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(71) **Applicants**: **INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)** [FR/FR]; 101 rue de Tolbiac, 75013 PARIS (FR). **UNIVERSITE PARIS SUD 11** [FR/FR]; 15 rue Georges Clémenceau, 91400 ORSAY (FR).

(72) **Inventors**: **LE NAOUR, François**; UMR-S1193 (Inserm/Univ Paris-Sud) 14 avenue Paul Vaillant Couturier, 94800 VILLEJUIF (FR). **CHIAPPINI, Franck**; SAS OTR 3 4 rue Française, 75001 PARIS (FR).

(74) **Agent**: **CABINET NONY**; 11 rue Saint-Georges, 75009 PARIS (FR).

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(54) **Title**: LIPID BIOMARKERS AND COMPOSITIONS

(57) **Abstract**: The present invention relates to the field of diagnosis, prognosis and treatment of liver conditions, including nonalcoholic steatohepatitis (NASH), in a human individual. The inventors have identified herein a lipid signature associated with the occurrence of liver conditions, by combining, in a global approach, lipidomics with an unbiased learning machine statistical analysis. Thus, the inventors provide herein a lipid signature, comprising a plurality of lipids selected from a group of up -regulated lipids and a group of down-regulated lipids. This plurality of lipids is further involved in the pathogenesis of liver conditions. Thus, according to another aspect, the invention also relates to said plurality of lipids, for medical uses and for the preparation of a medicament.

TITLE OF THE INVENTION

Lipid biomarkers and compositions

FIELD OF THE INVENTION

The present invention relates to the field of diagnosis, prognosis and treatment of liver conditions, including nonalcoholic steatohepatitis (NASH), in a human individual.

BACKGROUND OF THE INVENTION

Non-alcoholic fatty liver disease (NAFLD) is a pathological condition exhibiting a wide spectrum of lesions, from nonalcoholic fatty liver (NAFL) also called steatosis to nonalcoholic steatohepatitis (NASH). It is also established that NASH may progress to hepatic fibrosis, cirrhosis and hepatocellular carcinoma (HCC).

NAFLD is a systemic disease associated with obesity, insulin resistance, type 2 diabetes mellitus and the metabolic syndrome. The dramatic increase in such incidences that concern currently more than 1 billion individuals, makes NAFLD the most common cause of chronic liver disease in Western countries and a major public health problem worldwide.

The hallmark of fatty liver disease is the intra-cellular accumulation of lipids in particular triglycerides (TG) resulting in the formation of lipid droplets in hepatocytes. This accumulation results from an imbalance between uptake, synthesis, export and oxidation of fatty acids. Fatty liver is a reversible and asymptomatic lesion that has long been considered as benign. However, it is now admitted that fatty liver is a precursor for steatohepatitis morphologically defined by the presence of steatosis, ballooning of hepatocytes, Mallory's bodies and lobular inflammation with infiltrated macrophages and leukocytes on liver histology.

In agreement with the assumption, it has been demonstrated using transcriptomic analysis that genes involved in inflammatory processes were significantly up-regulated in patients with bland steatosis (**Chiappini *et al.***; Exploration of global gene expression in human liver steatosis by high-density oligonucleotide microarray. *Lab Invest.* 2006; 86(2):154-165).

This has led to the idea that NAFL is not benign (**Adams & Ratziu**; Non-alcoholic fatty liver - perhaps not so benign. *J Hepatol.* 2015; 62(5):1002-1004).

Also, it has been proposed that NAFLD may be associated with numerous changes in the lipid composition of the liver (**Puri *et al.***; A Lipidomic Analysis of Nonalcoholic Fatty Liver Disease. Hepatology. 2007; 46(4):1081-1090).

However, none of these studies was able to characterize a specific lipid signature of NASH. Therefore, the identification of lipids with potential toxicity related to the progression of fatty liver diseases, including NASH, is still an unmet need.

In particular, there remains a need for methods and tools for determining the occurrence of a nonalcoholic steatohepatitis (NASH) condition.

There also remains a need for methods and tools for discriminating between a nonalcoholic fatty liver (NAFL) condition and a nonalcoholic steatohepatitis (NASH) condition.

There also remains a need for methods for the diagnosis and prognosis of liver diseases, including nonalcoholic steatohepatitis (NASH) condition.

There also remains a need for methods for the screening of compounds for treating, preventing, or reducing the likelihood of occurrence of liver diseases.

There also remains a need for methods for treating or preventing, or reducing the likelihood of occurrence of liver diseases.

The invention has for purpose to meet the aforementioned needs.

SUMMARY OF THE INVENTION

The invention relates to a method for determining the occurrence of a fatty liver disease, in particular of a nonalcoholic steatohepatitis (NASH) condition, in a human individual.

Thus, the invention also relates to a method for determining the occurrence of a fatty liver disease, in particular of a nonalcoholic steatohepatitis (NASH) condition in a human individual, comprising the steps of:

a) determining in a biological sample from said individual the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0),

TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0); and/or

- a set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2, phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1);

b) comparing each lipid level determined at step a) with a reference value;

c) determining the occurrence of a fatty acid liver disease in said individual based on the comparison of step b).

The invention also relates to a method for discriminating between a nonalcoholic fatty liver (NAFL) condition and a nonalcoholic steatohepatitis (NASH) condition in a human individual.

In particular, the invention relates to a method for discriminating between a nonalcoholic fatty liver (NAFL) condition and a nonalcoholic steatohepatitis (NASH) condition in a human individual, comprising the steps of:

a) determining in a biological sample from said individual the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-

7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0); and/or

- a set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2, phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1);

b) comparing each lipid level determined at step a) with a reference value;

c) determining the occurrence of a NASH or NAFL condition in said individual based on the comparison of step b).

The methods disclosed herein may further comprise determining in said biological sample, a lipid level of at least one lipid selected from the group consisting of:

- a set of **up-regulated** lipids selected from: diglyceride (16:16) or DG (16:16); and/or

- a set of **down-regulated** lipids selected from: PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0).

Accordingly, the invention also relates to a method for determining the occurrence of a fatty liver disease, in particular of a nonalcoholic steatohepatitis (NASH) condition in a human individual, comprising the steps of:

a) determining in a biological sample from said individual the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0),

TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0), diglyceride (16:16) or DG (16:16); and/or

- a set of **down-regulated** lipids selected from: PC(16:0/18:0), PC(16:0/18:1), PC(18:0/18:1), PC(18:0/20:3), PC(18:0/20:4), PC(18:2/18:2), PE(16:1/16:1), PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), PS(18:0/20:3), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0);

b) comparing each lipid level determined at step a) with a reference value;

c) determining the occurrence of a fatty acid liver disease in said individual based on the comparison of step b).

Accordingly, the invention also relates to a method for discriminating between a nonalcoholic fatty liver (NAFL) condition and a nonalcoholic steatohepatitis (NASH) condition in a human individual.

In particular, the invention relates to a method for discriminating between a nonalcoholic fatty liver (NAFL) condition and a nonalcoholic steatohepatitis (NASH) condition in a human individual, comprising the steps of:

a) determining in a biological sample from said individual the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0), diglyceride (16:16) or DG (16:16); and/or

- a set of **down-regulated** lipids selected from: PC(16:0/18:0), PC(16:0/18:1), PC(18:0/18:1), PC(18:0/20:3), PC(18:0/20:4), PC(18:2/18:2), PE(16:1/16:1), PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), PS(18:0/20:3), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0);

b) comparing each lipid level determined at step a) with a reference value;

c) determining the occurrence of a NASH or NAFL condition in said individual based on the comparison of step b).

The invention also relates to a plurality of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2, phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1); for use for treating, preventing, or reducing the likelihood of the occurrence of a fatty liver disease, in particular a nonalcoholic steatohepatitis (NASH) condition, in an individual.

The plurality of **down-regulated** lipids may further comprise at least one lipid selected from: PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0).

The invention also relates to a plurality of **down-regulated** lipids selected from: PC(16:0/18:0), PC(16:0/18:1), PC(18:0/18:1), PC(18:0/20:3), PC(18:0/20:4), PC(18:2/18:2), PE(16:1/16:1), PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), PS(18:0/20:3), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0); for use for treating, preventing, or reducing the likelihood of the occurrence of a fatty liver disease, in particular a nonalcoholic steatohepatitis (NASH) condition, in an individual.

The invention also relates to a method for screening compounds for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising at least the steps of:

a) providing a biological sample from an individual (i) to whom at least one candidate compound has been administered, or alternatively (ii) wherein said sample was brought into contact with at least one candidate compound;

b) determining in said biological sample the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0); and/or

- a set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2), phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1);

c) comparing each lipid level determined at step b) with a reference value;

d) selecting the candidate compound as suitable for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease based on the comparison of step c).

The method for screening compounds disclosed herein may further comprise determining in said biological sample, a lipid level of at least one lipid selected from the group consisting of:

- a set of **up-regulated** lipids selected from: diglyceride (16:16) or DG (16:16); and/or

- a set of **down-regulated** lipids selected from: PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0).

The invention also relates to a method for screening compounds for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising at least the steps of:

a) providing a biological sample from an individual (i) to whom at least one candidate compound has been administered, or alternatively (ii) wherein said sample was brought into contact with at least one candidate compound;

b) determining in said biological sample the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0), diglyceride (16:16) or DG (16:16); and/or

- a set of **down-regulated** lipids selected from: PC(16:0/18:0), PC(16:0/18:1), PC(18:0/18:1), PC(18:0/20:3), PC(18:0/20:4), PC(18:2/18:2), PE(16:1/16:1), PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), PS(18:0/20:3), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0);

c) comparing each lipid level determined at step b) with a reference value;

d) selecting the candidate compound as suitable for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease based on the comparison of step c).

The invention also relates to a method for screening compounds for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising at least the steps of:

a) providing a biological sample from an individual (i) to whom at least one candidate compound has been administered, or alternatively (ii) wherein said sample was brought into contact with at least one candidate compound;

b) determining in said biological sample (i) an expression of a nucleic acid encoding Fatty Acid Desaturase 1 (FADS1), and/or (ii) a content of FADS1, and/or (iii) an activity of FADS1;

c) comparing the expression, content and/or activity determined at step b) with a reference value;

d) selecting the candidate compound as suitable for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease based on the comparison of step c).

The invention also relates to a mixture of lipids comprising, or consisting of, a plurality selected from: (C(14:0);C(16:0); C(18:0); C(16:1n-7); C(18:1n-7); and C(18:1n-9).

The invention also relates to a mixture of lipids comprising, or consisting of, a plurality selected from: ceramide (d18:1/20:0) or CE(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2), phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1).

The mixture of lipids may further comprise at least one lipid selected from: PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0).

The invention also relates to a mixture of lipids comprising, or consisting of, a plurality selected from: PC(16:0/18:0), PC(16:0/18:1), PC(18:0/18:1), PC(18:0/20:3), PC(18:0/20:4), PC(18:2/18:2), PE(16:1/16:1), PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), PS(18:0/20:3), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A-C: Hepatic levels of the up-regulated lipids discriminating NASH. 18 fatty acids are up-regulated in the NASH group. Data are represented as boxplot. *p<0.05, **p<0.01 and ***p<0.001 by unpaired *t*-test compared to the 4 other groups. +p<0.05 by unpaired *t*-test compared to Control and NAFL1 groups. ‡p<0.05 by unpaired *t*-test compared to Control, NAFL1 and NAFL2 groups. Unpaired *t*-test was done after ANOVA test. CE: cholesteryl ester, NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis; TG: triglyceride.

Figure 2: Hepatic levels of down-regulated lipids discriminating NASH. Summary of the 45 down-regulated phospholipids discriminating NASH group. Phosphatidylcholines: PC(14:0/14:0) + PC(14:0/16:0) + PC(16:0/16:0) + PC(16:0/18:0) + PC(16:0/18:1) + PC(16:1/16:1) + PC(16:0/18:2) + PC(16:1/18:2) + PC(18:0/18:1) + PC(18:0/20:2) + PC(18:0/20:3) + PC(18:0/20:4) + PC(18:0/22:3) + PC(18:1/18:1) + PC(18:1/18:2) + PC(18:2/18:2); Phosphatidylethanolamines: PE(16:0/16:0) + PE(16:1/16:1) + PE(18:0/18:1) + PE(18:1/18:1) + PE(18:0/20:2) + PE(18:0/22:6) + PE(18:1/20:4) + PE(18:2/20:4); Phosphatidylserines: PS(16:0/18:0) +PS(16:0/18:2) + PS(18:0/20:2) + PS(18:0/20:3) + PS(18:1/18:1) + PS(18:1/18:2); Phosphatidylinositols: PI(18:0/18:1) + PI(18:0/20:1) + PI(18:0/22:3) + PI(18:1/20:4); Sphingomyelins: SM(18:1/14:0) + SM(18:1/16:0) + SM(18:1/16:1) + SM(18:1/18:0) + SM(18:1/18:1) + SM(18:1/20:0) + SM(18:1/20:1) + SM(18:1/22:0) + SM(18:1/22:1).

Figure 3A-E: Activities of elongases and desaturases in NAFLD patients and murine models. In order to evaluate enzyme activities, a ratio between product to substrate for each reaction has been used in human groups (left panel) and mouse models (right panel). **(A)** Evaluation of ELOVL5 activity using C20:2n-6 to C18:2n-6 ratio, **(B)** Evaluation of ELOVL6 activity using C18:0 to C16:0 ratio, **(C)** evaluation of FADS2 activity using C18:3n-3 to C18:2n-6 ratio, **(D)** evaluation of SCD1 activity using C16:1 to C16:0 ratio and **(E)** evaluation of FADS1 activity using C20:4n-6 to C20:3n-6 ratio. Data are shown as means ± SEM. *p<0.05 by unpaired *t*-test compared to each other groups, +<0.05 by unpaired *t*-test compared to Control and §p<0.05 by unpaired *t*-test compared to Control, NAFL1 and NAFL2 after ANOVA analysis. Control patients n=7; NAFL1 n=9; NAFL2 n=11; NAFL3 n=12; NASH n=13. Control mice n=10; HFD n=5; MCD n=5. ELOVL: elongase of very long chain fatty acid; FADS: fatty acid desaturase; FASN: fatty acid synthase; HFD: high fat diet; MCD diet: methionine-choline deficient diet; NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis; SCD: stearoyl-CoA desaturase.

Figure 4A-C: Levels of eicosanoid precursors in NAFLD patients and murine models. Hepatic levels of **(A)** arachidonic acid (C20:4n-6), **(B)** eicosapentanoic acid (C20:5n-3), and **(C)** docosahexaenoic acid (C22:6n-3) in patients (left panel) and mouse models (right panel). Data are means ± SEM. *p<0.05, by unpaired *t*-test compared to Control group and †p<0.05, by unpaired *t*-test compared to NAFL groups after ANOVA analysis. Patients: Control n=7; NAFL1 n=9; NAFL2 n=11; NAFL3 n=12; NASH n=13. Murine models: Control n=10; High fat diet (HFD) n=5; Methionine choline deficient (MCD) diet n=5 mouse males. NAFL: nonalcoholic fatty liver; NASH nonalcoholic steatohepatitis.

Figure 5A-H: Gene expression of elongases and desaturases involved in fatty acid synthesis. **(A)** *ELOVL5,* **(B)** *ELOVL6*, **(C)** *FADS2*, **(D)** *SCD1*, **(E)** *FADS1,* **(F)** *SREBP1c*, **(G)** *FASN and* **(H)** *ACC1* gene expression from human liver biopsies analyzed by RT-Q-PCR. Data are mean ± SEM. Control patients (n=6), NAFL2 patients (n=6) were matched to NASH patients (n=8). NASH are matched to NAFL2 regarding to the total lipid content and no difference in age, gender and BMI between the two groups. *p≤ 0.05

compared to Control, †p≤0.05 compared to NAFL2 by unpaired *t*-test. ACC: Acetyl-CoA Carboxylase; ELOVL: elongase of very long-chain; FADS: fatty acid desaturase; FASN: fatty acid synthase; NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis; SCD: stearoyl-CoA desaturase.

Figure 6A-B: Expression of genes involved in cholesterol biosynthesis in human livers. (A) *SREBP2* and **(B)** *HMGCR* genes expression assessed in human liver biopsies assessed by RT-Q-PCR from control patients (Control n=6), NAFL2 patients (n=6) matching the same grade of triglycerides that the patients with NASH (n=8). Data are mean ± SEM. *p≤ 0.05 compared to Control, †p≤0.05 compared to NAFL2 by unpaired *t*-test. HMGCR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; NAFL: non-alcoholic fatty liver; NASH: non-alcoholic steatohepatitis; SREBP: sterol regulatory element binding protein.

Figure 7: Membrane integrity surrogate and distribution of lipids by mass spectrometry imaging. (A) Membrane integrity was evaluated by the ratio phosphatidylcholine (PC) to phosphatidylethanolamine (PE) in patients (left panel) and mouse models (right panel). Data are means ± SEM. *p<0.05, by unpaired *t*-test compared to Control group and †p<0.05, by unpaired *t*-test compared to NAFL groups after ANOVA analysis. Patients: Control n=7; NAFL1 n=9; NAFL2 n=11; NAFL3 n=12; NASH n=13. Murine models: Control n=10; High fat diet (HFD) n=5; Methionine choline deficient diet (MCD) n=5 mouse males. NAFL: nonalcoholic fatty liver; NASH nonalcoholic steatohepatitis.

Figure 8: Toxicity of lipid mixtures on human hepatoma cells and primary hepatocytes. Percentage of toxicity over control cells (non-treated) assessed by total ATP content into HepG2 human hepatoma cell line and human primary hepatocytes. **(A)** HepG2 cell and **(B)** human primary hepatocytes treated with individual lipids at different concentrations. **(C)** HepG2 cell and **(D)** human primary hepatocytes treated with lipid mixes at different concentrations. Cells are treated in triplicate during 24h with 50, 100, 250, 500 and 1000 µM final concentration of lipids: myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1n-7), vaccenic acid (C18:1n-7), oleic

acid (18:1n-9) or quadruplets with lipid mixes (Control Mix, NAFLD Mix, NASH Mix) based on the percentage of C14:0, C16:0, C18:0, C16:1n-7, C18:1n-7, C18:1n-9 found in liver tissues of control, NAFL2/3 and NASH patients. Two independent experiments were done. Data are mean ± SEM. *p<0.05 by unpaired *t*-test compared to Control Mix and NAFL Mix at the same concentration, after ANOVA analysis. NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis.

Figure 9: Total hepatic triglycerides clustered the four groups of patients with different grades of NAFL based on cluster analysis regression tree (CART). (A) Data are represented as a regression tree where each node of the tree corresponds to the total triglycerides (TG): Ranges of Total TG liver content determine the four groups of patients. **(B)** Total TG content from human liver biopsies in clustered patients based on the CART analysis. Data are represented as boxplot. *p<0.05, by unpaired *t*-test compared to each other groups after ANOVA analysis. Control n=7; NAFL1 n=9; NAFL2 n=11; NAFL3 n=12. NAFL: nonalcoholic fatty liver.

Figure 10: Decision tree for discriminating between five groups of individuals. The five groups consist, from left to right, in control, NAFL1, NAFL2, NAFL3 and NASH groups. The critical thresholds for discriminating between each group are indicated for each node in nmol/mg of tissue. TG: triglycerides. PI: phosphatidylinositol. The series of numbers below each group (in the format X/X/X/X/X) indicate the number of individuals belonging to each leaf. Overall, of the eighteen tested fatty acids, three (TG(16:0/16:0/16:0)TG(16:0/16:0/18:0)PI(18:0/18:0) are sufficient for discriminating between the five groups.

Figure 11: Decision tree for discriminating between three groups of individuals. The three groups consist, from left to right, in control, NAFL and NASH groups. The critical thresholds for discriminating between each group are indicated for each node in nmol/mg of tissue. TG: triglycerides. PI: phosphatidylinositol. The series of numbers below each group (in the format X/X/X) indicate the number of individuals belonging to each leaf. Overall, of the eighteen tested fatty acids, two (TG(16:0/16:0/16:0) PI(18:0/18:0) are sufficient for discriminating between the three groups.

Figure 12: Decision tree for discriminating between five groups of individuals. The five groups consist, from left to right, in control, NAFL1, NAFL2, NAFL3 and NASH groups. The critical thresholds for discriminating between each group are indicated for each node in nmol/mg of tissue. C: fatty acids. PI: phosphatidylinositol. The series of numbers below each group (in the format X/X/X/X/X) indicate the number of individuals belonging to each leaf. Overall, of the thirteen tested fatty acids (not including the five triglycerides), four (C18:3n-3 ; C16:0 ; C18:1n-9 ; PI(18:0/18:0)) are sufficient for discriminating between the five groups.

Figure 13: Decision tree for discriminating between three groups of individuals. The three groups consist, from left to right, in control, NAFL and NASH groups. The critical thresholds for discriminating between each group are indicated for each node in nmol/mg of tissue. C: fatty acids. PI: phosphatidylinositol. The series of numbers below each group (in the format X/X/X) indicate the number of individuals belonging to each leaf. Overall, of the thirteen tested fatty acids, two (C18:2n-6 ; PI(18:0/18:0)) are sufficient for discriminating between the three groups.

Figure 14: ROC curve analysis of a set of fatty acid markers. The ROC curve provides on the X-axis and Y axis, respectively the specificity and sensitivity rate. The X-axis scale is from 1.0 to 0.0; and the Y-axis scale is from 0.0 to 1.0. Fatty acids are describes according to the usual nomenclature **(A)** comparison control vs 3 grades of steatosis "NAFL1, NAFL2 and NAFL3". **(B)** comparison control and 3 grades of steatosis "NAFL1, NAFL2 and NAFL3" vs NASH.

Figure 15: ROC curve analysis of a set of cholesterol, cholesterol ester compounds, triglycerides and phosphatidylinositol markers. The ROC curve provides on the X-axis and Y axis, respectively the specificity and sensitivity rate. The X-axis scale is from 1.0 to 0.0; and the Y-axis scale is from 0.0 to 1.0. TG: triglycerides. PI: phosphatidylinositol. CE: cholesterol ester. **(A)** comparison control vs 3 grades of steatosis "NAFL1, NAFL2 and NAFL3". **(B)** comparison control and 3 grades of steatosis "NAFL1, NAFL2 and NAFL3" vs NASH.

Figure 16: Activities of elongases and desaturases in NAFLD patients. **(A)** Evaluation of ELOVL5 activity using C22:5n-6 to C20:5n-3; **(B)** Evaluation of FADS2 activity using C18:3n-6 to C18:2n-6 ratio; ratio, **(C)** Evaluation of SCD1 using C18:1n-9 to C18:0 ratio **(D)** both ELOVL5 and ELOVL6 activities using C18:1n-7 to C16:1n-7; **(E)** global ELOVL5, ELOVL6 and SCD1 activities using C18:1n-7 to C16:0 ratio; **(F)** both ELOVL5 and FADS1 activities using C20:4n-6 to C18:3n-6 ratio and **(G)** global ELOVL5, FADS1 and FADS2 activities using 20:4n-6 to C18:2n-6 ratio towards the n-6 pathway . **(H)** Hepatic n-6 to n-3 ratio and n-3 index from the different study groups. In order to evaluate enzyme activities, a ratio between product to precursor of each reaction has been used**.** Data are shown as means ± SEM. *p<0.05 by unpaired *t*-test compared to each other groups ‡<0.05 by unpaired *t*-test compared to Control, NAFL1 and NAFL2, +<0.05 by unpaired *t*-test compared to Control and NAFL1, †<0.05 by unpaired *t*-test compared to Control, NAFL2 and NAFL3 after ANOVA analysis. ELOVL: elongase of very long chain fatty acid; FADS: fatty acid desaturase; NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis; stearoyl CoA desaturase.

Figure 17: Metabolic pathway leading to fatty acids, eicosanoids and phospholipids biosynthesis. The long chain saturated fatty acids and unsaturated fatty acids of the n-3, n-6, n-7 and n-9 series can be synthesized from lauric acid (C12:0), myristic acid (C14:0) and palmitic acid (C16:0) produced by ACC and FASN. ACC: acetyl-CoA carboxylase; ELOVL: elongase of very long chain fatty acid; FASN: fatty acid synthase; FADS: fatty acid desaturase; SCD: stearoyl-CoA desaturase.

DETAILED DESCRIPTION OF THE INVENTION

The inventors have now established a lipid signature for the prognosis and follow-up of fatty liver diseases, including nonalcoholic fatty liver diseases (NAFLD), based on the quantification of a plurality of lipids belonging to a selection of 63 lipids.

This selection of 63 lipids is defined hereafter as the "*lipid signature*".

Narrower and/or distinct selections of lipid markers are further disclosed.

Additional, novel, lipid markers are further disclosed.

Also, a narrower selection of 32 lipids is disclosed herein, which includes those additional lipid markers.

Accordingly, the determination of lipid levels of a plurality (two or more) of lipids belonging to this lipid signature provides good specificity and sensitivity for detecting the occurrence of fatty acid diseases in an individual.

The identification of such a complex signature highlights the major interest of combining a global approach as lipidomics with an unbiased learning machine statistical approach such as random forest. Indeed, none of the lipids identified allowed by itself (alone) the discrimination of NAFL or NASH. In contrast, this complex lipid signature allows to discriminate the grades of nonalcoholic fatty liver (NAFL) as well as nonalcoholic steatohepatitis (NASH). The robustness of the lipid signature of NASH is further underlined by 100% specificity and 100% sensitivity on samples from two independent hospitals centers.

Thus, the inventors provide herein a lipid signature, comprising a plurality of lipids selected from a group of up-regulated lipids and a group of down-regulated lipids.

Accordingly, the inventors provide herein methods which include a step of determining, in a biological sample from an individual, the lipid levels for a plurality of lipids belonging to said lipid signature.

Most of the lipids belonging to this lipid signature share the common unifying feature of being associated to the metabolic pathway disclosed in **figure 17**, involving the desaturase FADS1.

Dysregulations of the metabolic pathway involved in synthesis of fatty acids are especially highlighted in NASH. The major impact of alterations in this metabolic pathway in the pathology is consistent with the similar biochemical features observed in human and in animal models by using mice fed with HFD and MCD diet.

Without wishing to be bound by the theory, the inventors are of the opinion that the decreased activity of the desaturase FADS1 creates a bottleneck leading to the accumulation upstream of fatty acids up to 20 carbons. This phenomenon is accentuated by increased *de novo* fatty acids synthesis as demonstrated by the increase in expression levels of *acetyl-CoA carboxylase 1 (ACC1)* and *fatty acid synthase (FASN).*

In addition, the decreased expression and activity of the elongase ELOVL6 also contributes to the marked increase of long-chain fatty acids culminating in NASH with the accumulation of myristic acid (C14:0). On the other hand, a consequence of the impaired expression and activity of the desaturase FADS1 is the extremely low amount of very long-chain polyunsaturated fatty acids added to the hepatic imbalance between n-6 and n-3 levels.

Indeed, the results demonstrate that n-6 to n-3 ratio is significantly increased, and associated with a significant decrease in n-3 index in livers of patients who suffer from NASH as well as in mice fed MCD diet.

Also, without wishing to be bound by the theory, the inventors are of the opinion that concomitant increase of saturated and unsaturated long-chain fatty acids with the significant decrease of very long-chain polyunsaturated fatty acids are resulting from the addition of several metabolic abnormalities.

Accordingly, the inventors are of the opinion that metabolic disorders, and thus the resulting lipid signature, constitute at least an early event in the progression of fatty liver diseases towards NASH.

