(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 April 2024 (11.04.2024)

WIPO | PCT

(10) International Publication Number
WO 2024/074685 A1

(51) International Patent Classification:
G01N 33/68 (2006.01)

(21) International Application Number:
PCT/EP2023/077721

(22) International Filing Date:
06 October 2023 (06.10.2023)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
22306513.7 07 October 2022 (07.10.2022) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

(54) Title: USE OF ALBUMIN ISOFORMS PROFILES FOR THE CHARACTERIZATION OF THE ETIOLOGY AND SEVERITY OF LIVER INJURIES

(57) **Abstract:** The inventors hypothesized that each type of liver injury can be revealed by a specific profile of HSA posttranslational modifications. Therefore, the aim of inventors was to study the pattern of albumin isoforms in rats intoxicated with acetaminophen (APAP), ethanol, and CCl_4. The second objective was to explore the potential of these isoforms as biomarkers of liver specific injuries. The results demonstrate that albumin posttranslational modifications (Alb-PTM) occur very early during the course of liver injuries induced by hepatotoxic substances. In 3 animal models, native albumin started to decrease in favor of other isoforms 24 hours after the administration of APAP, ethanol or CCl_4. Interestingly, the nature and the intensity of isoforms were different depending on the hepatotoxic substance. In a cohort of cirrhotic patients, the inventors were able to identify up to 14 albumin isoforms, all of which were also present in control patients. However, the inventors observed that the increase in the HSA-DA isoform was specific to patients with cirrhosis due to alcohol abuse, HSA+SGGS and HSA+2Glyc were increased specifically in NASH patients, and HSA-DA+Cys with HSA+SO2H were increased only in patients with the mixed form. In addition, we did not observe a specific isoform able to clearly discriminate the different stages of liver disease, but principal component analysis of the MS dataset perfectly separated cirrhosis patients with different Child-Pugh scores and control patients. The present invention thus relates to the use of albumin isoforms profiles for the characterization of the etiology and severity of liver injuries.

WO 2024/074685 A1

USE OF ALBUMIN ISOFORMS PROFILES FOR THE CHARACTERIZATION OF THE ETIOLOGY AND SEVERITY OF LIVER INJURIES

FIELD OF THE INVENTION:

The present invention is in the field of medicine, in particular hepatology.

BACKGROUND OF THE INVENTION:

The pathogenesis of liver injuries is complex and involves numerous cellular, molecular, immune and hormonal disturbances (1). This makes the diagnosis of liver diseases a challenging process. The intricacy of these disturbances has led to the examination of a multitude of potential serum biomarkers that reflect underlying disease through cellular pathways, including hepatocellular apoptosis, inflammation or oxidative stress. However, despite the utility of these serum biomarkers (called 'conventional liver biomarkers' thereafter) in the assessment of liver injuries and chronic liver diseases, they have limited usefulness in the early stages of liver injuries (2-4).

The use of process-related biomarkers has evolved to the use of panels of biomarkers in order to reflect a bigger picture of events occurring in the hepatocytes and/or in the organ tissues. Currently, because of their lack of sensitivity and specificity when taken individually, serum markers are now gathered in panel tests, sometimes together with interpretation algorithms (Fib4, FibroMeter, FibroTest, FIBROSpect, Hepascore…) or with imaging tests (FibroMeter-VCTE algorithm) to help diagnosing and staging fibrosis and/or cirrhosis. However, it has been recently argued that these tests "show limited accuracy in individual patients and do not reflect disease progression or treatment response and do not provide a mechanistic understanding of injury patterns"(3). Other biomarkers, such as molecules involved in the fibrosis process including protein-based biomarkers, microRNA or collagens, have been extensively studied. Although some are promising, they have not yet demonstrated the diagnostic or predictive performances required for early dysfunctions or for mid- or long-term liver injuries (5). Therefore, noninvasive (or less invasive), simple, sensitive and specific biomarkers for the early detection of dysfunctions able to lead to liver failure are still needed.

Recently, human serum albumin (HSA) isoforms have gained great interest as biomarkers of advanced liver diseases (6-16). Due to the exclusive synthesis of HSA by liver cells, its great

chemical reactivity and its abundance (60% of all plasma proteins), it is now clear that not only the quantity, but also the quality of HSA may reflect liver dys/functions. HSA may undergo several posttranslational modifications including truncations, acetylation, cysteinylation, homocysteinylation, glutathionylation, glycation, nitrosylation, nitration, phosphorylation and oxidation (17). The clinical relevance of some of these modifications has been recently investigated in advanced liver diseases (15, 17). The oxidation of the Cys34 residue has been the most studied. It was characterized on the basis of the redox state of Cys34 as follows: (i) Human mercaptalbumin (HMA), the reduced HSA (70 - 80% of total HSA in healthy subjects), (ii) Nonmercaptalbumin 1 (HNA1), a reversibly oxidized form (20 – 30%) and (iii) Nonmercaptalbumin 2 (HNA2) the irreversible oxidized form of albumin (< 5%) (17). The increase in HNA1 and HNA2 has been well documented in end-stage liver pathologies, with a progressive increase of these isoforms in severe cirrhosis correlated to a very high short-term mortality (9, 14, 15). Interestingly, it has been reported that HNA1 plays a pejorative role in decompensated cirrhosis (18) while native HSA has a protective role by reducing the proinflammatory environment present in patients with acutely decompensated cirrhosis (19). At present, it is not fully established whether other albumin isoforms are mostly inactive or possess some not yet characterized biological properties. Structural alterations involving sites others than Cys34 were reported. Indeed, N- or C-terminal truncated as well as glycated forms were found in plasma samples from patients with acutely decompensated cirrhosis or severe alcoholic hepatitis (9). Most importantly, these findings suggest that HSA modifications are directly related to liver injuries and that HSA isoforms are very likely produced because of the chemical environment into the hepatocytes.

This bundle of arguments strongly suggest that albumin modifications detected in blood may reflect the dys/function of hepatocytes and represent a versatile tool for the diagnosis and the prognosis of liver injuries and/or diseases.

SUMMARY OF THE INVENTION:

The present invention is defined by the claims. In particular, the present invention relates to the use of albumin isoforms profiles for the characterization of the etiology and severity of liver injuries.

DETAILED DESCRIPTION OF THE INVENTION:

The inventors hypothesized that each type of liver injury can be revealed by a specific profile of HSA posttranslational modifications. Since the cellular chemical environment at the origin of liver injuries is different, this logically should lead to the apparition of specific HSA isoform patterns in blood. Since HSA is continuously synthesized in hepatocytes and secreted into the bloodstream, the second hypothesis is that HSA modifications occur at early stages of cell injuries. Therefore, the aim of inventors was to study the pattern of albumin isoforms in rats intoxicated with acetaminophen (APAP), ethanol, and CCl_4. The second objective was to explore the potential of these isoforms as biomarkers of liver specific injuries. The results demonstrate that albumin posttranslational modifications (Alb-PTM) occur very early during the course of liver injuries induced by hepatotoxic substances. In 3 animal models, native albumin started to decrease in favor of other isoforms 24 hours after the administration of APAP, ethanol or CCl4. Interestingly, the nature and the intensity of isoforms were different depending on the hepatotoxic substance. In a cohort of cirrhotic patients, the inventors were able to identify up to 14 albumin isoforms, all of which were also present in control patients. However, the inventors observed that the increase in the HSA-DA isoform was specific to patients with cirrhosis due to alcohol abuse, HSA+SGGS and HSA+2Glyc were increased specifically in NASH patients, and HSA-DA+Cys with HSA+SO2H were increased only in patients with the mixed form. In addition, we did not observe a specific isoform able to clearly discriminate the different stages of liver disease (data not shown), but principal component analysis of the MS dataset perfectly separated cirrhosis patients with different Child-Pugh scores and control patients.

Main definitions:

As used herein, the term "**subject**" as used herein refers to any mammal organism. The term subject includes, but is not limited to, humans, nonhuman primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs, and the like. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered.

As used herein, the term "**injury**" refers to any damage that directly or indirectly affects the normal functioning. An insult may have a variety of causes including, but not limited to physiological injuries, chemical injuries or physical injuries. The term encompasses acute and

chronic injuries. As used herein, the term "**acute injury**" includes injuries that have recently occurred. For example, an acute injury may have very recently occurred, may have occurred within an hour or less, may have occurred within a day or less, may have occurred within a week or less, or may have occurred within two weeks or less. As used herein, the term "**chronic injury**" is an injury that has persisted for a period of time. For example, a chronic injury may have occurred more than two weeks ago, may have occurred more than three weeks ago, may have occurred more than two months ago, or may have occurred more than three months ago.

As used herein, the term "**liver injury**" refers to a state in which the liver function is decreased relative to a normal state. Hepatic dysfunction is characteristic of liver diseases. A number of acute or chronic pathological conditions leads to liver injury. These include, but are not limited to liver abscess, liver cancer, either primary or metastatic, cirrhosis, such as cirrhosis caused by the alcohol consumption or primary biliary cirrhosis, amebic liver abscess, autoimmune hepatitis, biliary atresia, coccidioidomycosis disseminated, portal hypertension hepatic infections (such as hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, or hepatitis E virus), hemochromatosis, hepatocellular carcinoma, pyogenic liver abscess, Reye's syndrome, sclerosing cholangitis, Wilson's disease, drug induced hepatotoxicity, or fulminant or acute liver failure.

As used herein, the term "**non-alcoholic fatty liver disease**" has its general meaning in the art and is intended to refer to the spectrum of disorders resulting from an accumulation of fat in liver cells in individuals with no history of excessive alcohol consumption. In the mildest form, NAFLD refers to hepatic steatosis. The term NAFLD is also intended to encompass the more severe and advanced form non-alcoholic steatohepatitis (NASH), cirrhosis, hepatocellular carcinoma, and virus- induced (e.g., HIV, hepatitis) fatty liver disease.

As used herein, the term "**NASH**" collectively refers to the state where the liver develops a hepatic disorder (e.g., inflammation, ballooning, fibrosis, cirrhosis, or cancer), or the state where the liver may induce such a pathological condition, and "NASH" is distinguished from "simple steatosis"; i.e., a condition in which fat is simply accumulated in the liver, and which does not progress to another hepatic-disorder-developing condition.

As used herein, the term "**cirrhosis**" refers to a consequence of chronic liver disease characterized by replacement of liver tissue by fibrosis, scar tissue and regenerative nodules

(lumps that occur as a result of a process in which damaged tissue is regenerated), leading to loss of liver function. The term "**alcohol-related cirrhosis**" indicates that cirrhosis is mostly caused by excessive alcohol consumption.

As used herein, the term "**etiology**" refers to the causes or origins, of diseases or abnormal physiological conditions.

As used herein, the term "**severity**" refers to the degree of symptom intensity experienced, ascertained, formally assessed or reported by a symptomatic subject with a liver injury. Typically, the severity correlates with the Child-Pugh score.

As used herein, the term the "**Child–Pugh score**" has its general meaning in the art and refers to the score used to assess the prognosis of chronic liver disease, mainly cirrhosis as described by Child CG, Turcotte JG (1964). "Surgery and portal hypertension". In Child CG (ed.). The liver and portal hypertension. Philadelphia: Saunders. pp. 50–64. Although it was originally used to predict mortality during surgery, it is now used to determine the prognosis, as well as the required strength of treatment and the necessity of liver transplantation. The score employs five clinical measures of liver disease including total bilirubin, serum albumin, prothrombin time prolongation (or INR), ascites and hepatic encephalopathy. Each measure is scored 1–3, with 3 indicating most severe derangement. Chronic liver disease is classified into Child–Pugh class A to C, as depicted in Table A.

Points	Class	One-year survival	Two-year survival
5–6	A	100%	85%
7–9	B	80%	60%
10–15	C	45%	35%

Table A: Child–Pugh score and significance.

As used herein, the term "**albumin**" has its general meaning in the art and refers to a globular protein that in humans is encoded by the *ALB* gene. Serum albumin is the most abundant plasma protein in mammals. Serum albumin is essential for maintaining the oncotic pressure needed for proper distribution of body fluids between intravascular compartments and body tissues. It also acts as a plasma carrier by non-specifically binding several hydrophobic steroid hormones

and as a transport protein for hemin and fatty acids. Furthermore, serum albumin has a very long half-life of about 19 days, and its metabolism is well-known. Albumin has also been widely used as a protein stabilizer in commercial pharmaceuticals (Sangastino et al. (2012), Blood, 120(12) 2405-2411). An exemplary amino acid sequence for human serum albumin (HSA) is represented by SEQ D NO:1 (UniProtKB/Swiss-Prot primary accession number P02768).

SEQ ID NO:1 >sp|P02768|ALBU_HUMAN Serum albumin OS=Homo sapiens OX=9606 GN=ALB PE=1 SV=2
MKWVTFISLLFLFSSAYSRGVFRRDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPF
EDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEP
ERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLF
FAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAV
ARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLK
ECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYAR
RHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFE
QLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVV
LNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTL
SEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLV
AASQAALGL

As used herein, the term "**isoform**" has its general meaning in the art and refers to the multiple molecular forms of a given protein, and includes proteins differing at the level of (1) primary structure (such as due to alternate RNA splicing, or polymorphisms); (2) secondary structure (such as due to different co- or posttranslational modifications); and/or (3) tertiary or quaternary structure (such as due to different sub-unit interactions, homo- or hetero- oligomeric multimerization). According to the present, invention the term "**isoform**" preferably refers to the multiple molecular forms of a given protein, and includes proteins secondary structure due to different co- or post translational modifications. Said post translational modifications include cysteinylation, homocysteinylation, glutathionylation, glycation, nitrosylation, nitration, oxidation and carbonylation.

As used herein, the term "**native albumin**" refers to the form of albumin that was not subjected to a modification, more particularly a post translational modification.

As used herein, the term "**Alb-DA**" refers to an albumin isoform characterized by a truncation of the N-terminus end.

As used herein, the term "**Alb-DA+Cys**" refers to an albumin isoform characterized by a truncation of the N-terminus end and a cysteinylation of the Cys34.

As used herein, the term "**Alb+SO2H**" refers to an albumin isoform characterized a deoxidation.

As used herein, the term "**Alb+SO3H**" or "**Alb-CysO3**" refers to an albumin isoform characterized by a trioxidation.

As used herein, the term "**Alb+Cys-DHA**" refers an albumin isoform characterized by a cysteinylation of the Cys34 and a transformation of a free cysteine to a dehydroalanine.

As used herein, the term "**Alb+Cys+SNO**" refers to an albumin isoform characterized by a cysteinylation of the Cys34 and a nitrosylation.

As used herein, the term "**Alb+Glyc**" refers to an albumin isoform characterized by a glycation/

As used herein, the term "**Alb+SO2H+Glyc**" refers to an albumin isoform characterized by a dioxidation and a glycation.

As used herein, the term "**Alb+SO3H+Glyc**" refers to an albumin isoform characterized by a trioxidation and a glycation.

As used herein, the term "**Alb+Cys+Glyc-DHA**" refer to an albumin isoform characterized by a cysteinylation of the Cys34, a glycation and a transformation of a free cysteine to a dehydroalanine.

As used herein, the term "**Alb+Cys+Glyc**" refers to an albumin isoform characterized by a cysteinylation of the Cys34 and a glycation.

As used herein, the term "**Alb-SGGS**" refers to an albumin isoform characterized by a glutathionylation.

As used herein, the term "**Alb+2Glyc**" refers to an albumin isoform characterized by two glycations.

As used herein, the term "**Alb+SO3H+2Glyc**" refers to an albumin isoform characterized by a trioxidation and two glycations.

As used herein, the term "**Alb+Cys+2Glyc**" refers to an albumin isoform characterized by a cysteinylation of the Cys34 and two glycations.

As used herein, the term "**profile**" means a pattern and relates to the magnitude and direction of change of a number of features. The profile may be interpreted stringently, i.e., where the variation in the magnitude and/or number of features within the profile displaying the characteristic is substantially similar to a reference profile or it may be interpreted less stringently, for example, by requiring a trend rather than an absolute match of all or a subset of feature characteristics.

As used herein, the term "**mass spectrometry**" or "**MS**" refers to an analytical technique to identify compounds by their mass. MS refers to methods of filtering, detecting, and measuring ions based on their m/z. MS technology generally includes (1) ionizing the compounds to form charged species (e.g., ions); and (2) detecting the molecular weight of the ions and calculating their m/z. The compounds may be ionized and detected by any suitable means. A "**mass spectrometer**" generally includes an ionizer and an ion detector. In general, one or more molecules of interest are ionized, and the ions are subsequently introduced into a mass spectrometric instrument where, due to a combination of magnetic and electric fields, the ions follow a path in space that is dependent upon mass ("m") and charge ("z"). See, e.g., U.S. Pat. No. 6,204,500, entitled "Mass Spectrometry From Surfaces;" U.S. Pat. No. 6,107,623, entitled "Methods and Apparatus for Tandem Mass Spectrometry;" U.S. Pat. No. 6,268,144, entitled "DNA Diagnostics Based On Mass Spectrometry;" U.S. Pat. No. 6,124,137, entitled "Surface-Enhanced Photolabile Attachment And Release For Desorption And Detection Of Analytes;" Wright et al., Prostate Cancer and Prostatic Diseases 2:264-76 (1999); and Merchant and Weinberger, Electrophoresis 21:1164-67 (2000).

