A DECREASE IN LIVER CARNITINE CONCENTRATION HAS AN UNFAVORABLE EFFECT ON NAFLD

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Index

Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) and obesity are two diseases that have become so important over time that they have become a global pandemic. NAFLD begins with simple inflammation, steatosis, to a more severe form such as non-alcoholic steatohepatitis (NASH), where other clinical signs are found in addition to possible mitochondrial dysfunction. Carnitines, compounds that support the transport of fatty acids into the mitochondria for beta-oxidation, have been linked to the progression of NAFLD as well as the degree of NASH.

Objectives: We sought to distinguish patients with NASH from patients without NASH by measuring carnitine concentrations in both plasma and liver, and to evaluate whether there may be an association between these species and mitochondrial dysfunction.

Results: Comparison of carnitine concentrations in liver of NASH patients with Non-NASH patients revealed that carnitine concentrations were found to be decreased in NASH. No differences were observed between the plasma carnitine concentrations of the different patients. In analyses to determine whether carnitines could be possible markers, we found that they could not be used as markers for this disease, as they were not specific enough to be so. Western blot results determined that both beta-oxidation and autophagy of mitochondria is highly correlated with carnitines and NAFLD.

Conclusions: Beta-oxidation and mitochondrial mitophagy and apoptosis have been shown to be altered as the disease has progressed. The carnitine profile is severely decreased in patients with the disease, an effect which causes the beta-oxidation of fatty acids in the mitochondria to be inactivated, and there is an increasing accumulation of fatty acids in the liver.

Abbreviations

ACC: acetyl CoA carboxylase

ACE-ARBS: angiotensin converting enzyme and angiotensin receptor blockers.

ALT: alanine aminotransferase

AST: aspartate aminotransferase

BB: gamma-butyrobetaine

BBD: gamma-butyrobetaine dioxygenase

BMI: Body mass index

CACT: carnitine acylcarnitine translocase

CoA: coenzyme A

CPT1: carnitine palmitoyltransferase 1

CPT2: carnitine palmitoyltransferase 2

CVD: cardiovascular diseases

DAP: diastolic arterial pressure

DLP: dyslipidemia

FABP: fatty acid binding protein

FASN: Fatty acid synthase

FAT/CD36: fatty acid translocase

FATP: fatty acid transporter protein

GGT: gamma-glutamyl transferase

HDL: high-density lipoproteins

HOMA-IR: homeostatic model assessment for insulin resistance

HSC: hepatic stellate cells

HTA: hypertension

HTML: 3-hydroxyTML

IKK-b: I-kappa-B kinase beta

JNK: cJun N-terminal kinase

LDL: low-density lipoproteins

MDD: major depressive disorder

MFN2: Mitofusin 2

NAFLD: Non-alcoholic Fatty Liver disease

NASH: Non-alcoholic steatohepatitis

OCTN2: high-affinity carnitine transporter

p62: ubiquitin-binding protein

pACC: phospho acetyl CoA carboxylase

PCOS: polycystic ovary syndrome

PINK: PTEN-induced kinase

SAP: systolic arterial pressure

T2DM: Type 2 diabetes mellitus

TGF-β: transforming growth factor beta

TMABA: 4-trimethylaminobutyraldehyde

TML: trimethyl-lysine precursor

Obesity

Obesity is becoming a global disease and has been defined by the World Health Organization (WHO) as "a condition of abnormal or excessive fat accumulation, to the extent that health may be impaired" (1), and since 1998 it is referred as a disease, for the epidemics proportions that has reached (2).

Causes

Obesity is a global health problem, which is caused by the combination of genetics, lifestyle and diet. The main factor is the negative energy expenditure (3). All these contributors affect the state of adipose tissue and its metabolism (4).

Prevalence and incidence of obesity

Obesity affects around 12 to 15% of worldwide population. The global prevalence of overweight and obesity has nearly doubled from 1980 to the present. It has increased in different age and gender groups and in all countries of the world, from the most underdeveloped to the least developed (5). In adults, a higher increase has been seen in women compared to men, and the maximum prevalence was between the ages of 60 and 64 years for women and between 50 and 55 years for men (Figure 1) (6). In children, as opposed to adults, no significant sex-specific evidence was found. Figure 1 shows how the prevalence of obesity drops at age 14, but then rises again dangerously (6).