Indeed, such metabolic alterations may generate broad effects since very long-chain polyunsaturated fatty acids represent substrates for the synthesis of eicosanoids and further phospholipids, thus impacting the properties of membranes. The very long-chain polyunsaturated fatty acids serve also as substrate precursors for the biosynthesis of lipid signaling molecules with either pro-inflammatory or anti-inflammatory properties. Therefore, the current study strengthens the central role of FADS1 in lipid homeostasis and positions this desaturase as a major player in NASH, thus leading to the identification of said lipid signature. It is also why FADS1 can be established as a therapeutic target, and why the increase of (i) the expression of a nucleic acid encoding FADS1, and/or (ii) the content of FADS1, and/or (iii) the activity of FADS1, is indicative of the efficiency of a candidate compound for treating, preventing, or reducing the likelihood of the occurence of a fatty liver disease.

According to a first embodiment, the invention relates to a method for determining the occurrence of a fatty liver disease in a human individual, comprising the steps of:

a) determining in a biological sample from said individual the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0); and/or

- a set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2, phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1);

b) comparing each lipid level determined at step a) with a reference value;

c) determining the occurence of a fatty liver disease in said individual based on the comparison of step b).

In the sense of the invention, a "*fatty liver disease*" (FLD), or "*hepatic steatosis*" encompasses both "*nonalcoholic fatty liver disease*" (NAFLD) and "*alcoholic fatty liver disease*".

According to a particular embodiment, the fatty liver disease is a nonalcoholic fatty liver disease (NAFLD).

NAFLD is an evolutive condition which may encompass different forms of lesions, ranging from simple steatosis (also referred herein as "*nonalcoholic fatty liver*", or NAFL) to nonalcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC).

According to a more particular embodiment, the fatty liver disease that is considered is a nonalcoholic fatty liver (NAFL) or a nonalcoholic steatohepatitis (NASH) condition.

According to a preferred embodiment, the fatty liver disease that is considered is a nonalcoholic steatohepatitis (NASH) condition.

According to a second embodiment, the invention relates to a method for discriminating between a nonalcoholic fatty liver (NAFL) condition and a nonalcoholic steatohepatitis (NASH) condition in a human individual, comprising the steps of:

a) determining in a biological sample from said individual the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0); and/or

- a set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2, phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0),

SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1);

b) comparing each lipid level determined at step a) with a reference value;

c) determining the occurrence of a NASH or NAFL condition in said individual based on the comparison of step b).

In the sense of the invention, "*determining the occurrence of a disease*" also encompasses "*detecting the* occurrence *of a disease*", "*determining the prognosis of a disease*", and "*determining the evolution of a disease*".

In the sense of the invention, "*discriminating between one disease and another*" also encompasses "*determining the likelihood of occurrence of one disease over another*".

In the sense of the invention, a "*lipid level*" represents the concentration of a given lipid in the biological sample. A lipid level is generally expressed either in nmol/mg of tissue (i.e. when the biological sample is a solid, such as a liver biopsy), or in nmol/ml (i.e. when the biological sample is a fluid, such as a blood sample), or in nmol/mg of protein for a sample (i.e. solid or fluid) in which the total amount of protein is determined.

In the sense of the invention, "*determining a lipid level*" also encompasses "*measuring a lipid level*". Methods for determining a lipid level are further detailed hereafter, and in the examples.

In the sense of the invention, "*at least one*", such as in "*at least one lipid*", may encompass "*one*" or a "*plurality*".

In the sense of the invention, a "*plurality*" means "*more than one*", or "*two or more*". Accordingly, a plurality (i.e. of lipids) encompasses 2, or more than 2, which includes 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, and, if applicable, more than 63.

In the sense of the invention, "*comprising*" encompasses "*consisting of*" and "*consisting essentially of*".

In the sense of the invention, a "*reference value*" refers to the expected lipid level in the biological sample of an individual that is either (i) a control individual which is not affected with a fatty liver disease, or (ii) an individual for whom the occurrence of a

given fatty liver disease is known or detectable. By extension, a reference value can be determined either from a single individual, or from a group of individuals.

The individuals which are considered herein are preferably human individuals.

In the sense of the invention, an "*individual having a NASH disease*" is defined by a nonalcoholic fatty liver disease activity score (NAS) superior to 5, as taught in **Kleiner et al.** (Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005; 41(6):1313-1321) and **Brunt et al**. (Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology*. 2011; 53(3):810-820).

In the sense of the invention, an "*individual having a NAFL disease*" can be characterized according to the total amount of triglycerides (TG) in a liver biopsy. Accordingly, this term may encompass 3 groups of NAFL individuals, namely:

- NAFL1 (having a total amount of TG ranging from about 41.7 to about 220 nmol/mg of protein);
- NAFL2 (having a total amount of TG ranging from about 220 to about 465.5 nmol/mg of protein);
- NAFL3 (having a total amount of TG superior to about 465.5 nmol/mg of tissue).

Plurality of Lipids – particular selections

Herein are described selections of lipids for which the lipid levels and/or lipid ratios can be determined in the methods of the invention. All the combinations of lipids which are disclosed in the Examples, are also explicitly considered for the methods of the invention.

According to one particular embodiment, the plurality of lipids comprises **at least one** lipid selected from the set of **up-regulated** lipids selected from: cholesterol, cholesteryl ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid

C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0).

According to one more particular embodiment, the plurality of lipids comprises **at least one** lipid selected from the set of **up-regulated** lipids selected from: cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0).

According to another particular embodiment, the plurality of lipids comprises **at least one** lipid selected from the set of **up-regulated** lipids selected from: cholesterol ester (16:0) or CE(16:0), CE(18:0), saturated fatty acid C(14:0), C(16:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0).

According to one particular embodiment, the plurality of lipids comprises **at least one** lipid selected from the set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2, phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1).

According to one more particular embodiment, the plurality of lipids comprises **at least one** lipid selected from the set of **down-regulated** lipids selected from: PC(16:0/18:0), PC(16:0/18:1), PC(18:0/20:3), PC(18:0/20:4), PC(18:2/18:2), PE(16:1/16:1), PS(18:0/20:3), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0).

According to one particular embodiment, the plurality of lipids comprises **at least one** lipid selected from the set of **up-regulated** lipids as defined above; and **at least one** lipid selected from the set of **down-regulated** lipids as also defined above.

For instance, according to one embodiment, the plurality of lipids comprises **at least one** lipid selected from the set of **up-regulated** lipids selected from: cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0); and

at least one lipid selected from the set of **down-regulated** lipids selected from: PC(16:0/18:0), PC(16:0/18:1), PC(18:0/20:3), PC(18:0/20:4), PC(18:2/18:2, PE(16:1/16:1), PS(18:0/20:3), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0).

According to some embodiments, the selection of a plurality of lipids may further comprise additional lipids, selected from:

- a set of **up-regulated** lipids selected from: diglyceride (16:16) or DG (16:16); and/or

- a set of **down-regulated** lipids selected from: PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0)

Narrower selections of **up-regulated** and **down-regulated** lipids are further disclosed here below.

According to one particular embodiment, the plurality of lipids is at least selected from the set of **up-regulated** lipids selected from: cholesterol, cholesteryl ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0), and optionally at least another **up-regulated** or **down-regulated** lipid as defined above, and here below..

According to one particular embodiment, the plurality of lipids is at least selected from the set of **up-regulated** lipids selected from: cholesterol, cholesterol ester

(16:0) or CE(16:0), CE(18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0), and optionally at least another **up-regulated** or **down-regulated** lipid as defined above, and here below.

According to one particular embodiment, the plurality of lipids is **at least** selected from the set of **up-regulated** lipids selected from (or consists of): saturated fatty acid C(14:0) and phosphatidyl-inositol (18:0/18:0), and optionally at least another **up-regulated** or **down-regulated** lipid as defined above, and here below.

This includes the plurality of lipids selected from: saturated fatty acid C(14:0), and phosphatidyl-inositol (18:0/18:0); and at least one selected from: cholesterol ester CE(16), CE(18), TG(14:0/16:0/16:0).

According to one particular embodiment, the plurality of lipids is selected at least from the set of **up-regulated** lipids selected from: TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), C(16:0), unsaturated fatty acid C(16:1n-7), C(18:1n-9), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0), and optionally at least another **up-regulated** or **down-regulated** lipid as defined above, and further below.

According to one particular embodiment, the plurality of lipids is selected at least from the set of **up-regulated** lipids selected from: saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), and C(18:1n-9), and optionally at least another **up-regulated** or **down-regulated** lipid as defined above, and further below.

According to one particular embodiment, the plurality of lipids is at least selected from the set of **up-regulated** lipids selected from: C(18:2n-6) and PI(18:0/18:0), and optionally at least another **up-regulated** or **down-regulated** lipid as defined above, and further below.

According to one particular embodiment, the plurality of lipids is at least selected from the set of **up-regulated** lipids selected from: C(18:3n-3), C(16:0), C(18:1n-9) and PI(18:0/18:0), and optionally at least another **up-regulated** or **down-regulated** lipid as defined above, and further below.

According to one particular embodiment, the plurality of lipids is at least selected from the set of **up-regulated** lipids selected from: TG(16:0/16:0/16:0),

TG(16:0/16:0/18:0) and PI(18:0/18:0), and optionally at least another **up-regulated** or **down-regulated** lipid as defined above, and further below.

According to one particular embodiment, the plurality of lipids is selected from the set of **up-regulated** lipids selected from: TG(16:0/16:0/16:0) and PI(18:0/18:0), and optionally at least another **up-regulated** or **down-regulated** lipid as defined above, and further below.

According to one particular embodiment, the plurality of lipids is selected from a set of **down-regulated** lipids selected from: total phosphatidyl-choline species, total phosphatidyl-ethanolamine species, total phosphatidyl-serine species, Total phosphatidyl-inositol species, and/or total sphingomyelin species (as detailed in **figure 2**).

In particular, it has been shown in **figure 2** that this set is sufficient for discriminating between a NASH and a NAFL condition in an individual.

According to one particular embodiment, the plurality of lipids is at least selected from a set of **down-regulated** lipids selected from: phosphatidyl-choline PC(16:0/18:2), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), phosphatidyl PE(18:0/22:6), phosphatidyl-inositol PI(18:0/18:1), sphingomyelin SM(18:1/20:0), and SM(18:1/22:1), and optionally at least another **up-regulated** or **down-regulated** lipid as defined above, and further below.

According to one particular embodiment, the methods of the invention further comprise a step of determining a phosphatidylcholine (PC) to phosphatidylethanolamine (PE) ratio or (PC/PE ratio) in the biological sample of said individual, and comparing it to the PC/PE ratio of a reference value; wherein a decrease of the PC/PE ratio is indicative of the occurrence of a fatty liver disease, especially a NASH disease, in said individual.

According to one particular embodiment, the methods of the invention further comprise a step of determining a ratio of lipid levels which are indicative of the enzyme activity of a set of elongases and desaturases (including ELOLV5, ELOLV6, FADS2, SCD1, FADS1, SREBP1c), as described in **figure 3**, and/or of determining the expression of a nucleic acid coding for said elongases and desaturases, as described in **figures 5-6** and **17**. Accordingly, the determination of lipid level ratios of the following lipids is explicitly considered:

- a C20:2n-6 / C18:2n-6 ratio, for determining the activity of ELOVL5 ;

- a C18:0/C16:0 ratio, for determining the activity of ELOVL6 ;
- a C18:3n-3/C18:2n-6 ratio, for determining the activity of FADS2 ;
- a C16:1/C16:0 ratio, for determining the activity of SCD1 ;
- a C20:4n-6/C20:3n-6 ratio, for determining the activity of FADS1.

According to some more particular embodiments, the invention further relates to a method for discriminating between a nonalcoholic fatty liver (NAFL) condition and a nonalcoholic steatohepatitis (NASH) condition in a human individual, comprising at least the steps of:

a1) determining in a biological sample from said individual the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: triglyceride TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), unsaturated fatty acid C(18:1n-9), C(18:2n-6), C(18:3n-3), phosphatidyl-inositol PI(18:0/18:0); and

a2) optionally a set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2, phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1);

b) comparing each lipid level determined at steps a1) and optionally a2) with a reference value;

c) determining the occurrence of a NASH or NAFL condition in said individual based on the comparison of step b).

Screening methods

As shown in the examples, the toxicity of the lipids identified in NASH was addressed. In particular, it has been shown that a specific mixture of up-regulated lipids which accumulated in NASH exhibited higher toxicity on hepatocytes.

Also, it has been shown that a set of lipids was down-regulated in individuals having a fatty liver disease, in particular a NASH condition.

In the sense of the invention, "*modulating the lipid levels of lipids belonging to the lipid signature*" encompasses:

- downregulating a set of up-regulated lipids; and/or
- upregulating a set of down-regulated lipids.

Accordingly, it is understood that a candidate compound that is efficient for **modulating** the lipid levels of lipids belonging to the lipid signature, in particular that is efficient for **down-regulating** a set of **up-regulated** lipids, in the biological sample of an individual, such as the lipids described above, is also predicted as efficient for treating, preventing, or reducing the likelihood of occurence of a fatty acid liver disease as also defined above, in particular a NASH condition, in said individual.

Thus, the invention also relates to a method for screening compounds for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising at least a step of determining in a biological sample of said individual the lipid levels for each of a plurality of up-regulated and down-regulated lipids as defined above.

The set of **up-regulated** lipids that is particularly considered includes at least one, in particular a plurality, and preferably all of a set selected from:

- myristic acid (C14:0);
- palmitic acid (C16:0);
- stearic acid (C18:0);
- palmitoleic acid (C16:1n-7);
- vaccenic acid (C18:1n-7); and
- oleic acid (C18:1n-9).

Preferably, the set of **up-regulated** lipids that is particularly considered includes at least one, in particular a plurality, and even more preferably all of a set selected from:

- myristic acid (C14:0);
- palmitic acid (C16:0);
- palmitoleic acid (C16:1n-7);
- vaccenic acid (C18:1n-7); and
- oleic acid (C18:1n-9).

Accordingly, said reference value, may be a biological sample that was not brought into contact with said at least one candidate compound; a biological sample from an individual (the same or not) prior to administration of the said at least one candidate compound; or alternatively that has been brought into contact with a different compound. Reference values and standards can be readily determined by the man skilled in the Art.

Accordingly, the biological sample may be selected from the group consisting of: a blood sample (which includes plasma and/or serum) or a liver biopsy.

According to one embodiment, the invention relates to a method for screening compounds for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising at least the steps of:

a) providing a biological sample from said individual to whom at least one candidate compound has been administered;

b) determining in said biological sample the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0); and/or
- a set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0),

PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2, phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1);

c) comparing each lipid level determined at step b) with a reference value, wherein said reference value is preferably determined on a biological sample from an individual to whom the at least one candidate compound has not been administered ;

d) selecting the candidate compound as suitable for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease based on the comparison of step c).

According to a particular embodiment, the invention relates to a method for screening compounds for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising at least the steps of:

a) providing a biological sample from said individual to whom at least one candidate compound has been administered;

b) determining in said biological sample the lipid levels for one or more, or even a plurality, and preferably all of lipids selected from a set of **up-regulated** lipids selected from:

- myristic acid (C14:0);
- palmitic acid (C16:0);
- stearic acid (C18:0);
- palmitoleic acid (C16:1n-7);
- vaccenic acid (C18:1n-7); and
- oleic acid (C18:1n-9);

c) comparing each lipid level determined at step b) with a reference value, wherein said reference value is preferably determined on a biological sample from an individual to whom the at least one candidate compound has not been administered ;

d) selecting the candidate compound as suitable for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease based on the comparison of step c).

According to one embodiment, the invention relates to a method for screening compounds for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising at least the steps of:

a) providing a biological sample from said individual, wherein said sample was brought into contact with at least one candidate compound;

b) determining in said biological sample the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0); and/or

- a set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2, phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1);

c) comparing each lipid level determined at step b) with a reference value;

d) selecting the candidate compound as suitable for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease based on the comparison of step c).

According to a particular embodiment, the invention relates to a method for screening compounds for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising at least the steps of:

a) providing a biological sample from said individual, wherein said sample was brought into contact with at least one candidate compound;

b) determining in said biological sample the lipid levels for one or more, or even a plurality, and preferably all of lipids selected from a set of **up-regulated** lipids selected from:

- myristic acid (C14:0);
- palmitic acid (C16:0);
- stearic acid (C18:0);
- palmitoleic acid (C16:1n-7);
- vaccenic acid (C18:1n-7); and
- oleic acid (C18:1n-9);

c) comparing each lipid level determined at step b) with a reference value;

d) selecting the candidate compound as suitable for treating, preventing, or reducing the likelihood of occurence of a fatty acid liver disease based on the comparison of step c).

Also, as described above and as further shown in the examples, it is proposed that the decreased activity of the desaturase FADS1 creates a bottleneck leading to the accumulation upstream of fatty acids up to 20 carbons. The FADS1 protein is encoded by a gene that is a member of the fatty acid desaturase (FADS) gene family.

Regarding FADS1, 21 transcripts have been identified, due to alternative splicing.

For reference, the nucleic acid sequence **SEQ ID N°25** corresponds to a nucleic acid encoding the human FADS1 protein, and is referenced under NCBI Reference Sequence NM_013402.

For reference, the sequence of a first variant of human *FADS1* protein corresponds to a polypeptide sequence **SEQ ID N°26**. Said polypeptide sequence correspond to the human FADS1 protein.

For reference, the sequence of a second variant of human *FADS1* protein corresponds to a polypeptide sequence **SEQ ID N°27**.

According to one embodiment, the invention relates to a method for screening compounds for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising at least the steps of:

a) providing a biological sample from said individual to whom at least one candidate compound has been administered;

b) determining in said biological sample (i) the expression of a nucleic acid encoding FADS1, and/or (ii) the content of FADS1, and/or (iii) the activity of FADS1;

c) comparing the expression, content and/or activity determined at step b) with a reference value;

d) selecting the candidate compound as suitable for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease based on the comparison of step c).

According to one embodiment, the invention relates to a method for screening compounds for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising at least the steps of:

a) providing a biological sample from said individual, wherein said sample was brought into contact with at least one candidate compound;

b) determining in said biological sample (i) the expression of a nucleic acid encoding FADS1, and/or (ii) the content of FADS1, and/or (iii) the activity of FADS1;

c) comparing the expression, content and/or activity determined at step b) with a reference value;

d) selecting the candidate compound as suitable for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease based on the comparison of step c).

The activity of an enzyme (i.e. FADS1) can be estimated by determining the ratio between its product and substrate (such as in **figure 17**).

In particular, the activity of FADS1 in a sample may be determined by:

I) providing a sample comprising FADS1, for which the activity of FADS1 has to be determined;

II) determining a C20:4n-6/C20:3n-6 ratio in said sample;

III) comparing the ratio determined at step II) with a reference value, wherein an increase of said ratio compared to said reference value is indicative of an increase of the activity of FADS1.

Alternatively, the activity of an enzyme (i.e. FADS1) may be determined by:

I) providing a sample comprising said enzyme, for which the activity of has to be determined;

II) bringing said sample into contact with a substrate, wherein said enzyme promotes the appearance of a reaction product in the presence of the substrate ;

III) determining the variation of the level of the substrate or product in said sample;

IV) comparing the level determined at step III) with a reference value, wherein a modulation of said level compared to said reference value is indicative of the activity of said enzyme.

Advantageously, the variation of the level of the substrate or product in said sample can be estimated by the product-to-substrate ratio.

Determination of lipid levels

Biological samples suitable for lipid profiling include blood, plasma, cerebral spinal fluid (CSF), brain tissue, fractionated cells or cell lysates, a tissue biopsy (i.e. a liver biopsy), surgical specimen, and autopsy material. A skilled artisan will recognize that the

use of each biological sample is associated with advantages and disadvantages and the appropriate biological sample type is dependent upon the intended use of the method. For example, while a blood or plasma sample can be obtained with little discomfort and risk, the lipids in the blood or plasma sample may be from other parts of the body.

According to one particular embodiment, the biological sample is selected from the group consisting of: a blood sample (which includes plasma and/or serum) or a liver biopsy.

According to one particular embodiment, the reference value is determined from a biological sample of an individual not affected with NASH or an individual having a nonalcoholic fatty liver (NAFL) condition.

The lipid levels for one given lipid may vary depending on (i) the biological sample (i.e. blood sample or liver biopsy); (ii) the age of the individual; (iii) the moment of the day. A skilled artisan will also recognize how such parameters may vary and adapt the reference values accordingly.

Protocols for determining a lipid level in a biological sample are known in the Art, and further detailed in the Material & Methods part.

Also, shotgun lipidomics methods are known in the art and described in the examples herein (Han, X., et al. (2006) Shotgun lipidomics of cardiolipin molecular species in lipid extracts of biological samples, *J Lipid Res* 47, 864-879; and Jiang, X., and Han, X. (2006) Characterization and direct quantitation of sphingoid base-1-phosphates from lipid extracts: A shotgun lipidomics approach, *J. Lipid Res.* 47, 1865-1873, both incorporated herein by reference). By way of example, a lipid sample may be extracted from a biological sample using any method known in the art such as chloroform-methanol based methods, isopropanol-hexane methods, the Bligh & Dyer lipid extraction method or a modified version thereof, or any combination thereof. Suitable modifications to the Bligh & Dyer method include treatment of crude lipid extracts with lithium methoxide followed by subsequent liquid-liquid extraction to remove generated free fatty acids, fatty acid methyl esters, cholesterol, and water-soluble components that may hinder the shotgun analysis of sphingolipidomes. Since sphingolipids are inert to the described base-treatment, the global analysis and accurate quantitation to assess low and even very low abundant sphingolipids is possible by using a modified Bligh & Dyer method.

Following lipid extraction, it may be beneficial to separate the lipids prior to mass spectrometric analysis. Methods for separating lipids are known in the art. Suitable methods include, but are not limited to, chromatography methods such as solid-phase extraction, high performance liquid chromatography (HPLC), normal-phase HPLC, or reverse-phase HPLC.

Advantageously, the resultant lipid extracts are then analyzed by mass spectrometric techniques.

Applications of Mass Spectrometry to Lipid Analysis is known in the Art, as disclosed for instance in the examples and/or in Murphy & Gaskel ("New Applications of Mass Spectrometry in Lipid Analysis"; The Journal of Biological Chemistry; Vol. 286, No. 29, pp. 25427-25433, 2011).

Protocols for identifying a given lipid, or alternatively for discriminating between two lipids may vary on a case to case basis, depending on the chemical nature of the lipids for which the level has to be determined. For example, chemical derivatization may be suitable for locating double bonds, by reacting them with appropriate reagents to give chemical derivatives that give distinctive fragmentations in a mass spectrometer.

Also, lipid profiles may be determined by gas or liquid chromatography, and compared to known internal standards.

Alternatively, lipid levels may be approximated based on the outcome of a combination of techniques, for example for determining lipid levels from a complex mixture, without departing from scope of the invention.

Accordingly, the determination of lipid levels may comprise a step of derivatization before quantification by gas or liquid chromatography, or mass spectrometry.

According to one particular embodiment, the lipid levels are determined by gas or liquid chromatography, optionally followed by a step of separation.

According to one particular embodiment, the lipid levels are determined by mass spectrometry.

According to one particular embodiment, the lipid levels are determined by gas or liquid chromatography and mass spectrometry.

Therapeutic methods, and pharmaceutical compositions

The invention also relates to a plurality of lipids selected from a set of **up-regulated** and **down-regulated lipids**, as defined above and below; and compositions thereof. In particular, said plurality of lipids, and compositions thereof, can be used for the preparation for a medicament.

When the plurality of lipids is considered in the form of a composition, this composition may comprise at least 50% by weight of the composition of the set of up-regulated and/or down-regulated lipids that is considered; which includes at least 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% by weight of the total weight of the composition; which includes 100%

Thus, the invention also relates to- a plurality of lipids selected from a set of **down-regulated lipids**, as defined above and here below; and compositions thereof; for use for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual; or alternatively for the preparation of a medicament, in particular for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual.

Thus, the invention also relates to- a plurality of lipids selected from a set of **down-regulated lipids** selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2), phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1); for use for treating, preventing, or

reducing the likelihood of the occurrence of a nonalcoholic steatohepatitis (NASH) condition in an individual.

Accordingly, the invention also relates to a method for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising a step of administering, to said individual,- a plurality of lipids selected from a set of **down-regulated lipids**, as defined above and further below.

The mixtures of lipids which are described herein, and compositions thereof can be suitable for any route of administration known in the Art, as previously defined.

In particular, the administration by the oral route is considered (i.e. in the form of encapsulated mixtures, and compositions thereof).

The sets **of down-regulated** lipids which are described herein are particularly convenient for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease, and preferably of a NASH condition.

According to one particular embodiment, the invention relates to a plurality of lipids selected from a set of **down-regulated lipids**, as defined above and further below; and compositions thereof; for use for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual by the oral route; or alternatively

for the preparation of a medicament suitable for oral administration, in particular for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual.

According to one embodiment, the invention relates to a mixture of a plurality of lipids, or a composition thereof, comprising (or consisting of) a plurality of **down-regulated** lipids, as above defined, which includes a set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or CE(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2), phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4),

PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1).

The above-mentioned sets of down-regulated lipids are particularly considered for the treatment, prevention and/or reduction of the likelihood of the occurrence of a fatty liver disease, and preferably of a NASH condition, in an individual.

Individuals for which a reduction of the likelihood of occurrence of a NASH condition is particularly considered include individuals having a fatty liver disease, such as a NAFL condition.

According to another embodiment, the invention also relates to a method for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising a step of administering, to said individual:

- a compound suitable for increasing (i) the expression of a nucleic acid encoding FADS1, and/or (ii) the content of FADS1, or (iii) the activity of FADS1 in said individual; and/or

Thus, the invention also relates to: a compound suitable for increasing (i) the expression of a nucleic acid encoding FADS1, and/or (ii) the content of FADS1, or (iii) the activity of FADS1 in said individual; for use for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual; or alternatively

for the preparation of a medicament, in particular for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual.

In the sense of the invention, "*increasing (i) the expression of a nucleic acid encoding FADS1, and/or (ii) the content of FADS1, or (iii) the activity of FADS1 in an individual*" can be achieved by

a) increasing the expression of a nucleic acid coding for FADS1; and/or

b) administering an exogenous FADS1.

According to another embodiment, the invention relates to a mixture of lipids, or a composition thereof, comprising (or consisting of) a plurality of a set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0).

In particular, the invention relates to a mixture of lipids, or a composition thereof, comprising (or consisting of) a plurality of up-regulated lipids, as defined above, selected from:

- myristic acid (C14:0);
- palmitic acid (C16:0);
- stearic acid (C18:0);
- palmitoleic acid (C16:1n-7);
- vaccenic acid (C18:1n-7); and
- oleic acid (C18:1n-9).

Preferably, the invention relates to a mixture of lipids, or a composition thereof, comprising (or consisting of) a plurality of up-regulated lipids, as defined above, selected from:

- myristic acid (C14:0);
- palmitic acid (C16:0);
- palmitoleic acid (C16:1n-7);
- vaccenic acid (C18:1n-7); and
- oleic acid (C18:1n-9).

Surprisingly, it has also been found that this mixture comprising a plurality of **up-regulated** lipids exhibited toxicity on hepatic cells, including HepG2 and HPH (human primary hepatocytes), by triggering 25% to 90% cell death (see the Examples), even at low concentrations.

Also surprisingly, mixtures corresponding to the up-regulated lipids observed in NASH, are significantly more toxic on hepatic cells.

According to some embodiments, the following amounts and daily doses of up-regulated and/or down-regulated lipids are considered:

- a mixture of lipids, or a composition thereof, comprising (or consisting of) a plurality of up-regulated or down-regulated lipids, as herein defined, in a daily dose of at least 50 mg of said lipids; which includes a daily dose of at least 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1100, 1200, 1300, 1400, 1500, or 2000 mg; and/or

- a mixture of lipids, or a composition thereof, comprising (or consisting of) a plurality of encapsulated up-regulated or down-regulated lipids, as herein defined, in a daily dose of at least 50 mg per capsule of said lipids; which includes a daily dose of at least 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1100, 1200, 1300, 1400, 1500, or 2000 mg per capsule of said lipids.