As used herein, the term "**blood sample**" means a whole blood, serum, or plasma sample obtained from the patient or the animal.

Methods of the present invention:

The first object of the present invention relates to a method of determining the etiology and severity of a liver injury in a subject comprising determining the profile of albumin isoforms in a blood sample obtained from the subject wherein the profile indicates the etiology and severity of the liver injury.

More particularly, the method of the present invention comprises the step of detecting a plurality of albumin isoforms. Even, more particularly, the method of the present invention comprises the step of detecting a plurality of albumin isoforms selected from **Table 4**. In some embodiments, the method of the present invention comprises the step of detecting a plurality of isoforms selected from the group consisting of Alb+SO2H, HSA-CysO3, Alb+Cys-DHA, Alb+Cys, Alb+Cys+SNO, Alb+SO2H+Glyc, Alb+SO3H+Glyc, HAS-SGGS, Alb+2Glyc, Alb+SO3H+2Glyc, and Alb+Cys+2Glyc.

In some embodiments, the method of the present invention comprises the steps of i) determining the profile of albumin isoforms in the blood sample obtained from the patient, and ii) comparing the profile to one or more reference profiles associated with various liver injuries.

The method of the present invention is particularly suitable for detecting any kind of liver injury. In some embodiments, the method of the present invention is particularly suitable for detecting chemical liver injuries. In some embodiments, the method of the present invention is particularly suitable for detecting physical liver injuries. In some embodiments, the method of the present invention is particularly suitable for detecting ischemic liver injuries.

The method of the present invention is particularly suitable for the early detection of a liver injury, i.e. the detection of a liver injury before the observation of a symptom.

The method of the present invention is particularly suitable for the early detection of early graft dysfunction or non-function in liver transplanted patients.

In particular, the method of the present invention is particularly suitable for detecting a chemical liver injury induced by a toxicant selected from the group consisting of alcohol, 2,2′ ,4,4′ ,5,5′ -hexachlorobiphenyl (PCB-153), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 2-bromoethylamine (BEA), 3-methylcholanthrene, 4-aminophenol (PAP), acetaminophen (APAP), adriamycin, allyl alcohol, amiodarone, amphotericin B, Aroclor 1254,

Aroclor 1260, arsenic, aspirin, astemizole, benzene, cadmium, carbamezipine, carbon tetrachloride (CCl4), ciprofibrate (cipro), clofibrate, cobalt chloride, corvastatin, cyclosporin A, diethylnitrosamine, dimethylformamide, dimethylhydrazine (DMH), diquat, ethosuximide, etoposide, famotidine, fluconazole, gemfibrozil, ganciclovir, hexachloro-1,3-butadiene (HCBD), HIV protease inhibitors, hydrazine, indomethacin, ketoconazole, lead acetate (PbAc), lipopolysaccharide (LPS), mercury(II) chloride ($HgCl_2$), methanol, methapyrilene, methotrexate, metronidazole, miconazole, monocrotaline, nitric oxide, ondansetron, pentamidine, phenobarbital, phenylhydrazine (phenylhyrzn), phenytoin, pravastatin, propulsid, puromycin aminonucleoside (PAN), quinolones, simvastatin, sodium fluoride (NaF), statins, thioacetamide, tocainidine, tricyclic antidepressants, troglitazone, tumor necrosis factor α (TNFα), uranyl nitrate, valproic acid, vincristine, Wy-16,463, zidovudine (AZT), α-naphthyl isothiocyanate (ANIT), β-naphthoflavone (BNF), asbestos, radon, cigarette smoke, glues, dioxin, nickel, arsenic, mercury, cement (chromium), polychlorinated biphenyls (PCBs), carbon tetrachloride, methylene chloride, vinyl chloride, mercury, chlorinated hydrocarbon solvents, carbon disulfide, cadmium, ozone, tobacco smoke, nitrates, methylene chloride, ethylene dibromide, and polychlorinated biphenyls.

The method of the present invention is particularly suitable for detecting a liver injury selected from the group consisting of liver abscess, liver cancer, either primary or metastatic, cirrhosis, such as cirrhosis caused by the alcohol consumption or primary biliary cirrhosis, amebic liver abscess, autoimmune hepatitis, biliary atresia, coccidioidomycosis disseminated, portal hypertension hepatic infections (such as hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, or hepatitis E virus), hemochromatosis, hepatocellular carcinoma, pyogenic liver abscess, Reye's syndrome, sclerosing cholangitis, Wilson's disease, drug induced hepatotoxicity, or fulminant or acute liver failure.

The method of the present invention is particularly suitable for detecting a non-alcoholic fatty liver disease, and in particular for detecting NASH.

The method of the present invention is particularly suitable for detecting liver fibrosis. The method of the present invention is particularly suitable for detecting cirrhosis.

In some embodiments, the method of diagnosing described herein is applied to a subject who presents symptoms of liver injury without having undergone the routine screening to rule out

all possible causes for liver injury. The methods described herein can be part of the routine set of tests performed on a subject who presents symptoms of liver injury such as jaundice, abdominal pain and swelling, swelling in the legs and ankles, itchy skin, dark urine color, pale stool color, bloody color stool, tar-colored stool, chronic fatigue, nausea or vomiting, loss of appetite, tendency to bruise easily… The method of the present invention can be carried out in addition of other diagnostic tools that include ultrasound evaluation (e.g. elastography), biopsy and/or quantification of at least one further biomarkers such as levels of blood AST, ALT, ALP, TTT, ZTT, total bilirubin, total protein, albumin, lactate dehydrogenase, choline esterase and the like.

A further object to the present invention relates to a method of predicting the worsening of a liver injury comprising the steps of determining the evolution of the profile of albumin isoforms in the blood sample obtained from the patient wherein said evolution predicts the worsening of the liver injury.

A further object of the present invention relates to a method of predicting an early allograft liver dysfunction or liver non-function in a liver-transplanted patient comprising the steps of determining the evolution of the profile of albumin isoforms in the blood sample obtained from the patient wherein said evolution predicts the early allograft dysfunction or non-function.

In some embodiments, the isoforms are detected by mass spectrometry. In some embodiments, the isoform is identified visually by its mass shift with respect to native albumin and by taking the maximum intensity in a mass interval of 10 Da around the observed peak of each isoform. Table 4 indicates the different mass shifts that are associated with Alb+SO2H, HSA-CysO3, Alb+Cys-DHA, Alb+Cys, Alb+Cys+SNO, Alb+SO2H+Glyc, Alb+SO3H+Glyc, HSA-SGGS, Alb+2Glyc, Alb+SO3H+2Glyc, and Alb+Cys+2Glyc.

Mass spectrometry is performed using a mass spectrometer, which includes an ion source for ionizing the fractionated sample and creating charged molecules for further analysis. Ionization sources used in various MS techniques include, but are not limited to, electron ionization, chemical ionization, electrospray ionization (ESI), photon ionization, atmospheric pressure chemical ionization (APCI), photoionization, atmospheric pressure photoionization (APPI), fast atom bombardment (FAB)/liquid secondary ionization (LSIMS), matrix assisted laser desorption ionization (MALDI), field ionization, field desorption, thermospray/plasmaspray

ionization, surface enhanced laser desorption ionization (SELDI), inductively coupled plasma (ICP) and particle beam ionization. The skilled artisan will understand that the choice of ionization method may be determined based on the analyte to be measured, type of sample, the type of detector, the choice of positive versus negative mode, etc. After the sample has been ionized, the positively charged ions thereby created may be analyzed to determine m/z. Suitable analyzers for determining m/z include quadrupole analyzers, ion trap analyzers, and time-of-flight analyzers. The ions may be detected using one of several detection modes. For example, only selected ions may be detected using a selective ion monitoring mode (SIM), or alternatively, multiple ions may be detected using a scanning mode, e.g., multiple reaction monitoring (MRM) or selected reaction monitoring (SRM). One may enhance the resolution of the MS technique by employing "tandem mass spectrometry," or "MS/MS." In this technique, a precursor ion (also called a parent ion) generated from a molecule of interest can be filtered in an MS instrument, and the precursor ion subsequently fragmented to yield one or more fragment ions (also called daughter ions or product ions) that are then analyzed in a second MS procedure. By careful selection of precursor ions, only ions produced by certain analytes are passed to the fragmentation chamber, where collision with atoms of an inert gas produce the fragment ions. Because both the precursor and fragment ions are produced in a reproducible fashion under a given set of ionization/fragmentation conditions, the MS/MS technique may provide an extremely powerful analytical tool. For example, the combination of filtration/fragmentation may be used to eliminate interfering substances, and may be particularly useful in complex samples, such as biological samples. Additionally, recent advances in technology, such as matrix-assisted laser desorption ionization coupled with time-of-flight analyzers ("MALDI-TOF") permit the analysis of analytes at femtomole levels in very short ion pulses. Mass spectrometers that combine time-of-flight analyzers with tandem MS are also well known to the artisan. Additionally, multiple mass spectrometry steps may be combined in methods known as "MS/MS". Various other combinations may be employed, such as MS/MS/TOF, MALDI/MS/MS/TOF, or SELDI/MS/MS/TOF mass spectrometry.

Typically the blood samples are processed to obtain preparations that are suitable for analysis by mass spectrometry. Such purification will usually include chromatography, such as liquid chromatography or capillary electrophoresis, and may also often involve an additional purification procedure that is performed prior to chromatography. Various procedures may be used for this purpose depending on the type of sample or the type of chromatography. Examples include filtration, centrifugation, combinations thereof and the like. The pH of the serum sample

may then be adjusted. The sample may be purified with a filtration. The filtrate from this filtration can then be purified by liquid chromatography and subsequently subjected to mass spectrometry analysis. Various methods have been described involving the use of high performance liquid chromatography (HPLC) for sample clean-up prior to mass spectrometry analysis. See, e.g., Taylor et al., Therapeutic Drug Monitoring 22:608-12 (2000) (manual precipitation of blood samples, followed by manual C18 solid phase extraction, injection into an HPLC for chromatography on a C18 analytical column, and MS/MS analysis); and Salm et al., Clin. Therapeutics 22 Supl. B:B71-B85 (2000). Commercially available HPLC columns include, but are not limited to, polar, ion exchange (both cation and anion), hydrophobic interaction, phenyl, C-2, C-8, C-18, and polar coating on porous polymer columns. During chromatography, the separation of materials is effected by variables such as choice of eluent (also known as a "mobile phase"), choice of gradient elution and the gradient conditions, temperature, etc.

One or more steps of the methods may be performed using automated machines. In some embodiments, one or more purification steps are performed on-line, and more preferably all of the LC purification and mass spectrometry steps may be performed in an on-line fashion.

In some embodiments, the method of the invention comprises the use of an algorithm. More particularly, the method of the present invention is a computer-implemented method comprising applying, on a set of values for each detected isoforms relative to the subject, a trained model configured to determine the etiology and severity of the liver injury based on the set of values. Typically, the set of values comprises the relative abundance of the different detected isoforms. In some embodiments, the model is preliminary trained by supervised learning on a training dataset comprising, for a plurality of individuals of a population, the etiologies and the severities of a liver injury. In some embodiments, the method of the invention thus comprises the use of a classification algorithm typically selected from Linear Discriminant Analysis (LDA), Topological Data Analysis (TDA), Neural Networks, Support Vector Machine (SVM) algorithm and Random Forests algorithm (RF). As used herein, the term "**classification algorithm**" has its general meaning in the art and refers to classification and regression tree methods and multivariate classification well known in the art such as described in US 8,126,690; WO2008/156617. Thus, in some embodiments, the method of the present invention comprises a) detecting the plurality of albumin isoforms; b) implementing a classification algorithm on data relative to the detected isoforms so as to obtain an algorithm output; c)

determining the liver injury and severity. The algorithm of the present invention can be performed by one or more programmable processors executing one or more computer programs to perform functions by operating on input data and generating output. The algorithm can also be performed by, and apparatus can also be implemented as, special purpose logic circuitry, e.g., an FPGA (field programmable gate array) or an ASIC (application-specific integrated circuit). Processors suitable for the execution of a computer program include, by way of example, both general and special purpose microprocessors, and any one or more processors of any kind of digital computer. Generally, a processor will receive instructions and data from a read-only memory or a random access memory or both. The essential elements of a computer are a processor for performing instructions and one or more memory devices for storing instructions and data. Generally, a computer will also include, or be operatively coupled to receive data from or transfer data to, or both, one or more mass storage devices for storing data, e.g., magnetic, magneto-optical disks, or optical disks. In some embodiments, the algorithm can be implemented in a computing system that includes a back-end component, e.g., as a data server, or that includes a middleware component, e.g., an application server, or that includes a front-end component, e.g., a client computer having a graphical user interface or a Web browser through which a user can interact with an implementation of the invention, or any combination of one or more such back-end, middleware, or front-end components.

The result given by the methods of the invention may be used as a guide in selecting a therapy or treatment regimen for the subject. Typically, the patient can be then eligible for intensive surveillance (e.g., referral to tertiary care centers; intensive control of risk factors), for a selected therapy or transplantation and for inclusion in clinical trials testing new drugs. According to the present invention, the treatment consists in any method or drug that could be suitable for the treatment of a liver injury. Some liver problems can be treated with lifestyle modifications, such as stopping alcohol use or losing weight, typically as part of a medical program that includes careful monitoring of liver function. Each liver disease will have its own specific treatment regimen. For example, hepatitis A requires supportive care to maintain hydration while the body's immune system fights and resolves the infection. Patients with gallstones may require surgery to remove the gallbladder. Other diseases may need long-term medical care to control and minimize the consequences of their disease. In patients with cirrhosis and end-stage liver disease, medications may be required to control the amount of protein absorbed in the diet. Other examples include operations required to treat portal hypertension. The patient can also

be eligible for administration of corticosteroids, pentoxifylline, or N-acetylcysteine; antiapoptotics, or vasoactive drugs) or even for liver transplantation.

The method of the present invention can also be useful for monitoring the subject. In some embodiments, the method of the present invention is also particularly suitable for determining whether a subject suffering from a liver injury achieves a response to a therapy. The method is thus particularly suitable for discriminating responder from non-responder. As used herein the term "**responder**" in the context of the present disclosure refers to a subject that will achieve a response, i.e. a subject who is under remission and more particularly a subject who does not suffer from liver injury. A "**non-responder**" subject includes subjects for whom the disease does not show reduction or improvement after the treatment (e.g. the liver injury remains stable or decreases). For instance, the nature and the abundance of the different isoforms can indeed be monitored during the treatment of the subject and thus can indicate whether the subject achieves a response to the therapy.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1. Evolution of albumin isoforms in the different groups of rats exposed to ethanol for different time durations. Native albumin is expressed as percent of the sum of all detected isoforms. Other isoforms are expressed in relative abundance to that of native albumin.

Figure 2. Evolution of albumin isoforms in the different groups of rats exposed to CCl_4 for different time durations. Native albumin is expressed as percent of the sum of all detected isoforms. Other isoforms are expressed in relative abundances of the native albumin.

Figure 3. Evolution of albumin isoforms in the different groups of rats exposed to APAP at different doses and for different durations. Native albumin is expressed as percent of the sum of all detected isoforms. Other isoforms are expressed in relative abundances of the native albumin.

Figure 4: A. median albumin profiles of rats intoxicated with CCl_4; **B.** median albumin profiles of rats intoxicated with Ethanol, **C.** median profiles of rats intoxicated with APAP. The different lines represent the control group for each model; the profiles of groups in which biochemistry tests started to be altered and the groups where albumin profiles were the most disturbed.

Figure 5. PCA-DA plot of the 3 animal models. Each dot represents the whole information of the albumin isoform profile for one rat. The different dots represent rats exposed to ethanol, rats exposed to APAP and are rats exposed to CCl_4.

Figure 6. Histograms of albumin isoforms in cirrhotic patients.

Figure 7. A. PCA-DA plot of the 4 groups of patients. Each dot represents the whole information of the albumin profile for one patient. Square dots are control patients, orange dots represent patients with ALD, circle dots are NASH patients and triangle dots patients with cirrhosis of mixed origin. **B.** PCA-DA plot of 3 groups of patients: Controls, NASH and mixed origin.

Figure 8. A. PCA-DA plot of the 4 groups of patients. Each dot represents the whole information of the albumin profile for one patient. Square dots are control patients, circle dots represent stage A, circles dots stage B, triangle dots represent stage C cirrhosis patients. **B.** PCA-DA plot of 3 groups of patients: stage A, B and C.

Figure 9.PLS-DA plot of 2 groups of patients. EAD+ are patients in whom early allograft dysfunction has been diagnosed based on the LGrAFT10 or the EAD scores. EAD- are patients with no graft dysfunction detected. Each dot represents the whole information of the albumin profile for one patient at V1 (24 hours before transplantation) subtracted from the whole information of the albumin profile at V6 (7 days after transplantation).

Figure 10. PLS-DA plot of 2 groups of patients. EAD+ are patients in whom early allograft dysfunction has been diagnosed based on the LGrAFT10 or the EAD scores. EAD- are patients with no graft dysfunction detected. Each dot represents the whole information of the albumin profile for one patient at V1 (24 hours before transplantation) subtracted from the whole information of the albumin profile at V3 (72 hours after transplantation).

