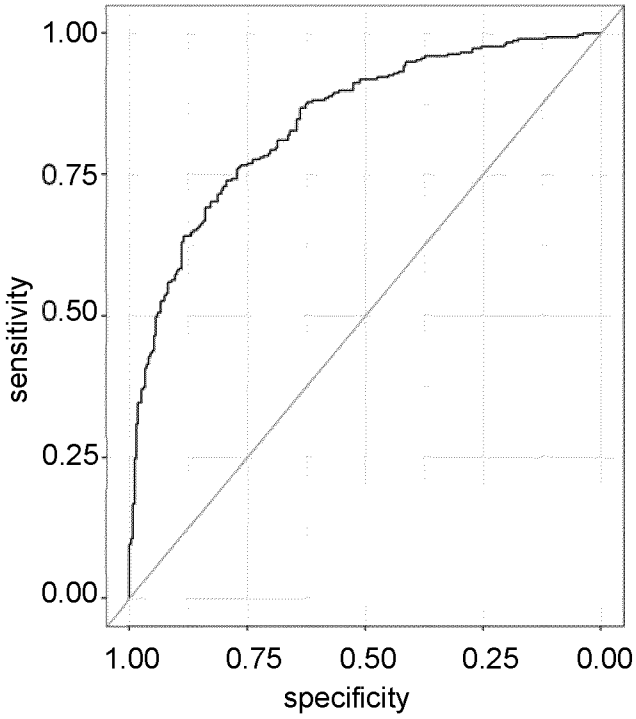

FIG 8A

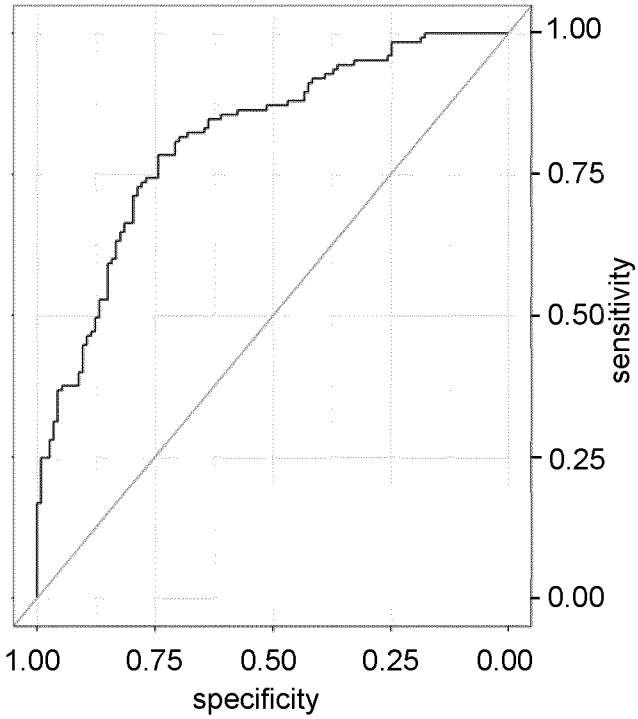

FIG 8B

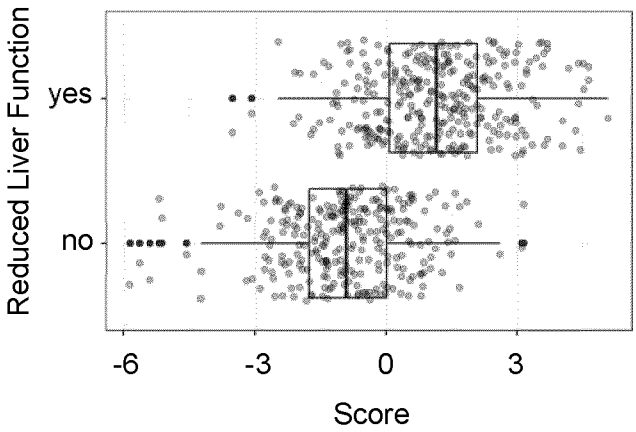

FIG 8C

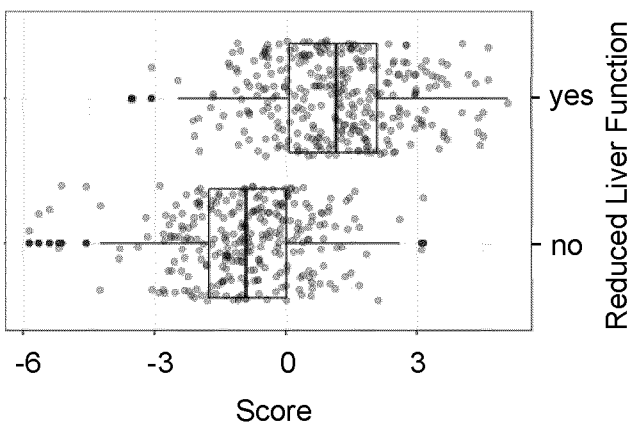