For example, the following proportions of up-regulated lipids are considered (as mass percent by weight, compared to the total weight of the mixture, or composition thereof):

- myristic acid (C14:0) in a proportion ranging from about 0.5% to about 50% by weight of the total weight of the composition, which includes of about 1% by weight of the total weight of the mixture, or composition thereof, and in particular of about 2.5% by weight of the total weight of the mixture, or composition thereof; and/or

- palmitic acid (C16:0) in a proportion ranging from about 20% to about 50% by weight of the total weight of the mixture, or composition thereof, which includes of about 40% by weight of the total weight of the mixture, or composition thereof; and/or

- stearic acid (C18:0) in a proportion ranging from about 10% to about 50% by weight of the total weight of the mixture, or composition thereof, which includes of about 27% by weight of the total weight of the mixture, or composition thereof; and/or

- palmitoleic acid (C16:1n-7) in a proportion ranging from about 0.5% to about 50% by weight of the total weight of the mixture, or composition thereof, which includes of about 4% by weight of the total weight of the mixture, or composition thereof; and/or

- vaccenic acid (C18:1n-7) in a proportion ranging from about 20% to about 50% by weight of the total weight of the mixture, or composition thereof, which includes of about 25% by weight of the total weight of the mixture, or composition thereof; and/or

- oleic acid (C18:1n-9) in a proportion ranging from about 0.5% to about 50% by weight of the total weight of the mixture, or composition thereof, which includes of about 3% by weight of the total weight of the mixture, or composition thereof.

In the sense of the invention any sub-interval of from 0.5 to 100%, as described above, is considered. This includes: 0.5; 1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 11; 12; 13; 14; 15; 16; 17; 18; 19; 20; 21; 22; 23; 24; 25; 26; 27; 28; 29; 30; 31; 32; 33; 34; 35; 36; 37; 38; 39; 40; 41; 42; 43; 44; 45; 46; 47; 48; 49; 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99% and 100%.

Advantageously, the cytotoxic effect of mixtures comprising up-regulated lipids, and compositions thereof, can be used:

- for targeting (i.e. killing or reducing the growth) of hepatic and endothelial cells; and/or

- as a tool for methods for screening compounds useful for treating or preventing or reducing the likelihood of a fatty acid liver disease, and/or for targeting (i.e. killing or reducing the growth) of hepatic and endothelial cells.

Accordingly, those mixtures of lipids can also be used for cell culture, or for administration to a human or non-human mammal. Administration of a mixture including up-regulated lipids, as defined above, to a non-human mammal may be useful in a method for generating an *in vivo* model of a fatty liver disease.

Such mixtures may also considered in the form of compositions, which includes pharmaceutical compositions (i.e. sterile compositions; and/or compositions which are suitable for injection in an individual).

According to one embodiment, such mixtures may be considered in the form of compositions, which includes pharmaceutical compositions as defined above; said

compositions being of natural or non-natural origin (i.e. not being directly obtained from a human or a non-human mammal).

For example, such mixtures can be dissolved in bovine serum albumin (10% BSA, Sigma) at the final ratio 1/10 (v/v) in William's E plus Glutamax™ medium or in OptiMEM™ (Gybco, Invitrogen) and warmed again at 55°C for 10 min before use.

Accordingly, the invention further relates to mixtures comprising a plurality of up-regulated lipids, as defined above, and compositions thereof, for targeting hepatic cells, and/or in screening methods, as previously described.

The compounds (i.e. nucleic acid coding for FADS1 and/or exogenous FADS1 and/or mixtures of lipids) which are considered for administration may be present either in a free form, or in an encapsulated form.

Encapsulation of active compounds for administration is known in the Art.

The route of administration may include: topical, enteral, parenteral administration; in a non-limitative manner, this may include oral administration, topical administration, intravenous administration, intraperitoneal administration, inhalation and/or injection.

Supplementation of lipids by the oral route is known in the Art, and has already been described, for instance, with **Omacor®/Lovaza** (Pronova Biopharma) capsules in **Scorletti *et al.*** (Hepatology ; Vol.60, No. 4 ; 2014).

Compounds and compositions which are suitable for injection, are most preferably sterile and/or apyrogenic.

EXAMPLES

A. MATERIAL & METHODS

Study Cohort

A total of 52 patients were enrolled in this study selected from two hospital centers (Paul Brousse hospital, n=45 and L'Archet hospital, n=7). The institutional review board approved the study and written informed consent was obtained from all patients. Access to this material was in agreement with French ethical laws. A single pathologist

expert (CG) reviewed all liver biopsies. Seven patients had a normal liver forming the control group (hepatic steatosis <5%). Forty-five patients have been recorded as affected with NAFLD. To differentiate NAFL and NASH, a histological discrimination was made based on a separate system of scoring the features of NAFLD called the NAFLD Activity Score (NAS) (see **Kleiner et al**. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005; 41(6):1313-1321).

By definition, a NAS<5 represents NAFL and a NAS≥5 represents NASH (see **Kleiner et al**. & **Brunt et al.** Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology*. 2011; 53(3):810-820).

Clinical and biological data on general status, metabolic syndrome and liver function were retrospectively recorded. Exclusion criteria were liver diseases such as viral hepatitis B, viral hepatitis C, primary biliary cirrhosis, sclerosing cholangitis, autoimmune hepatitis, hemochromatosis, Wilson's disease, α1-antitrypsin deficiency, drug-induced liver disease and alcohol consumption more than 20 g/day for women and 30 g/day for men.

Animal Models

Male C57Bl/6J mice were fed on chow diet, high fat diet (HFD) and methionine/choline deficient (MCD) diet develop. Mice fed with HFD and MCD developed NAFL and NASH, respectively (see **Tanaka et al.** Disruption of phospholipid and bile acid homeostasis in mice with nonalcoholic steatohepatitis. *Hepatology*. 2012; 56(1):118-129; see also **Takahashi et al.** Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol*. 2012; 18(19):2300-2308).

A total of 20 animals underwent chow diet (n=10, Teklad Rodent Diet no. 5053; 5% kcal from fat; 3.1 kcal/g), HFD (n=5; 15 weeks on diet, Research Diet D12492i; 60% kcal from fat; 5.24 kcal/g) and MCD diet (n=5; 5 weeks on diet, TekladRef# TD.90262). Mice were housed at room temperature (22–24°C) with a 12-hour light/12-hour dark cycle. Food and water were provided *ad libitum*. Animal protocols were accepted by the Institutional Animal Care and Use Committee (IACUC) in Main (Jackson Laboratories) and by the "Comité d'Ethique pour l'Expérimentation Animale" registered to

the "Comité National de Réflexion Ethique sur l'Expérimentation Animale 05" (Protocol # Ce5/2012/075).

Lipid Profiling

Lipidomic analysis was performed by gas or liquid chromatography coupled to mass spectrometry on 52 human liver biopsies (5-10 mg). This analysis allowed the identification and relative quantifications of hepatic lipid content (expressed in nmol/mg of total proteins).

Lipids were extracted from the liver tissue and further identified by gas phase or liquid phase chromatography coupled to mass spectrometry. Investigations were conducted on 104 lipid species such as cholesterol, cholesterol esters (CE, n=3), triglycerides (TG, n=5), fatty acids (n=21), ceramides (Cer, n=4), phosphatidylcholines (PC, n=18), phosphatidylethanolamines (PE, n=16), phosphatidylinositols (PI, n=14), phosphatidylserines (PS, n=11) and sphingomyelins (SM, n=11). The protocol for lipidomic analysis is further detailed hereafter.

Liver biopsies (5-10 mg) were homogenized in 2 mL of methanol/EGTA (2:1 v/v) with FAST-PREP (MP Biochemicals) tissue lyser for further lipid analyses. Also, the equivalent of 0.5 mg of tissues was evaporated. The dry pellets were dissolved in 0.25 mL of NaOH (0.1M) overnight and proteins were measured with the Bio-Rad assay. The quantification of the lipids was expressed in nmol/mg of total proteins. Briefly, lipids were extracted from liver tissues according to **Bligh and Dyer** (A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* 1959; 37(8):911-917) in dichloromethane/methanol/water (2.5:2.5:2.1, v/v/v), in the presence of the internal standards (stigmasterol, cholesteryl heptadecanoate, glyceryl trinonadecanoate) to quantify neutral lipids. Dichloromethane phase were evaporated to dryness, and the residue dissolved in 20 µL of ethyl acetate. The lipid extract (1 µL) was analyzed by gas-liquid chromatography on a FOCUS Thermo Electron system using a Zebron-1 Phenomenex fused silica capillary columns coupled to mass spectrometry according to **Barrans** (Hepatic lipase induces the formation of pre-beta 1

high density lipoprotein (HDL) from triacylglycerol-rich HDL2. A study comparing liver perfusion to in vitro incubation with lipases. *J Biol Chem.* 1994; 269(15):11572-11577).

Phospholipids for relative quantification were extracted as neutral lipids but with 2% acetic acid in the presence of the internals standards (Cer(d18:1/15:0) 16 ng; PE(12:0/12:0) 180 ng; PC(13:0/13:0) 16 ng ; SM(d18:1/12:0) 16 ng ; PI(16:0/17:0) 30 ng ; PS(12:0/12:0) 156.25 ng). After centrifugation the organic phase was collected and dried under azote, then dissolved in 50 µL of methanol. Lipids were separated using a Kinetex HILIC column with a mobile phase A of acetonitrile and B with 10 mM ammonium formate in water at pH 3.2 following a gradient and the injection volume was 5 µL. Sample solutions were analyzed using an Agilent 1290 UPLC system coupled to a G6460 triple quadrupole mass spectrometer (Agilent Technologies) and using MassHunter software (Agilent Technologies) for data acquisition and analysis. Data were treated using QqQ Quantitative (version B.05.00) and Qualitative analysis software (version B.04.00).

Time-of-Flight Secondary Ion Mass Spectrometry Imaging

Time-of-flight-secondary ion mass spectrometry (ToF-SIMS) with a bismuth cluster ion source was used to address the distribution of lipids directly on frozen liver sections, as previously described (See **Debois et al.** In situ lipidomic analysis of nonalcoholic fatty liver by cluster TOF-SIMS imaging. *Anal Chem.* 2009; 81(8):2823-2831; See also **Le Naour et al.** Chemical imaging on liver steatosis using synchrotron infrared and ToF-SIMS microspectroscopies. *PLoS One.* 2009; 4(10):e7408; See also **Petit et al.** Multimodal spectroscopy combining time-of-flight-secondary ion mass spectrometry, synchrotron-FT-IR, and synchrotron-UV microspectroscopies on the same tissue section. *Anal Chem.* 2010; 82(9):3963-3968).

We selected 3 patients in each group of control, NAFL1, NAFL2 and NAFL3 as well as 4 patients with NASH. The sum of the diacylglycerol (DAG) served to localize the lipid droplets into the liver tissue. C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C20:4 were investigated using ToF-SIMS procedure in different areas of the liver. The size

of each patch was 500 µm x 500 µm. The protocole for ToF-SIMS Imaging is further detailed hereafter.

A subset group of patients with the four grades of liver steatosis (3 patients per group) and a group of patients with NASH (4 patients) underwent for ToF-SIMS imaging analyses. Briefly, each sample was cut at -20°C with a CM3050-S cryostat (Leica Microsystèmes SAS, France). Tissue sections of 10 µm thickness were deposited on a gold coated glass slide (Mirr IR®, Kevley Technologies, OI, US). Before analysis, tissue sections were placed under vacuum at a pressure of a few hPa during 10 min in order to eliminate water. Before analysis, tissue samples were examined and optical images were recorded with a microscope (Olympus BX 51, Olympus France, SAS, Rungis, France) equipped with a ColorView I camera monitored by Cell^{B} software (Soft Imaging System GmbH, Münster, Germany). No further sample preparation was required before introduction in the mass spectrometer.

A TOF-SIMS IV mass spectrometer (ION-TOF GmbH, Münster, Germany), equipped with a Liquid Metal Ion Gun (LMIG) filled with bismuth and allowing delivery of Bi_3^+ cluster ion beam was used to localized lipids of interest directly on the liver tissues as previously described in **Debois D, et al.** (In situ lipidomic analysis of nonalcoholic fatty liver by cluster TOF- SIMS imaging. *Anal Chem.* 2009; 81(8):2823-2831). Briefly, a set of images was acquired without sample stage movement, just by rastering the primary ion beam, with a field of view of 500µm × 500µm. For these images, the number of pixels was chosen as 256 × 256 to obtain a ~2 µm pixel size. Under these conditions the flow was fixed to 3×10^{11} ions/cm^2 for all the acquisitions, allowing acquisition time of about 10 minutes. Each area was scanned twice in order to record both positive and negative ion images. Due to the very low initial kinetic energy distribution of the secondary ions, the relationship between the time-of-flight and the square root of *m/z* is always linear over the whole mass range. Consequently, the mass calibration was made with H^-, C^-, CH^-, CH_2^-, CH_3^-, C_2^-, C_3^- and $C4H^-$ ions for the negative ion mode, and H+, H_2^+, H_3^+, C^+, CH^+, CH_2^+, CH_3^+ and $C_2H_5^+$ for the positive ion mode, respectively. To refine the mass calibration,ion peaks of cholesterol and vitamin E were used in positive ion mode, and fatty acid carboxylate ions

in negative ion mode based on previous studies reported on biological samples, as described in **Debois D, et al.** (In situ lipidomic analysis of nonalcoholic fatty liver by cluster TOF- SIMS imaging. *Anal Chem.* 2009; 81(8):2823-2831), in **Le Naour F, et al.** (Chemical imaging on liver steatosis using synchrotron infrared and ToF-SIMS microspectroscopies. *PLoS One.* 2009; 4(10):e7408) and in **Petit VW, et al.** (Multimodal spectroscopy combining time-of-flight-secondary ion mass spectrometry, synchrotron-FT-IR, and synchrotron-UV microspectroscopies on the same tissue section. *Anal Chem.* 2010; 82(9):3963-3968).

Data processing was achieved using Surface Lab 6.2 software (ION-TOF GmbH, Münster, Germany). This software allows extraction of ion spectra and images from the raw data. In order to compare the relative intensity of species in the first set of experiments, a normalization of their respective mass spectrum intensities had to be performed: the intensity of the mass spectrum from each stage scan was normalized against the area of the smallest one, given that all the data had been acquired under the same experimental conditions, as described in **Debois D et al.** (see reference above), in **Touboul et al.** (Mass spectrometry imaging: Towards a lipid microscope? *Biochimie.* 2011; 93(1):113-119), in **Touboul et al.** (Micrometric molecular histology of lipids by mass spectrometry imaging. *Curr Opin Chem Biol.* 2011; 15(5):725-732), and in **Malmberg et al.** (High-resolution, imaging TOF-SIMS: novel applications in medical research. *Anal Bioanal Chem.* 2011; 399(8):2711-2718).

Real-time quantitative PCR of genes involved in lipid metabolism.

Total RNA was extracted from liver biopsies using RNA-STAT 60 reagent (AMS Biotechnology Europe LTD, UK). Quantity and quality of RNA were assessed using NanoDrop®-ND1000 (Thermo Scientific). cDNAs were generated by using the RivertAid® First Strand cDNA Synthesis (Thermo Scientific, France), and Syber Green from FastStart Essential DNA Green Master mixes (Roche, Life Science) were used to quantify hepatic mRNA levels with specific primers of each gene described in **Table 1:**

Table 1: List of primers used for Q-RT-PCR

Genes	Accession number	NCB	TM
ELOVL6_human	AK027031	NM_024090	58,99
ELOVL5_human	AF231981	NM_021814	59,1
ELOVL3_human	BC034344	NM_152310	59,1
SCD1 (delta(9)-desaturase)_human	AF097514	NM_005063	59
FADS2 (delta(6)-desaturase)_human	AF126799	NM_004265	59
FADS1 (delta(5)-desaturase)_human	AF199596	NM_013402	59
SREBP1c_human	AB373959	NM_004176	59
SREBP2_human	BC051385	NM_004599	58,9
FASN_human	BC063242	NM_004104	59
HMGCR_human	M11058	NM_000859	59
ACC1_human	AY315627	NM_198834	59
Actin Beta_human	X00351	NM_001101	59

with ACC: acetyl CoA carboxylase; ELOVL: elongase of very long chain fatty acid; FADS; fatty acid desaturase; FASN: fatty acid synthesis; SCD: staroyl CoA desaturase; SREBP: sterol-regulated transcription factors (see also Sequence Listing).

Q-RT-PCR was performed using LightCycler® 96 Instrument (Roche, Life Science). Gene expression levels were normalized to actin RNA levels and data analyzed with LightCycler® 96 SW 1.1 software (Roche, Life Science). For each sample, the gene to actin ratio was calculated based on an arbitrary value of copies determined by the standard curve for each gene, as previously described (See **Chiappini et al.** Ventromedial hypothalamus-specific Ptpn1 deletion exacerbates diet-induced obesity in female mice. *J Clin Invest*. 2014; 124(9):3781-3792).

Isolation and primary culture of human hepatocytes

Normal liver tissue was obtained from adult patients undergoing partial hepatectomy at Saint Antoine Hospital (Paris, France) (generous gift from Dr. Filomena Conti and Pr. Yvon Calmus). The first donor was a 63 years old woman treated for liver metastasis for colorectal adenocarcinoma. The second donor was a 36 years old female treated for hepatocellular carcinoma developed on normal liver. The third patient is a 65 years old man treated for liver metastasis of pancreatic cancer. Experimental procedures were performed in accordance with French laws and regulations. Human primary

hepatocytes isolation was made based on previous protocol (58, 59). Briefly, immediately after hepatectomy liver resection specimen was stored in Celsior solution (IMTIX-SangStat), followed by a 2-steps perfusion method, less than 3h after resection. Visible vessels were first perfused with Liver Perfusion Medium (Invitrogen) at 37°C to eliminate blood cells. A second perfusion then was performed with collagenase- and dispase-containing Liver Digest Medium (Invitrogen) at 37°C, at constant flow rate until the tissue was fully digested. Liver fragments were shaken gently in Hepatocyte Wash Medium (Invitrogen) to free loose cells, and then were filtered before centrifugation. The fibroblast- and Küpffer cell–containing supernatant was discarded, and hepatocytes were washed a second time before assessing viability by trypan blue dye exclusion. Cells were re-suspended in complete hepatocyte medium.

The complete hepatocyte medium is a William's E plus Glutamax™ medium (Gybco, Invitrogen) supplemented with 10% (v/v) FBS (fœtal bovine serum), 100 units/mL penicillin, and 100 g/mL streptomycin. For assessment of lipid levels, the fœtal bovine serum is removed.

Cells were seeded at a density of $5x10^5$ viable cells per well onto 96-well plates that had been pre-coated with a solution type I collagen from calf skin between 1 and 10 hours before plaiting cells. The medium was replaced 16–20 hours later with fresh complete hepatocyte medium supplemented with 1 mol/L hydrocortisone hemi-succinate and 100 units/mL penicillin, and 100 g/mL streptomycin, as described in **Aoudjehane et al.** (Interleukin-4 induces human hepatocyte apoptosis through a Fas-independent pathway. *FASEB J.* 2007; 21(7):1433-1444) and **Podevin et al.** (Production of infectious hepatitis C virus in primary cultures of human adult hepatocytes. *Gastroenterology.* 2010; 139(4):1355-1364).All cell cultures were maintained at 37°C in a 5% CO_2 atmosphere.

Cell culture

HepG2 cells, derived from differentiated human hepatoblastoma (54), were obtained from ATCC (Manassas, VA). Cells were cultured in DMEM containing 10% (v/v) FBS, 100 units/mL penicillin, and 100 g/mL streptomycin. The medium was changed 12h before treatment. All cell cultures were maintained at 37°C in a 5% CO_2 atmosphere.

Cell toxicity of the lipids

Myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) palmitoleic acid (C16:1n-7), vaccenic acid (C18:1n-7) and oleic acid (C18:1n-9) were all obtained from Sigma (Ref# M3128, 43051, S4751, P9417, V0384, O1257, respectively). Fatty acids (FA) were dissolved in absolute ethanol at a concentration of 40mM stock solutions, sonicated 15 min and then warmed at 70°C for 15 min, for complete dissolution. FA solutions were filtered through a 0.22 µm filter before use and stored at -20°C. Then, FA were dissolved in bovine serum albumin (10% BSA, Sigma) at the final ratio 1/10 (v/v) in William's E plus Glutamax™ medium or in OptiMEM™ (Gybco, Invitrogen) and warmed again at 55°C for 10 min before use, as described in **Huang et al.** (Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. *J Lipid Res.* 2012; 53(9):2002-2013). The final concentration of ethanol did not exceed 1%. The final concentrations of FA were 50, 100, 250, 500 and 1000 µM. HPH were treated in William's E plus Glutamax™ and HepG2 in OptiMEM™ medium (Gibco, Invitrogen, France) during 24h. Three mixes so-called Control, NAFL2/3 and NASH were prepared based on the percentage of myristic acid, palmitic acid, stearic acid, palmitoleic acid, vaccenic acid and oleic acid found in liver of patients (**Table 2**). HepG2 cells or human primary hepatocytes were treated with each lipid individually or with the lipid mix during 24h. Lipotoxicity was assessed by the content of total ATP into the cells (CellTiter-Glo® Luminescent Cell Viability Assay, Promega, France).

Statistical Analysis

All calculations were performed using R v.3.2.2 software, as described in **Team RC**. (R: A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing*. 2012). To analyze the homogeneity of the groups of patients with NAFL, recursive partitioning and regression trees ("rpart") approach was used to build a regression tree based on the predict values from the lipidomic data leading to obtain a classification analysis and a regression tree (CART). CART was applied on lipid families such as total TG, total DG, Total cholesterol, Total CE, Total SFA, Total USFA, Total MUFA, Total PUFA, Total PC, Total PE, Total PI, Total PS, Total Cer and Total SM.

In order to identify the specific dependent variables (lipids) that contributed to the significant overall effect (between different NAFL groups and between NAFL and

NASH groups), a random forests analysis was used with the following R packages "randomForest" and "varSelRF" leading to obtain a narrow numbers of markers. Briefly, RF consisted of a collection of tree predictors where each tree depended on the value of a random vector of measured variables sampled independently and with the same distribution for all trees in the forest. RF classified a case by assigning the input vector of variables to each tree of the forest. Each tree gave a classification, *i.e.* a classis voted, and the forest chose the class with the most votes from all the trees in the forest (31, 61).

RF analysis was an effective tool in prediction without over-fitting and multiclass classification, as described in Breiman L. (Random forests. *Machine Learning*. 2001; 45(1):27), in **Pang H, et al**. (Pathway analysis using random forests classification and regression. *Bioinformatics*. 2006; 22(16):2028-2036), in **Furey TS, et al**. (Support vector machine classification and validation of cancer tissue samples using microarray expression data. *Bioinformatics*. 2000; 16(10):906-914) and in **Svetnik V, et al**. (Random forest: a classification and regression tool for compound classification and QSAR modeling. *J Chem Inf Comput Sci*. 2003; 43(6):1947-1958).

As in many statistical analyses leading to a lot of variables and few groups (as we face here: 104 lipids and 5 groups of patients), a crucial problem was variables not significantly relevant to explain the analyzed phenomenon (*i.e.* occurrence of NASH) and missing values, but could create a random noise which hided the main effects and the relevant predictors, as shown in **Breiman L**. (Random forests. *Machine Learning*. 2001; 45(1):27).

Thus to determine the most discriminant lipids, RF were applied using "randomForest" package in R. To determine the best number of predictors (*mtry*) was used for each split of the tree and *tune RF* function was used to determine the lowest *mtry* to the lowest out-of-bag (OOB) error data that was used to get a running unbiased estimate of the classification error as trees were added to the forest. Also *ntree* (number of trees to be built) was set up at 1040 corresponding to the number of the columns (variable) of the matrix multiply by ten. During the analysis, the mean decreased accuracy (MDA) and the mean decreased Giny (MDG) were determined. MDA was determined during the OOB error calculation phase and lipids with a large MDA were more important for classification of the data. In addition, MDG that was a measure of how each variable contributes to the homogeneity of the nodes and leaves in the resulting RF was assessed. Lipids that resulted

in nodes with higher purity had a higher MDG. Dendrogram was clustered with DIANA (DIvisiveANAlysis Clustering) approach with Euclidian distance based on RF analysis. Results were expressed as a matrix view of lipid expression data represented by heatmap. To compute clustering and graphical representations (heatmap and dendrogram), "clValid", "heatmap3", "gplots" and "RColorBrewer" packages were used.

Individual variables among the different groups of patients were shown as boxplot and tested with analysis of variance (ANOVA-test) followed by unpaired *t*-test. Type I error-set was 5%. Kruskal-Wallis rank sum test was used to compare gender repartition between the 5 groups of patients.

B. RESULTS

Clinical, biochemical and histological characteristics of the study groups

In the present study, the approach led to discriminate 3 groups of nonalcoholic fatty livers based on total amount of triglycerides (TG), namely NAFL1 (41.7<TG<220 nmol/mg; n=9), NAFL2 (220<TG<465.5 nmol/mg; n=11) and NAFL3 (TG>465.5 nmol/mg; n=12) **(Figure 9)**. The 13 liver biopsies corresponding to NASH were obtained from two different hospital centers, Paul Brousse hospital (NASH1, n=6) and L'Archet hospital (NASH2, n=7). Patients in the NASH2 group were obese (n=4 android obesity; n=3 gynoid obesity) with a higher BMI compared to NASH1 groups ($p<0.01$). All patients were Caucasian. The control group was significantly younger with a lower BMI compared to other groups ($p< 0.05$). The only difference between NAFL and NASH groups was BMI. Thus, six well-defined groups were designed.

Lipidomic and machine learning analysis revealed a lipid signature of NAFLD.

The study was first focused on the comparison of the lipid composition in NASH obtained from the two different hospitals. A random forests analysis was performed on the 104 lipids quantified in NASH1 and NASH2 groups. Only 2 lipids out of 104 exhibited a significant variation in concentration. Indeed, cholesterol was significantly higher in NASH1 group (164.8 ± 17.9 *vs.* 52.5 ± 5.3 nmol/mg, $p< 0.01$) and oleic acid (C18:1n-9) was significantly higher in NASH2 group (1340.9 ± 197.2 *vs.* 537.1 ± 111.4

nmol/mg, $p < 0.01$). Beside these two lipids, the data showed that both groups were highly similar. Furthermore, the random forest analysis of NASH groups extended to NAFL groups showed that NASH1 and NASH2 were distinct to NAFL groups. Therefore, NASH1 and NASH2 groups were further fused and considered as a unique group of NASH.

Investigations were further conducted on the whole set of data corresponding to the 104 variables from the 52 liver biopsies distributed in 5 groups including normal liver (n=7), NAFL1 (n=9), NAFL2 (n=11), NAFL3 (n=12) and NASH (n=13). The random forest analysis led to the characterization of a signature constituted by 63 lipids. Such a signature allowed well discriminating the 5 groups with each other (**Figure 9, 10 and 12**). Indeed, normal livers were distinguished from low level of steatosis NAFL1 along the first dimension that is related with the higher variance. The three groups of NAFL (NAFL1; NAFL2 and NAFL3) were well discriminated. Finally, the NASH patients appeared as a compact group onto the two dimensions of the plot that was completely separated from the other groups (**Figure 11 and 13**). A heatmap representation using unsupervised cluster analysis confirmed that NASH group was correctly assembled (100%, n=13/13). Among the 63 discriminant lipids, 45 lipids were significantly decreased in NASH, mainly ceramides, phospholipids and sphingomyelins. On the other hand, 18 lipids exhibited an increased tendency in NAFL or NASH such as phosphatidylinositol PI(18:0/18:0), cholesterol and 2 cholesterol ester compounds (CE(16:0) and CE(18:0)), 5 species of triglycerides and 9 fatty acids such as myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n-7), stearic acid (C18:0), vaccenic acid (C18:1n-7), oleic acid (C18:1n-9), linoleic acid (C18:2n-6), α-linoleic acid (C18:3n-3) and eicosadienoic acid (C20:2n-6) (**Figure 1, 2, 14 and 15**).