EXAMPLE:

Methods

Materials and methods

1. Animals

All of the experimental procedures were performed in male, adult (6 weeks old) albino Wistar rats (Janvier Labs, France) weighing an average of 250 grams and maintained under normal temperature (21 ° C) and humidity and a 12-hour light-dark cycle with unrestricted access to food and water. Rats were acclimatized to the conditions of our animal facility for 1 week before the start of the investigations.

All animal experiments had been approved by the Ethics Committee for experiments at the University of Limoges and by the French Ministry of National Education, Higher Education and Research (APAFiS # 20354-2019042414581742 v1).

2. Experimental procedure

2.1. Model of ethanol (EtOH) hepatotoxicity:

In the EtOH model, hepatotoxicity was induced by oral administration (gavage) of 2 mL of a 50% EtOH solution prepared in physiological saline (0.9% NaCl) to evaluate the time-dependent changes in biochemical markers and histological liver injuries. Six different groups of 6 to 9 rats were followed for 1, 3, 7, 10, or 14 days, respectively. All rats received the daily dose of EtOH. The animals were sacrificed 24 hours after their last intake of EtOH. "Control" rats were followed throughout the duration of the protocol, ie 14 days, and received by gavage only physiological serum (0.9% NaCl).

2.2. Model of carbon tetrachloride (CCl_4) hepatotoxicity:

In the CCl_4 model, hepatotoxicity was induced by oral administration (gavage) of 1 mL / kg body weight of a 30% solution of CCl_4 diluted in olive oil. Five groups of rats were followed for 1, 3, 7, 10 days after daily administration of CCl_4, respectively. The animals were sacrificed 24 hours after their last intake of CCl_4. "Control" rats were followed for 10 days and received by gavage only olive oil.

2.3. Model of paracetamol (APAP) hepatotoxicity:

In the APAP model, rats were randomly divided into eleven groups, each group containing six rats. Depending on the group, the animals received APAP suspensions prepared in 1% carboxymethylcellulose at doses D1 = 1 g/kg, D2= 2g/kg, D3= 3 g/kg or D4= 4g/kg. APAP was administrated for either 1 day or 3 days. "Control" rats were followed for 3 days and received by gavage only a single dose of 1% carboxymethylcellulose. The animals were sacrificed 24h after the last dose of APAP.

During the experimental period, body weight was recorded daily until sacrifice. For each experimental model, the animals were sacrificed by intraperitoneal injection of pentobarbital (150 mg/kg).

Blood samples were collected in Vacutainer® lithium heparin tube for trace elements (Beckton Dickinson, France) and then centrifuged at 3000 rpm for 10 minutes. The plasma samples were then stored at -80 ° C until analysis. Rat livers were quickly removed and fixed in formalin for histological analysis.

3. Analytical procedures

3.1. Biochemistry analyzes

From the collected plasma, measurements of classic biochemistry parameters such as albumin (ALB), total (BILIT) and conjugated (BILID) bilirubin, aspartate aminotransferases (ASAT), alanine aminotransferases (ALAT) and alkaline phosphatases (PAL) were evaluated in the Biochemistry and Molecular Genetics laboratory of Limoges University Hospital using a COBAS® 8000 automaton (Roche, Germany).

3.2. Pathological analysis of the liver

At the end of each sacrifice, the livers were cut into sections of 1 to 1.5 cm perpendicular to the major axis to allow homogeneous fixation in a 4% formalin solution for a maximum of 7 days. Samples were stained for light microscopy with hematoxylin and eosin staining and Masson's trichrome stain. The pathologist performed histological analysis blindly, with no knowledge of the different experimental groups.

3.3. Proteomic analysis of the plasma samples (identification-quantification of the different isoforms of albumin)

Standards and reagents

Organic solvents and reagents were of analytical grade. Acetonitrile was obtained from Merck (Molsheim, France), ammonium formate and formic acid from Sigma (Saint-Quentin-Fallavier, France). Deionized water was prepared on a Direct-Q laboratory plant (Millipore, Molsheim, France).

Sample preparation

A volume of 20 µL of plasma was diluted with 980 µL of an aqueous 20mM ammonium formate solution with 0.1 % formic acid, and then vortex-mixed. The mixture was centrifuged at 10°C and 14 000 g, and 300 µl of the supernatant was then filtered on a 0.22 µm cellulose acetate filter before injection.

Liquid chromatography - mass spectrometry

Chromatographic separation was performed using a Nexera LC40 system (Shimadzu, Noisiel, France) equipped with a thermostated column compartment and a thermostated autosampler with a six-port switching valve. Samples were analyzed without chromatographic separation under isocratic conditions using a 2mM aqueous ammonium formate solution containing 0.1% formic acid as mobile phase A and a mixture of acetonitrile/mobile phase A (90:10, by volume) as mobile phase B, programmed as follows: 0–3 min, 50% B.

Mass spectrometric detection was performed using a Q-TOF mass spectrometer (TripleTOF® 5600+, Sciex, Concord, Canada) equipped with a DuoSpray ion source and operated in the positive ionization mode. A beta-galactosidase solution was used for internal calibration. The source conditions were as follows: temperature, 200 °C; declustering potential (DP), 250 eV; curtain gas (CUR), 40 units; ion source gas (GS1, GS2), respectively 70 and 10 units; and ion-spray voltage floating, 5.5 kV. All MS parameters were controlled by Analyst® TF 1.7 (Sciex). Data were processed with PeakView® 2.2 software (Sciex). m/z ratios were scanned using a TOF MS scan from m/z 900 and 1800 with an accumulation time of 500 ms.

Spectra deconvolution

The LC-MS data were processed using PeakView® 2.2 software and its Bio Tool Kit 2.2.0 feature (Sciex). The input MS spectra selected from 1300 to 1600 was then deconvolved using a low resolution (5000) from m/z 1000 to 200000. Deconvolution spectra (or profiles) are expressed as intensity versus mass in Da.

Estimation of the relative abundance of isoforms.

The relative abundance of each isoform was determined after calculating the ratio between each centroided peak and the total area represented by the sum of the centroid peaks between 65500 and 67000 for rat serum albumin or 66000 to 67500 for human serum albumin.

Identification and quantification (intensity and relative abundance) of isoforms

Each isoform was identified visually by its mass shift with respect to native albumin and by taking the maximum intensity in a mass interval of 10 Da around the observed peak of each isoform.

The relative proportion of the intensity of each isoform was calculated by dividing the intensity or area obtained from its deconvoluted spectrum by the summed intensity of all isoforms (between 65500 and 67000) and multiplying it by 100. Data were gathered in an Excel file then analyzed using GraphPad® for the potential isoforms.

Relative abundance was calculated as the ratio between the maximum intensity of each identified isoform compared that of the native isoform.

4. Patients and samples

Patient plasma samples were all leftovers after biochemistry laboratory tests prescribed according to the standard of care. In accordance with French regulations and Good Clinical Practice for biomedical studies, patients were informed of, and were able to oppose to, the use of the leftovers of their blood samples at any time (CSP article L1211-2).

The cohort was composed of cirrhotic patients and of patients with no liver dysfunction as controls. Patients were considered as free from liver dysfunction on the basis of their clinical diagnosis and their liver function biochemical tests, namely, aspartate transaminase, alanine transaminase, alkaline phosphatase, ɤ-glutamyltransferase, free and total bilirubin, and albumin levels.

Cirrhotic patients were included based on an hepatologist's diagnosis, their liver function biochemical tests and their Child-Pugh scores.

Patient plasma samples were prepared as previously described and albumin isoforms were analyzed and identified as previously described for rat plasma samples.

5. Statistical analyses

The results of the biochemical analyses are presented as the median [min-max].
Statistical probabilities of $p < 0.01$ (*) and $p < 0.05$ (**) were considered significant. All analyses and figures were performed using GraphPad Prism 7.0a software (GraphPad Software, San Diego, USA).

Results

Precocity of albumin modifications

SEB test in acute models (EtOH and CCl_4):
In rats exposed to EtOH and CCl_4, hepatic cytolysis was observed starting at D7. In the EtOH model, it was further increased, together with a significant increase in ALT levels, on D10 and D14. In this model, a significant increase in AST level was observed at D7, D10 and D14 (**Table 1**). In the CCl_4 model, a significant increase in AST and ALT levels at all time points and a significant increase in bilirubin (BILIT & BILID) at D7 and D10 were detected (**Table 2**), suggesting hepatic cytolysis and cholestasis. Histologically, only minor inflammation was detected in some of the rats receiving ethanol. Rats exposed to CCl_4 showed steatosis starting at D3 and only 2 rats had a fibrotic liver tissue (1 in the D3 group and 1 in the D10 group).

SEB test and albumin isoforms in the acute hepatotoxicity models:
Most ligands of the SEB test increased starting D1 in both models except for Cd that increased only in EtOH model at D14 (**Figure 1**). A normalization of Au, L-Thyroxine was observed at D7 in both models followed by secondary increase at D10 or D14 for Au and dansylsarcosine.

In the EtOH model, In addition to the native albumin with a mass of 65870, 12 potential isoforms were identified, namely Alb+SO_2H, Alb-$CysO_3$, Alb+Cys-DHA, Alb+Cys, Alb+Cys+SNO, Alb+Glyc, Alb+SO_2H+Glyc, Alb+SO_3H+Glyc, Alb+Cys+Glyc+DHA, Alb+Cys+Glyc, Alb+2Glyc and, Alb+Cys+2Glyc. All isoforms except Alb+$CysO_3$ and Alb+Cys+Glyc were also detected in the CCl_4 model, in addition to another isoform, Alb+SO_3H+2Glyc (isoforms and corresponding mass shifts are listed in **Table 4**).

In both models, native albumin decreased rapidly after the administration of the hepatotoxic compound, as soon as D1 for some. It increased again at D3 in the CCl_4 model and at D7 in the

EtOH model then dropped again. These modifications were paralleled by the increase of several isoforms as shown in **Figures 1 and 2**.

Gradual acute model (APAP):

In rats acutely intoxicated with APAP for 2 (D2), 3 (D3) or 4 (D4) days (with doses of 2, 3 and 4g/kg per day, respectively), classical biochemistry markers were not different from those in the control groups (**Table 3**) except a transient increase of AST in the D2g-D1 group due to an outlier and a slight but significant decrease of total albumin. Histological examination of liver tissues revealed inflammation in some rats, mostly those sacrificed 72h after receiving 4g/kg of APAP (D4g-D3). Necrosis was observed only in the group D4g-D1 (5 rats out of 6) but was not visible in the group D4g-D3.

Albumin isoforms analysis of APAP exposed rats:

In addition to native albumin, we have identified 11 isoforms in the serum of rats exposed to APAP, namely, Alb+SO_2H, HSA-$CysO_3$, Alb+Cys-DHA, Alb+Cys, Alb+Cys+SNO, Alb+SO_2H+Glyc, Alb+SO_3H+Glyc, HSA+SGGS, Alb+2Glyc, Alb+SO_3H+2Glyc, Alb+Cys+2Glyc. All the isoforms were also present in the control group. However, native albumin decreased dramatically in all groups in respect to control group, particularly in the groups sacrificed 3 days after APAP initiation, namely, groups D2gD3, D3gD3 and D4gD3 (**Figure 3**). Albumin in all rats was intensely modified. The percentage of native albumin gradually dropped from 12% in the control group to less than 1% in the groups sacrificed at D3. In parallel, several isoforms increased dramatically, up to 10^3-fold, in some groups (**Figure 3**). This is principally due to the huge decrease of native albumin in this model. For example, Alb+Cys intensities were 2-fold higher than native Alb in the control group; after 3 days of daily APAP administration, this isoform increased to more than 200-times the concentration of native Alb.

Albumin isoform profiles as a signature of the nature and the intensity of liver injuries

Animal experiments:

As shown in the previous paragraphs, the decrease of native albumin concentration was associated with the increase of certain isoforms, varying with the toxicant (**Table 4**) and resulting in profiles of albumin isoforms that were visually different **(Figure 4**).

Therefore, we then considered the whole MS profile for each model. A supervised principal component analysis (PCA-DA) revealed a clear clustering of the 3 different groups (**Figure 5**).

Albumin isoform profiles in cirrhotic patients:

Albumin isoforms were characterized for 38 cirrhotic patients and 52 control patients (with no liver injuries). Among the cirrhotic patients, 18 were diagnosed with alcoholic liver disease (ALD), 5 had a nonalcoholic steatosis (NASH) and 16 were diagnosed with a mixed origin of ALD and NASH. The isoforms detected are depicted in **Figure 6** where we can observe a decrease of native albumin in all cirrhotic patients. This decrease was the most important in NASH patients. Generally, the increase of the different isoforms was not homogenous among the three groups of patients (**Table 5**). HSA-DA+Cys and HSA+SO_2H were only significantly increased in patients with cirrhosis of mixed origin. HSA+SGGS and HSA+2Glyc were increased only in NASH patients. When considering the whole profile for each patient, PCA-DA showed a clear clustering between the 4 groups, namely, control patients, NASH patients, ALD patients and the mixed-cirrhosis patients (**Figure 7**).

Among the recruited patients, 11 were diagnosed with stage A (calculated on the basis of Child-Pugh score), 12 stage B and 14 stage C cirrhosis. Interestingly, PCA-DA showed a clear clustering of the different stages of cirrhosis and the control patients (**Figure 8**).

Prediction of early allograft dysfunction by the evolution of the profiles of albumin isoforms:

Albumin isoforms were characterized in 38 liver-transplanted patients at different times: V1 (24h before the transplantation), V2 (during the transplantation), V3 (24h after the transplantation), V4 (48h after the transplantation), V5 (72h after the transplantation), V6 (7 days after the transplantation). Among the 38 patients, 3 experienced an early allograft dysfunction, diagnosed clinically and objectivized by the LGrAFT10 or the EAD scores. Although, albumin isoforms profiles, when taken individually at each time, were not able to discriminate patients with an early allograft dysfunction, the subtraction of the profiles obtained at V6 –V1 or V6-V3 allowed a clear clustering of these patients as shown in figure 9 and figure 10 respectively.

Discussion:

Precocity of albumin modifications:

Our results demonstrate that albumin posttranslational modifications (Alb-PTM) occur very early during the course of liver injuries induced by hepatotoxic substances. In 3 animal models, native albumin started to decrease in favor of other isoforms 24 hours after the administration of APAP, ethanol or CCl_4. Interestingly, the nature and the intensity of isoforms were different depending on the hepatotoxic substance.

Concerning ethanol, it was already known that the daily dose chosen (50% w/v, a very high daily dose) induces inflammation, focal necrosis and finally perivenular fibrosis, within 4 to 29 weeks- depending on the animal model and the experimental protocol (20, 21). In our experimental conditions, inflammation was detected in a few rats (2 out of 7) 24 h after the first administration of ethanol and no more than 33% of rats had inflammation after 2 weeks of daily administration. However, AST and ALT started to increase significantly at D10. The pathogenesis of alcoholic liver disease (ALD) remains poorly understood but is likely a multihit pathophysiological process (22). Ethanol is mostly metabolized into acetaldehyde by ADH, and to a lesser extent, by CYP2E1. A tertiary pathway for the oxidation of ethanol involves catalase, a peroxisomal enzyme that also catalyses the removal of reactive oxygen species (e.g. H_2O_2). Acute exposure to EtOH leads to an adaptive increase in EtOH metabolism within 2 to 3 hours, in both rodent and human livers. This involves the so-called swift increase in alcohol metabolism (SIAM), defined experimentally as a rapid increase in hepatic alcohol metabolism and mitochondrial respiration after a single high dose of alcohol. High demand for O_2 during SIAM leads to zones of hypoxia, especially in pericentral (centrilobular) regions of liver lobules, which may contribute to liver injury. Interestingly, the very rapid posttranslational modifications of albumin in our EtOH model are consistent with the rapid onset liver injury described here.

Other mechanisms are at play in longer-term EtOH induced liver injury. EtOH changes the gut microbiome, causing bacterial overgrowth and increasing formation of toxic/proinflammatory products. EtOH consumption also promotes hepatic ROS and RNS formation. EtOH increases CYP2E1, largely by a posttranscriptional mechanism involving stabilization against proteolysis. CYP2E1 generates superoxide ($O_2^{\cdot -}$), which then forms highly reactive peroxynitrite ($ONOO^-$) by reaction with NO, and hydroxyl radical ($^{\cdot}OH$) by the Fenton

reaction. In the presence of EtOH, the 1-hydroxyethyl radical is also formed. Therefore, ROS, RNS and other radical species increase after EtOH ingestion. These radicals attack and damage proteins, lipids, and DNA, induce mitochondrial permeability transition (MPT), cause cell death, and trigger inflammatory processes.

Since albumin is the most abundant protein produced in hepatocytes and due to its great reactivity, it is naturally the most prone to posttranslational modifications due to the above-described reactions, which leads to the increase of albumin isoforms as observed in our ethanol model. However, it is still difficult to explain the increase of each isoform individually, since the chemical environment and the reactions at stake are not yet fully understood.