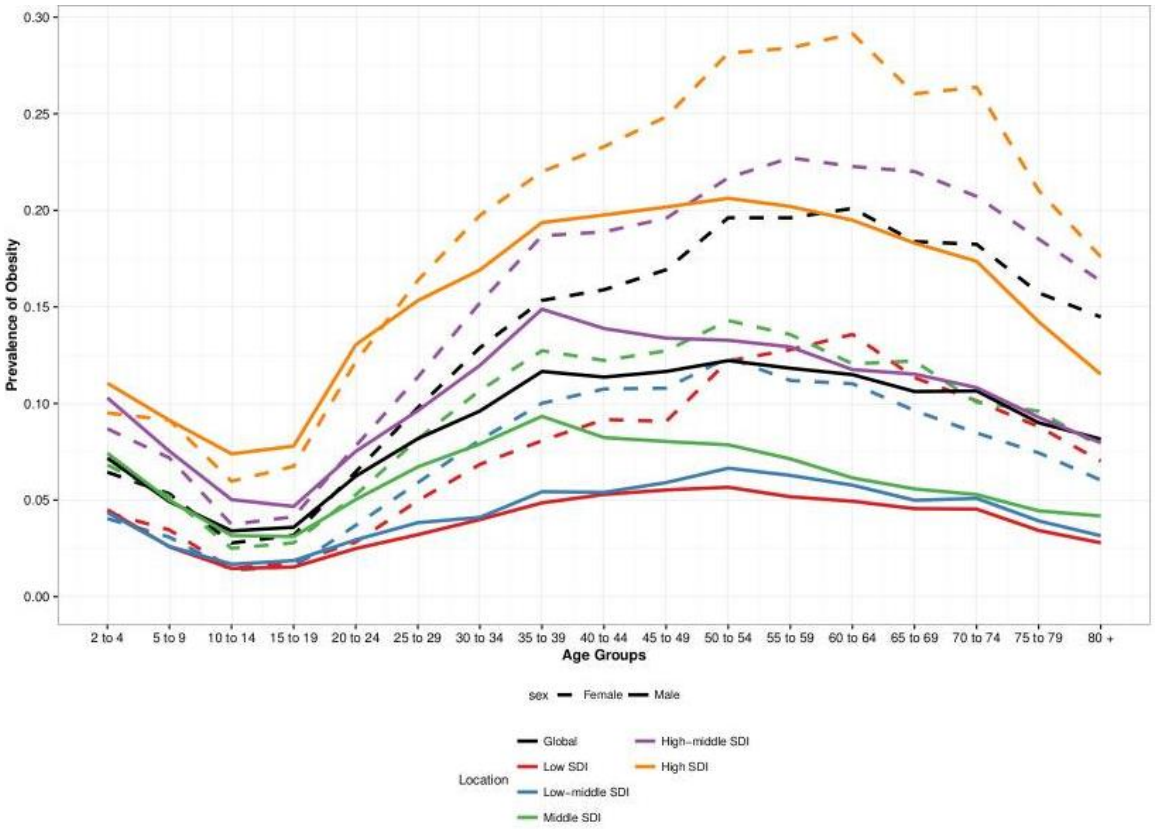

Figure 1: Prevalence of obesity by age in 2015. (SDI: sociodemographic index). Extracted from GBD Obesity collaborators (2017) (6)

Although it is a global disease, America and Europe have more prevalence than other continents. Each of the countries had a percentage of increase since 1980 (figure 2). In Europe and America, the highest prevalence rates were found in countries such as Turkey and the USA, while France and Colombia had the lowest rates (5). The Eastern Mediterranean and Africa showed high variability in prevalence among their countries and the Western Pacific had the lowest prevalence results of all regions (5).