Altogether, these results demonstrated that the random forest mathematical approach allowed characterizing a lipid signature of the grades of fatty liver diseases, in particular of NASH.

Lipid signature of NASH was related to dysregulations along fatty acid synthesis pathway

The metabolic pathways deregulated in NASH were investigated. Interestingly, the 9 fatty acids increased in NASH (C14:0, C16:0, C18:0, C16:1n-7, C18:1n-7, C18:1n-8, C18:2n-6, C18:3n-3 and C20:2n-6) belong to the metabolic pathway involved in synthesis of long-chain and very-long chain fatty acids (**Figure 17**). Thus, investigations were performed on the activity of the enzymes along this metabolic pathway, mainly elongases and desaturases. The activity of each enzyme was estimated by measuring the ratio between its product and substrate based on our lipidomic analysis.

The study was first focused on the two main elongases ELOVL5 and ELOVL6 (elongation of very long chain fatty acids) of the metabolic pathway. The activity of ELOVL5 was significantly increased (**Figure 3**), whereas the activity of ELOVL6 was significantly decreased (**Figure 3**). The elongase ELOVL6 is involved in the elongation of lauric acid (C12:0) to stearic acid (C18:0) (**Figure 17**). The decreased activity of ELOVL6 in NASH was consistent with the marked increase in long-chain fatty acids in NASH culminating with the accumulation of myristic acid (C14:0) (**Figure 1**). Investigations were further performed on the activity of the desaturases FADS1 (fatty acid desaturase 1), FADS2 (fatty acid desaturase 2) and SCD1 (steroyl-CoA desaturase 1). FADS2 and SCD1 activities were increased in accordance with the increase of monounsaturated (C16:1n-7 and C18:1n-9) and polyunsaturated (C18:2n-6 and C18:3n-3) fatty acids in NASH (**Figure 3 and 16**). In contrast, a significant decrease in FADS1 activity was observed in NASH (**Figure 3 and 16**). Furthermore, the estimation of amount of substrate and product on multiple steps suggested that ELOVL6 and FADS1 were limiting enzymes along the metabolic pathway. Indeed, the decrease activity of these two enzymes was driving the global activity of the pathway significantly down in NASH patients (**Figure 16**). The decrease in FADS1 desaturase activity in NASH may constitute a bottleneck leading to the accumulation of fatty acids upstream.

As a consequence, the synthesis of lipids downstream of this enzyme such as the eicosanoid precursors arachidonic acid (AA, C20:4n-6), eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) exhibited a significant decrease in livers of NASH patients as compared to controls (**Figure 4**). It should be noted that eicosanoid are involved in the synthesis of phospholipids. Therefore, the deficiency in the synthesis of long-chain polyunsaturated fatty acids has to be related with the extremely low representation of 45 phospholipid species in NASH.

To determine if changes in desaturase and elongase activities were related with their expression levels, liver mRNA gene expressions of *ELOVL5, ELOVL6, FADS1*, *FADS2* and *SCD1* were investigated. Studies were performed by RT-Q-PCR on NASH as compared to normal livers as well as NAFL2. Indeed, patients from NAFL2 group matched to NASH patients based on total amount of lipids. *ELOVL5* mRNA expression was slightly but significantly increased in NASH compared to NAFL2 group (**Figure 5**) whereas *ELOVL6* mRNA liver expression was significantly decreased in NASH patients compared to normal liver and NAFL2 (**Figure 5**) that was consistent with low enzyme activity observed in NASH. Regarding *FADS1,* liver mRNA gene expression was similar in NASH compared to control group and only a slight decrease is observed in NASH compared to NAFL2 group (**Figure 5**). The gene expression of *FADS2* and *SCD1* were significantly increased in NASH patients compared to NAFL2, according with the observed enzyme activities. Studies were further focused on genes related to *de novo* fatty acids synthesis such as sterol regulatory element-binding proteins 1c (*SREBP1c*), fatty acid synthase (*FASN*) and acetyl-CoA carboxylase 1 (*ACC1*) (**Figure 17**). As previously reported in human livers, *SREBP1c* expression was significantly decreased in NAFL patients compared to control. We observe that SREBP1c mRNA level was increased in NASH patients compared to NAFL2 (**Figure 5F**). Interestingly, the expression levels of *FASN* and *ACC1* genes were significantly decreased in NAFL group but significantly increased in NASH compared to control and NAFL groups suggesting that *de novo* fatty acid synthesis may contribute to long-chain fatty acids accumulation in NASH (**Figure 5G and H**).

Altogether, these results highlighted the major impact in NASH of the alterations of the metabolic pathway involved in synthesis of fatty acids.

Changes in lipid composition in NASH resulted of the additional effect of the increase in *de novo* synthesis of short-chain fatty acids, increase in desaturase activities of FADS2 and SCD1 as well as decrease in elongase ELOVL6 and desaturase FADS1 activities.

These results also positioned FADS1 as a bottleneck leading upstream to the accumulation of long-chain fatty acids, and downstream to the deficiency in very long-chain fatty acids and thus in phospholipids further.

Dysregulations along fatty acid synthesis pathway were confirmed in animal models.

The metabolic features observed in human were investigated using animal models. Nonalcoholic fatty liver and NASH can be induced in mice by using specific diets, respectively high fat diet (HFD) and methionine choline deficient (MCD) diet. We generated HFD (n=5) and MCD diet (n=5) mice as well as control mice (n=10). Mice fed HFD developed steatosis without any histological sign of inflammation whereas mice fed MCD diet after 5 weeks developed steatosis (less pronounced than HFD mice) exhibiting ballooning hepatocytes, Mallory hyaline bodies and inflammatory infiltrates (data not shown). Additional lipidomic analyses were performed from liver biopsies of animals focusing on fatty acids. The activity of each enzyme along the fatty acid synthesis pathway was estimated by measuring the ratio between its product and substrate as described above. Interestingly, the activities of the elongase Elovl5 as well as the desaturases Fads2 and Scd1 were increased in MCD diet mice. In contrast, the activities of the elongase Elovl6 and the desaturase Fads1 were decreased (**Figure 3**). The amount of lipids synthesized downstream Fads1 such as the eicosanoid precursors arachidonic acid (AA, C20:4n-6), eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) were dramatically decreased in livers of mice fed MCD diet as compared to HFD or control mice (**Figure 4**).

These observations demonstrated the common metabolic dysregulation along the fatty acid synthesis pathway in both human patients and animal models, leading to the development of NASH.

It should be noted that mice fed MCD diet for only 5 weeks did not develop fibrosis meaning that the differences with HFD mice could not be due to a long inflammatory and fibrotic process.

Therefore, the metabolic changes observed in MCD diet mice may represent early steps in the development of NASH.

NASH was related to inadequate cholesterol synthesis

A significant increase in cholesterol and cholesterol esters was observed in NASH as compared to the other groups. We focused on transcription factors possibly involved in cholesterol synthesis such as SREBP2 and hydroxymethylglutaryl-CoA reductase (*HMGCR*). The gene expression levels were investigated in human by RT-Q-PCR. Gene expression of *SREBP2* and *HMGCR* were decreased in NAFL2 patients compared to control group whereas these genes were both significantly increased in NASH patients (**Figure 6**), leading to increase the hepatic cholesterol level.

Hence, these results suggest a deregulation of cholesterol synthesis in NASH.

In NAFL, the decrease in mRNA levels of genes involved in this metabolic pathway was known and explained by a negative feedback thus limiting lipid accumulation. The increase in mRNA levels in NASH patients demonstrated inadequacy to regulate cholesterol synthesis in this pathology.

Mass spectrometry imaging on tissue section revealed spreading of lipids in NASH.

A major feature of NASH revealed by our lipidomic analysis was the failure in phospholipids. The extremely low amount of phospholipids may have an impact on cellular membranes in which phospholipids are important components. Furthermore, it has been reported that the ratio between phosphatidylcholine (PC) and phosphatidylethanolamine (PE) can be used as a surrogate to assess cell membrane integrity. Investigations were performed in both human patients and mouse model thus demonstrating a significant decrease of the ratio PC to PE in NASH (**Figure 7**). These observations suggested cell membrane impairments thus leading to a possible spreading of hepatocyte content into hepatic parenchyma. In order to address the distribution of lipids in the liver tissue, experiments were performed using time-of-flight-secondary ion mass spectrometry (ToF-SIMS). This approach allows investigating the lipid composition at the subcellular level. By rastering a tissue section, the distribution of lipids can be visualized. Mass spectrometry imaging using ToF-SIMS was performed on tissue sections from

patients with NAFLD. The distribution of fatty acids (C14:0, C16:0, C18:0, C16:1, C18:1, C18:2 and C20:4) was addressed. The distribution of diacylglycerols (DAG) corresponding mostly to the fragmentation of triglycerides under mass spectrometry analysis was also addressed. In NAFL, lipids were accumulated into lipid droplets (**Figure 7B and C**). In patients with NASH, the lipids were also accumulated into lipid droplets but an important diffusion was observed into hepatic parenchyma (**Figure 7B and C**). It should be noted that the comparison in the lipid repartition between NAFL and NASH was performed from images exhibiting similar amount of the lipid species studied as attested by the total count (TC) values ($TC_{NAFL3}=2.25x10^5$ vs $TC_{NASH}=2.37x10^5$), thus strengthening a real difference in terms of distribution.

These results suggested compromised cell membrane integrity in NASH leading to leak of hepatocyte content that was spreading into the parenchyma.

Specific mixture of lipids accumulated in NASH exhibited higher toxicity on hepatocytes

The toxicity of the lipids identified in NASH was addressed. Studies were focused on 6 fatty acids available for cell culture out of the 9 fatty acids accumulated in NASH. Thus, the toxicity of myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), palmitoleic acid (C16:1n-7), vaccenic acid (C18:1n-7) and oleic acid (C18:1n-9) was investigated in cell culture on HepG2 cells as well as on human primary hepatocytes (HPH). The overall toxicity after 24h was assessed quantifying cell death by total ATP concentration. Lipotoxicity was first addressed for each individual lipid at various concentrations such as 50, 100, 250, 500 and 1000 µM. The HepG2 cell line and HPH showed different sensitivities to lipids most likely due to differences in the metabolism of such cells. Moreover, the 6 lipids at the highest concentration exhibited toxicity on both HepG2 cells and HPH by triggering 25% to 90% cell death (**Figure 8A and B**). The toxicity of lipids in combination was further investigated. Three mixes corresponding to the composition and proportion of the 6 fatty acids into the normal liver, NAFL2/3 and NASH respectively were composed based on the mean concentrations obtained from our lipidomic analysis (**Table 2**). HepG2 cells and HPH were incubated with such mixes at the same final concentrations from 50 µM to 1000 µM. Interestingly, NASH mix was

significantly more toxic on both hepatic cells. Furthermore, lipotoxicity of the NASH mix was also observed at low concentration (**Figure 9C and D**).

Table 2. Concentrations and proportions of six discriminant fatty acids in human livers

	Patients	Myristic acid	Palmitic acid	Stearic acid	Palmitoleic acid	Vaccenic acid	Oleic acid	Total
FA concentration* in human livers (nmol/mg of proteins)	**Control**	0	138.04	160.01	10.54	52.52	14.32	321.42
	NAFL2/3	14.04	626.36	421.50	66.24	388.83	36.23	1553.20
	NASH	71.82	1148.53	512.93	237.64	969.89	77.47	3018.28
% of FA in liver tissues	**Control**	0	43	33	3	16	5	100
	NAFL2/3	1	40	27	4	25	3	100
	NASH	2.5	38	17	8	32	2.5	100

FA: fatty acid; NAFL: nonalcoholic fatty liver; NASH: nonalcoholic steatohepatitis. * Mean of each lipid assessed by lipidomic analysis found in each group of patients: Control (n=7), NAFL 2 and NAFL3 (n=23), NASH (n=13).

These results demonstrated the potent toxicity of the mixture of lipids accumulated in NASH.

Concluding Remarks

In conclusion, we characterized a specific and sensitive lipid signature of NASH. The use of this lipid signature, as early diagnosis of NASH in an individual, is considered.

This study also highlights dysregulations of the metabolic pathway involved in the synthesis of fatty acids and eicosanoids precursors. In particular, our study positioned the elongase ELOVL6 and the desaturase FADS1 as major players in the progression to NASH. Finally, the current study also suggests a direct role of lipids accumulated in NASH in the progression of the pathology due to their toxicity. This opens up new avenues for further development of therapeutic approaches.

SEQUENCE LISTING

SEQ N°	Genes	Sequence	Tm
Primer Forward 5'-3'			
1	hELOVL6	GCAAACACAAAACCCAAGGC	58,99
2	hELOVL5	GGACTCACACTGCTGTCTCT	59,1
3	hELOVL3	AACCTCATTCCCCATAGCCC	59,1
4	hSCD1 (delta(9)-desaturase)	TGAAAGCCAACAACTCTGCC	59
5	hFADS2 (delta(6)-desaturase)	TTCCAAGGAGCAGAGAGGTG	59
6	hFADS1 (delta(5)-desaturase)	TGCAATGTCCACAAGTCTGC	59
7	hSREBP1c	ACACAGCAACCAGAAACTCAAG	59
8	hSREBP2	GCCTGTAATGATGGGGCAAG	58,9
9	hFASN	CCCTCATCTCCCCACTCATC	59
10	hHMGCR	CACGATGCATAGCCATCCTG	59
11	hACC1	TTGACTCCTCCATCAACCCC	59
12	hActin Beta	CATCCGCAAAGACCTGTACG	59
Primer Reverse 5'-3'			
13	hELOVL6	TGGCTTGCTTTTGTTCTCCC	58,99
14	hELOVL5	GTTGTTCTTGCGCAGGATGA	59,1
15	hELOVL3	AGCACACGGTTTGCTTTAGG	59,1
16	hSCD1 (delta(9)-desaturase)	GCTGGACACTGAGCAAAGAC	59
17	hFADS2 (delta(6)-desaturase)	CCCTATGAACCCCAAGAGCA	59
18	hFADS1 (delta(5)-desaturase)	AGCTGCCCTGACTCCTTTAG	59
19	hSREBP1c	AGTGTGTCCTCCACCTCAGTCT	59
20	hSREBP2	TTCCTCAGAACGCCAGACTT	58,9
21	hFASN	CAGCGTCTTCCACACTATGC	59
22	hHMGCR	GTGCTTGCTCTGGAAAGGTC	59
23	hACC1	AATTCCTCCCGCTCCTTCAA	59
24	hActin Beta	CCTGCTTGCTGATCCACATC	59
FADS1 gene and protein			
25	hFADS1 mRNA (NCBI gene NM_013402)		
26	hFADS1 protein variant 1 MGTRAARPAGLPCGAENPARRRLALGARQQIHSWSPRTPSTRLTAPAGP		

	ARGVARPAMAPDPVAAETAAQGPTPRYFTWDEVAQRSGCEERWLVID RKVYNISEFTRRHPGGSRVISHYAGQDATDPFVAFHINKGLVKKYMNSL LIGELSPEQPSFEPTKNKELTDEFRELRATVERMGLMKANHVFFLLYLLH ILLLDGAAWLTLWVFGTSFLPFLLCAVLLSAVQAQAGWLQHDFGHLSV FSTSKWNHLLHHFVIGHLKGAPASWWNHMHFQHHAKPNCFRKDPDIN MHPFFFALGKILSVELGKQKKKYMPYNHQHKYFFLIGPPALLPLYFQWY IFYFVIQRKKWVDLAWMITFYVRFFLTYVPLLGLKAFLGLFFIVRFLESN WFVWVTQMNHIPMHIDHDRNMDWVSTQLQATCNVHKSAFNDWFSGH LNFQIEHHLFPTMPRHNYHKVAPLVQSLCAKHGIEYQSKPLLSAFADIIH SLKESGQLWLDAYLHQ
27	hFADS1 protein variant 2 (UniprotKB/Swiss-Prot O60427): MAPDPVAAETAAQGPTPRYFTWDEVAQRSGCEERWLVIDRKVYNISEF TRRHPGGSRVISHYAGQDATDPFVAFHINKGLVKKYMNSLLIGELSPEQP SFEPTKNKELTDEFRELRATVERMGLMKANHVFFLLYLLHILLLDGAAW LTLWVFGTSFLPFLLCAVLLSAVQAQAGWLQHDFGHLSVFSTSKWNHL LHHFVIGHLKGAPASWWNHMHFQHHAKPNCFRKDPDINMHPFFFALGK ILSVELGKQKKKYMPYNHQHKYFFLIGPPALLPLYFQWYIFYFVIQRKK WVDLAWMITFYVRFFLTYVPLLGLKAFLGLFFIVRFLESNWFVWVTQM NHIPMHIDHDRNMDWVSTQLQATCNVHKSAFNDWFSGHLNFQIEHHLF PTMPRHNYHKVAPLVQSLCAKHGIEYQSKPLLSAFADIIHSLKESGQLWL DAYLHQ

CLAIMS

1. A method for determining the occurrence of a fatty liver disease in a human individual, comprising the steps of:

a) determining in a biological sample from said individual the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0); and/or

- a set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2, phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1);

b) comparing each lipid level determined at step a) with a reference value;

c) determining the occurrence of a fatty liver disease in said individual based on the comparison of step b).

2. The method according to claim 1, wherein said fatty liver disease is a nonalcoholic steatohepatitis (NASH) condition.

3. The method according to claim 1, wherein the plurality of lipids comprises at least one lipid selected from the group consisting of the set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride

(14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0).

4. The method according to claim 1, wherein the plurality of lipids is at least selected from the set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0).

5. The method according to claim 1, wherein the plurality of lipids is at least selected from the set of **up-regulated** lipids selected from: saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), and C(18:1n-9).

6. The method according to claim 1, wherein the plurality of lipids is at least selected from the set of **up-regulated** lipids selected from: C(18:2n-6) and PI(18:0/18:0).

7. The method according to claim 1, wherein the plurality of lipids is at least selected from the set of **up-regulated** lipids selected from: C(18:3n-3), C(16:0), C(18:1n-9) and PI(18:0/18:0).

8. The method according to claim 1, wherein the plurality of lipids is at least selected from the set of **up-regulated** lipids selected from: TG(16:0/16:0/16:0), TG(16:0/16:0/18:0) and PI(18:0/18:0).

9. The method according to the preceding claim, wherein the plurality of lipids is at least selected from the set of **up-regulated** lipids selected from: TG(16:0/16:0/16:0) and PI(18:0/18:0).

10. The method according to any one of the preceding claims, wherein the biological sample is selected from the group consisting of: a blood sample or a liver biopsy.

11. The method according to any one of the preceding claims, wherein the lipid levels are determined by gas or liquid chromatography coupled to mass spectrometry.

12. A method for discriminating between a nonalcoholic fatty liver (NAFL) condition and a nonalcoholic steatohepatitis (NASH) condition in a human individual, comprising the steps of:

a) determining in a biological sample from said individual the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0); and/or

- a set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2), phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1);

b) comparing each lipid level determined at step a) with a reference value;

c) determining the occurrence of a NASH or NAFL condition in said individual based on the comparison of step b).

13. A plurality of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2), phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3),

PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1); for use for treating, preventing, or reducing the likelihood of the occurence of a fatty liver disease, in particular of a nonalcoholic steatohepatitis (NASH) condition, in an individual.

14. The plurality of **down-regulated** lipids according to the preceding claim, further comprising at least one lipid selected from: PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0).

15. A method for screening compounds for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising at least the steps of:

a) providing a biological sample from an individual (i) to whom at least one candidate compound has been administered, or alternatively (ii) wherein said sample was brought into contact with at least one candidate compound;

b) determining in said biological sample the lipid levels for each of a plurality of lipids selected from the group consisting of:

- a set of **up-regulated** lipids selected from: cholesterol, cholesterol ester (16:0) or CE(16:0), CE(18:0), triglyceride (14:0/16:0/16:0) or TG(14:0/16:0/16:0), TG(16:0/16:0/16:0), TG(16:0/16:0/18:0), TG(16:0/18:0/18:0), TG(18:0/18:0/18:0), saturated fatty acid C(14:0), C(16:0), C(18:0), unsaturated fatty acid C(16:1n-7), C(18:1n-7), C(18:1n-9), C(18:2n-6), C(18:3n-3), C(20:2n-6), phosphatidyl-inositol (18:0/18:0) or PI(18:0/18:0); and/or

- a set of **down-regulated** lipids selected from: ceramide (d18:1/20:0) or Cer(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1), PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2, phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0),

SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1);

c) comparing each lipid level determined at step b) with a reference value;

d) selecting the candidate compound as suitable for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease based on the comparison of step c).

16. The method according to any one of claims 1 to 12 and 15, further comprising determining in said biological sample, a lipid level of at least one lipid selected from the group consisting of:

- a set of **up-regulated** lipids selected from: diglyceride (16:16) or DG (16:16); and/or

- a set of **down-regulated** lipids selected from: PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0).

17. A method for screening compounds for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease in an individual, comprising at least the steps of:

a) providing a biological sample from an individual (i) to whom at least one candidate compound has been administered, or alternatively (ii) wherein said sample was brought into contact with at least one candidate compound;

b) determining in said biological sample (i) an expression of a nucleic acid encoding Fatty Acid Desaturase 1 (FADS1), and/or (ii) a content of FADS1, and/or (iii) an activity of FADS1;

c) comparing the expression, content and/or activity determined at step b) with a reference value;

d) selecting the candidate compound as suitable for treating, preventing, or reducing the likelihood of occurrence of a fatty acid liver disease based on the comparison of step c).

18. A mixture of lipids comprising, or consisting of, a plurality selected from: C(14:0); C(16:0); C(18:0); C(16:1n-7); C(18:1n-7); and C(18:1n-9).

19. A mixture of lipids comprising, or consisting of, a plurality selected from: ceramide (d18:1/20:0) or CE(d18:1/20:0), phosphatidyl-choline (14:0/14:0) or PC(14:0/14:0), PC(14:0/16:0), PC(16:0/16:0), PC(16:0/18:0), PC(16:0/18:1),

PC(16:1/16:1), PC(16:0/18:2), PC(16:1/18:2), PC(18:0/18:1), PC(18:0/20:2), PC(18:0/20:3), PC(18:0/20:4), PC(18:0/22:3), PC(18:1/18:1), PC(18:1/18:2), PC(18:2/18:2), phosphatidyl-ethanolamine (16:0/16:0) or PE(16:0/16:0), PE(16:1/16:1), PE(18:0/18:1), PE(18:1/18:1), PE(18:0/20:2), PE(18:0/22:6), PE(18:1/20:4), PE(18:2/20:4), phosphatidyl-serine (16:0/18:0) or PS(16:0/18:0), PS(16:0/18:2), PS(18:0/20:2), PS(18:0/20:3), PS(18:1/18:1), PS(18:1/18:2), phosphatidyl-inositol (18:0/18:1) or PI(18:0/18:1), PI(18:0/20:1), PI(18:0/22:3), PI(18:1/20:4), sphingomyelin (18:1/14:0) or SM(18:1/14:0), SM(18:1/16:0), SM(18:1/16:1), SM(18:1/18:0), SM(18:1/18:1), SM(18:1/20:0), SM(18:1/20:1), SM(18:1/22:0), SM(18:1/22:1), SM(18:1/24:1).

20. The mixture of lipids according to the preceding claim, further comprising at least one lipid selected from: PS(16:0/16:0), PS(16:0/18:1), PS(18:0/18:1), ceramide (18:1/16:0) or Cer (18:1/16:0), Cer (18:1/24:0).