CCl_4 is metabolized in the liver by CYP2E1 and CYP2B1 enzymes and is converted into a highly reactive trichloromethyl ($CCl_3\cdot$) radical, ultimately leading to hepatotoxic damage, inflammation and fibrosis within 6 to 12 weeks. In our CCl_4 model, steatosis was detected in all rats after 3 days of daily administration. AST and ALT were significantly increased as soon as D1, and BILID after 7 days. The $CCl_3\cdot$ radical, produced after exposure to CCl_4, can bind to cellular molecules (nucleic acid, protein, lipid), impairing crucial cellular processes such as lipid metabolism, with the potential outcome of fatty degeneration leading to steatosis. $CCl_3\cdot$ reacts with oxygen to form the trichloromethylperoxy radical $CCl_3OO\cdot$, another highly reactive species. $CCl_3OO\cdot$ initiates a chain reaction of lipid peroxidation, which attacks and destroys polyunsaturated fatty acids, in particular those associated with phospholipids. These affect the permeability of mitochondrial, endoplasmic reticulum, and plasma membranes. At the molecular level, CCl_4 activates tumor necrosis factor (TNF)alpha, nitric oxide (NO), and transforming growth factors (TGF)-alpha and -beta in the cell, processes that appear to direct the cell primarily toward (self-)destruction or fibrosis (23).

Despite differences in cellular chemical reactions and environment involved in the mechanism of toxicity of EtOH and CCl_4, the albumin isoforms that we were able to identify in both models were the same with only 3 exceptions: Alb+SO_3H+2Glyc was specific of CCl_4 and Alb+$CysO_3$ and Alb+Cys+Glyc were specific of EtOH model.

In both models, after a decrease, native albumin increased to reach the level of the control groups at D7, before dropping again. This could be explained by a short regeneration phase, which has been extensively reported for CCl_4 animal models but much less for EtOH (24).

Paracetamol is metabolized by CYP2E1 and 1A2 to form -acetyl-p-benzoquinone imine (NAPQI). High concentrations of NAPQI deplete cellular glutathione stores and subsequently form APAP protein adducts, especially with mitochondrial proteins. NAPQI is very reactive and enhances the generation of free radicals such as superoxide. This reacts with nitric oxide (NO) within the mitochondria to produce highly reactive peroxynitrite, which nitrates mitochondrial proteins such as manganese superoxide dismutase (MnSOD). This alters mitochondrial antioxidant defenses, causing mitochondrial oxidant stress and oxidation of proteins such as mitochondrial thioredoxin. In the cytosol, oxidation of thioredoxin results in its detachment from its binding partner apoptosis signal-regulating kinase 1 (ASK1), which is then activated. ASK1, along with activated mixed-lineage kinase 3 (MLK3) then activate c-jun N-terminal kinase (JNK) to its phosphorylated form through MKK4 phosphorylation. Phosphorylated JNK translocates to the mitochondria and binds to Sab on the outer mitochondrial membrane, which, through a Src-mediated pathway, further inhibits mitochondrial electron transport. This amplifies mitochondrial oxidant stress, which is further exacerbated by translocation of Bax and glycogen synthase kinase-3β (GSK-3β) from the cytosol to the mitochondria. These events activate the mitochondrial permeability transition, which releases mitochondrial intermembrane proteins such as endonuclease G and apoptosis-inducing factor (AIF), along with cytochrome c and Smac. Translocation of AIF and endonuclease G to the nucleus then induces nuclear DNA fragmentation, which, along with activation of receptor-interacting protein kinases 3/1 (RIP3/RIP1), finally induce programmed necrosis. The extensive cell necrosis after an APAP overdose leads to release of damage-associated molecular patterns (DAMPs) including mitochondrial DNA, nuclear DNA fragments, high-mobility group box 1 (HMGB1) protein, and many others. DAMPs bind to pattern recognition receptors such as toll-like receptors (TLRs) on inflammatory cells and transcriptionally activate cytokine formation in inflammatory cells (25). APAP hepatotoxicity is a time-dependent event involving a number of different phases critical for the injury and recovery process: i) the metabolism phase that includes NAPQI production and glutathione depletion occurs within 0 to 3 hours after APAP administration; ii) the early injury phase that includes JNK activation and mitochondrial translocation, mitochondrial BAX translocation, mitochondrial superoxide formation, MPT, glutathione recovery and ALT/AST increase in plasma occurs within 2 to 6 h; iii) the late injury/early recovery phase that includes ALT/AST increase in plasma, necrosis, innate immune response occurs within 12 to 24h; and finally iv) the regeneration phase where resolution of necrosis could be encountered occurs within 24 to

96 h (26). In our APAP model, necrosis was mainly observed in rats given 4 g/kg APAP, and inflammation was noted for all doses. Native albumin in this model did not decrease 24 hours post APAP and was even barely detectable at 72h , which is consistent with the mechanism of APAP toxicity described here. The sharp decrease of native albumin was associated with an increase in other isoforms. Among them, Alb+SGGS was specific to this APAP model. As we did not observe any normalization of native albumin, we can assume that the regeneration phase was not reached in our experimental conditions.

Overall, these preliminary results support the hypothesis that albumin posttranslational modifications (Alb-PTM) occur early in the course of induced liver injury, suggesting that the use of Alb-PTM as an early biomarker to characterize drug-induced injury would be relevant.

Albumin profile as a signature

Although most of the isoforms characterized in this research are the result of oxidative reactions, albumin can undergo several chemical modifications on several sites of its amino acids chain, including: cysteinylation, homocysteinylation, glutathionylation, glycation, nitrosylation, nitration, oxidation and carbonylation. With our analytical method, we were able to characterize, after the deconvolution of the MS spectra, up to 17 isoforms (Table 4). Our analytical strategy, which could be seen as a simplified top-down analysis, is based on the simple injection of diluted serum into the LC-MS instrument. It has the advantage of preserving the structural and chemical modifications of interest in the native form of the protein. Theoretically, the deconvoluted MS spectrum is capable of identifying unknown modifications because the addition or subtraction of a structural modification result in a characteristic mass shift, equal to the mass of the specific modification (for example, a mass difference of 163 Da for a glycation). However, the 17 isoforms identified had to be “visually detectable” to be included, increasing the risk of leaving behind minor isoforms with low signals. Therefore, to investigate the differences in isoform distribution among the 3 animal models, we decided to integrate the whole information from each spectrum in PCA-DA. The profiles obtained with the 3 models were nicely separated (Figure 5), suggesting that the Alb profile of each model depends on the different chemical environment and mechanisms of intracellular reactions generated by each substance, as previously explained.

Interestingly, the same approach allowed us to demonstrate that the albumin profiles observed in cirrhotic patients were able to discriminate them from control patients and to discriminate the origins and stages of liver injury among them (**Figures 7 and 8**). This suggests that these isoform profiles may be used as a very valuable tool in the diagnosis and monitoring of liver diseases. In the clinical arena, it is frequently of paramount importance to know whether steatohepatitic liver injury is related to alcohol or NAFLD because this distinction may influence patient management and candidacy for liver transplantation (27).

In our cohort of cirrhotic patients, we could identify up to 14 albumin isoforms, all of which were also present in control patients. However, we observed that the increase in the HSA-DA isoform was specific to patients with cirrhosis due to alcohol abuse, HSA+SGGS and HSA+2Glyc were increased specifically in NASH patients, and HSA-DA+Cys with HSA-SO_2H were increased only in patients with the mixed form. Despite these specificities, the use of the whole information from the MS spectra should increase the discrimination performance of our tool as described above. In addition, we did not observe a specific isoform able to clearly discriminate the different stages of liver disease (data not shown), but principal component analysis of the MS dataset perfectly separated cirrhosis patients with different Child-Pugh scores and control patients.

Overall, albumin isoform profiles represent rich data that could help diagnosing liver diseases, staging them and sorting out their origin.

Prediction of early allograft dysfunction by the evolution of the profiles of albumin isoforms:

The rationale for using the albumin isoforms profiles at 2 different times was to compare the evolution and distribution of isoforms between V6 and V3 or V1. The evolutionary nature of this criterion in relation to a baseline situation was intended to use albumin isoform analysis as a functional test, with changes in isoform proportions increasing as the risk of allograft dysfunction increased and graft function was compromised. This is consistent with the fact that the production of less modified albumin after transplantation reflects good graft function. However, because of the low number of patients experiencing an early allograft dysfunction, this observation has to be confirmed in a larger cohort of liver transplanted patients.

TABLES:

Table 1: Effect of EtOH on biochemical markers expressed as the median [min-max] (* p < 0,05).

Biomarkers	Controls	D1	D3	D7	D10	D14
ALB (g/L)	14,4 [12,7-17,6]	13,8 [13,2-14,1]	**12,4* [11,8-14,2]**	**12,2* [11,0-13,8]**	16,35 [12,4-17,7]	**12,9* [10,2-13,5]**
AST (UI/L)	70,8 [61-75]	78 [70-85]	80 [52-144]	**96* [75-157]**	**92* [81-157]**	**90* [85-294]**
ALT (UI/L)	57 [46-61]	63 [52-71]	62 [46-123]	69 [52-97]	**78* [68-98]**	**82* [65-104]**
PAL (UI/L)	196 [101-325]	232 [153-304]	180 [121-329]	159 [84-263]	172 [100-222]	230 [110-327]
BILID (µM)	0,75 [0,6-1]	0,7 [0,3-1]	0,7 [0,5-1,3]	0,7 [0,4-4,4]	0,8 [0,5-1,1]	0,6 [0,4-0,7]
BILIT (µM)	0,75 [0,4-1,3]	0,8 [0,4-1,6]	0,8 [0,2-1,7]	1,0 [0,2-1,9]	1,0 [0,8-1,6]	0,9 [0,5-1,8]
Histology		2/7 Inflammation	1/6 inflammation	4/7 inflammation	2/8 inflammation	3/9 inflammation

Table 3: Effect of CCl_4 on administration on biochemical markers expressed as the median [min-max] (* p < 0,05; ** p < 0,01).

Biomarkers	Controls	D1	D3	D7	D10
ALB (g/L)	**14,4 [12,7-17,6]**	**14,2 [12,9-14,5]**	**13,0* [11,8-14,2]**	**12,9* [10,4-14,5]**	**16,0 [15,9-16,7]**
AST (UI/L)	70,8 [61-75]	**123* [90-251]**	**151* [82-214]**	**232** [168-305]**	**337** [192-473]**
ALT (UI/L)	**57 [46-61]**	**77* [67-181]**	**90* [78-208]**	**200** [107-638]**	**355** [166-538]**
PAL (UI/L)	196 [101-325]	232 [192-270]	237 [174-329]	248 [117-341]	205 [149-250]
BILID (µM)	**0,75 [0,6-1,0]**	**1,1 [0,7-1,5]**	**1,2 [0,3-1,3]**	**1,6** [1,2-2,0]**	**2,5** [1,8-2,9]**
BILIT (µM)	0,75 [0,4-1,3]	1,4 [0,4-2,0]	1,3 [0,6-3,5]	**2,3** [1,8-4,5]**	**3,5** [2,4-5,3]**
Histology			**Steatosis 5/5 1/5 fibrosis**	**Steatosis 6/6**	**6/6 steatosis 1/6 fibrosis**

Table 3: Effect of APAP administration on biochemical markers expressed as the median [min-max] (* $p < 0,05$; ** $p < 0,01$) and histology examination. D2g-D1 is the group of rats that were sacrificed 24h after receiving a dose of 2 g/kg of APAP. D2g-D3 is the group of rats that were sacrificed 72h after receiving a dose of a dose of 2g/kg. D3g-D1 is the group of rats that were sacrificed 24h after receiving 3g/kg of APAP.

Biomarkers	Controls	D2g-D1	D2g-D3	D3g-D1	D3g-D3	D4g-D1	D4g-D3
ALB (g/L)	13,3 [12,6-14]	12,65 [10,6-14,3]	**11,2* [8,3-12,3]**	**10,4** [9,6-11,8]**	**11,1* [9,9-11,4]**	**10,9* [10,4-12]**	**10,95* [10,2-12,0]**
AST (UI/L)	54,35 [49,5-58]	**205,2* [113-803]**	75,95 [31,8-259]	87,1 [67-100,2]	44,3 [38-95]	198,8 [49,1-600]	62,9 [56-103]
ALT (UI/L)	34	53	24	31	32,5	44,5	25

	[30-36]	[41-376]	[6-89]	[28-51]	[18-44]	[26-134]	[17-28]
PAL (UI/L)	101 [82-113]	134 [89-243]	122,5 [90-157]	106 [72-167]	103,5 [73-142]	149 [109-241]	96 [88-111]
BILID (µM)	0,6 [0,04-1,2]	0,5 [0,2-1,1]	0,34 [0,1-0,99]	0,1 [0,1-1,5]	0,2 [0,15-0,6]	0,18 [0,08-0,5]	0,3 [0,13-0,35]
BILIT (µM)	0,7 [0,1-1,8]	0,75 [0,3-1,4]	0,4 [0,1-1,8]	0,3 [0,2-2,5]	0,5 [0,3-0,9]	0,3 [0,2-0,9]	0,5 [0,4-3]
Histology		1/ necrosis	1/6 necrosis 1/6 inflammation 2/6 F1	3/6 portal dilatation 2/6 inflammation	1/6 portal dilatation 1/6 F1	5/6 necrosis	4/6 portal inflammation 3/6 F1

Table 4. Isoforms of albumin and mass shifts of native albumin in rats and humans.

Isoformes ID	Rats	Humans	Mass shift	ISOFORMES	APAP	EtOH	CCl4
A	65685	66253	-185	HSA-DA	0	0	0
B	65803	66371	-67	HSA-DA+Cys	0	0	0
C	65870	66438	0	HSA Native	1	1	1
D	65901	66469	31	HSA+SO2H	1	1	1
E	65918	66486	48	HSA+CysO3	1	1	0
F	65966	66534	96	HSA+Cys-DHA	1	1	1
G	65989	66557	119	HSA+Cys	1	1	1
H	66018	66586	148	HSA+Cys+SNO	1	1	1
I	66033	66601	163	HSA+Glyc	0	1	1
J	66065	66633	195	HSA+SO2H+Glyc	1	1	1
K	66081	66649	211	HSA+SO3H+Glyc	1	1	1
L	66118	66686	248	HSA+Cys+Glyc-DHA	0	1	1
M	66152	66720	282	HSA+Cys+Glyc	0	1	0
N	66175	66743	305	HSA+SGGS	1	0	0
O	66196	66764	326	HSA+2Glyc	1	1	1
P	66242	66810	372	HSA+SO3H+2Glyc	1	0	1
Q	66310	66878	440	HSA+Cys+2Glyc	1	1	0

Table 5. Variations of the different albumin isoforms in the different groups of patients as compared to the control group.

	Controls	EtOH	NASH	EtOH-NASH
HSA-DA	0,1 %	↗ 0,4 %	- 0,07 %	↘ 0,03 %
HSA-DA+Cys	0,14 %	- 0,14 %	- 0,08 %	↗↗↗ 0,5 %
HSA Native	37 %	↘ 26 %	↘↘ 14 %	↘ 29 %
HSA SO2H	0,3 %	✖	✖	↗↗↗ 2 %
HSA SO3H	3 %	- 1,6 %	✖ 0,2 %	- 3,5 %
HSA+Cys-DHA	1 %	- 1 %	↘* 0,5 %	- 0,8 %
HSA+Cys	27 %	↗↗ 42 %	↗↗ 47 %	↗↗ 47 %
HSA+Cys+SNO	4,7 %	↗	↗	↗

		6,8 %	6,7 %	8,4 %
HSA+Glyc	10 %	- 10 %	- 10 %	- 10 %
HSA+SO2H+Glyc	1 %	↘* 0,7 %	↘* 0,6 %	- 1 %
HSA+Cys+Glyc	8 %	- 7 %	↗↗ 18 %	- 8 %
HSA+SGGS	5 %	5 %	↗* 8,5 %	5 %
HSA+2Glyc	0,4 %	↘ 0,15 %	↗ 0,7 %	↘ 0,14
HSA+Cys+2Glyc	0,13 %	↗↗ 0,4 %	↗↗↗ 2 %	↗↗ 0,3

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method of determining the etiology and severity of a liver injury in a subject comprising determining the profile of albumin isoforms in a blood sample obtained from the subject wherein the profile indicates the etiology and severity of the liver injury.

2. The method of claim 1 that comprises the step of detecting a plurality of albumin isoforms.

3. The method of claim 1 that comprises the step of detecting a plurality of albumin isoforms selected from Table 4.

4. The method of claim 1 that comprises the step of detecting a plurality of isoforms selected from the group consisting of Alb+SO2H, HSA-CysO3, Alb+Cys-DHA, Alb+Cys, Alb+Cys+SNO, Alb+SO2H+Glyc, Alb+SO3H+Glyc, HAS-SGGS, Alb+2Glyc, Alb+SO3H+2Glyc, and Alb+Cys+2Glyc.

5. The method of claim 1 that comprises the steps of i) determining the profile of albumin isoforms in the blood sample obtained from the patient, and ii) comparing the profile to one or more reference profiles associated with various liver injuries.

6. The method of the present invention of claim 1 for detecting chemical liver injuries, for detecting physical liver injuries, for detecting ischemic liver injuries, for the early detection of a liver injury, and/or for the early detection of early graft dysfunction or non-function in liver transplanted patients.