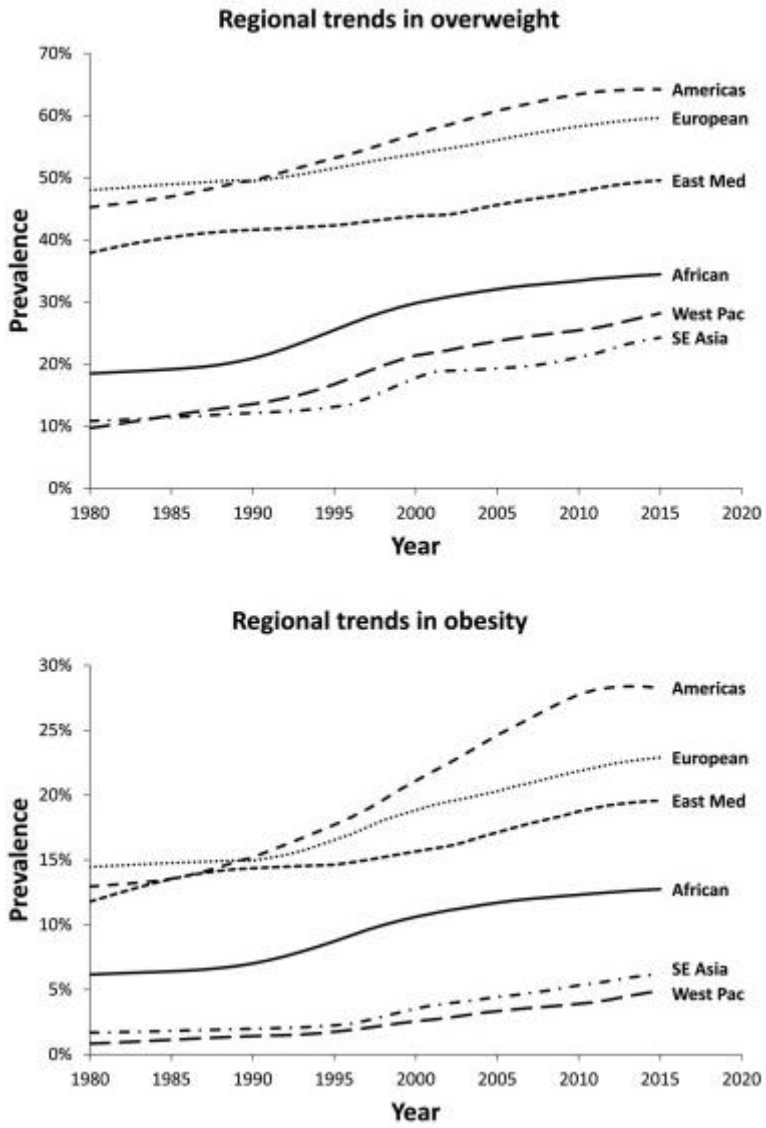

Figure 2: Prevalence of obesity and overweight over the years in the different regions. Extracted from Chooi, Yu Chung (2019) (5)

Overweight and obesity are considered risk diseases because they cause high mortality. Obesity in middle age is estimated to be able to reduce life expectancy by 4 to 7 years.

Physiopathogenesis

Currently, the diagnosis of obesity is based on the body mass index (BMI) of the patient, which can be divided into 5 categories: underweight (BMI 18.5 kg /m^2), normal weight (BMI 18.5-24.9 kg/m^2), obesity class I - overweight (BMI 25.0-29.9 kg/m^2), obesity class II - obesity (BMI 30.0-39.9 kg/m^2), obesity class III - extreme obesity (BMI> 40 kg/m^2) (3). This assessment is complemented by the waist circumference (considering obesity when waist

circumference is greater than 102 cm in men, and 88 cm in women) and the waist-to-hip ratio (0.85-0.90), as these measures inform about body fat distribution, and give clues of possible comorbidities that may be developing. For example, visceral fat, which is the adipose tissue found under the abdominal area, can be considered as ectopic fat accumulation when there is an excessive quantity, for example, it increases the risk of suffering from T2DM (7). Therefore, we should not only base it on body weight but also on body composition and energy expenditure, which is closely related to metabolism (3). Depending on hormone levels, the distribution of fat accumulation in the body can occur in two patterns: gynoid bodies, which there is an accumulation of fat in the lower part of the body, and android bodies, in which the accumulation of fat is distributed in the higher part of the core. There is a clear difference between men and women, as men tend to accumulate fat in the upper part of the body, being an android body, while women have a tendency to accumulate fat in the thighs and hips, being a gynoid body (3).

Obesity associated-comorbidities

When fat accumulates in adipose tissue due to these circumstances, adipogenesis is promoted. Adipose tissue grows until it reaches its full capacity, and then it starts to malfunction. Hormonal homeostasis is impaired and ectopic fat accumulation starts. For example, there is an increase of visceral adipose tissue size, and the liver captures the fat that no longer can be stored in adipose tissue. All these provokes lipotoxicity, and ultimately immune system is involved (3).

The imbalances caused by this situation cause disease such as metabolic syndrome, hypertension (HTA), type 2 diabetes mellitus (T2DM), non-alcoholic fatty liver disease (NAFLD), dyslipidemia (DLP), cardiovascular diseases (CVD) and some cancers, decreasing patients' life quality and can lead to reduced life expectancy (1).

Over the years, obesity has been found to be associated with many other diseases. Among the best known are HTA and T2DM, in which no ethnic differences are observed. People who suffer from obesity where the fat is in the central part of the body are more susceptible to suffer from

hyperlipidemia, especially with triglycerides and cholesterol. We may also find heart failure or cancer, both of which increase the mortality rate independently of other environmental factors (8).