FIG 8D

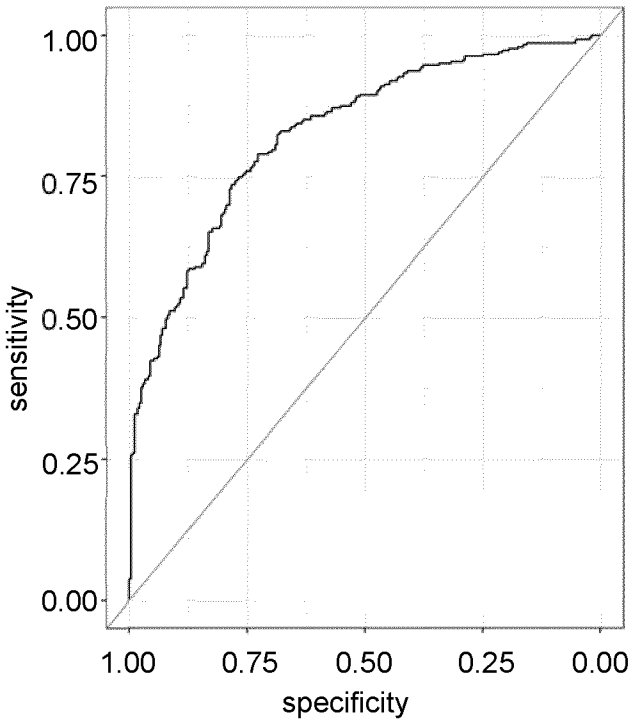

FIG 9A

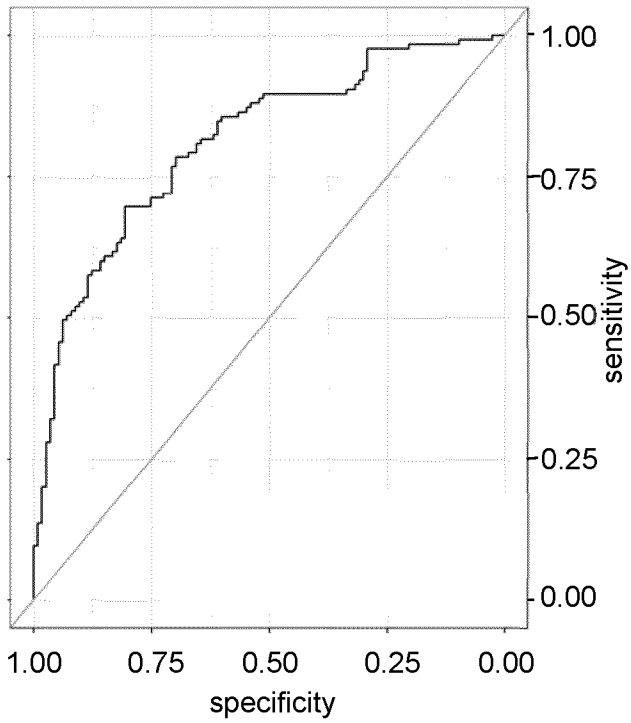

FIG 9B

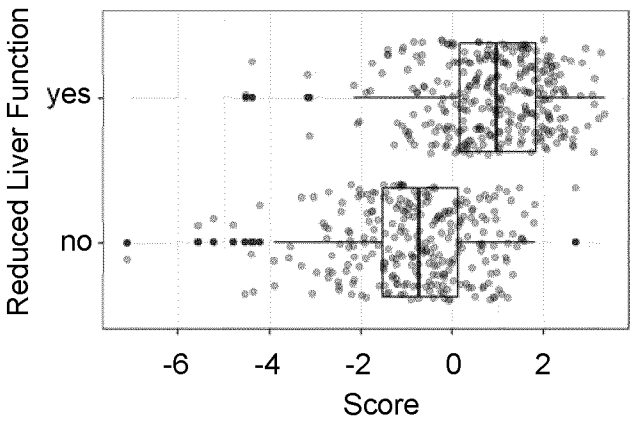

FIG 9C