7. The method of claim 6 for detecting a non-alcoholic fatty liver disease, and in particular for detecting NASH.

8. The method of claim 6 for detecting liver fibrosis, and in particular for detecting cirrhosis.

9. A method of predicting the worsening of a liver injury comprising the steps of determining the evolution of the profile of albumin isoforms in the blood sample obtained from the patient wherein said evolution predicts the worsening of the liver injury.

10. A method of predicting an early allograft liver dysfunction or liver non-function in a liver-transplanted patient comprising the steps of determining the evolution of the profile of albumin isoforms in the blood sample obtained from the patient wherein said evolution predicts the early allograft dysfunction or non-function.

11. The method according to any one of claims 1 to 10 wherein the isoforms are detected by mass spectrometry.

12. The method according to any one of claims 1 to 11 that comprises the use of an algorithm.

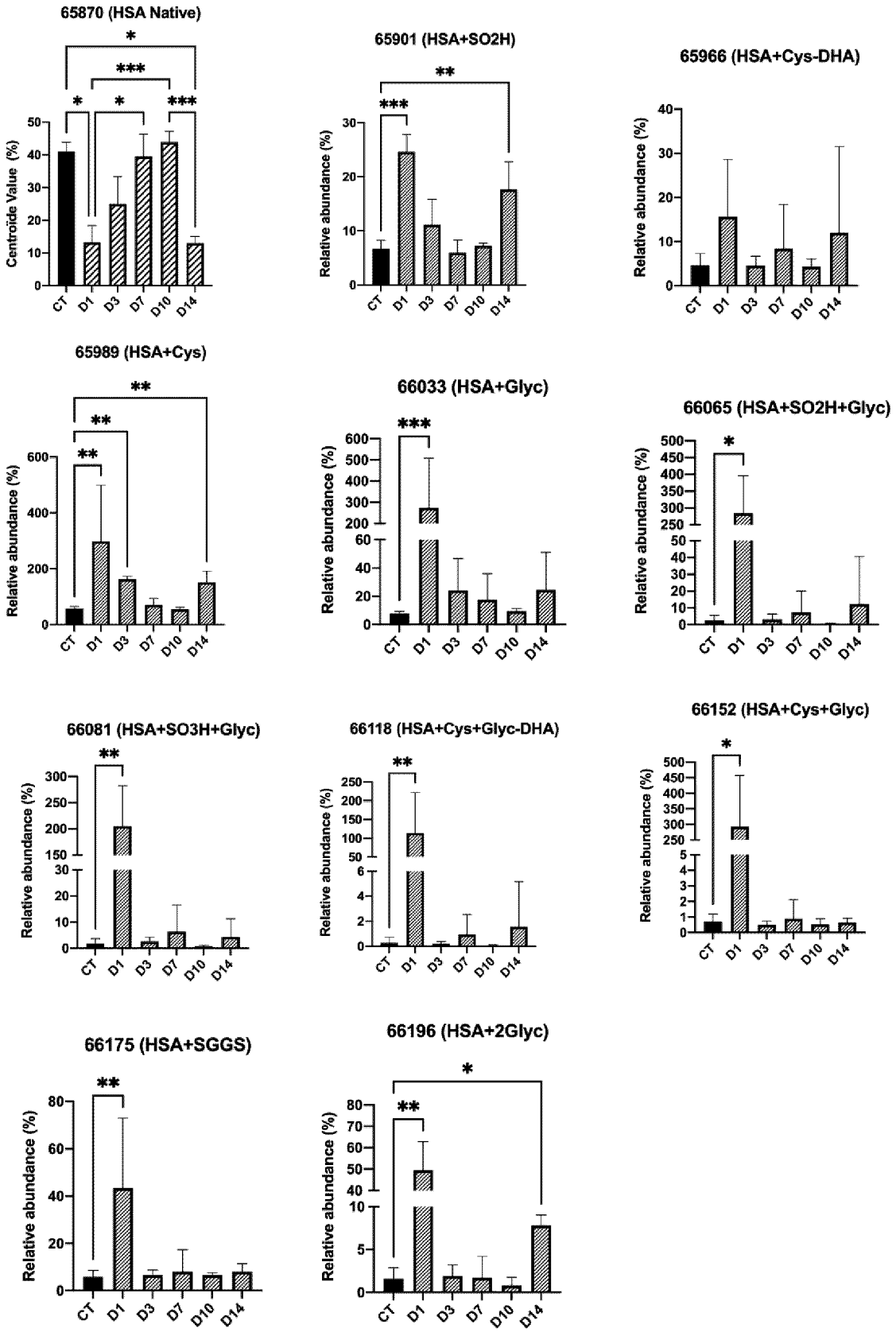
13. The method of claim 12 that is a computer-implemented method comprising applying, on a set of values for each detected isoforms relative to the subject, a trained model configured to determine the etiology and severity of the liver injury based on the set of values.

14. The method of claim 12 that the set of values comprises the relative abundance of the different detected isoforms.

15. The method of claim 12 wherein the model is preliminary trained by supervised learning on a training dataset comprising, for a plurality of individuals of a population, the etiologies and the severities of a liver injury.

16. The method of claim 12 wherein that comprises the use of a classification algorithm.

17. The method according to any one of claim 1 to 16 for determining whether a subject suffering from a liver injury achieves a response to a therapy.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Relative abundance (%)
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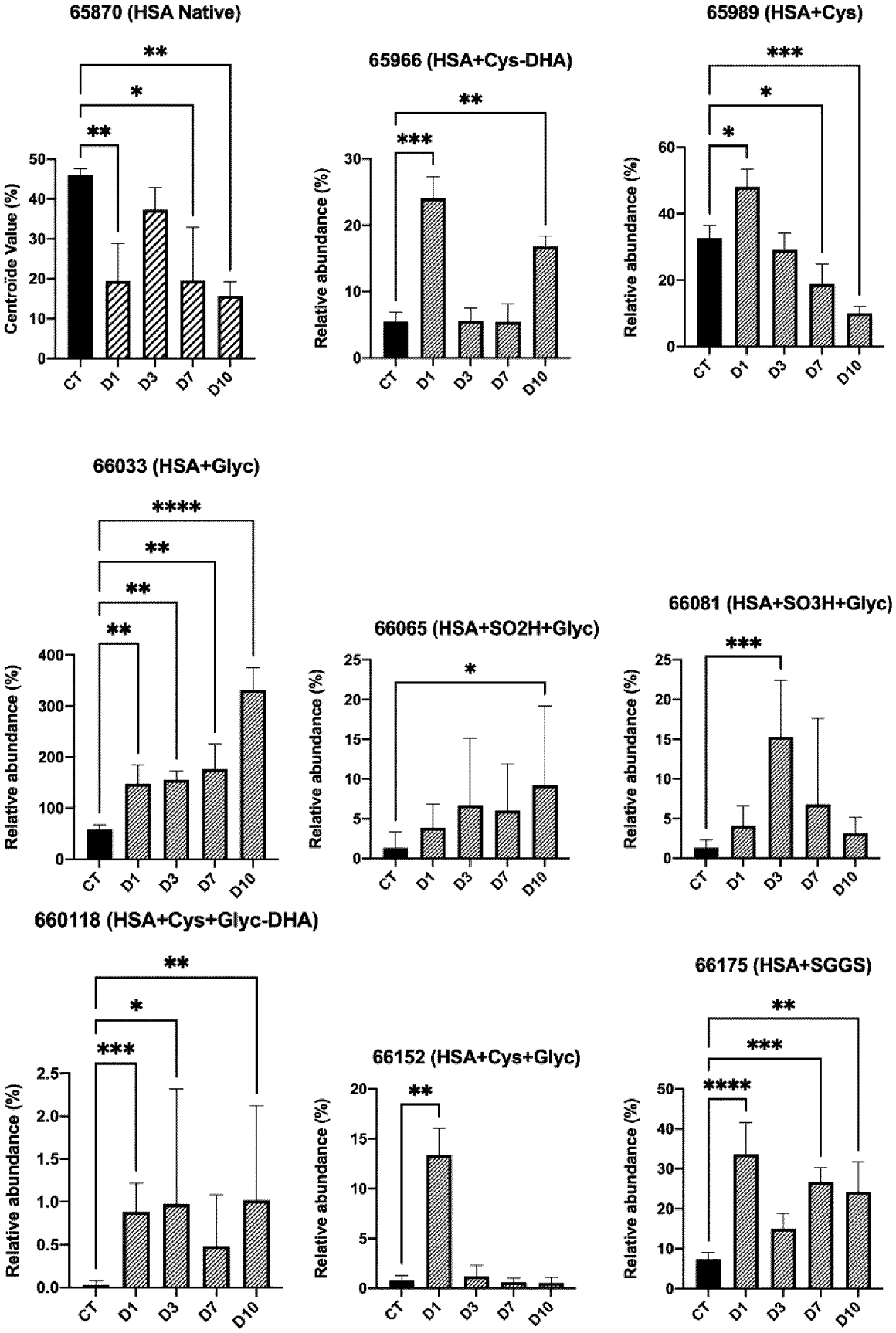
Relative abundance (%)
66033 (HSA+Glyc)
Relative abundance (%)
66065 (HSA+SO2H+Glyc)
Relative abundance (%)
66081 (HSA+SO3H+Glyc)
Relative abundance (%)
66118 (HSA+Cys+Glyc-DHA)
Relative abundance (%)
66152 (HSA+Cys+Glyc)
Relative abundance (%)
66175 (HSA+SGGS)
Relative abundance (%)
66196 (HSA+2Glyc)
Relative abundance (%)
CT
D1
D3
D7
D10
D14

Figure 1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Relative abundance (%)
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Relative abundance (%)
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Relative abundance (%)
66065 (HSA+SO2H+Glyc)
Relative abundance (%)
66081 (HSA+SO3H+Glyc)
Relative abundance (%)
660118 (HSA+Cys+Glyc-DHA)
Relative abundance (%)
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Relative abundance (%)
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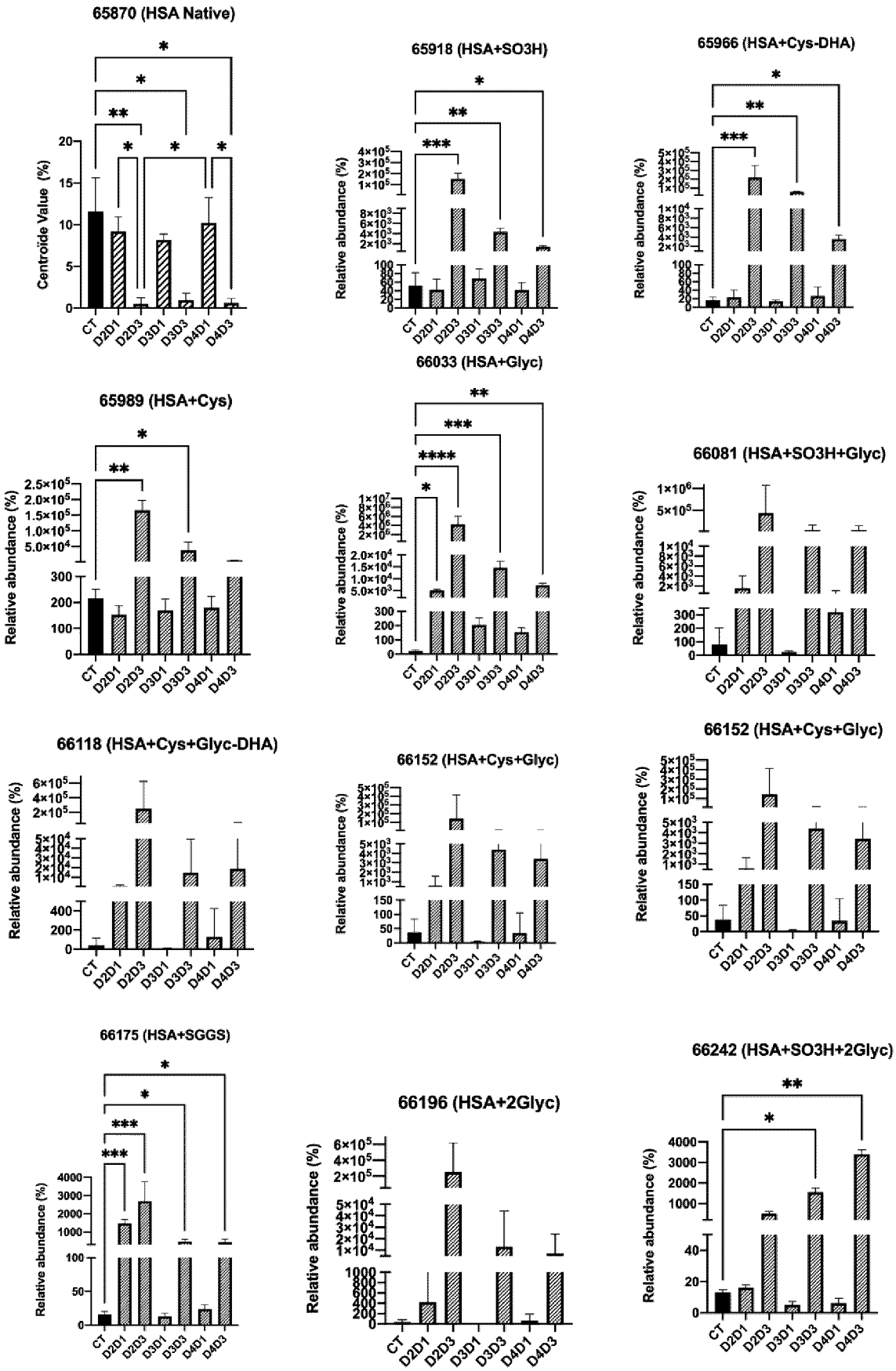
Relative abundance (%)
CT
D1
D3
D7
D10

Figure 2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66118 (HSA+Cys+Glyc-DHA)
66152 (HSA+Cys+Glyc)
66152 (HSA+Cys+Glyc)
66175 (HSA+SGGS)
66196 (HSA+2Glyc)
66242 (HSA+SO3H+2Glyc)
Relative abundance (%)
CT
D2D1
D2D3
D3D1
D3D3
D4D1
D4D3