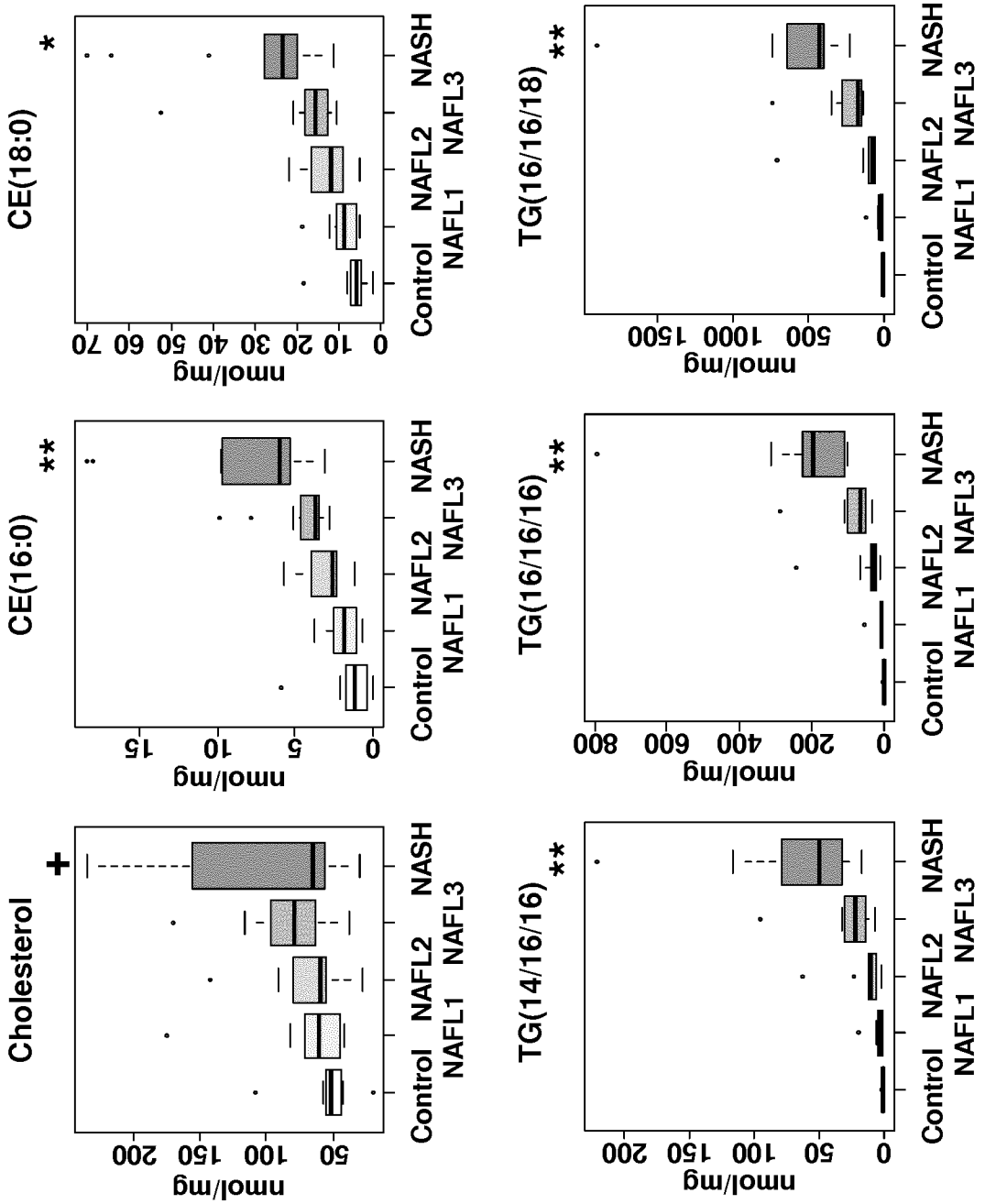

Cholesterol
+
nmol/mg
50 100 150 200
Control NAFL1 NAFL2 NAFL3 NASH
CE(16:0)
**
nmol/mg
0 5 10 15
Control NAFL1 NAFL2 NAFL3 NASH
CE(18:0)
*
nmol/mg
0 10 20 30 40 50 60 70
Control NAFL1 NAFL2 NAFL3 NASH
TG(14/16/16)
**
nmol/mg
0 50 100 150 200
Control NAFL1 NAFL2 NAFL3 NASH
TG(16/16/16)
**
nmol/mg
0 200 400 600 800
Control NAFL1 NAFL2 NAFL3 NASH
TG(16/16/18)
**
nmol/mg
0 500 1000 1500
Control NAFL1 NAFL2 NAFL3 NASH

Figure 1A

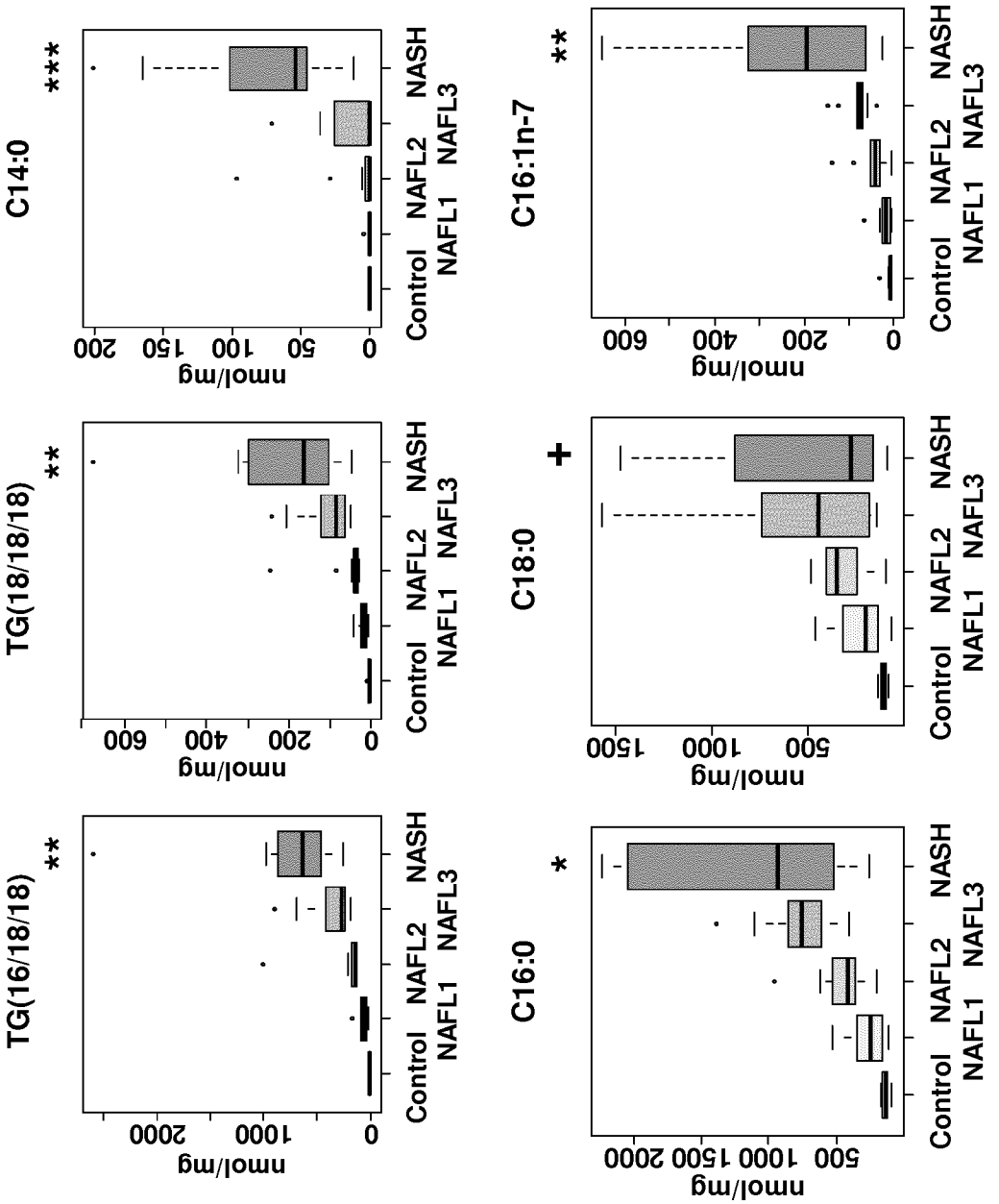

C14:0
***
nmol/mg
0 50 100 150 200
Control NAFL1 NAFL2 NAFL3 NASH
C16:1n-7
**
nmol/mg
0 200 400 600
Control NAFL1 NAFL2 NAFL3 NASH
TG(18/18/18)
**
nmol/mg
0 200 400 600
Control NAFL1 NAFL2 NAFL3 NASH
C18:0
+
nmol/mg
500 1000 1500
Control NAFL1 NAFL2 NAFL3 NASH
TG(16/18/18)
**
nmol/mg
0 1000 2000
Control NAFL1 NAFL2 NAFL3 NASH
C16:0
*
nmol/mg
500 1000 1500 2000
Control NAFL1 NAFL2 NAFL3 NASH

Figure 1B

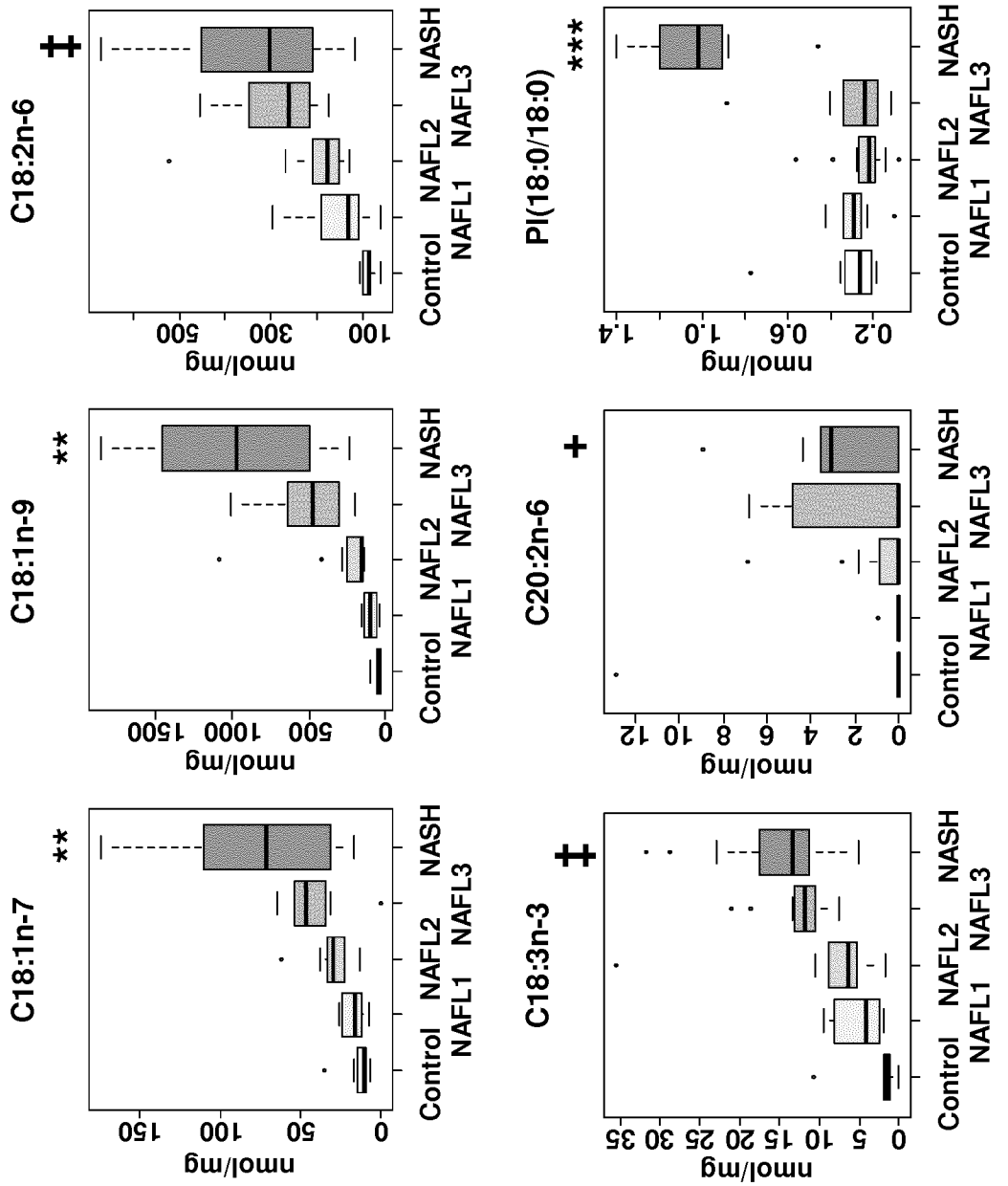

C18:1n-7
**
nmol/mg
0
50
100
150
Control NAFL2 NASH
NAFL1 NAFL3
C18:1n-9
**
nmol/mg
0
500
1000
1500
Control NAFL2 NASH
NAFL1 NAFL3
C18:2n-6
++
nmol/mg
100
300
500
Control NAFL2 NASH
NAFL1 NAFL3
C18:3n-3
++
nmol/mg
0
5
10
15
20
25
30
35
Control NAFL2 NASH
NAFL1 NAFL3
C20:2n-6
+
nmol/mg
0
2
4
6
8
10
12
Control NAFL2 NASH
NAFL1 NAFL3
PI(18:0/18:0)
***
nmol/mg
0.2
0.6
1.0
1.4
Control NAFL2 NASH
NAFL1 NAFL3

Figure 1C

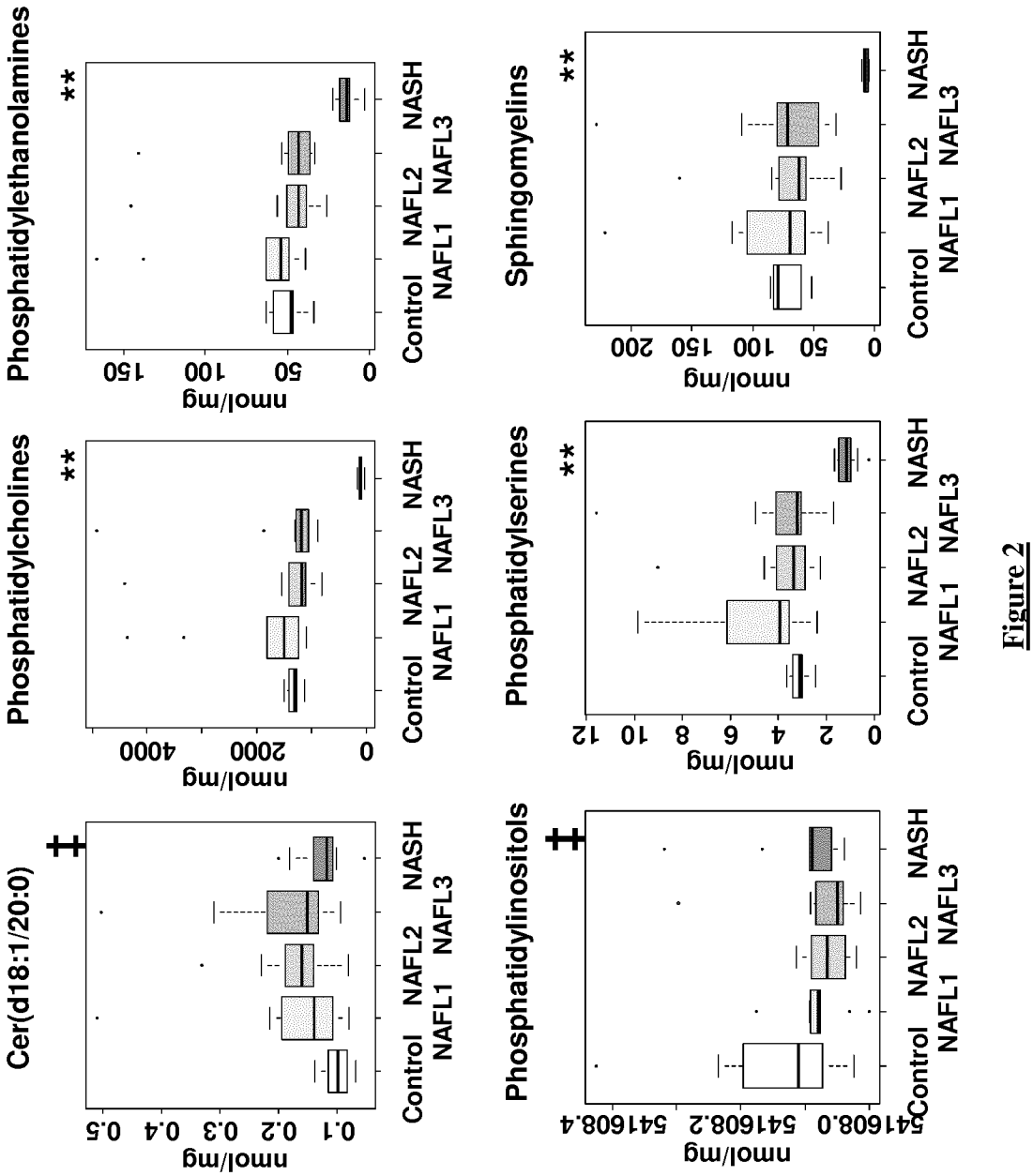

Cer(d18:1/20:0)
nmol/mg
0.1 0.2 0.3 0.4 0.5
++
Control NAFL1 NAFL2 NAFL3 NASH
Phosphatidylcholines
nmol/mg
0 2000 4000
**
Control NAFL1 NAFL2 NAFL3 NASH
Phosphatidylethanolamines
nmol/mg
0 50 100 150
**
Control NAFL1 NAFL2 NAFL3 NASH
Phosphatidylinositols
nmol/mg
541608.0 541608.2 541608.4
++
Control NAFL1 NAFL2 NAFL3 NASH
Phosphatidylserines
nmol/mg
0 2 4 6 8 10 12
**
Control NAFL1 NAFL2 NAFL3 NASH
Sphingomyelins
nmol/mg
0 50 100 150 200
**
Control NAFL1 NAFL2 NAFL3 NASH

Figure 2

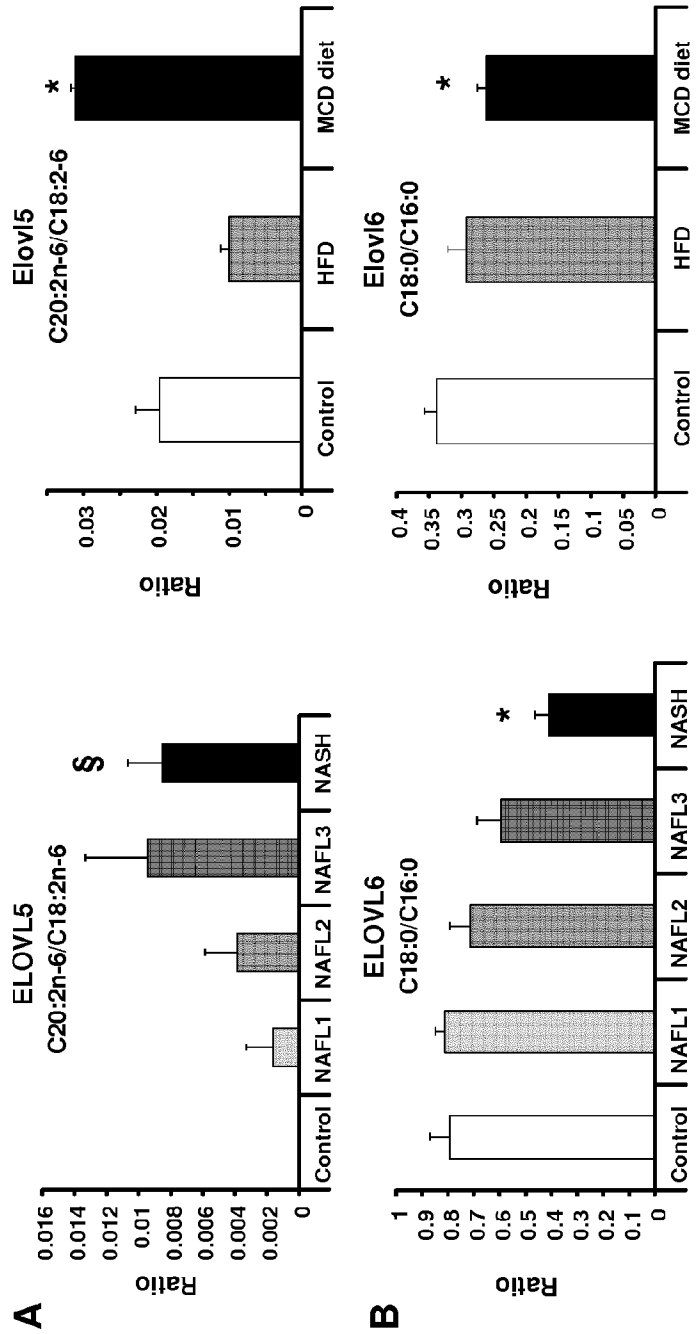

A
ELOVL5
C20:2n-6/C18:2n-6
Ratio
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
§
Control
NAFL1
NAFL2
NAFL3
NASH
B
ELOVL6
C18:0/C16:0
Ratio
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Control
NAFL1
NAFL2
NAFL3
NASH
Elovl5
C20:2n-6/C18:2-6
Ratio
0.03
0.02
0.01
0
*
Control
HFD
MCD diet
Elovl6
C18:0/C16:0
Ratio
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
Control
HFD
MCD diet

Figure 3A-B

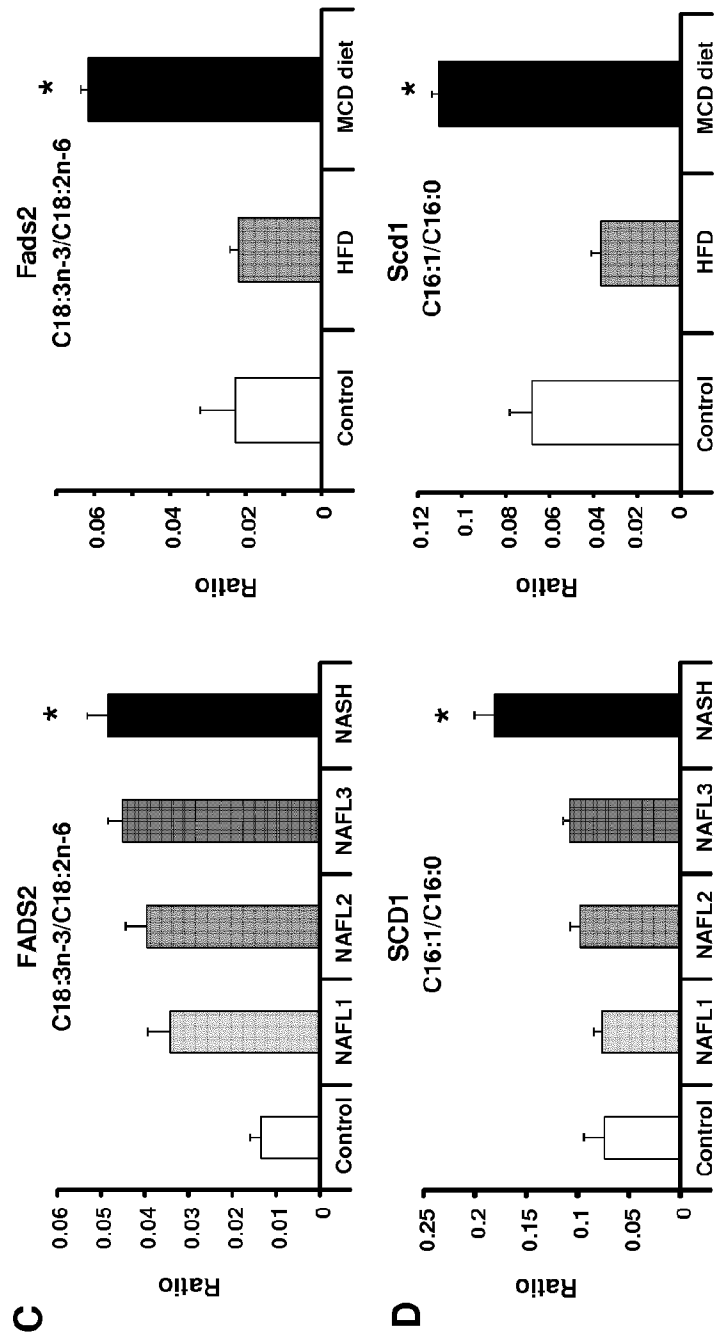

C
FADS2
C18:3n-3/C18:2n-6
Ratio
0.06
0.05
0.04
0.03
0.02
0.01
0
Control
NAFL1
NAFL2
NAFL3
NASH
*
D
SCD1
C16:1/C16:0
Ratio
0.25
0.2
0.15
0.1
0.05
0
Control
NAFL1
NAFL2
NAFL3
NASH
*
Fads2
C18:3n-3/C18:2n-6
Ratio
0.06
0.04
0.02
0
Control
HFD
MCD diet
*
Scd1
C16:1/C16:0
Ratio
0.12
0.1
0.08
0.06
0.04
0.02
0
Control
HFD
MCD diet
*

Figure 3C-D

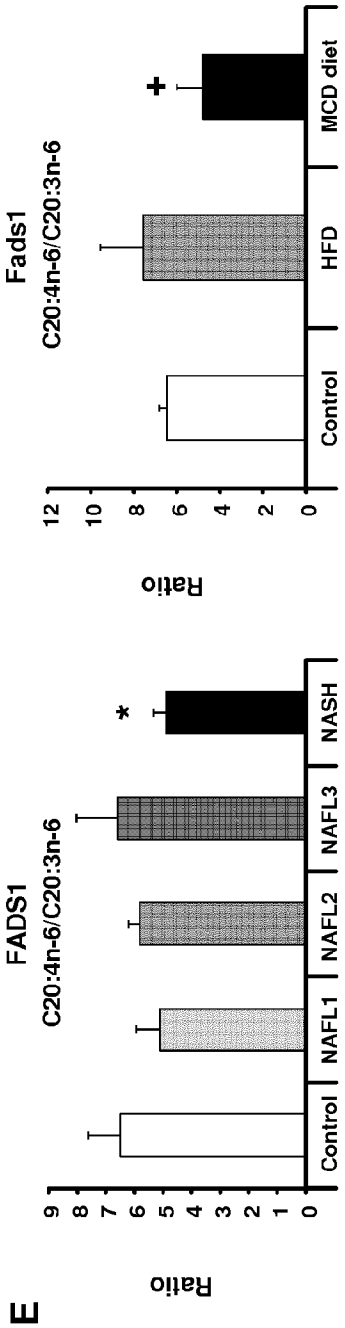

E
FADS1
C20:4n-6/C20:3n-6
Ratio
0
1
2
3
4
5
6
7
8
9
Control
NAFL1
NAFL2
NAFL3
NASH
*
Fads1
C20:4n-6/C20:3n-6
Ratio
0
2
4
6
8
10
12
Control
HFD
MCD diet
+

Figure 3E

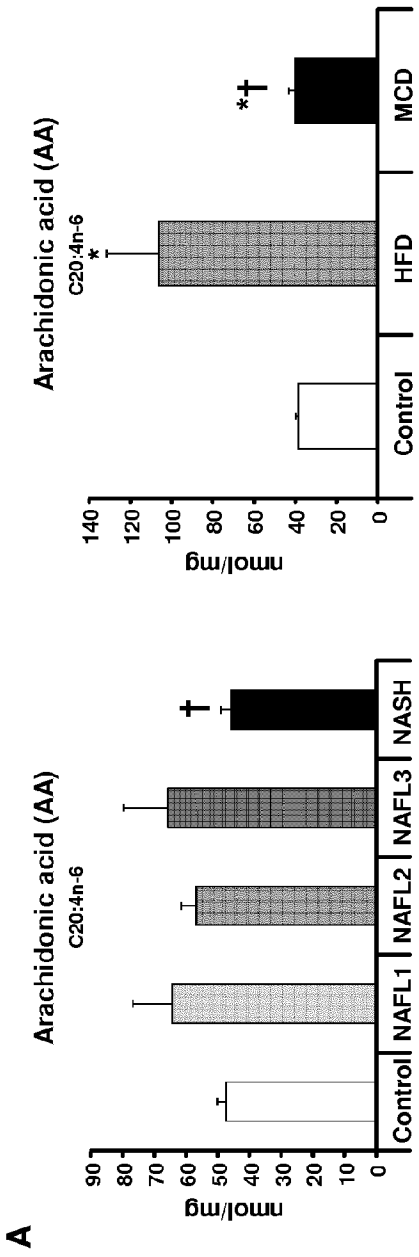

A
Arachidonic acid (AA)
C20:4n-6
nmol/mg
0
10
20
30
40
50
60
70
80
90
Control
NAFL1
NAFL2
NAFL3
NASH
+
Arachidonic acid (AA)
C20:4n-6
nmol/mg
0
20
40
60
80
100
120
140
Control
HFD
MCD
*
*+

Figure 4A

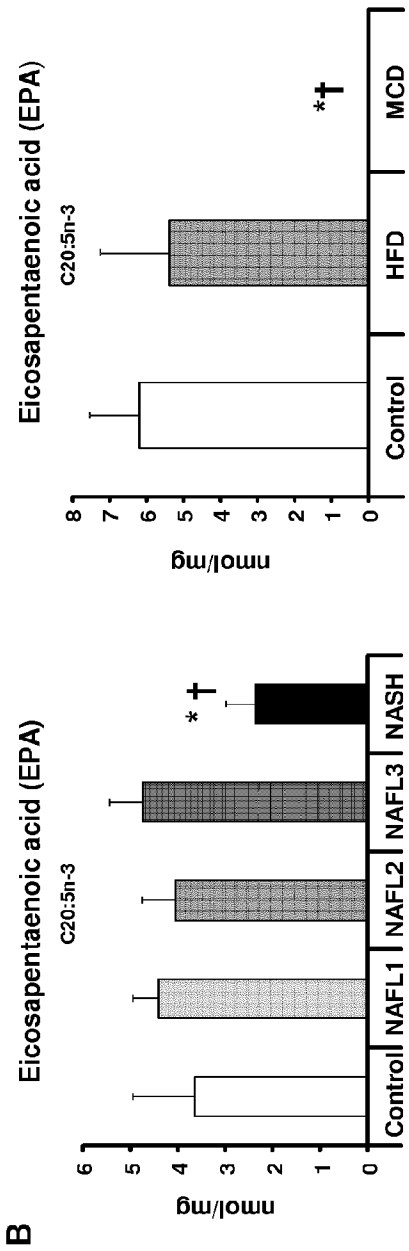

B
Eicosapentaenoic acid (EPA)
C20:5n-3
nmol/mg
0
1
2
3
4
5
6
Control
NAFL1
NAFL2
NAFL3
NASH
*†
Eicosapentaenoic acid (EPA)
C20:5n-3
nmol/mg
0
1
2
3
4
5
6
7
8
Control
HFD
MCD
*†