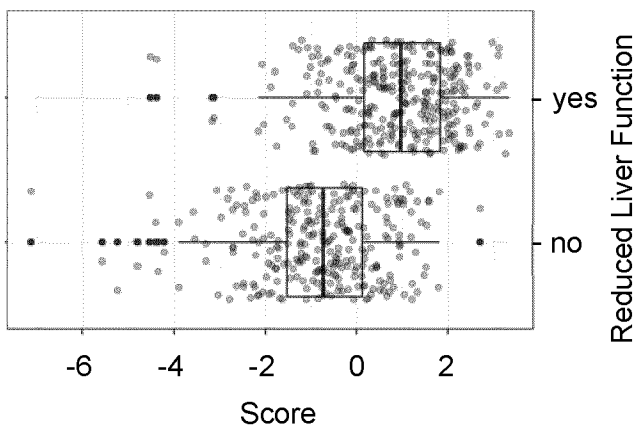

FIG 9D

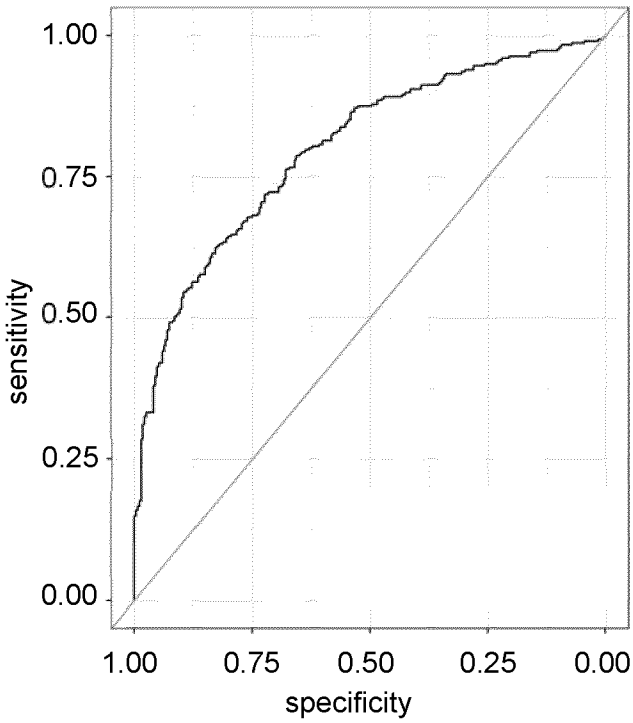

FIG 10A

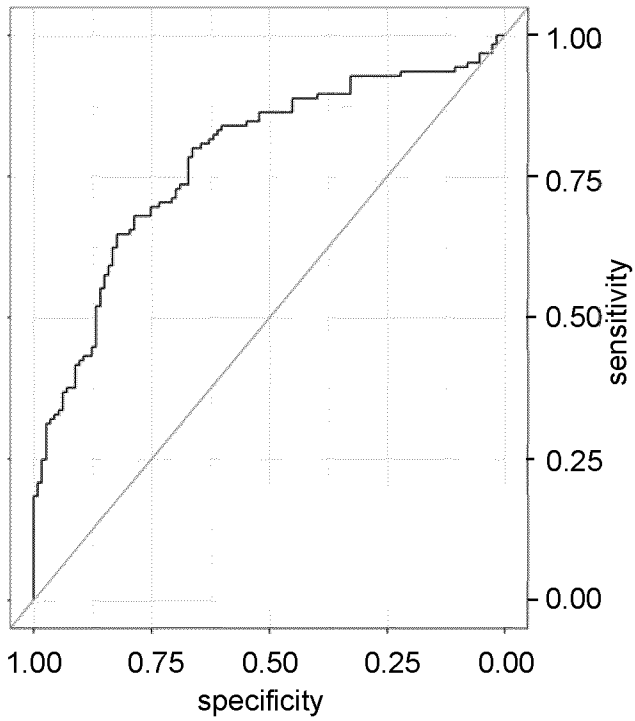

FIG 10B