Figure 3

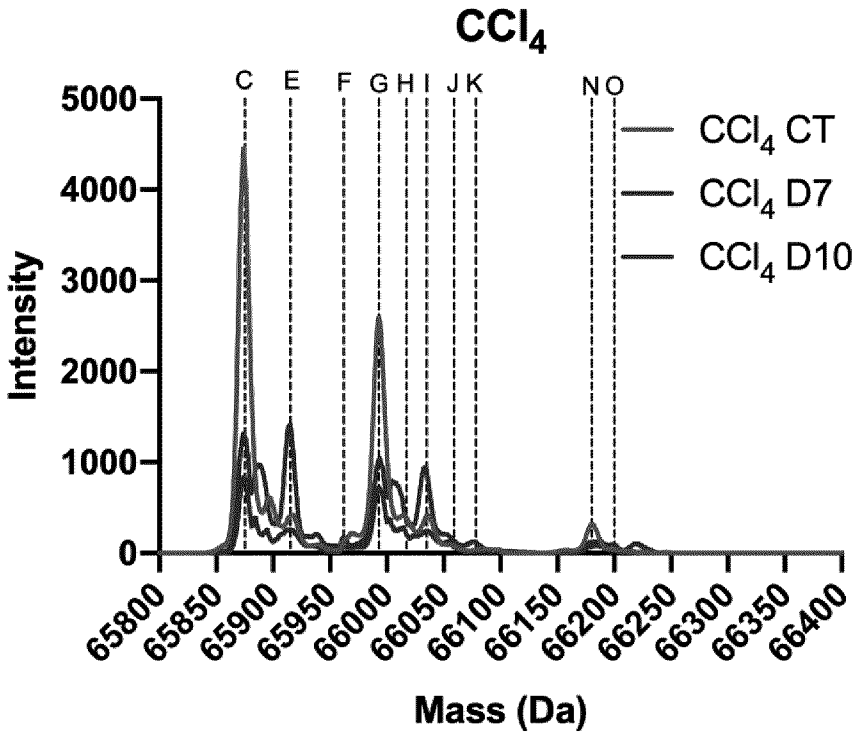

Figure 4A

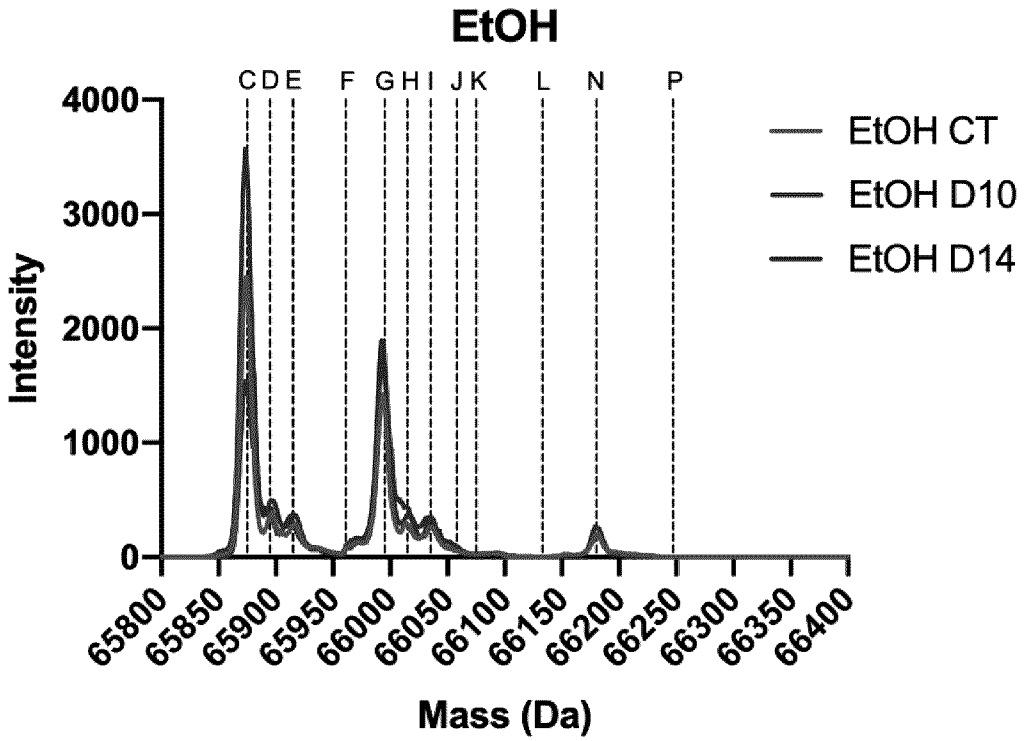

Figure 4B

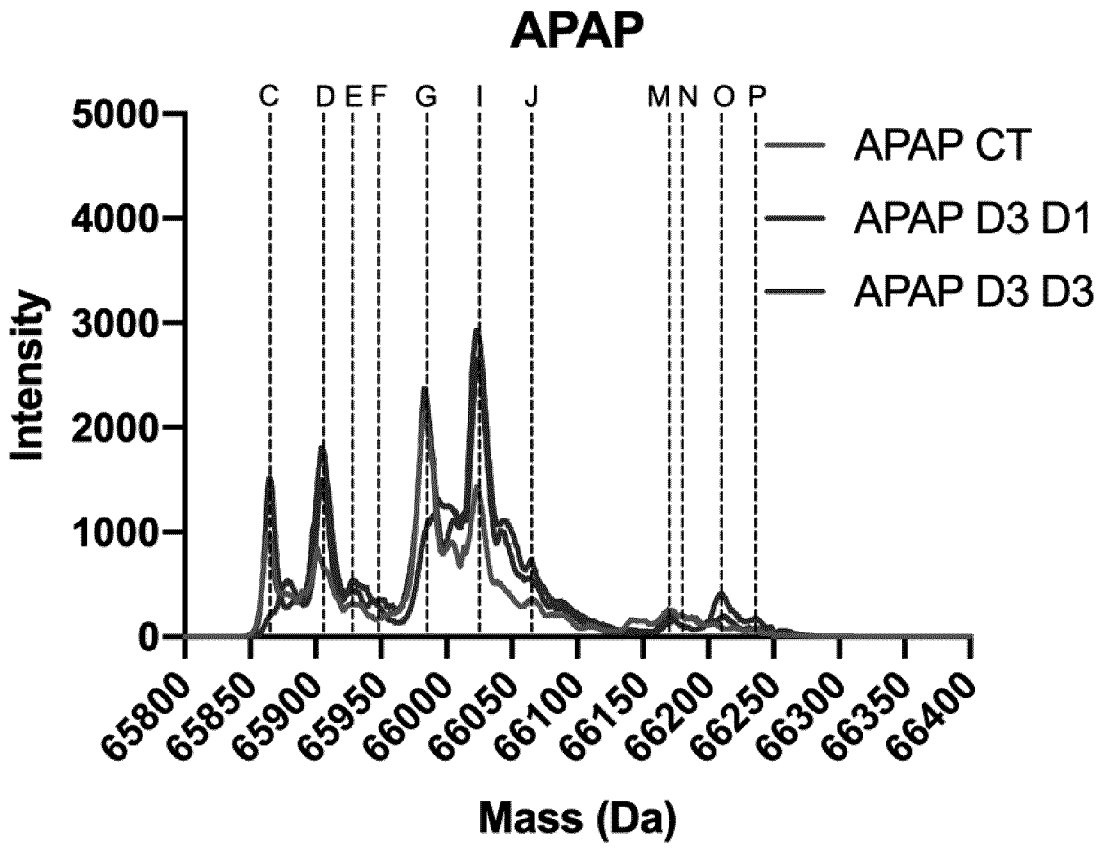
APAP
C
D
E
F
G
I
J
M
N
O
P
APAP CT
APAP D3 D1
APAP D3 D3
Intensity
5000
4000
3000
2000
1000
0
65800
65850
65900
65950
66000
66050
66100
66150
66200
66250
66300
66350
66400
Mass (Da)

Figure 4C

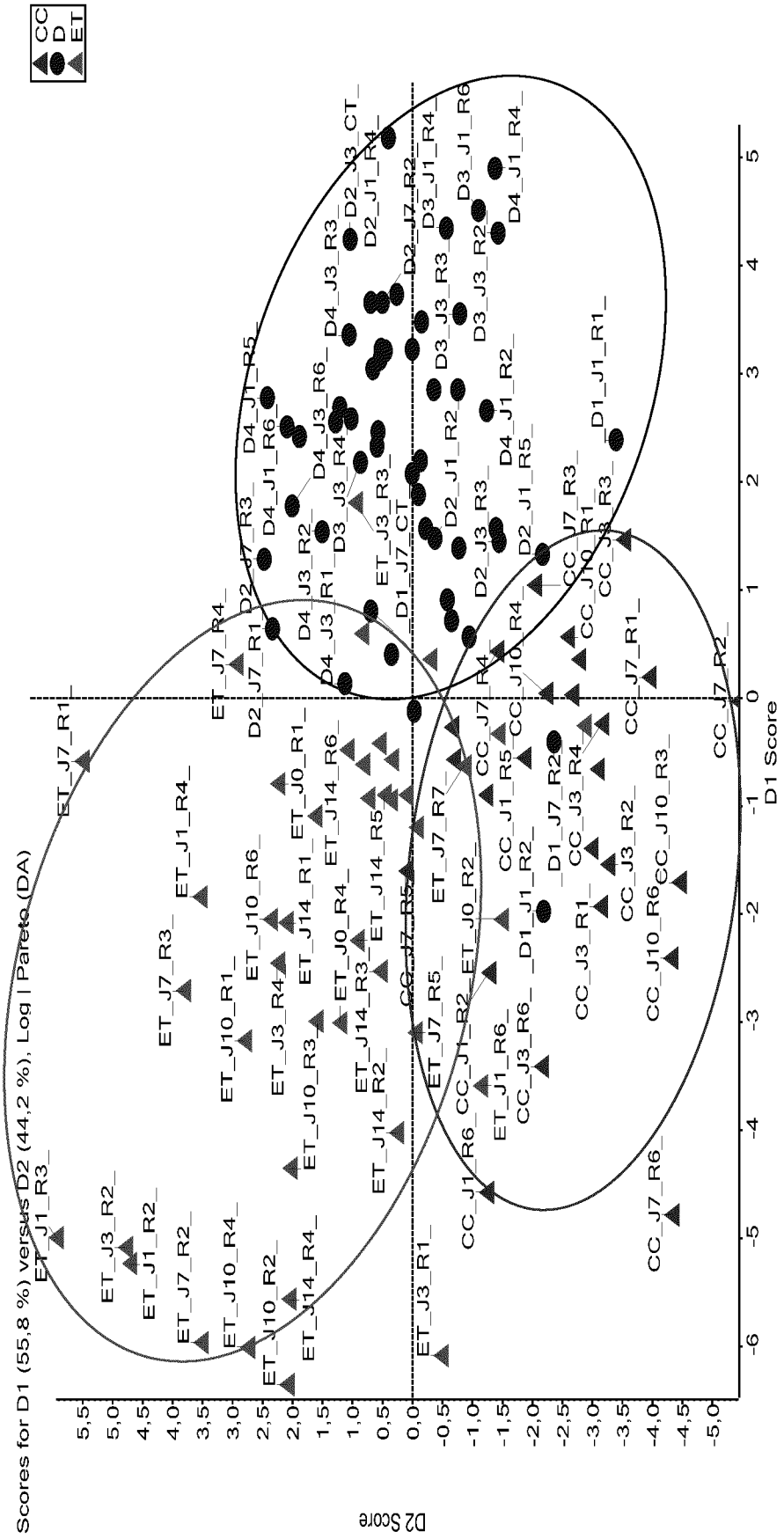
CC
D
ET
Scores for D1 (55,8 %) versus D2 (44,2 %), Log | Pareto (DA)
D2 Score
D1 Score

Figure 5

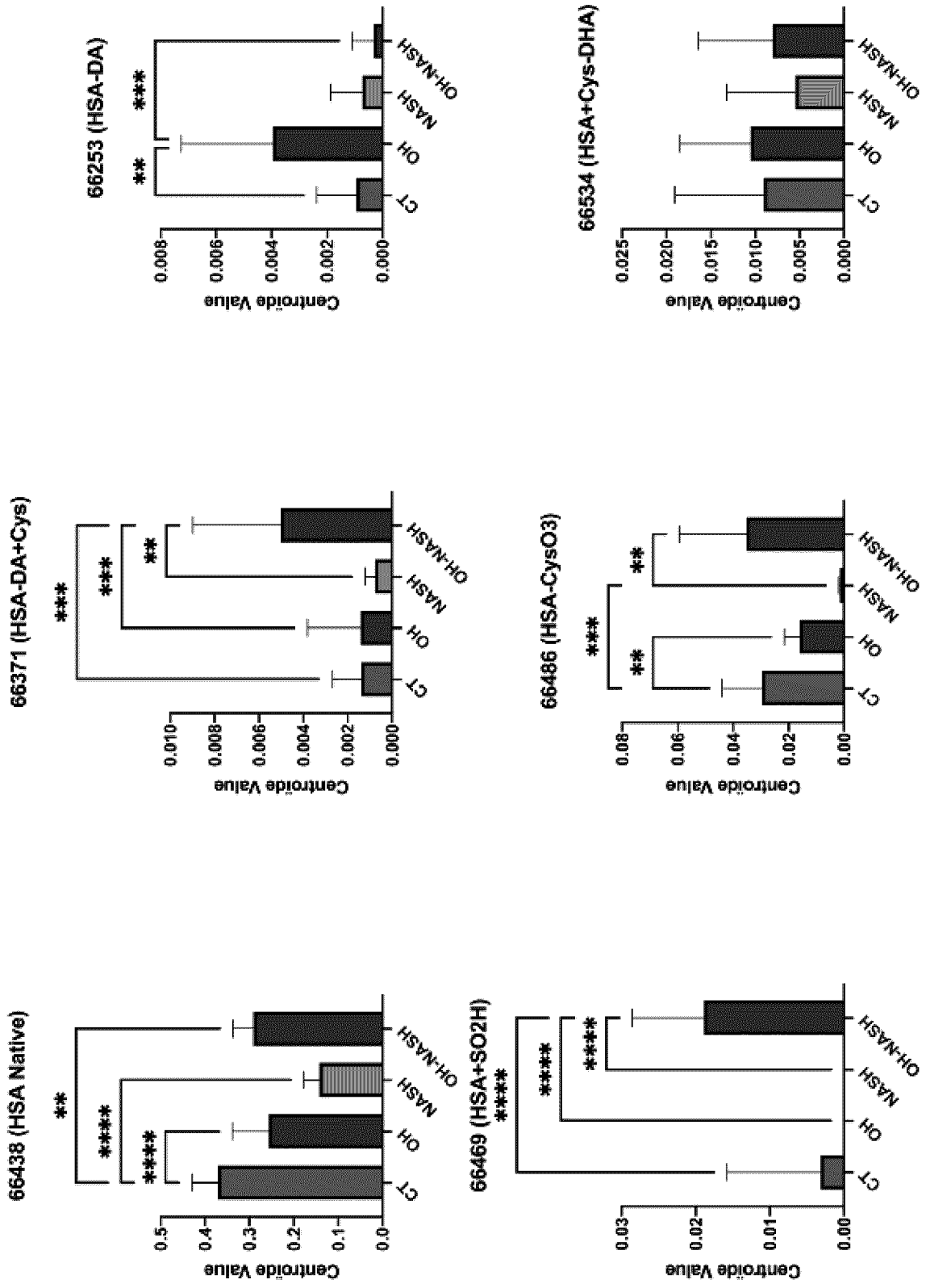

Figure 6 (part 1/3)

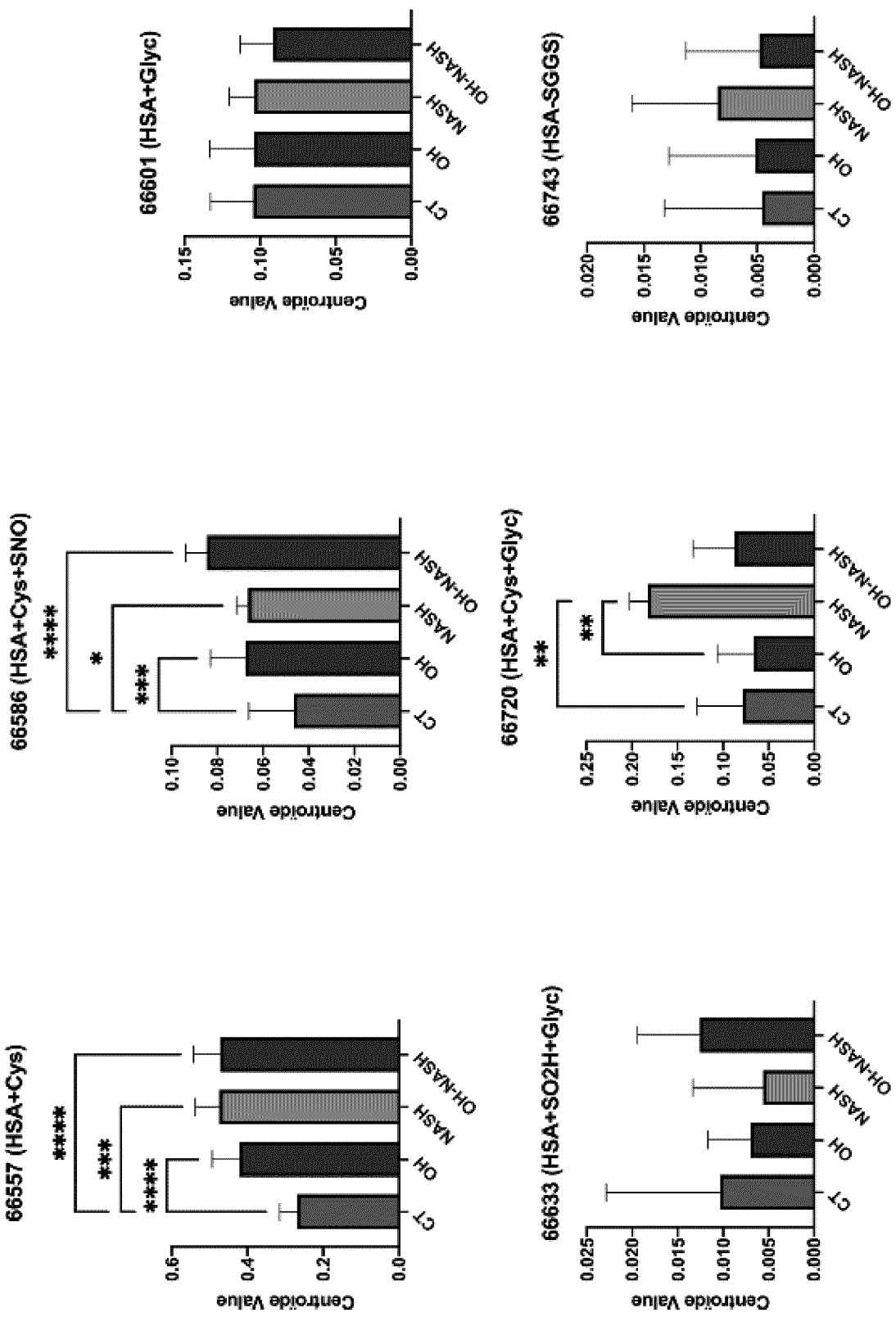

Figure 6 (part 2/3)