Figure 4B

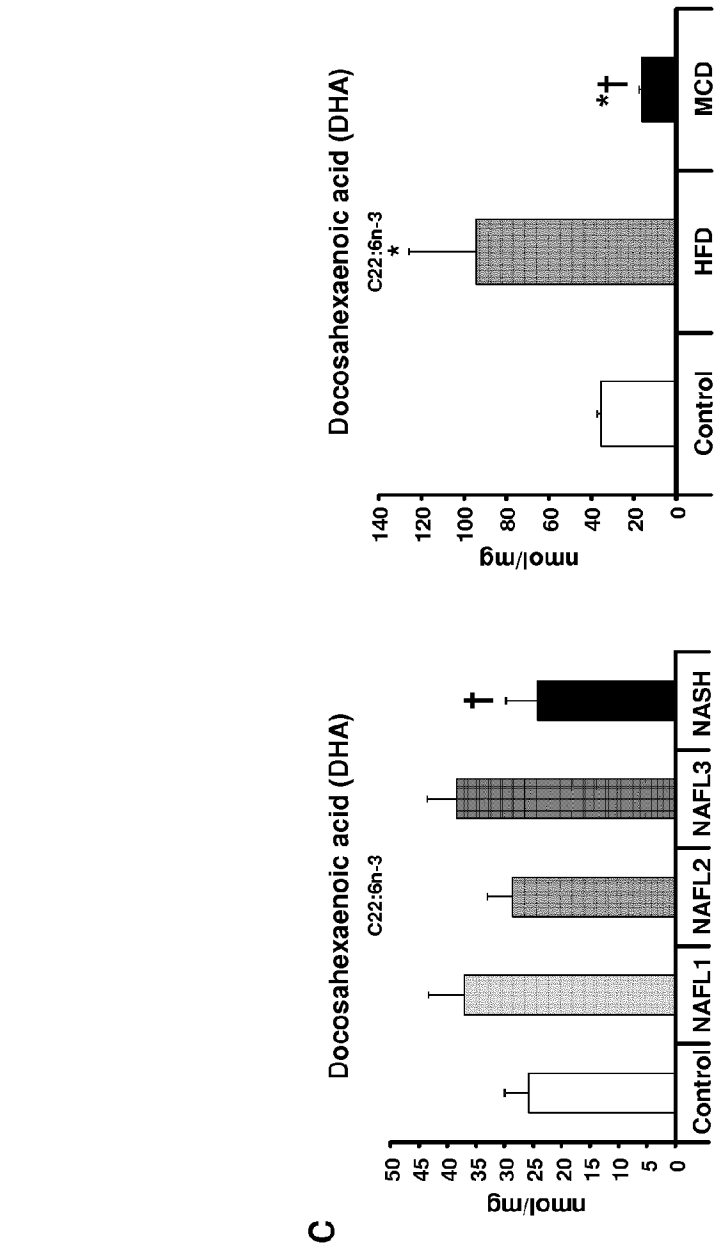

C
Docosahexaenoic acid (DHA)
C22:6n-3
nmol/mg
0
5
10
15
20
25
30
35
40
45
50
Control
NAFL1
NAFL2
NAFL3
NASH
+
Docosahexaenoic acid (DHA)
C22:6n-3
nmol/mg
0
20
40
60
80
100
120
140
Control
HFD
MCD
*
*+

Figure 4C

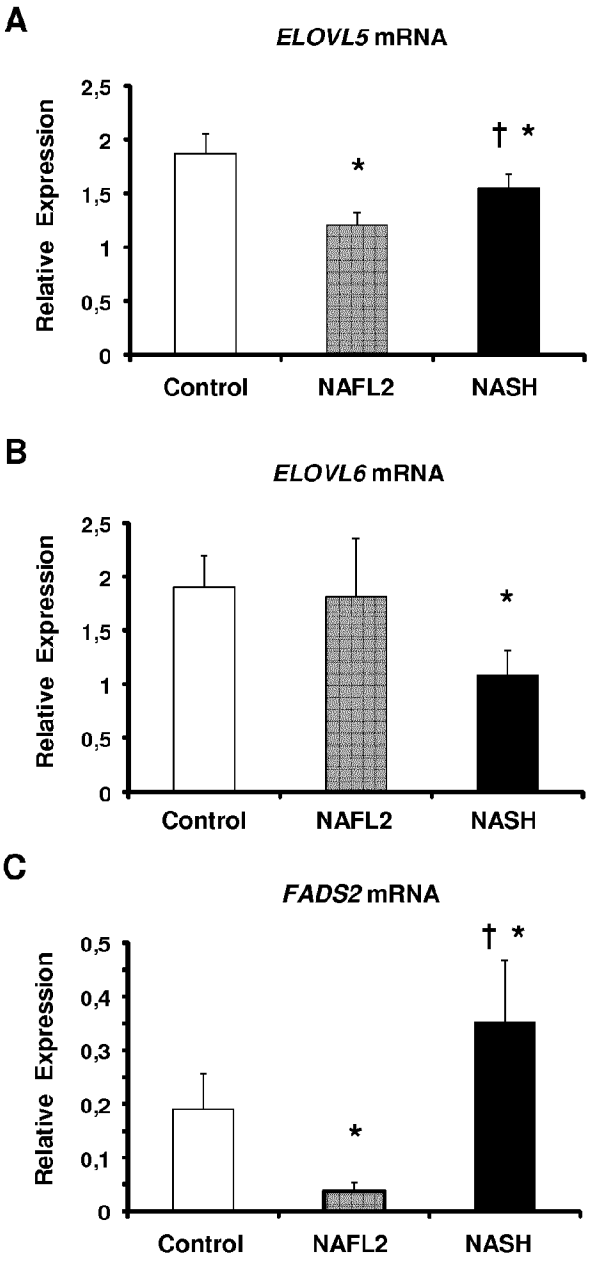

A
ELOVL5 mRNA
Relative Expression
2,5
2
1,5
1
0,5
0
*
† *
Control
NAFL2
NASH
B
ELOVL6 mRNA
Relative Expression
2,5
2
1,5
1
0,5
0
*
Control
NAFL2
NASH
C
FADS2 mRNA
Relative Expression
0,5
0,4
0,3
0,2
0,1
0
*
† *
Control
NAFL2
NASH

Figure 5A-C

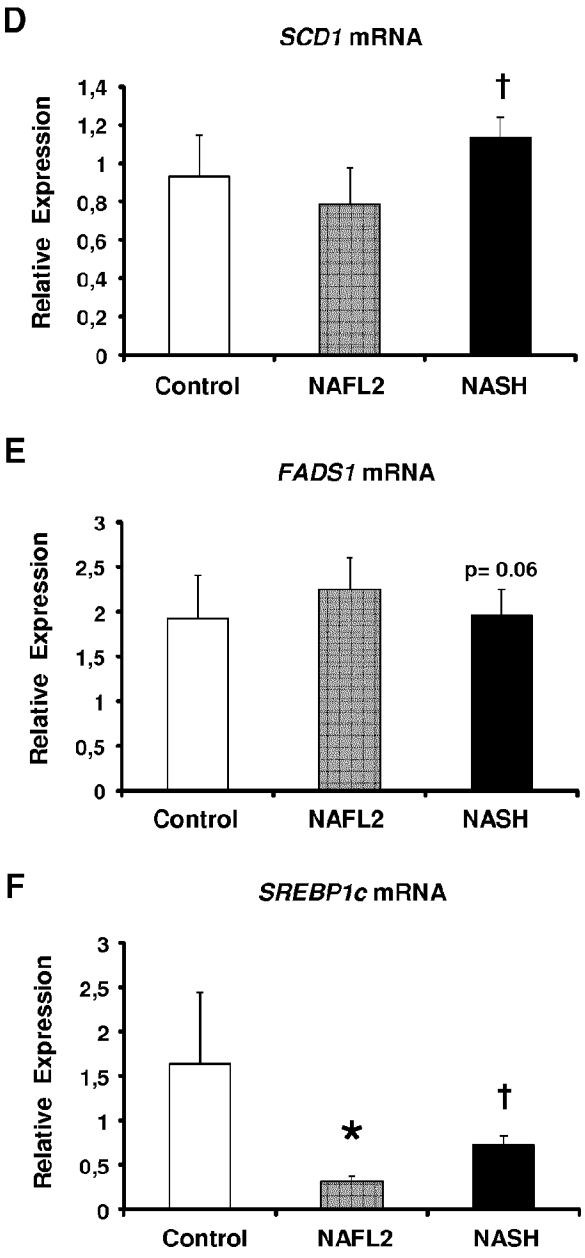

D
SCD1 mRNA
Relative Expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
†
Control
NAFL2
NASH
E
FADS1 mRNA
Relative Expression
3
2,5
2
1,5
1
0,5
0
p= 0.06
Control
NAFL2
NASH
F
SREBP1c mRNA
Relative Expression
3
2,5
2
1,5
1
0,5
0
*
†
Control
NAFL2
NASH

Figure 5D-F

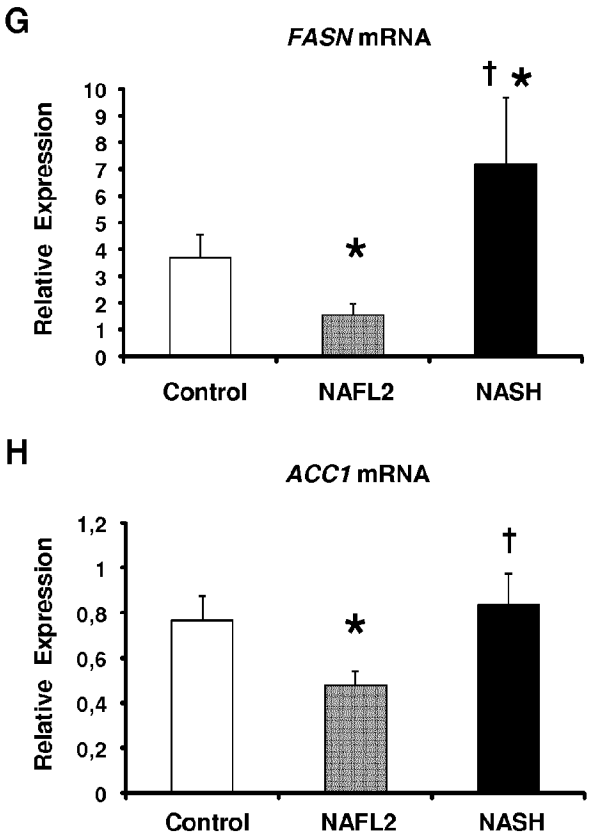
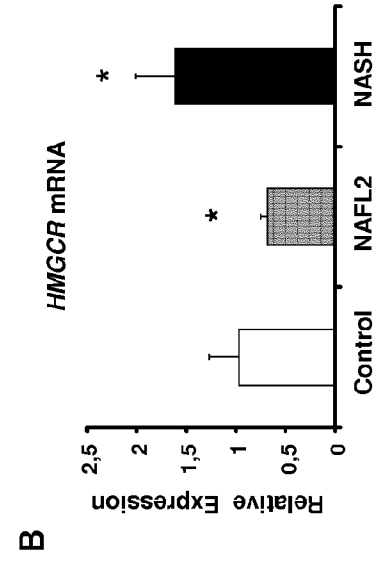

G
FASN mRNA
Relative Expression
10
9
8
7
6
5
4
3
2
1
0
† *
*
Control
NAFL2
NASH
H
ACC1 mRNA
Relative Expression
1,2
1
0,8
0,6
0,4
0,2
0
†
*
Control
NAFL2
NASH

Figure 5G-H


B
HMGCR mRNA
Relative Expression
2,5
2
1,5
1
0,5
0
*
*
Control
NAFL2
NASH

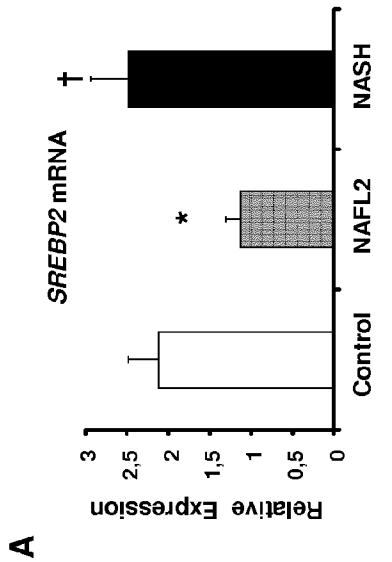

A
SREBP2 mRNA
Relative Expression
3
2,5
2
1,5
1
0,5
0
*
†
Control
NAFL2
NASH

Figure 6A-B

5

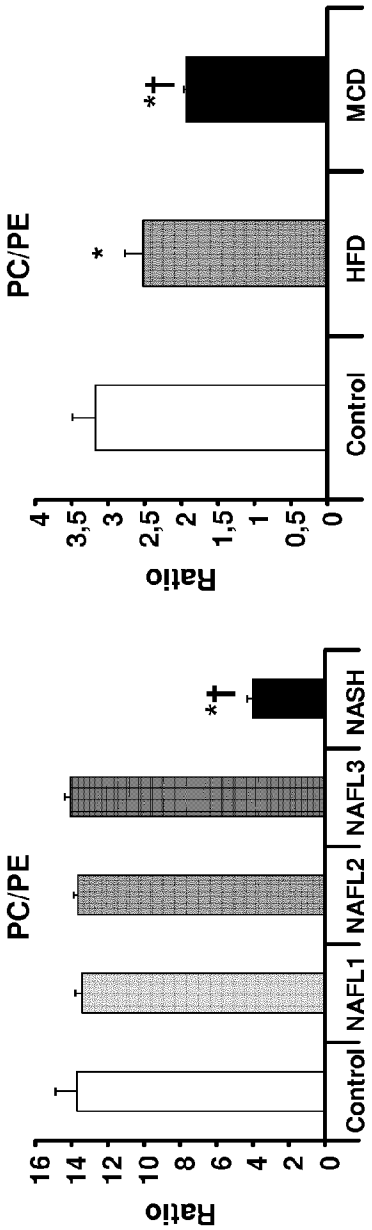

PC/PE
Ratio
0
2
4
6
8
10
12
14
16
Control
NAFL1
NAFL2
NAFL3
NASH
*†
PC/PE
Ratio
0
0,5
1
1,5
2
2,5
3
3,5
4
Control
HFD
MCD
*
*†

Figure 7

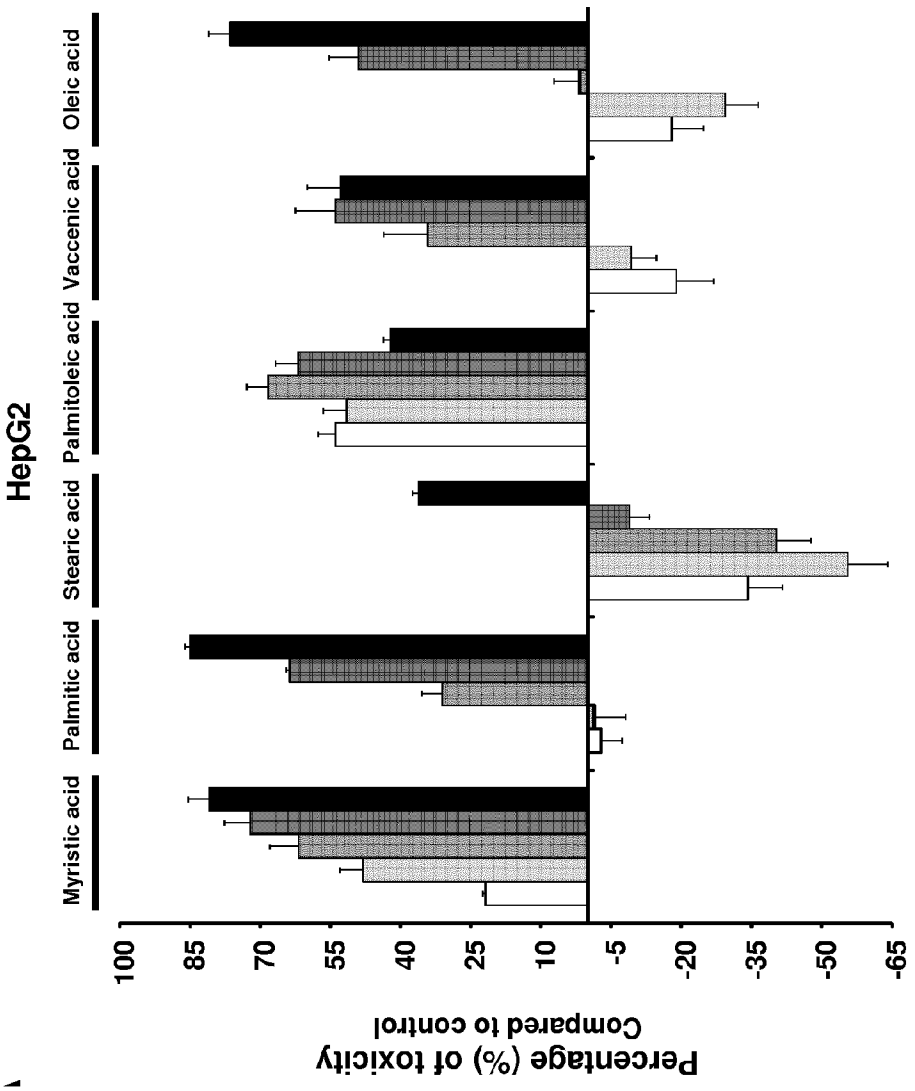

HepG2
Myristic acid
Palmitic acid
Stearic acid
Palmitoleic acid
Vaccenic acid
Oleic acid
100
85
70
55
40
25
10
-5
-20
-35
-50
-65
Percentage (%) of toxicity
Compared to control

Figure 8A

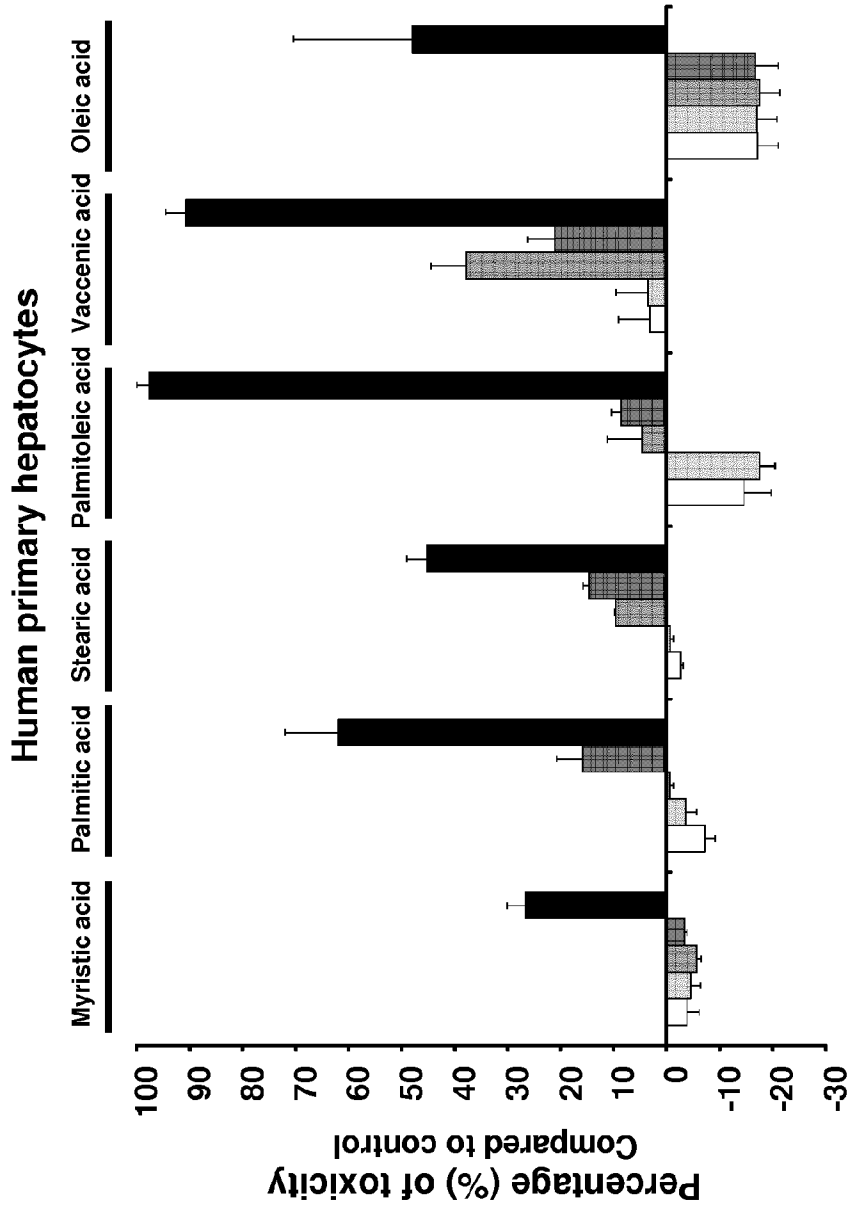

Human primary hepatocytes
Myristic acid
Palmitic acid
Stearic acid
Palmitoleic acid
Vaccenic acid
Oleic acid
Percentage (%) of toxicity
Compared to control
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30

Figure 8B

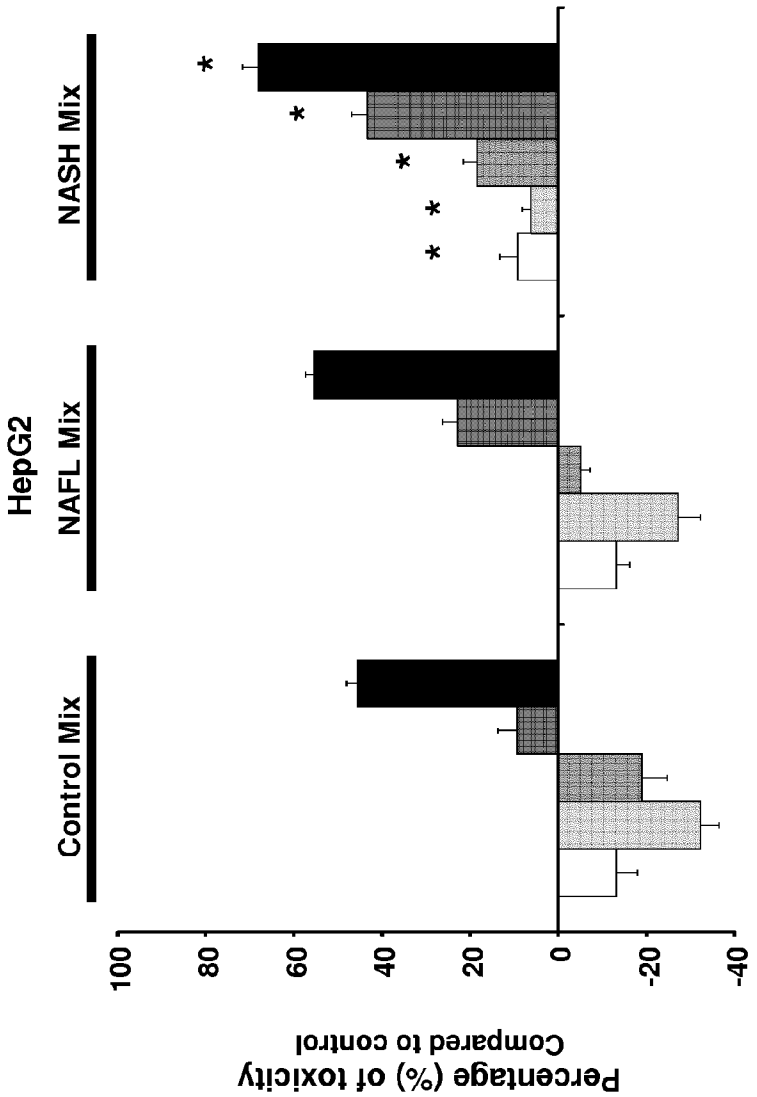

HepG2
Control Mix
NAFL Mix
NASH Mix
Percentage (%) of toxicity
Compared to control
100
80
60
40
20
0
-20
-40
*
*
*
*
*

Figure 8C

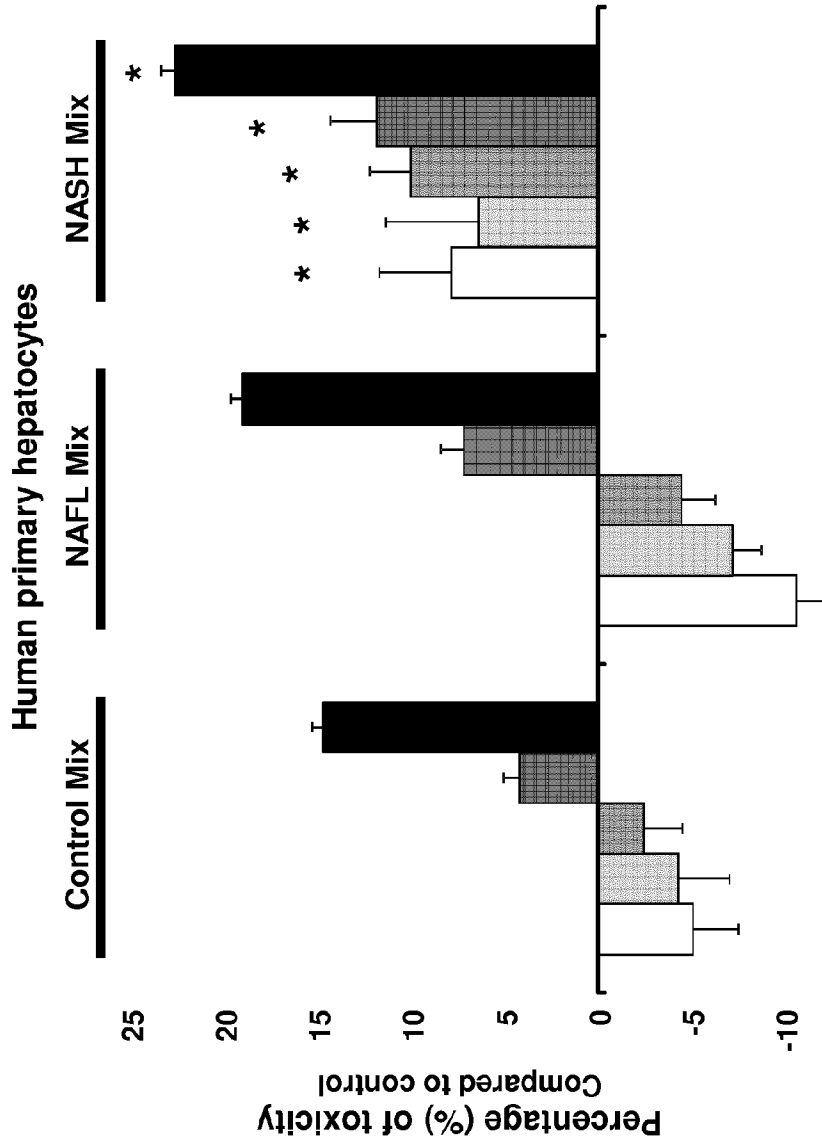

Human primary hepatocytes
Control Mix
NAFL Mix
NASH Mix
Percentage (%) of toxicity
Compared to control
25
20
15
10
5
0
-5
-10
*
*
*
*
*