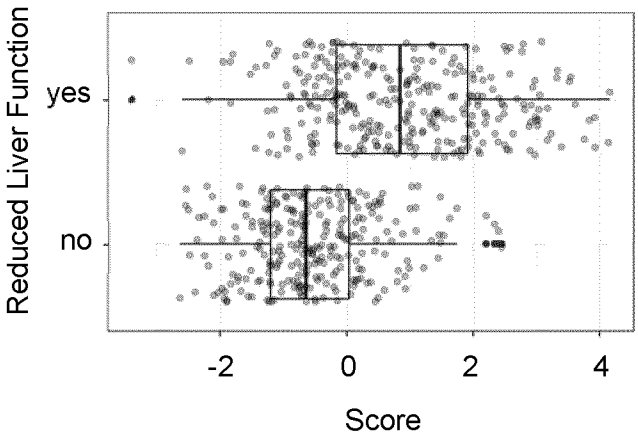

FIG 10C

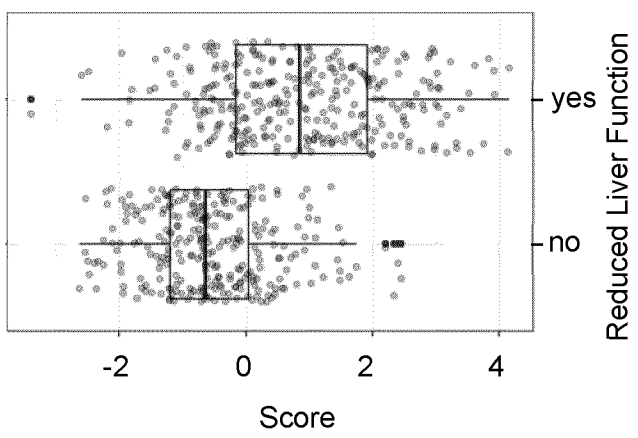

FIG 10D

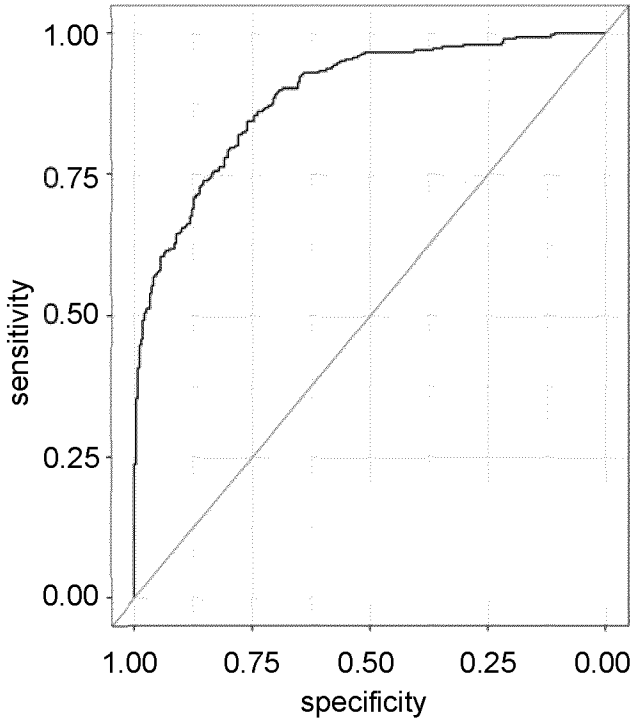

FIG 11A

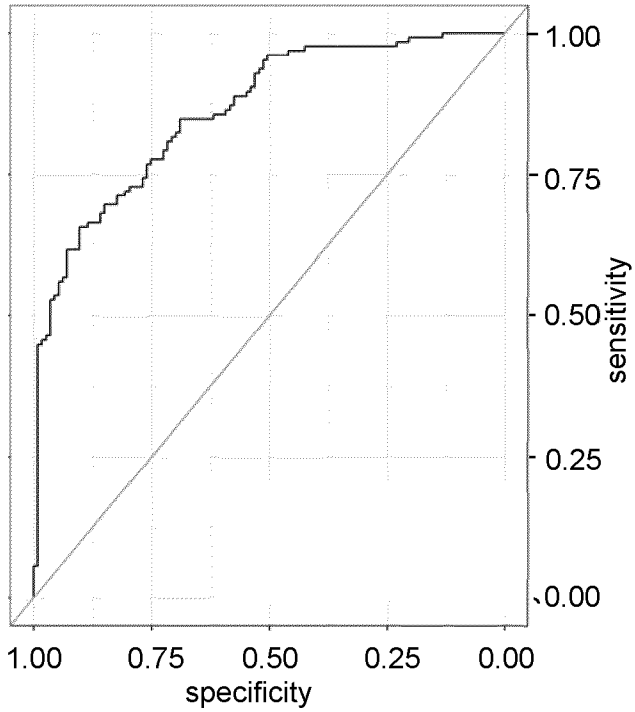