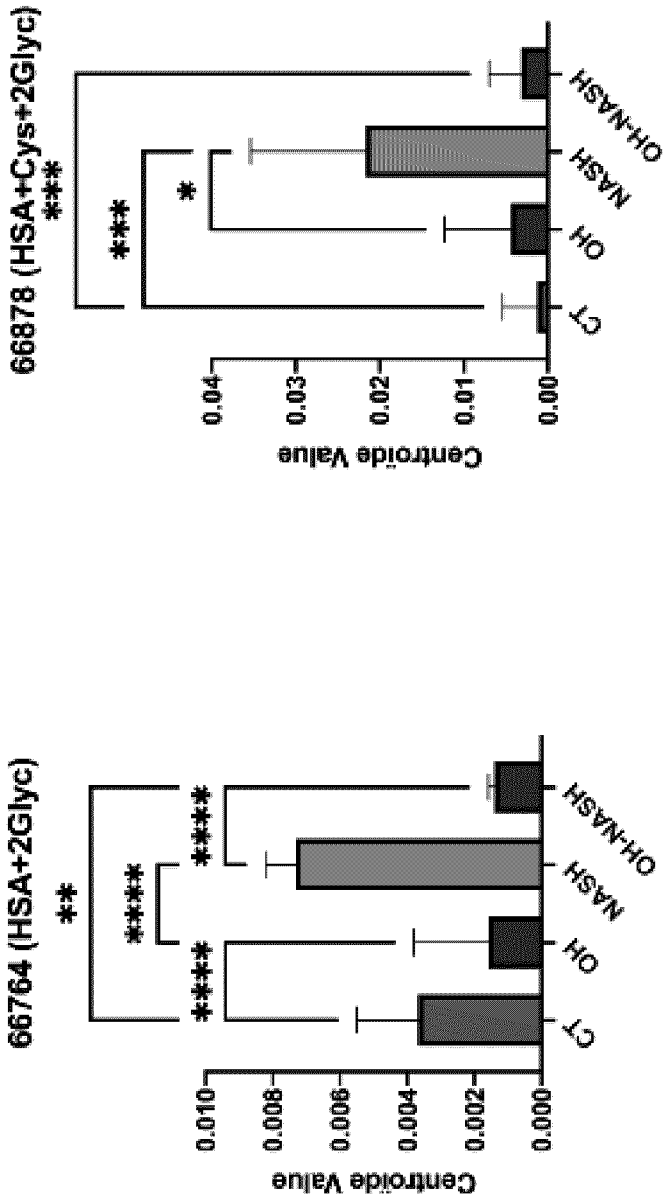

Figure 6 (part 3/3)

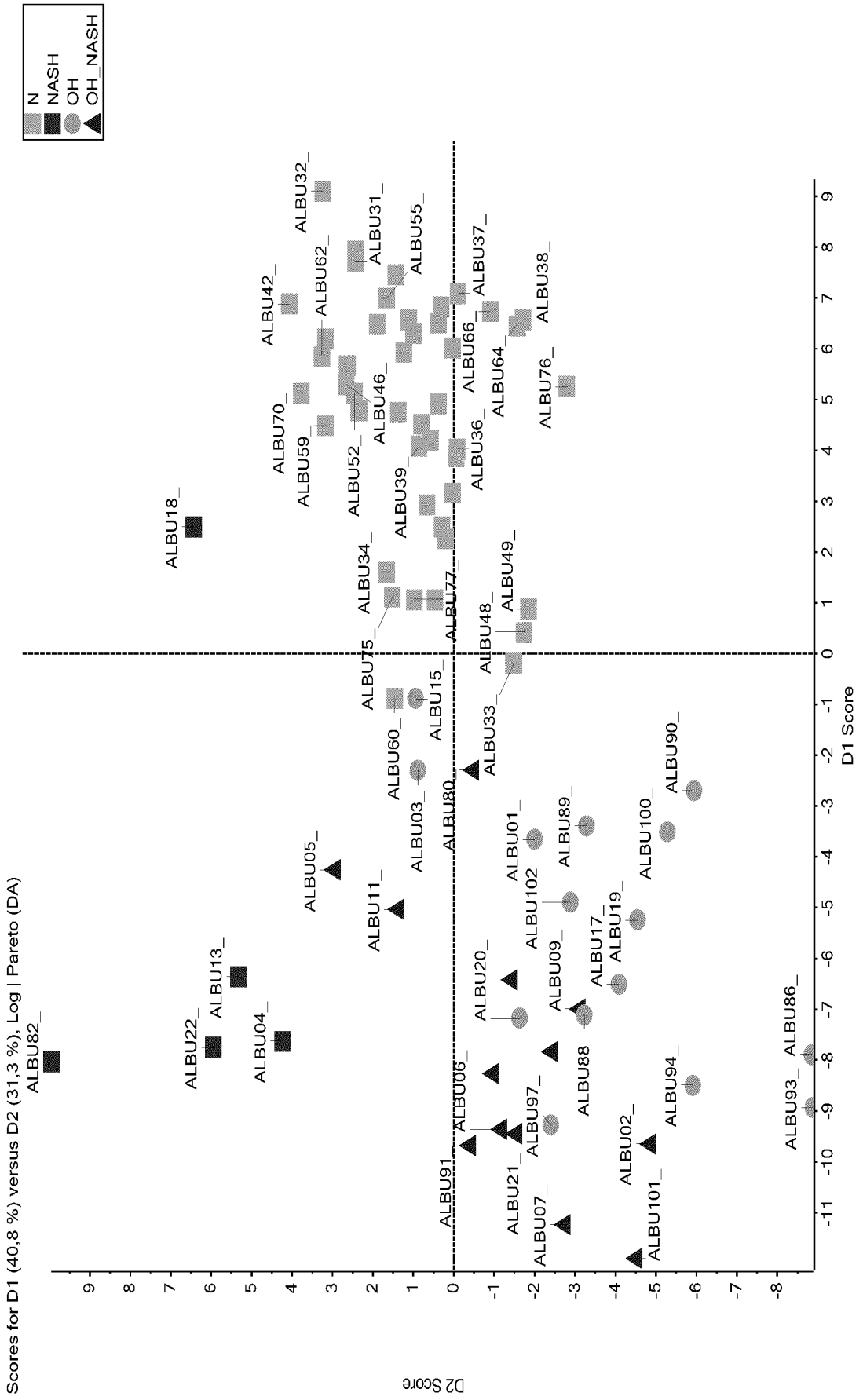
N
NASH
OH
OH_NASH
Scores for D1 (40,8 %) versus D2 (31,3 %), Log | Pareto (DA)
D1 Score
D2 Score
ALBU82_
ALBU22_
ALBU13_
ALBU04_
ALBU18_
ALBU42_
ALBU32_
ALBU62_
ALBU70_
ALBU59_
ALBU31_
ALBU55_
ALBU52_
ALBU46_
ALBU39_
ALBU34_
ALBU75_
ALBU77_
ALBU36_
ALBU66_
ALBU37_
ALBU64_
ALBU38_
ALBU76_
ALBU48_
ALBU49_
ALBU33_
ALBU15_
ALBU60_
ALBU03_
ALBU80_
ALBU05_
ALBU11_
ALBU01_
ALBU102_
ALBU89_
ALBU09_
ALBU20_
ALBU17_
ALBU19_
ALBU100_
ALBU90_
ALBU88_
ALBU97_
ALBU06_
ALBU91
ALBU21_
ALBU07_
ALBU02_
ALBU101_
ALBU94_
ALBU93_
ALBU86_

Figure 7A

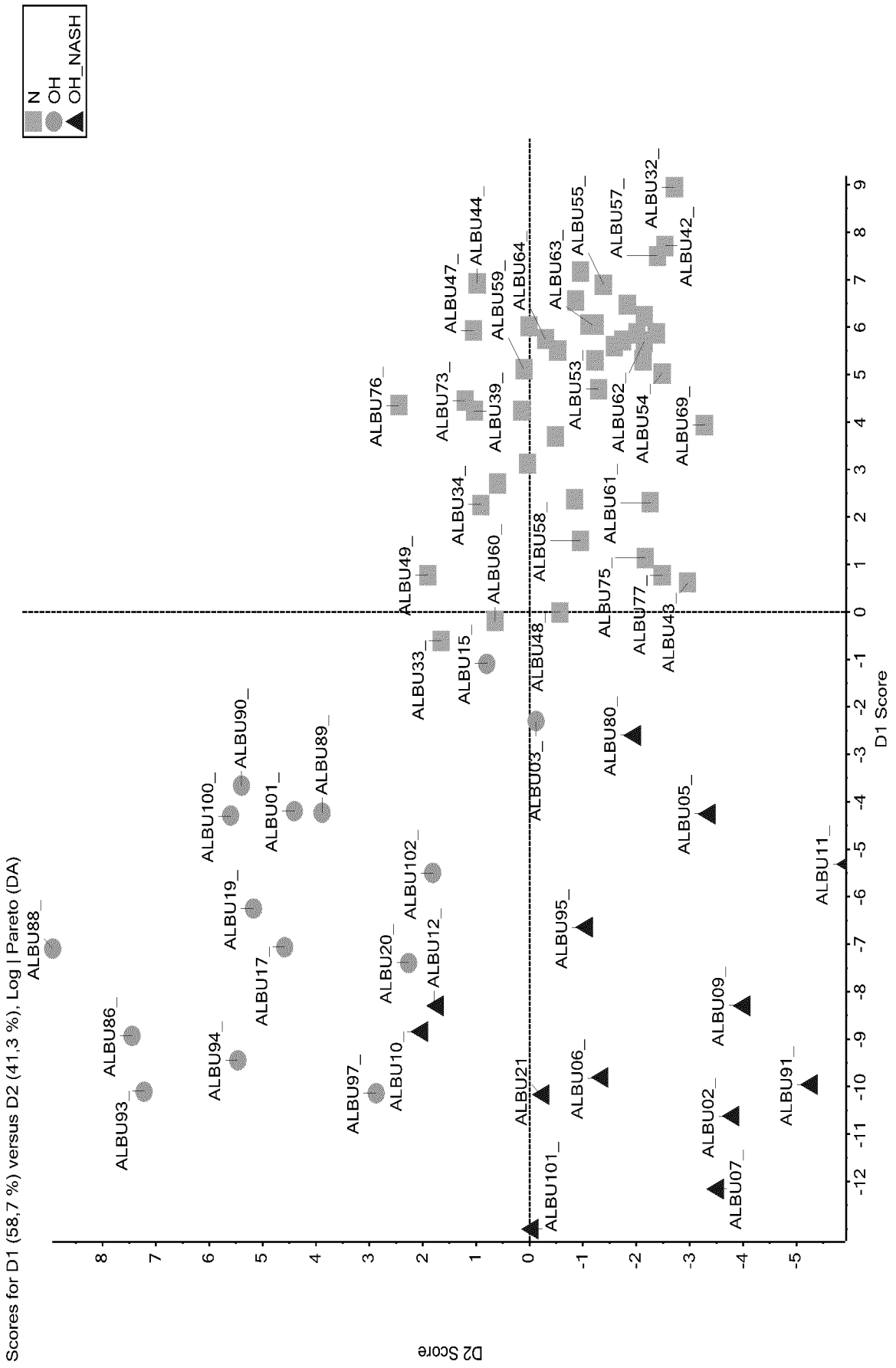
N
OH
OH_NASH
Scores for D1 (58,7 %) versus D2 (41,3 %), Log | Pareto (DA)
D1 Score
D2 Score
ALBU88_
ALBU86_
ALBU93_
ALBU94_
ALBU19_
ALBU17_
ALBU100_
ALBU90_
ALBU01_
ALBU89_
ALBU97_
ALBU10_
ALBU20_
ALBU102_
ALBU12_
ALBU21
ALBU101_
ALBU06_
ALBU95_
ALBU02_
ALBU07_
ALBU09_
ALBU91_
ALBU11_
ALBU03_
ALBU48_
ALBU80_
ALBU05_
ALBU33_
ALBU15_
ALBU49_
ALBU76_
ALBU60_
ALBU34_
ALBU58_
ALBU75_
ALBU61_
ALBU77_
ALBU43_
ALBU73_
ALBU39_
ALBU47_
ALBU44_
ALBU59_
ALBU64_
ALBU53_
ALBU63_
ALBU55_
ALBU62_
ALBU54_
ALBU57_
ALBU69_
ALBU32_
ALBU42_

Figure 7B

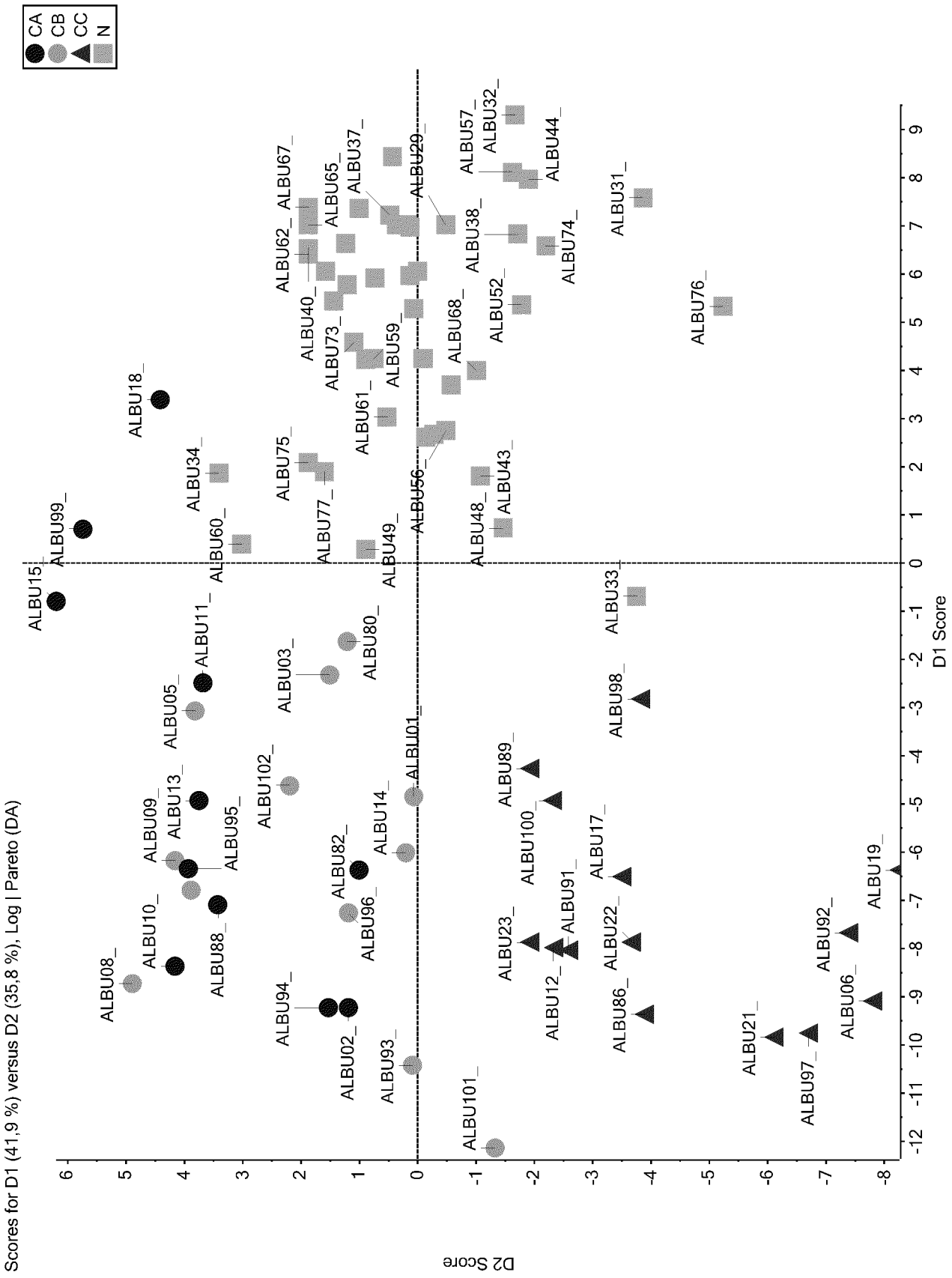
CA
CB
CC
N
Scores for D1 (41,9 %) versus D2 (35,8 %), Log | Pareto (DA)
D1 Score
D2 Score
ALBU15_
ALBU99_
ALBU18_
ALBU34_
ALBU60_
ALBU11_
ALBU75_
ALBU77_
ALBU62_
ALBU67_
ALBU40_
ALBU65_
ALBU73_
ALBU37_
ALBU61_
ALBU59_
ALBU29_
ALBU68_
ALBU49_
ALBU56_
ALBU48_
ALBU43_
ALBU57_
ALBU32_
ALBU38_
ALBU44_
ALBU52_
ALBU74_
ALBU31_
ALBU76_
ALBU33_
ALBU03_
ALBU80_
ALBU05_
ALBU01_
ALBU102_
ALBU89_
ALBU98_
ALBU08_
ALBU09_
ALBU13_
ALBU14_
ALBU100_
ALBU17_
ALBU10_
ALBU95_
ALBU82_
ALBU91_
ALBU88_
ALBU96_
ALBU23_
ALBU22_
ALBU12_
ALBU94_
ALBU86_
ALBU21_
ALBU92_
ALBU02_
ALBU93_
ALBU06_
ALBU19_
ALBU97_
ALBU101_