Figure 8D

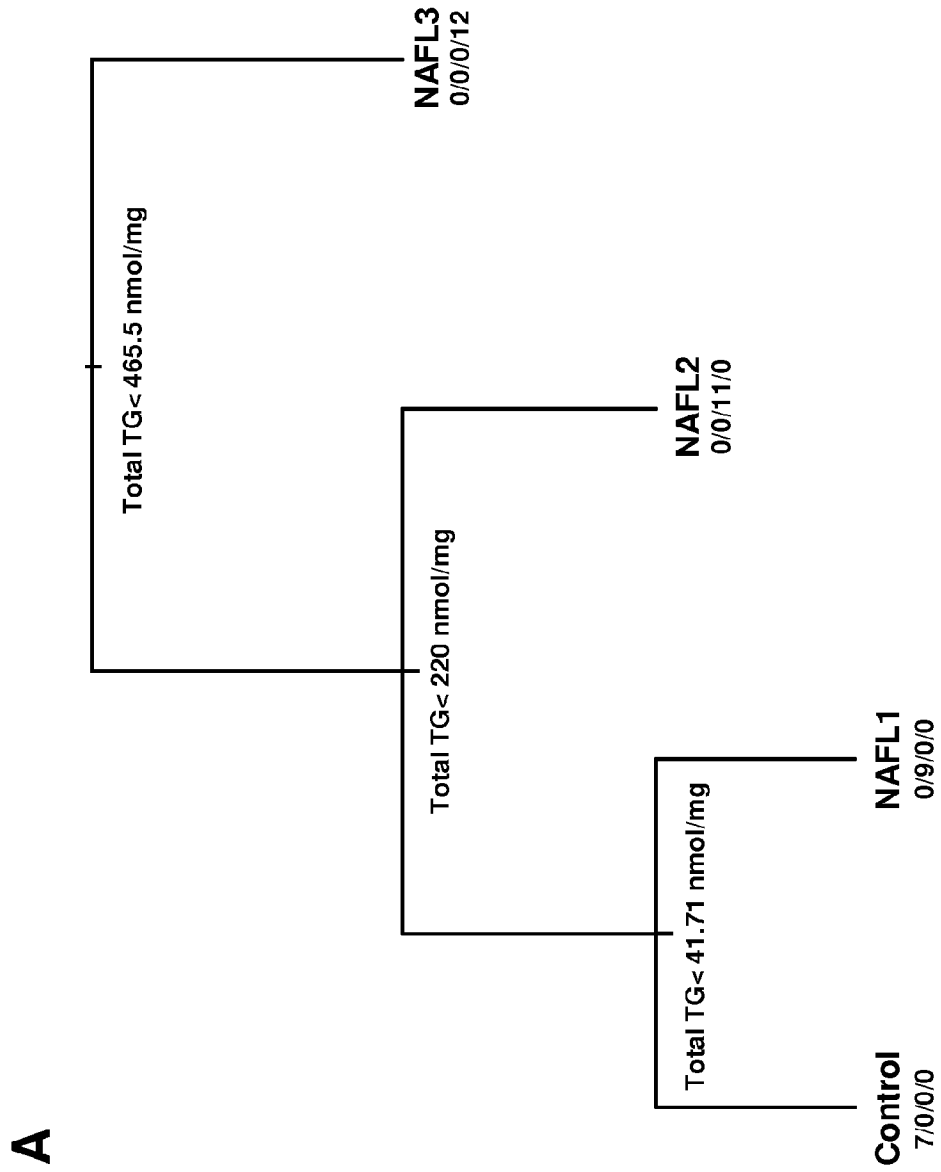

A
Total TG< 465.5 nmol/mg
Total TG< 220 nmol/mg
Total TG< 41.71 nmol/mg
NAFL3
0/0/0/12
NAFL2
0/0/11/0
NAFL1
0/9/0/0
Control
7/0/0/0

Figure 9A

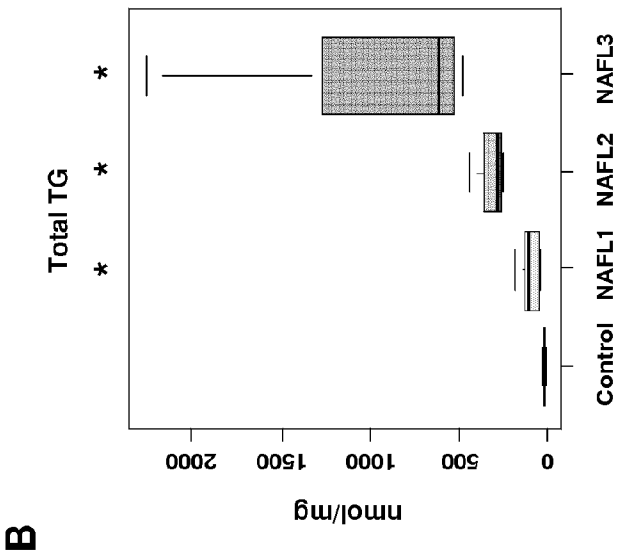

B
Total TG
nmol/mg
0
500
1000
1500
2000
Control
NAFL1
NAFL2
NAFL3
*
*
*

Figure 9B

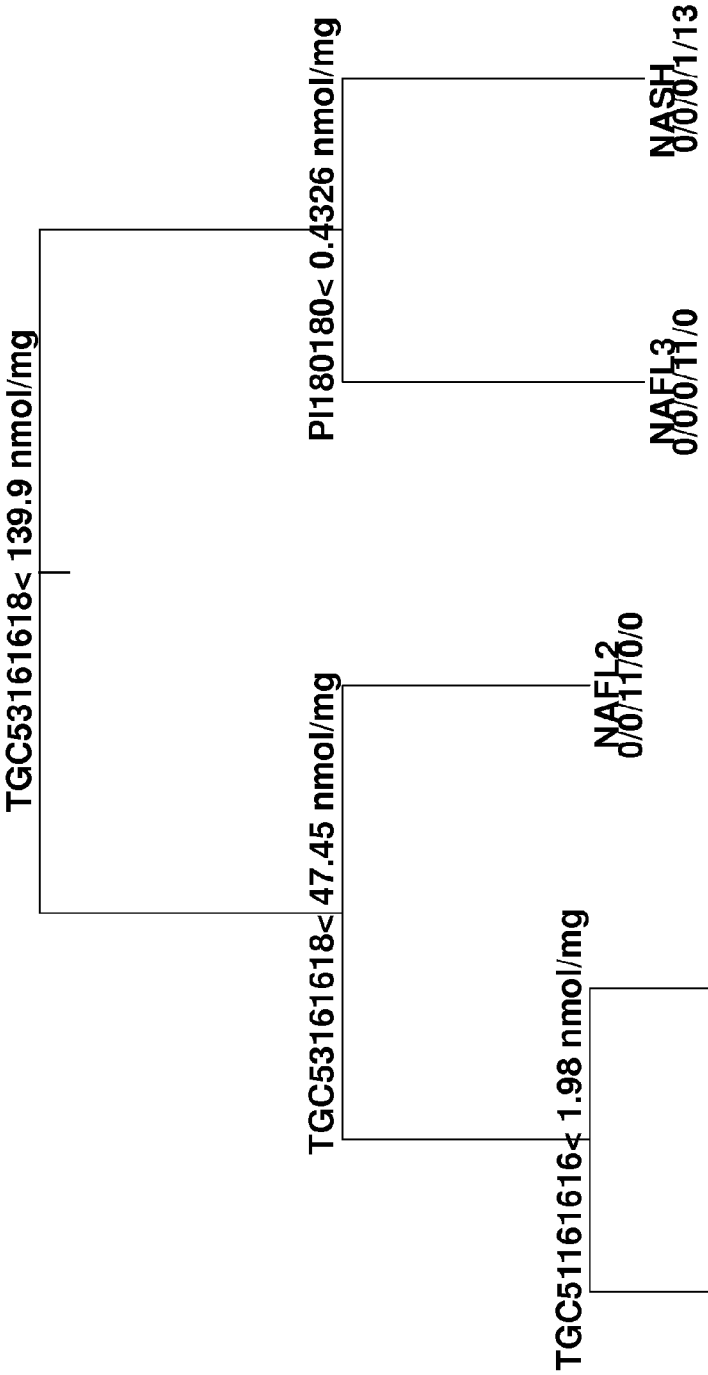

TGC53161618< 139.9 nmol/mg
PI180180< 0.4326 nmol/mg
NASH
0/0/0/1/13
NAFL3
0/0/0/11/0
TGC53161618< 47.45 nmol/mg
NAFL2
0/0/11/0/0
TGC51161616< 1.98 nmol/mg
NAFL1
0/9/0/0/0
Control
7/8/0/0/0

Figure 10

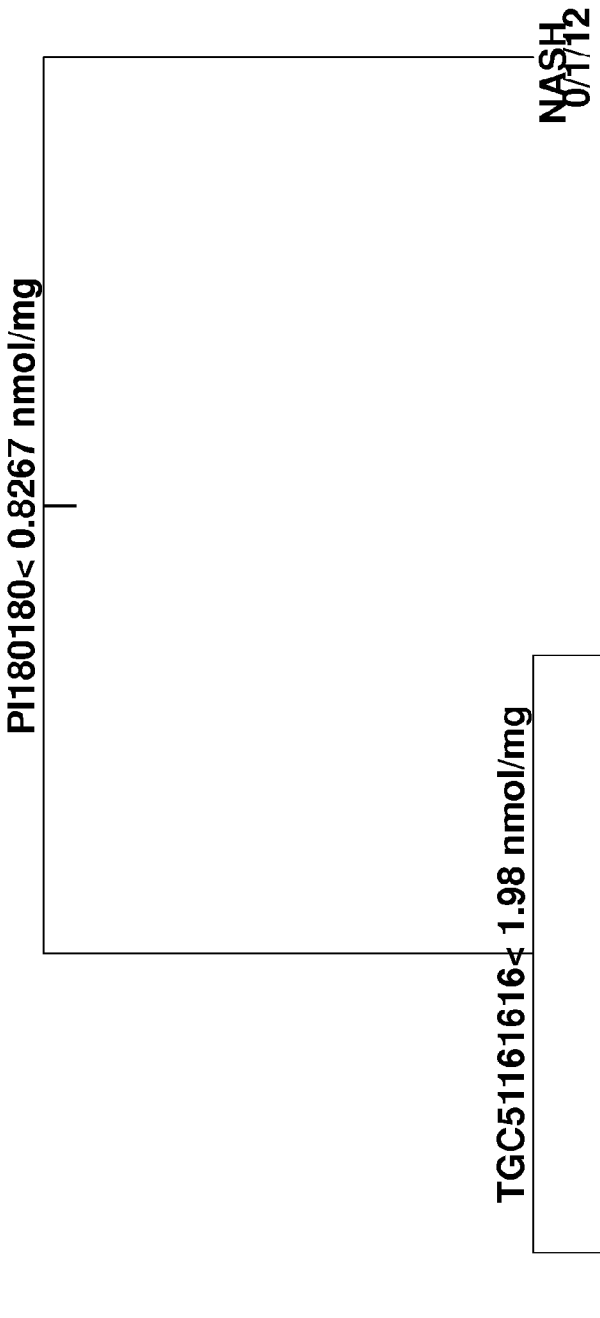

PI180180< 0.8267 nmol/mg
NASH
0/1/12
TGC51161616< 1.98 nmol/mg
NAFL
0/3/11
Control
7/0/0

Figure 11

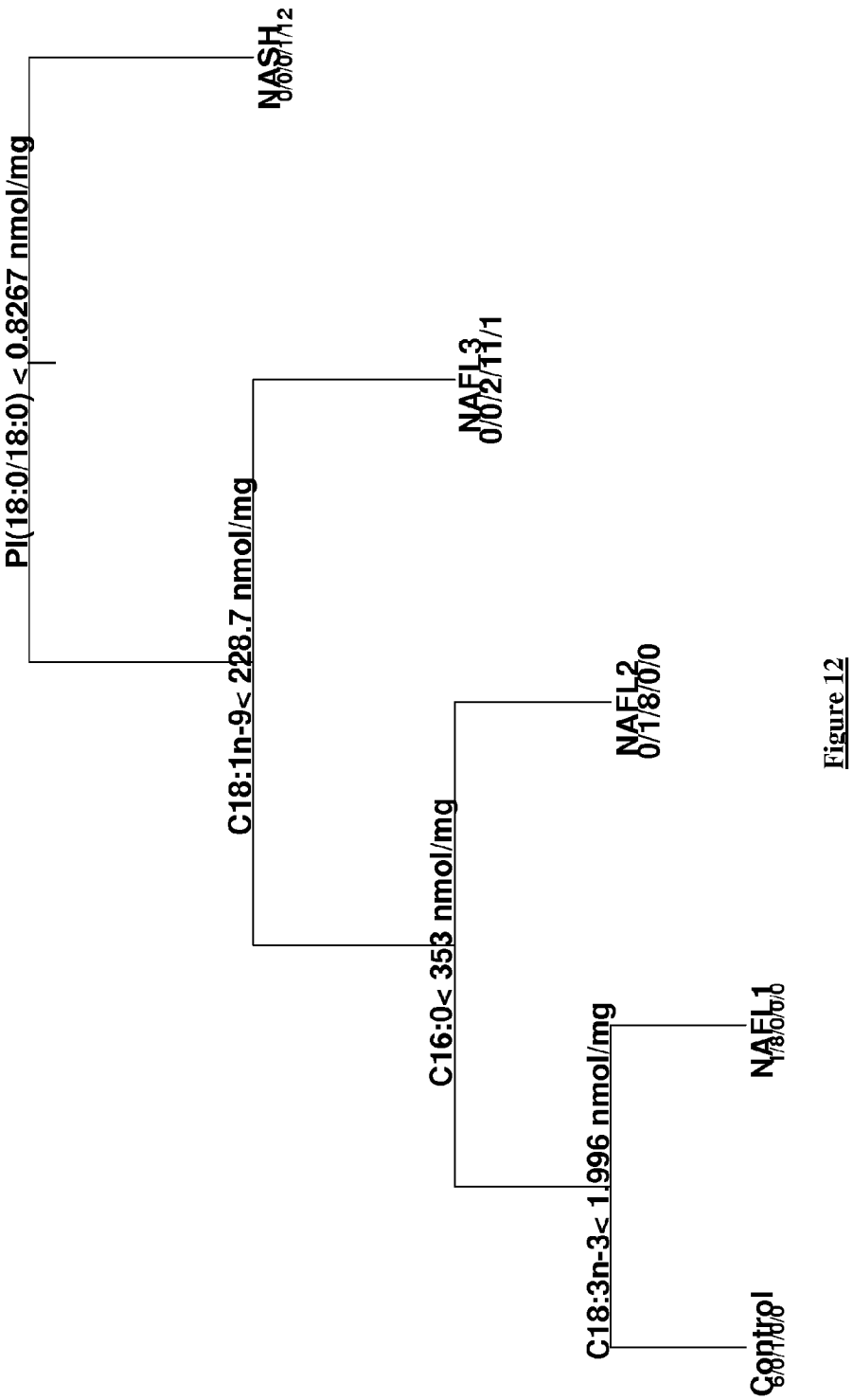

PI(18:0/18:0) < 0.8267 nmol/mg
NASH
0/6/1/2
C18:1n-9< 228.7 nmol/mg
NAFL3
0/0/2/1/1
C16:0< 353 nmol/mg
NAFL2
0/1/8/0/0
C18:3n-3< 1.996 nmol/mg
NAFL1
1/8/0/0/0
Control
6/8/1/0/0

Figure 12

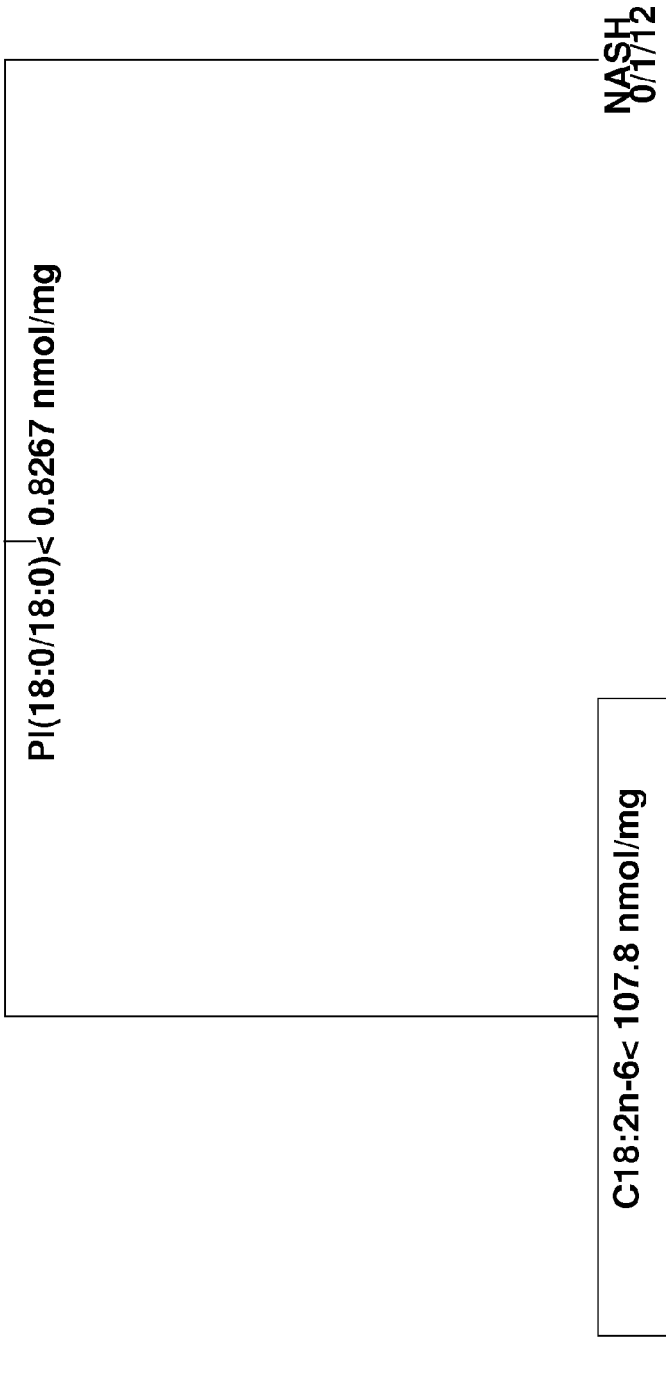

NASH
0/1/12
PI(18:0/18:0)< 0.8267 nmol/mg
C18:2n-6< 107.8 nmol/mg
Control

Figure 13

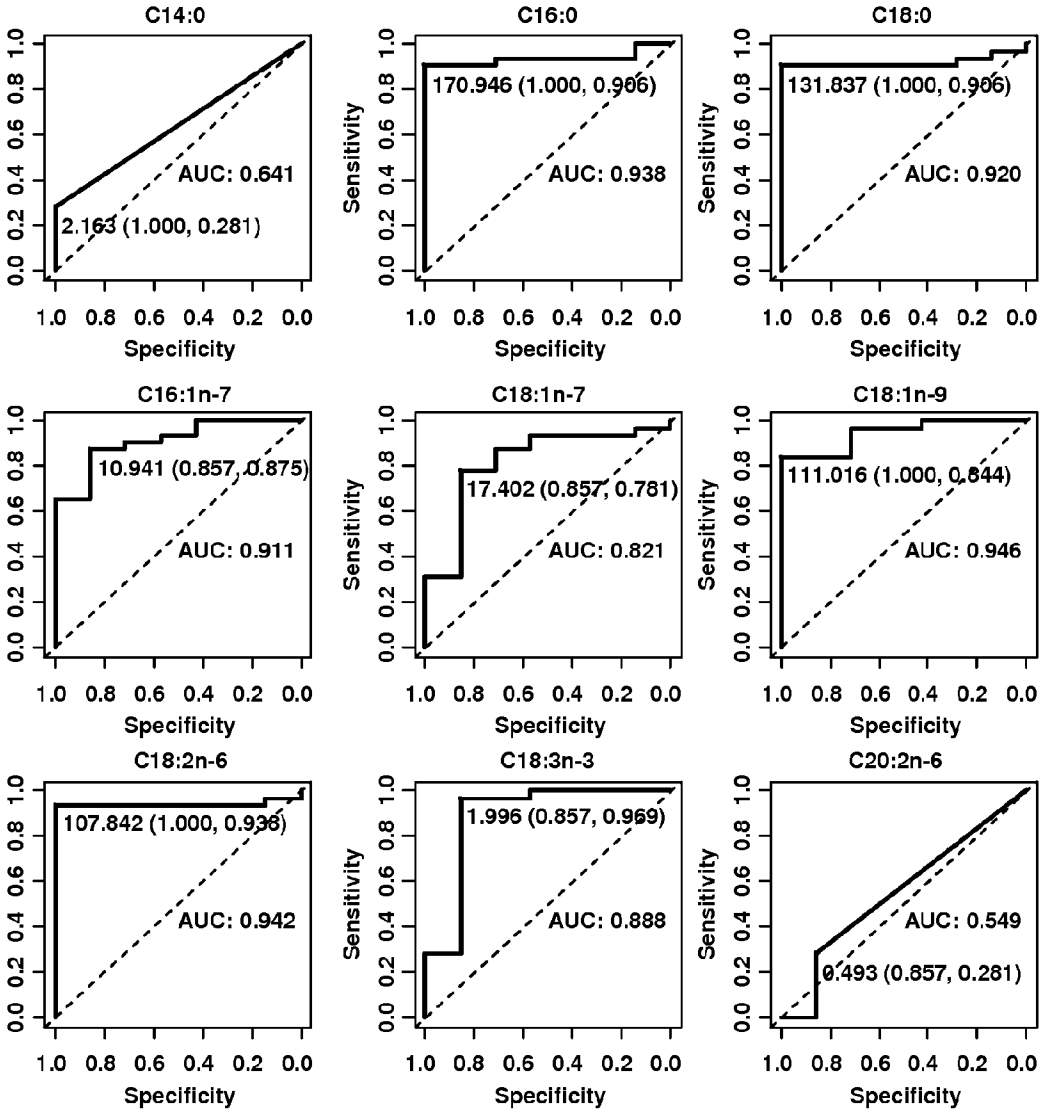

C14:0
AUC: 0.641
2.163 (1.000, 0.281)
C16:0
170.946 (1.000, 0.906)
AUC: 0.938
C18:0
131.837 (1.000, 0.906)
AUC: 0.920
C16:1n-7
10.941 (0.857, 0.875)
AUC: 0.911
C18:1n-7
17.402 (0.857, 0.781)
AUC: 0.821
C18:1n-9
111.016 (1.000, 0.844)
AUC: 0.946
C18:2n-6
107.842 (1.000, 0.938)
AUC: 0.942
C18:3n-3
1.996 (0.857, 0.969)
AUC: 0.888
C20:2n-6
AUC: 0.549
0.493 (0.857, 0.281)
Sensitivity
Specificity
1.0 0.8 0.6 0.4 0.2 0.0
0.0 0.2 0.4 0.6 0.8 1.0

Figure 14A

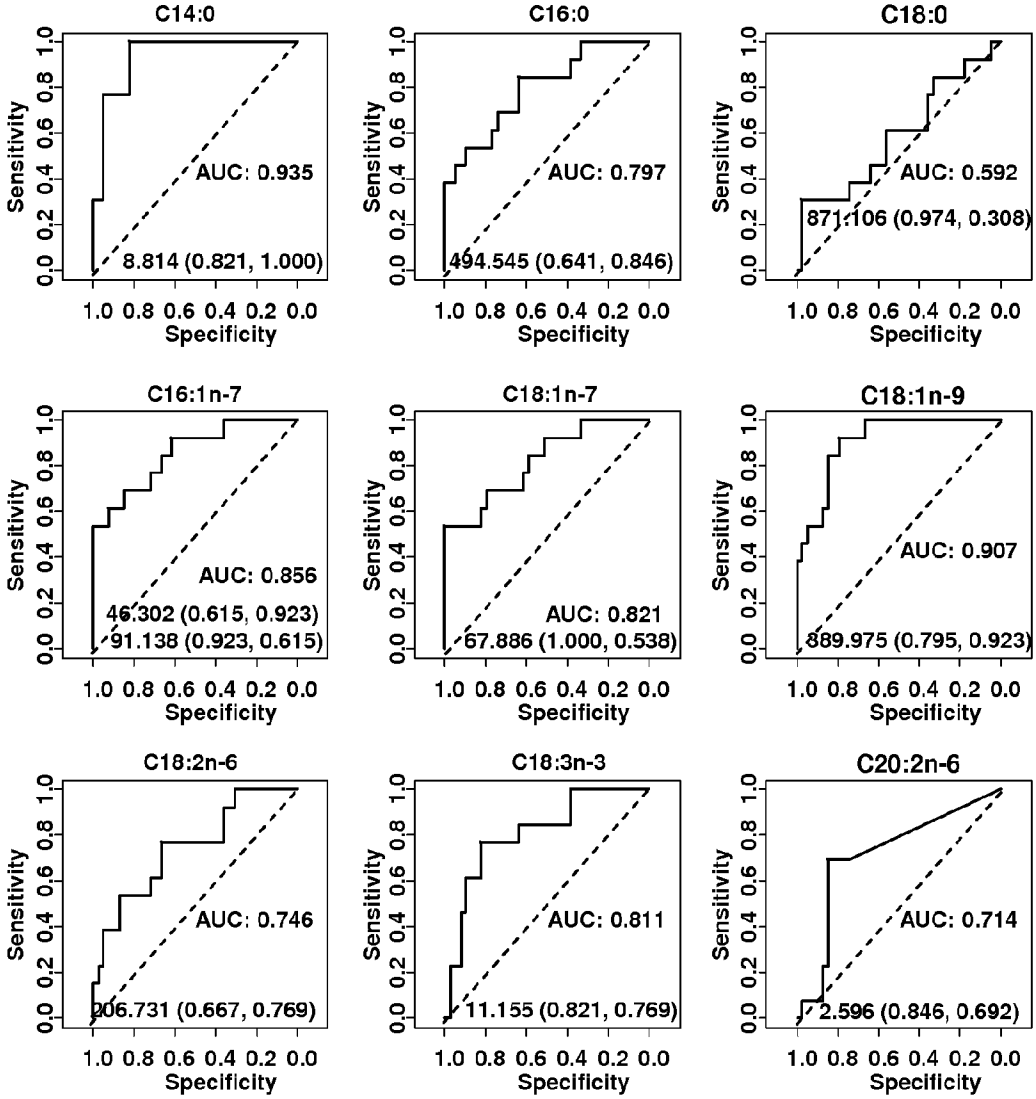

C14:0
AUC: 0.935
8.814 (0.821, 1.000)
C16:0
AUC: 0.797
494.545 (0.641, 0.846)
C18:0
AUC: 0.592
871.106 (0.974, 0.308)
C16:1n-7
AUC: 0.856
46.302 (0.615, 0.923)
91.138 (0.923, 0.615)
C18:1n-7
AUC: 0.821
67.886 (1.000, 0.538)
C18:1n-9
AUC: 0.907
889.975 (0.795, 0.923)
C18:2n-6
AUC: 0.746
206.731 (0.667, 0.769)
C18:3n-3
AUC: 0.811
11.155 (0.821, 0.769)
C20:2n-6
AUC: 0.714
2.596 (0.846, 0.692)
Sensitivity
Specificity
1.0 0.8 0.6 0.4 0.2 0.0
0.0 0.2 0.4 0.6 0.8 1.0