FIG 11B

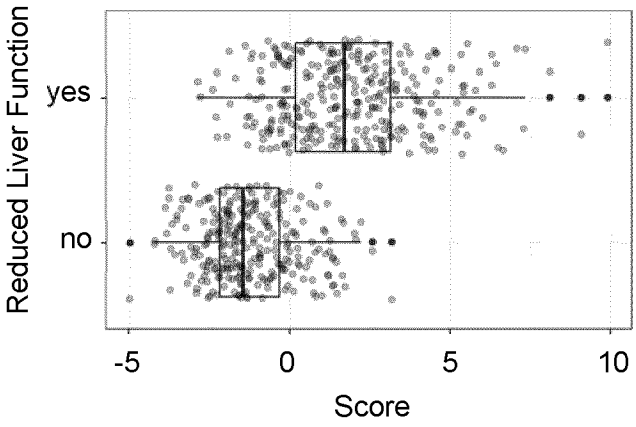

FIG 11C

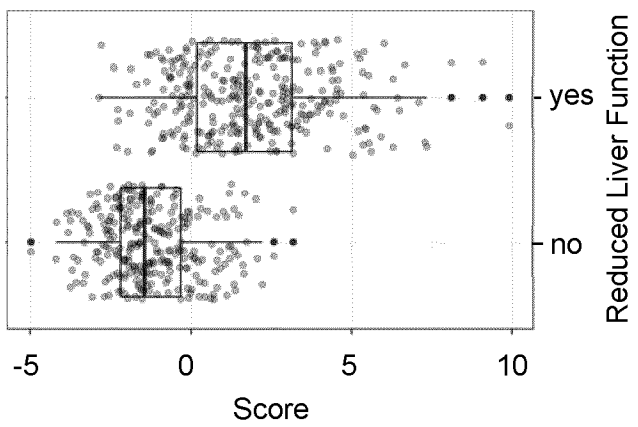

FIG 11D

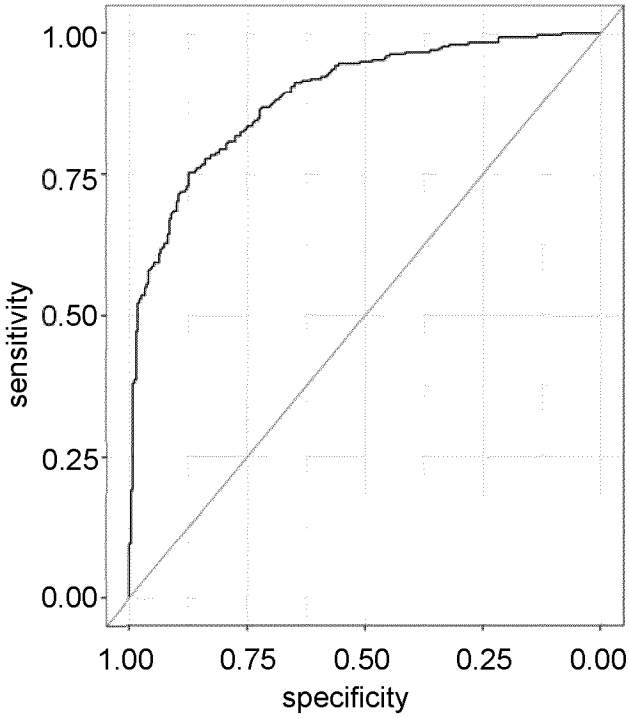

FIG 12A