Figure 8A

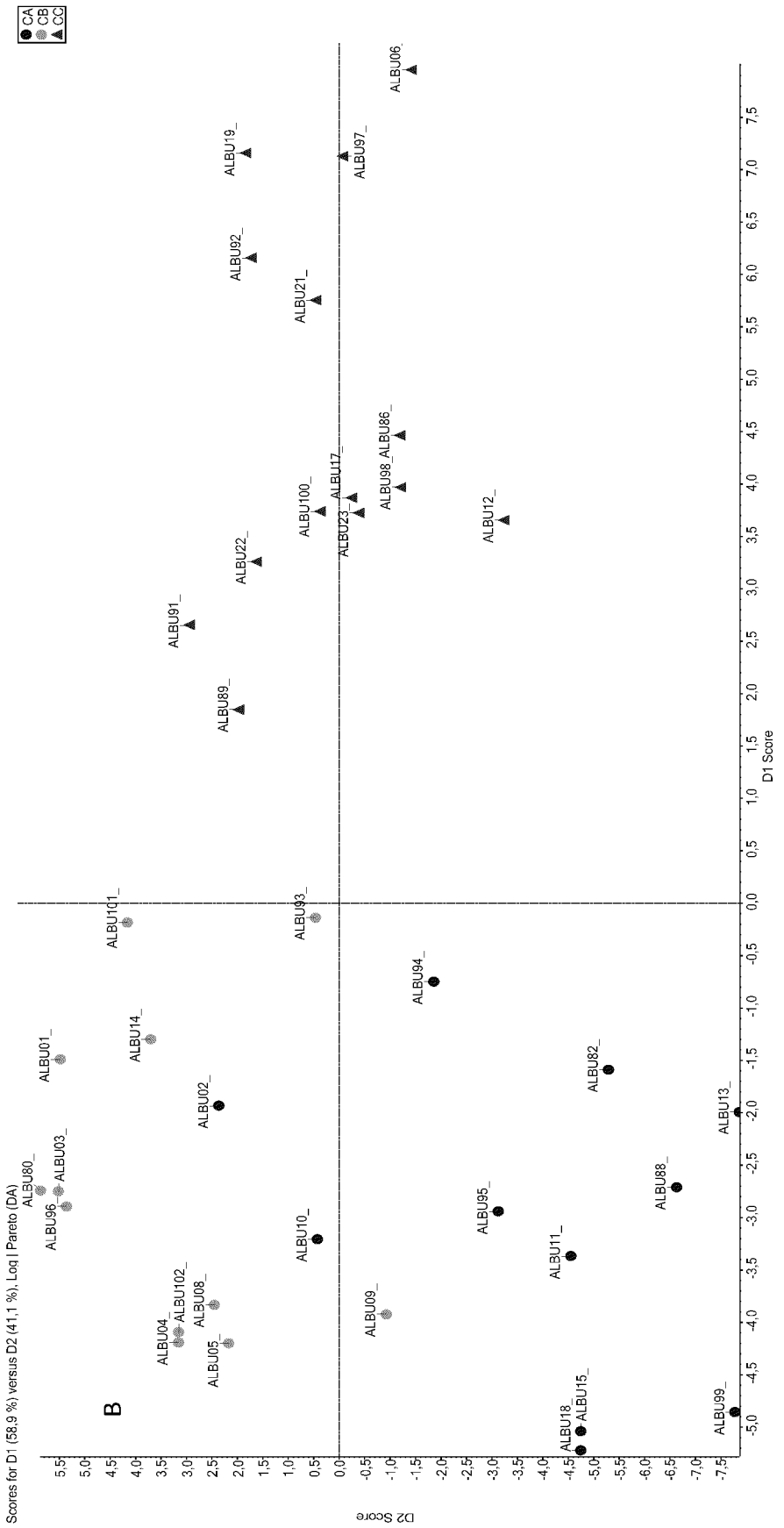
CA
CB
CC
Scores for D1 (58,9 %) versus D2 (41,1 %), Log | Pareto (DA)
B
D1 Score
D2 Score
ALBU01_
ALBU02_
ALBU03_
ALBU04_
ALBU05_
ALBU06_
ALBU08_
ALBU09_
ALBU10_
ALBU11_
ALBU12_
ALBU13_
ALBU14_
ALBU15_
ALBU17_
ALBU18_
ALBU19_
ALBU21_
ALBU22_
ALBU23_
ALBU80_
ALBU82_
ALBU86_
ALBU88_
ALBU89_
ALBU91_
ALBU92_
ALBU93_
ALBU94_
ALBU95_
ALBU96_
ALBU97_
ALBU98_
ALBU99_
ALBU100_
ALBU101_
ALBU102_

Figure 8B

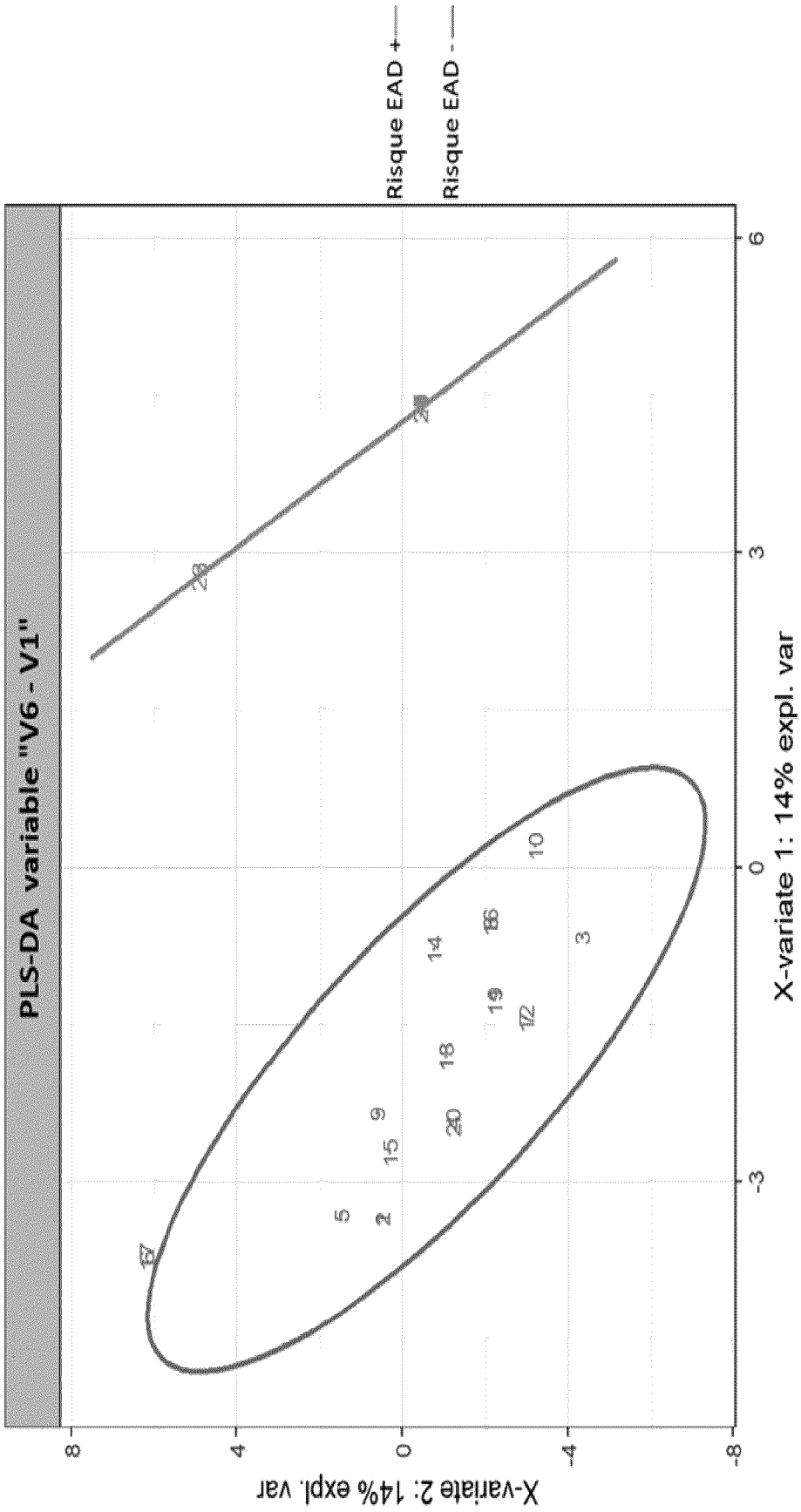
PLS-DA variable "V6 - V1"
Risque EAD +
Risque EAD -
X-variate 1: 14% expl. var
X-variate 2: 14% expl. var
8
4
0
-4
-8
6
3
0
-3

Figure 9

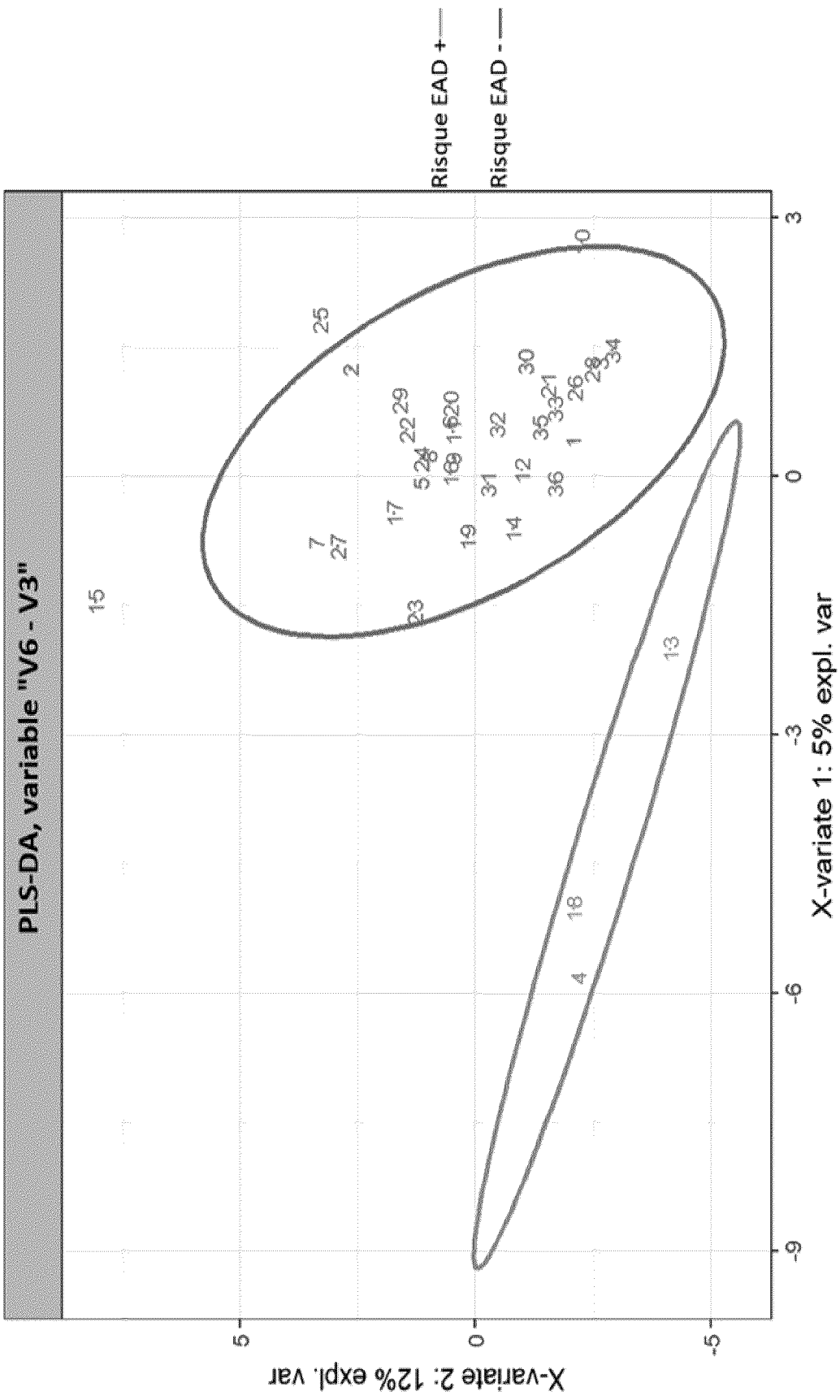
PLS-DA, variable "V6 - V3"
Risque EAD +
Risque EAD -
X-variate 1: 5% expl. var
X-variate 2: 12% expl. var
3
0
-3
-6
-9
5
0
-5

Figure 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/077721

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2022/018852 A1 (EL BALKHI SOULEIMAN [FR] ET AL) 20 January 2022 (2022-01-20)	1,9,10
Y	the whole document paragraphs [0005], [0013] - [0019], [0024], [0032], [0053], [0056] - [0058], [0063], [0066], [0074] - [0078], [0081] - [0083], [0086] - [0098]; claims 1-17; figures 1-8 paragraphs [0102] - [0128], [0130] - [0132]; tables 1-2	2-8, 11-17

-/--

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 October 2023	03/11/2023

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Boiangiu, Clara

3

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Baldassarre Maurizio ET AL: "2058 LIVER FAILURE/CIRRHOSIS/ PORTAL HYPERTENSION ¦ Hepatology Determination of Effective Albumin in Patients With Decompensated Cirrhosis: Clinical and Prognostic Implications", , 1 January 2021 (2021-01-01), pages 2058-2073, XP093032274, DOI: 10.1002/hep.31798/suppinfo Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8518406/pdf/HEP-74-2058.pdf [retrieved on 2023-03-16] the whole document abstract figure 14; tables 1-4 -----	1-17
X	SPINELLA ROSARIA ET AL: "Albumin in chronic liver disease: structure, functions and therapeutic implications", HEPATOLOGY INTERNATIONAL, SPRINGER INDIA, INDIA, vol. 10, no. 1, 29 September 2015 (2015-09-29), pages 124-132, XP035954121, ISSN: 1936-0533, DOI: 10.1007/S12072-015-9665-6 [retrieved on 2015-09-29] cited in the application the whole document -----	1-17
X	SUKANTA DAS ET AL: "Hyperoxidized albumin modulates neutrophils to induce oxidative stress and inflammation in severe alcoholic hepatitis", HEPATOLOGY, JOHN WILEY & SONS, INC, US, vol. 65, no. 2, 19 December 2016 (2016-12-19), pages 631-646, XP071563920, ISSN: 0270-9139, DOI: 10.1002/HEP.28897 cited in the application the whole document ----- -/--	1-17

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARCO DOMENICALI ET AL: "Posttranscriptional changes of serum albumin: Clinical and prognostic significance in hospitalized patients with cirrhosis", HEPATOLOGY, JOHN WILEY & SONS, INC, US, vol. 60, no. 6, 3 November 2014 (2014-11-03), pages 1851-1860, XP071562263, ISSN: 0270-9139, DOI: 10.1002/HEP.27322 cited in the application the whole document	1-17
X	SUN LEJIA ET AL: "Impaired albumin function: a novel potential indicator for liver function damage?", ANNALS OF MEDICINE , vol. 51, no. 7-8 17 November 2019 (2019-11-17), pages 333-344, XP093031849, SE ISSN: 0785-3890, DOI: 10.1080/07853890.2019.1693056 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7877890/pdf/IANN_51_1693056.pdf the whole document	1-17
Y	KLAMMT SEBASTIAN ET AL: "Albumin-binding function is reduced in patients with decompensated cirrhosis and correlates inversely with severity of liver disease assessed by model for end-stage liver disease", EUROPEAN JOURNAL OF GASTROENTEROLOGY AND HEPATOLOGY, LIPPINCOTT WILLIAMS AND WILKINS, UK, vol. 19, no. 3, 1 January 2007 (2007-01-01), pages 257-263, XP009505681, ISSN: 0954-691X, DOI: 10.1097/MEG.0B013E3280101F7D cited in the application the whole document	1-17

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NALDI MARINA ET AL: "Structural and functional integrity of human serum albumin: Analytical approaches and clinical relevance in patients with liver cirrhosis", JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS, ELSEVIER B.V, AMSTERDAM, NL, vol. 144, 18 April 2017 (2017-04-18), pages 138-153, XP085152051, ISSN: 0731-7085, DOI: 10.1016/J.JPBA.2017.04.023 cited in the application the whole document	1-17
Y	US 2015/377883 A1 (OMARY M BISHR [US] ET AL) 31 December 2015 (2015-12-31) the whole document	1-17
Y	JAGDISH RAKESH KUMAR ET AL: "Albumin in Advanced Liver Diseases: The Good and Bad of a Drug!", HEPATOLOGY , vol. 74, no. 5 12 September 2021 (2021-09-12), pages 2848-2862, XP093032262, US ISSN: 0270-9139, DOI: 10.1002/hep.31836 Retrieved from the Internet: URL:https://onlinelibrary.wiley.com/doi/full-xml/10.1002/hep.31836 the whole document	1-17
Y	LEBLANC YANN ET AL: "Human serum albumin presents isoform variants with altered neonatal Fc receptor interactions", PROTEIN SCIENCE , vol. 28, no. 11 18 October 2019 (2019-10-18), pages 1982-1992, XP093033265, US ISSN: 0961-8368, DOI: 10.1002/pro.3733 Retrieved from the Internet: URL:https://onlinelibrary.wiley.com/doi/full-xml/10.1002/pro.3733 the whole document	1-17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/077721

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2023/077721

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2022018852 A1	20-01-2022	EP 3884280 A1	29-09-2021
		US 2022018852 A1	20-01-2022
		WO 2020104458 A1	28-05-2020
US 2015377883 A1	31-12-2015	US 2015377883 A1	31-12-2015
		US 2018031558 A1	01-02-2018