Figure 14B

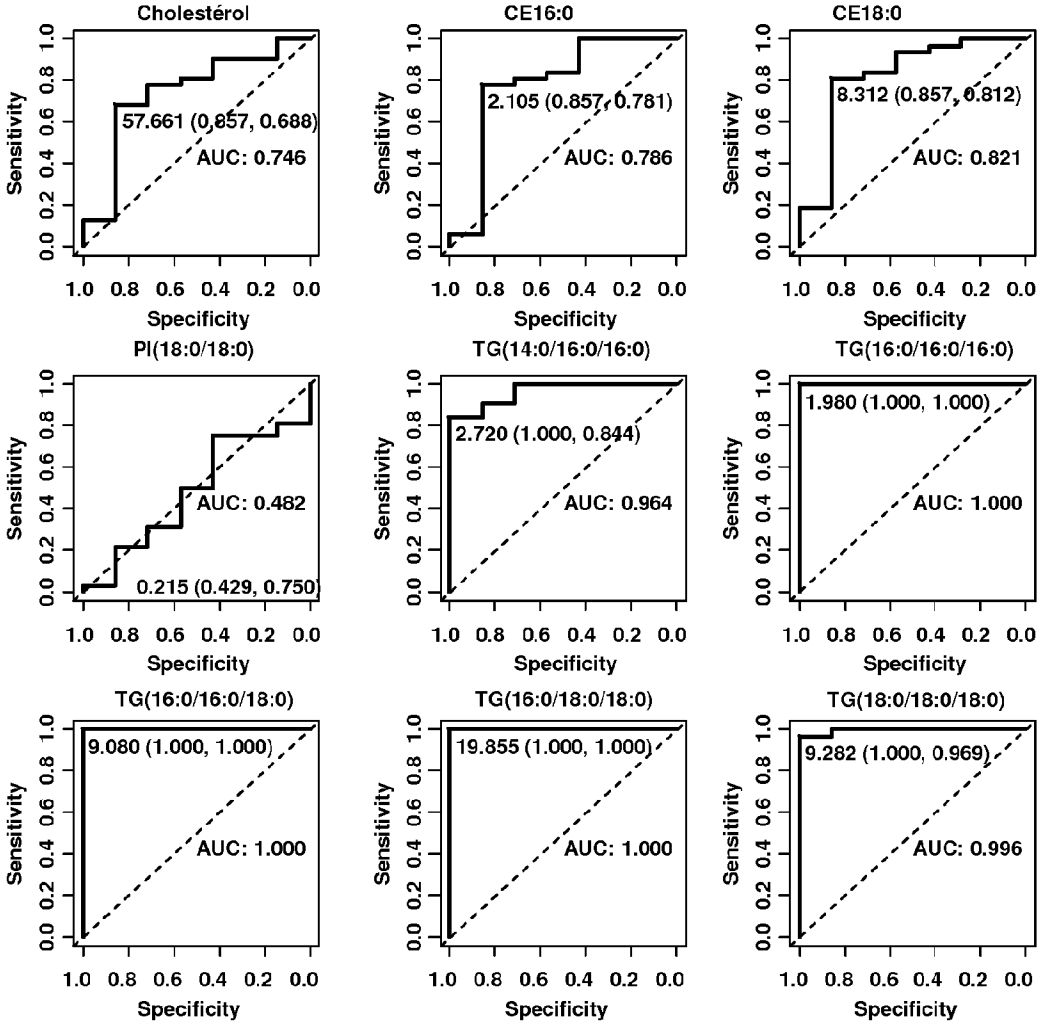

Cholestérol
57.661 (0,857, 0.688)
AUC: 0.746
CE16:0
2.105 (0.857, 0.781)
AUC: 0.786
CE18:0
8.312 (0.857, 0.812)
AUC: 0.821
PI(18:0/18:0)
AUC: 0.482
0.215 (0.429, 0.750)
TG(14:0/16:0/16:0)
2.720 (1.000, 0.844)
AUC: 0.964
TG(16:0/16:0/16:0)
1.980 (1.000, 1.000)
AUC: 1.000
TG(16:0/16:0/18:0)
9.080 (1.000, 1.000)
AUC: 1.000
TG(16:0/18:0/18:0)
19.855 (1.000, 1.000)
AUC: 1.000
TG(18:0/18:0/18:0)
9.282 (1.000, 0.969)
AUC: 0.996
Sensitivity
Specificity
0.0 0.2 0.4 0.6 0.8 1.0
1.0 0.8 0.6 0.4 0.2 0.0

Figure 15A

5

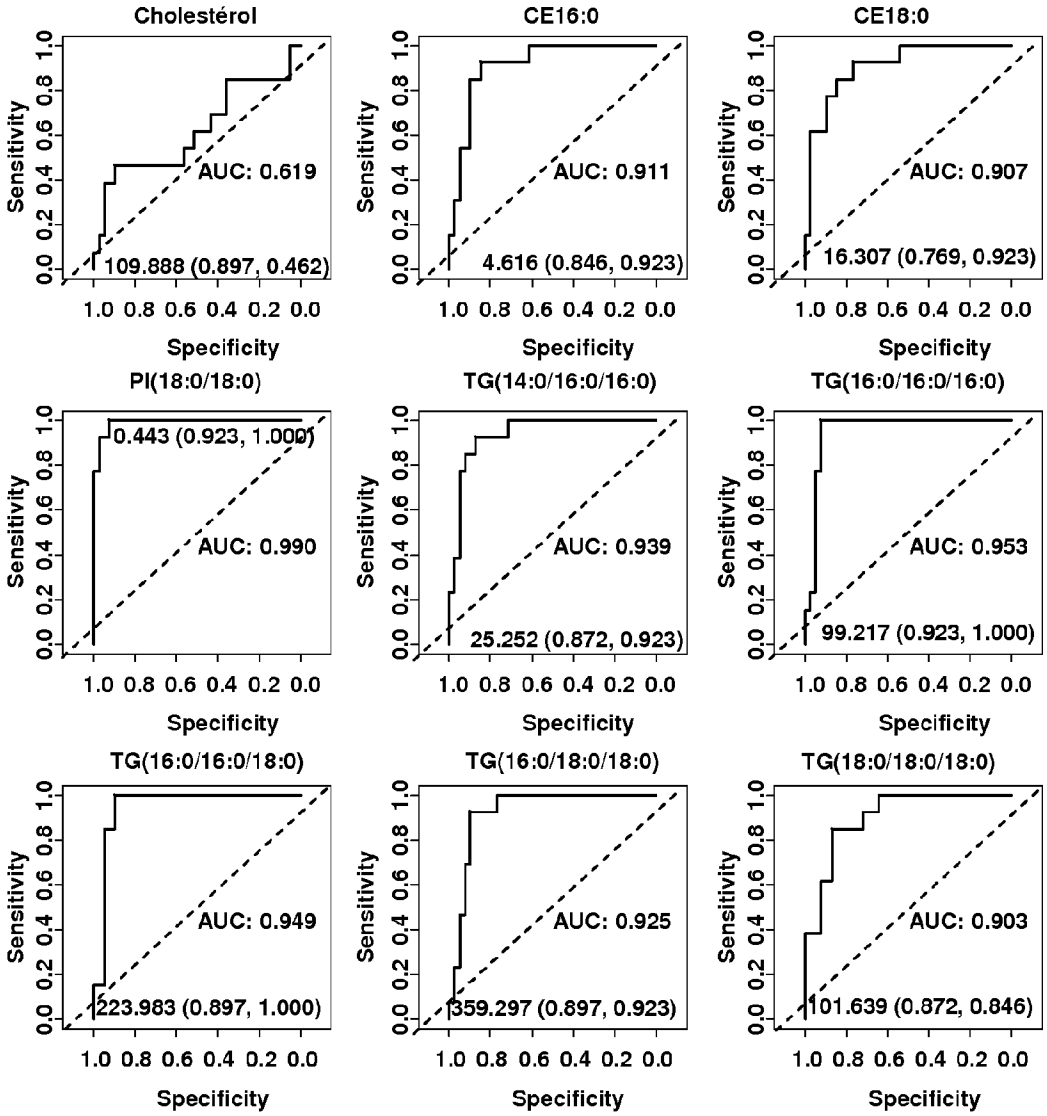

Cholestérol
AUC: 0.619
109.888 (0.897, 0.462)
CE16:0
AUC: 0.911
4.616 (0.846, 0.923)
CE18:0
AUC: 0.907
16.307 (0.769, 0.923)
PI(18:0/18:0)
0.443 (0.923, 1.000)
AUC: 0.990
TG(14:0/16:0/16:0)
AUC: 0.939
25.252 (0.872, 0.923)
TG(16:0/16:0/16:0)
AUC: 0.953
99.217 (0.923, 1.000)
TG(16:0/16:0/18:0)
AUC: 0.949
223.983 (0.897, 1.000)
TG(16:0/18:0/18:0)
AUC: 0.925
359.297 (0.897, 0.923)
TG(18:0/18:0/18:0)
AUC: 0.903
101.639 (0.872, 0.846)
Sensitivity
Specificity
0.0 0.2 0.4 0.6 0.8 1.0
1.0 0.8 0.6 0.4 0.2 0.0

Figure 15B

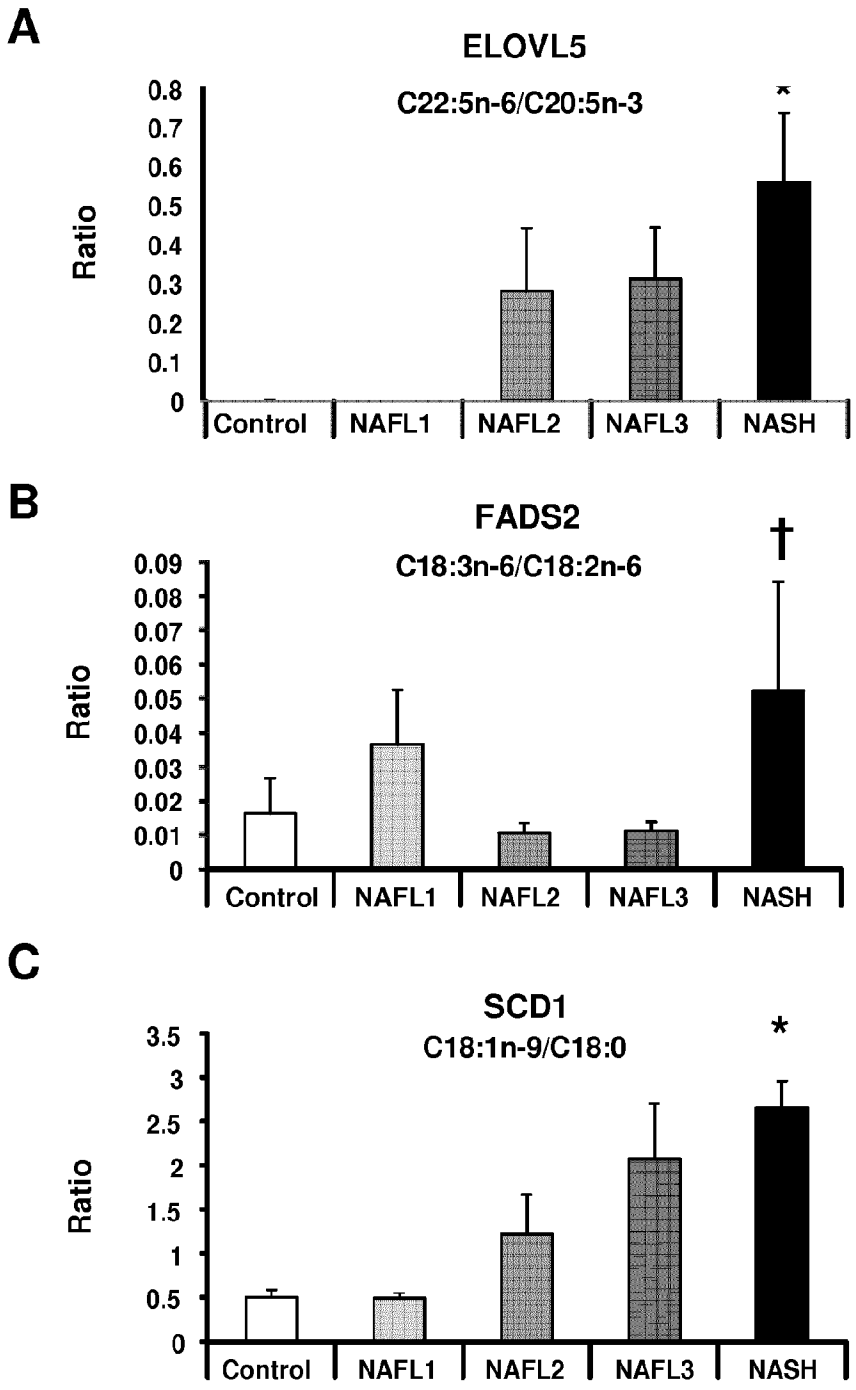

A
ELOVL5
C22:5n-6/C20:5n-3
Ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
NAFL1
NAFL2
NAFL3
NASH
B
FADS2
C18:3n-6/C18:2n-6
Ratio
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Control
NAFL1
NAFL2
NAFL3
NASH
C
SCD1
C18:1n-9/C18:0
Ratio
3.5
3
2.5
2
1.5
1
0.5
0
Control
NAFL1
NAFL2
NAFL3
NASH

Figure 16A-C

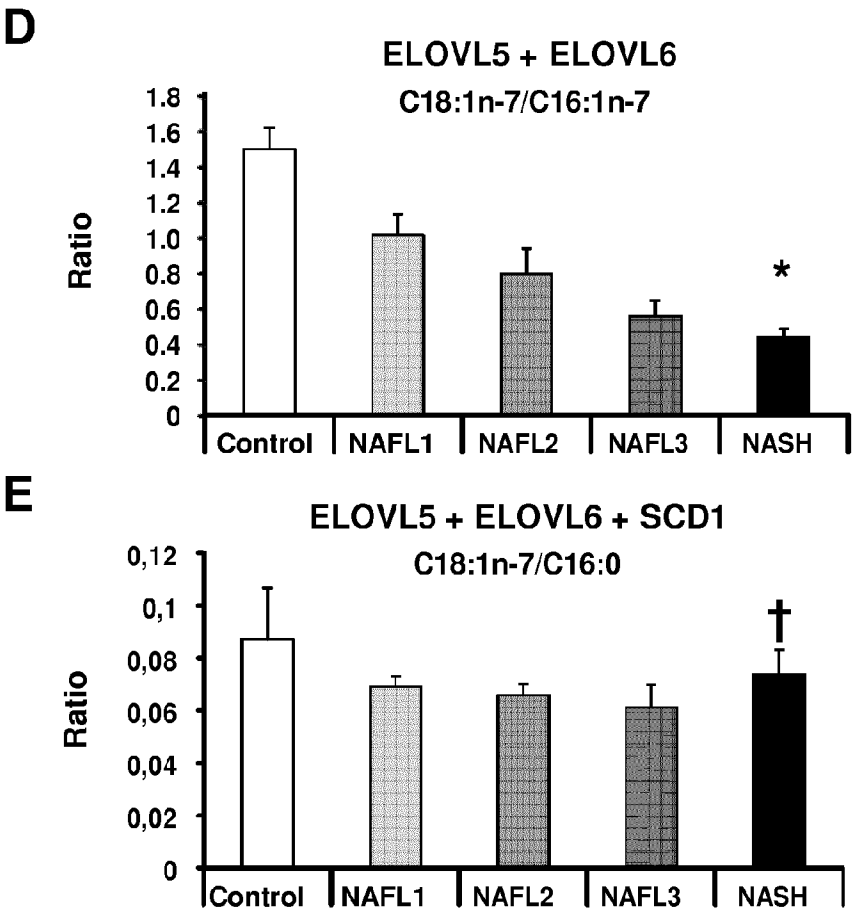

D
ELOVL5 + ELOVL6
C18:1n-7/C16:1n-7
Ratio
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Control
NAFL1
NAFL2
NAFL3
NASH
E
ELOVL5 + ELOVL6 + SCD1
C18:1n-7/C16:0
Ratio
0,12
0,1
0,08
0,06
0,04
0,02
0
†
Control
NAFL1
NAFL2
NAFL3
NASH

Figure 16D-E

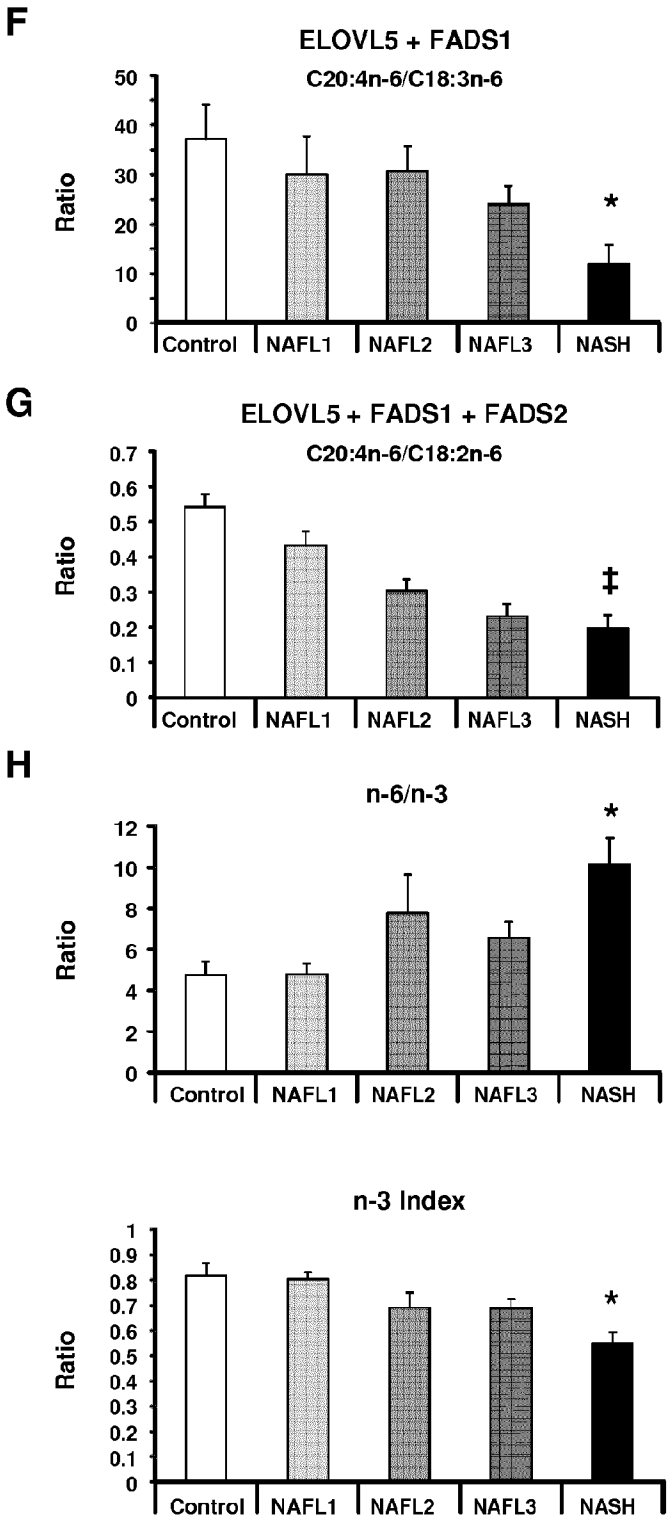

F
ELOVL5 + FADS1
C20:4n-6/C18:3n-6
Ratio
50
40
30
20
10
0
*
Control
NAFL1
NAFL2
NAFL3
NASH
G
ELOVL5 + FADS1 + FADS2
C20:4n-6/C18:2n-6
Ratio
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
‡
Control
NAFL1
NAFL2
NAFL3
NASH
H
n-6/n-3
Ratio
12
10
8
6
4
2
0
*
Control
NAFL1
NAFL2
NAFL3
NASH
n-3 Index
Ratio
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Control
NAFL1
NAFL2
NAFL3
NASH

Figure 16F-H

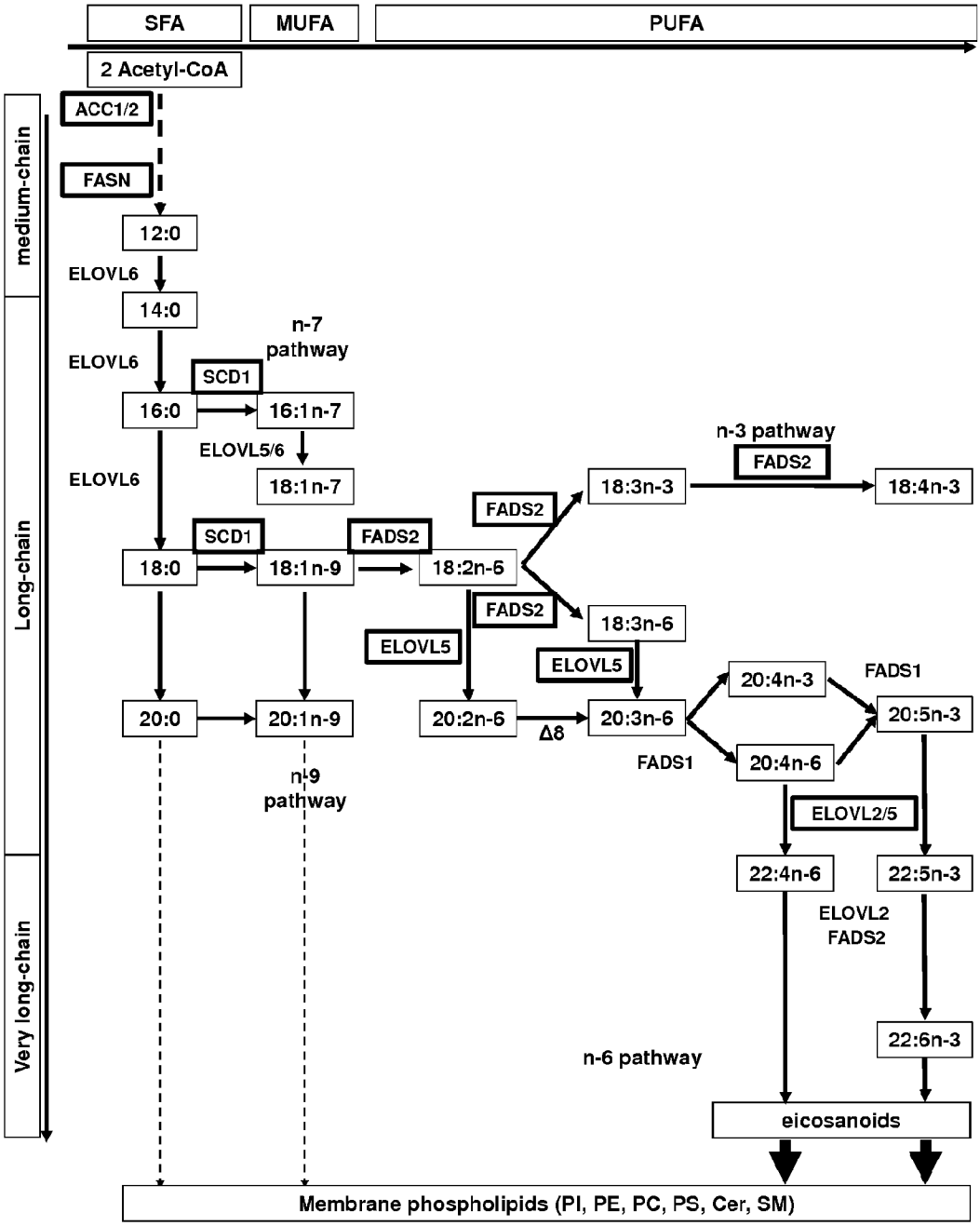

SFA
MUFA
PUFA
2 Acetyl-CoA
ACC1/2
FASN
12:0
ELOVL6
14:0
ELOVL6
n-7 pathway
SCD1
16:0
16:1n-7
ELOVL5/6
18:1n-7
ELOVL6
SCD1
FADS2
18:0
18:1n-9
18:2n-6
FADS2
18:3n-3
n-3 pathway
FADS2
18:4n-3
FADS2
18:3n-6
ELOVL5
ELOVL5
20:0
20:1n-9
20:2n-6
Δ8
20:3n-6
20:4n-3
FADS1
20:5n-3
FADS1
20:4n-6
n-9 pathway
ELOVL2/5
22:4n-6
22:5n-3
ELOVL2
FADS2
22:6n-3
n-6 pathway
eicosanoids
Membrane phospholipids (PI, PE, PC, PS, Cer, SM)
medium-chain
Long-chain
Very long-chain

Figure 17

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/057447

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 G01N33/92
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)
C12Q 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, BIOSIS, CHEM ABS Data, Sequence Search, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/113992 A1 (TEKNOLOGIAN TUTKIMUSKESKUS VTT [FI]) 8 August 2013 (2013-08-08) claims; examples	1,3, 10-12,19
X	ANJANI KAVYA ET AL: "Circulating phospholipid profiling identifies portal contribution to NASH signature in obesity", JOURNAL OF HEPATOLOGY, vol. 62, no. 4, 8 November 2014 (2014-11-08), pages 905-912, XP029205792, ISSN: 0168-8278, DOI: 10.1016/J.JHEP.2014.11.002 introduction; page 907, column 1; page 908, figure 2F	1,2, 4-10,12, 14,16, 19,20

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[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
1 June 2017	31/07/2017

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	Hennard, Christophe

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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.
X	PAOLA STIUSO ET AL: "Serum Oxidative Stress Markers and Lipidomic Profile to Detect NASH Patients Responsive to an Antioxidant Treatment: A Pilot Study", OXIDATIVE MEDICINE AND CELLULAR LONGEVITY, vol. 2, no. 1, 1 January 2014 (2014-01-01) , pages 95-8, XP055298842, US ISSN: 1942-0900, DOI: 10.1161/01.ATV.17.7.1258 introduction; page 6, table 4	1,2,10, 15,19
X	FONG ET AL: "Protein and lipid composition of bovine milk-fat-globule membrane", INTERNATIONAL DAIRY JOURNAL, ELSEVIER APPLIED SCIENCE, BARKING, GB, vol. 17, no. 4, 8 January 2007 (2007-01-08), pages 275-288, XP005824820, ISSN: 0958-6946, DOI: 10.1016/J.IDAIRYJ.2006.05.004 abstract; tables	13,18,19
Y	WO 2015/089102 A1 (UNIV CALIFORNIA [US]) 18 June 2015 (2015-06-18) claims	1
Y	Rohit Loomba ET AL: "Polyunsaturated fatty acid metabolites as novel lipidomic biomarkers for noninvasive diagnosis of nonalcoholic steatohepatitis", Journal of lipid research, 1 January 2015 (2015-01-01), pages 185-192, XP055298849, United States DOI: 10.1194/jlr.P055640 Retrieved from the Internet: URL:http://www.jlr.org/content/56/1/185.full.pdf+html page 188, table2	1
X	Hannele Yki-Järvinen: "Nutritional Modulation of Non-Alcoholic Fatty Liver Disease and Insulin Resistance", Nutrients, 1 November 2015 (2015-11-01), pages 9127-9138, XP055298850, Switzerland DOI: 10.3390/nu7115454 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4663582/pdf/nutrients-07-05454.pdf page 9132, item 3	13

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3

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 1 739 431 A1 (AJINOMOTO KK [JP]) 3 January 2007 (2007-01-03) claim 1; example, Table 2, page 6 -----	1,16
X	WO 2008/021192 A2 (LIPOMICS TECHNOLOGIES INC [US]; WATKINS STEVEN M [US]; WIEST MICHELLE) 21 February 2008 (2008-02-21) claims; examples -----	1-16, 18-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2017/057447

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-16, 18-20

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-16, 18-20

concern method for determining the occurrence of fatty liver disease; a method for discriminating between NAFL and NASH; lipids; a method for screening a compound and mixtures comprising lipids characterised in that the lipids and their level in a sample are used in the methods.

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2. claim: 17

concerns a method for screening compounds for treating, preventing or reducing the likelihood of occurrence of fatty liver disease in an individual characterised in that the expression level or the content of FADS1 is determined in a sample.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/057447

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013113992 A1	08-08-2013	EP 2810079 A1	10-12-2014
		US 2015011424 A1	08-01-2015
		WO 2013113992 A1	08-08-2013
WO 2015089102 A1	18-06-2015	AU 2014364327 A1	09-06-2016
		CA 2933034 A1	18-06-2015
		CN 105980861 A	28-09-2016
		EP 3080613 A1	19-10-2016
		JP 2016540218 A	22-12-2016
		KR 20160096618 A	16-08-2016
		US 2016305925 A1	20-10-2016
		WO 2015089102 A1	18-06-2015
EP 1739431 A1	03-01-2007	EP 1739431 A1	03-01-2007
		JP 4682980 B2	11-05-2011
		JP WO2005109006 A1	21-03-2008
		US 2007037287 A1	15-02-2007
		WO 2005109006 A1	17-11-2005
WO 2008021192 A2	21-02-2008	AU 2007284733 A1	21-02-2008
		BR PI0715402 A2	26-11-2013
		CA 2662987 A1	21-02-2008
		CN 101523221 A	02-09-2009
		EP 2057473 A2	13-05-2009
		EP 2607902 A1	26-06-2013
		ES 2527438 T3	26-01-2015
		HK 1131209 A1	27-11-2015
		JP 2010500566 A	07-01-2010
		US 2010233724 A1	16-09-2010
		WO 2008021192 A2	21-02-2008