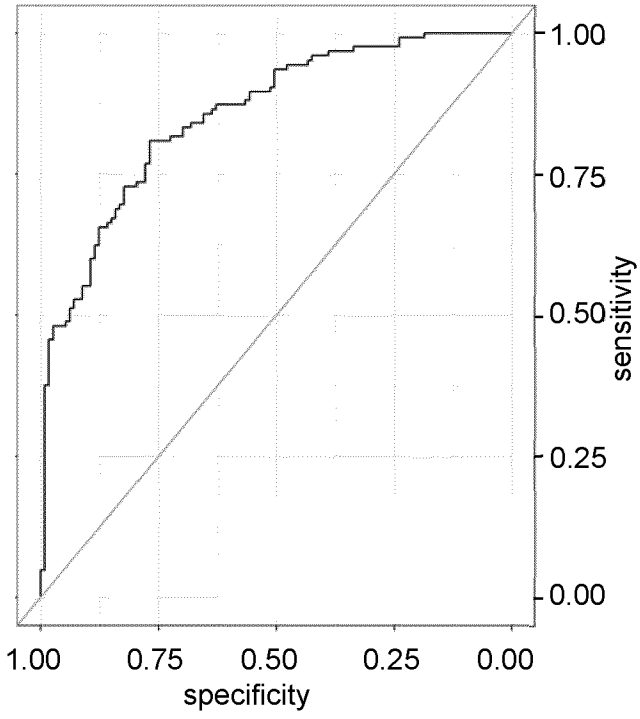

FIG 12B

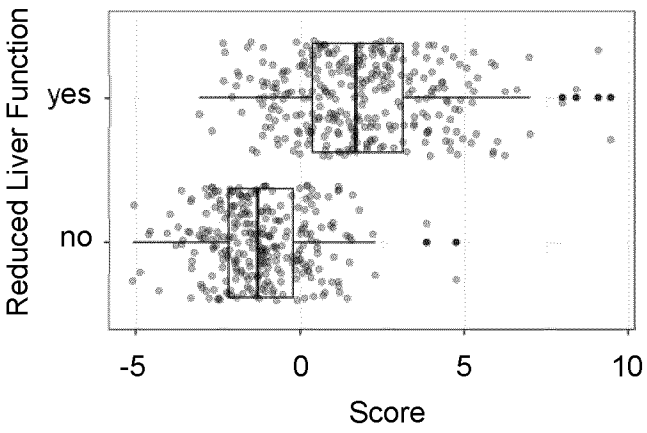

FIG 12C

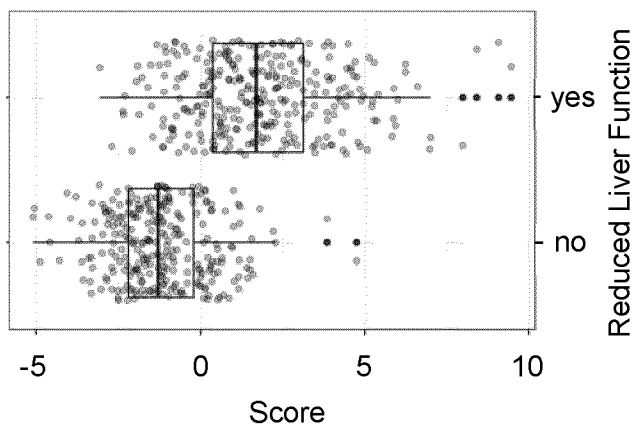

FIG 12D

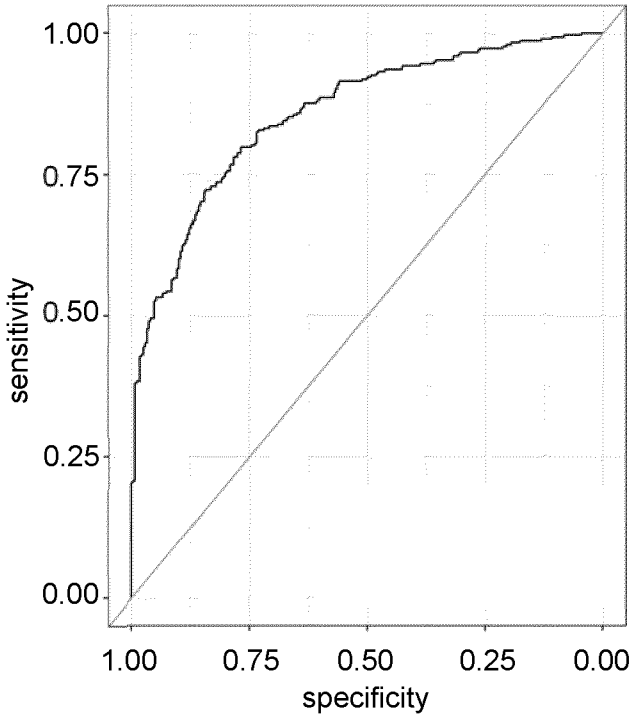

FIG 13A

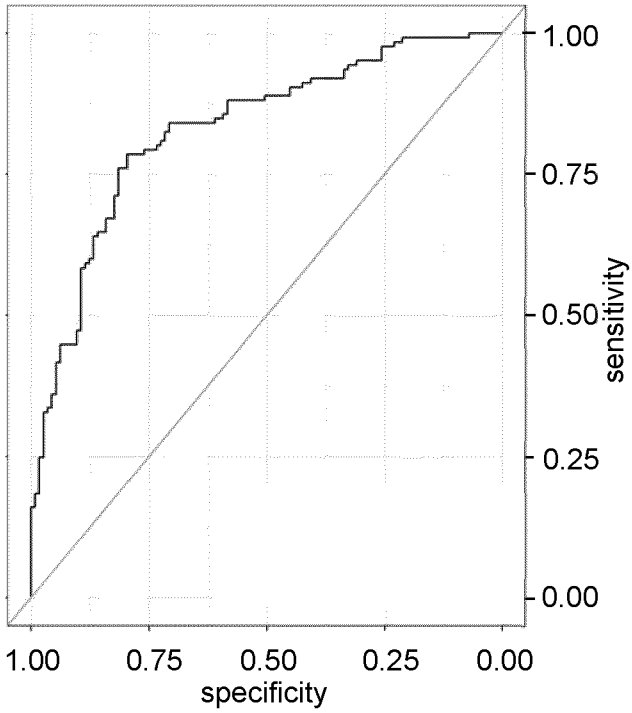

FIG 13B

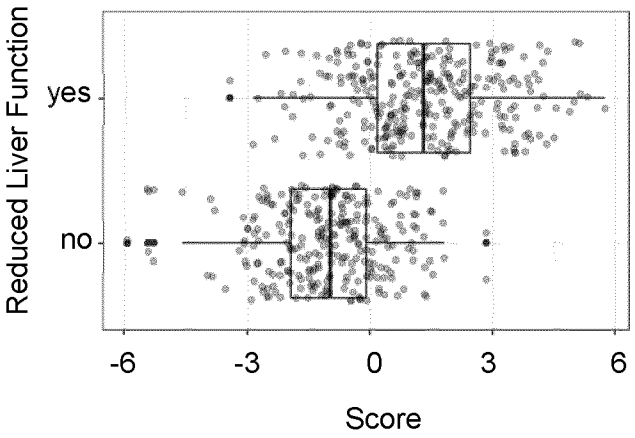

FIG 13C

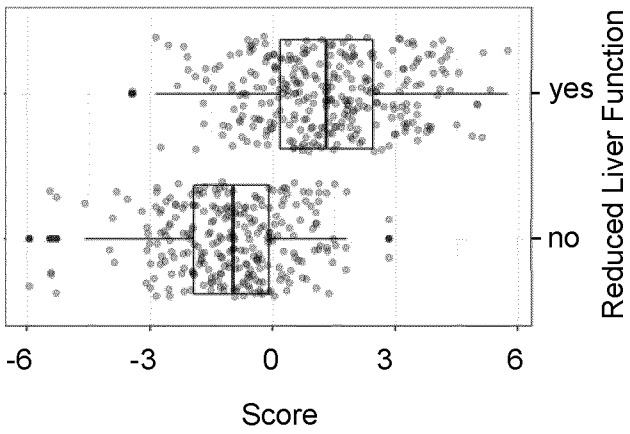

FIG 13D