But not only we can see comorbidities with these diseases, we can also find other diseases such as rheumatoid arthritis, non-allergic rhinitis, major depressive disorder (MDD), polycystic ovary syndrome (PCOS) or hypothyroidism. All these diseases have been shown to be more prevalent in obese people than in people with a normal BMI. In rheumatoid arthritis an increase of 13% is shown with a relative risk of 1.21 for obese patients (1.05 in normal weight patients), and happens the same in non-allergic rhinitis with 1.43% for adults and 0.88% for children. Women with a BMI of 30 kg/m^2 have a higher predisposition (5,25%) of to develop T2DM as well as SOP, in which 80% of women are obese (4).

Thyroid hormones are linked to lipid metabolism and thermogenesis. An increase in thyroid stimulating hormone goes along with an increase in BMI, which we can deduce that obesity has an impact on the thyroid, and vice versa (4).

The fact that obesity is so closely linked to other diseases, increases mortality from these associated diseases. A study revealed that mortality rates in the US had increased by 78.9% for hypertensive diseases and 114% for obesity. Deaths also increased (over 30,000 deaths in 1990) in other organ system diseases (9).

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is the ectopic accumulation of fat, in form of triglycerides, in the hepatic tissue, originated in an impaired adipose tissue function and insulin resistance. Patients with NAFLD suffer from this disease underdiagnosed until it reaches nonreversible severity stages, like cirrhosis and hepatocellular carcinoma.

Prevalence and incidence

NAFLD affects 25% of the world's population, with a lower prevalence in Africa and higher prevalence in Europe and in the Middle East (10). The prevalence

of NAFLD not only diversifies with the different regions, we also found differences between the various age groups (Table 1) (11). Table 1 shows how prevalence increases with advancing age (11). It has been found to be closely associated with obesity, and with diseases such as metabolic syndrome, T2DM and insulin resistance (12). One third of the NAFLD population have developed non-alcoholic steatohepatitis (NASH), which is also associated with different comorbidities, just like NAFLD patients (11). One of the closely related diseases is obesity, which 51% and 82% of NAFLD and NASH patients were suffering from. T2DM is a risk factor in people with NAFLD for developing NASH, which is why it affects twice as many people with NASH (47%) as people with NAFLD (23%). Metabolic syndrome together with different DLP are also associated with NAFLD and NASH, with 41% and 71% of people with NAFLD and NASH suffering from metabolic syndrome, respectively (11).

Mean Age	*N*	*Prevalence (%)*	*95% CI (%)*
30-39	3	22.43	(15.38-31.52)
40-49	14	26.53	(22.37-31.16)
50-59	11	27.40	(19.56-36.93)
60-69	4	28.90	(19.25-40.94)
70-79	1	33.99	(32.08-35.95)
Overall	41	24.29	(20.96-27.96)

Table 1: Prevalence of NAFLD based on age. Adapted from Younossi, Zobair M. et al. (2016) (11).

Diagnosis

The gold standard method to diagnose the severity of NAFLD is by hepatic biopsy, a very invasive procedure that requires patients' hospitalization and is very painful (10). It was found that the previous diagnosis that was used through liver enzyme analysis did not give a true diagnosis, as it gave reduced results in reference to other types of diagnosis (11). To diagnose the progression of NAFLD and the degree of NASH we should look at a number of features as a whole rather than controlling for a single characteristic. To determine this progression, liver biopsies stained with Hematoxylin and Eosin and Masson's Trichrome are needed (13). The criteria to diagnose NASH implies the evaluation of histological characteristics, that the pathologist give

a punctuation depending on their severity status. This characteristics are: steatosis (0-3), ballooning (0-2) and lobular inflammation (0-3). The sum of all scores will result in the NAFLD Activity Score (NAS score), i.e. from 0 to 8 (0-2 non-NASH, 3-4 Uncertain, 5-8 NASH) (13). Fibrosis, despite being very important, as it creates dysfunctional tissue, is not considered a variable for the NAS score (13).

Physiopathology

This disease has different degrees, which can go from NAFLD to NASH in which the phenotype is reversible. Ultimately, fibrosis present in the severe stages of the disease can lead to cirrhosis and, if the liver is not transplanted at this step, hepatocellular carcinoma may be developed (14). NAFLD is characterized by steatosis (equal to or higher than 5%), which refers to an excess accumulation of fat in the hepatocytes, without being caused by any external factor such as alcohol. When this steatosis is accompanied by (non-alcoholic) steatohepatitis, which is always associated with inflammation and hepatocellular damage, we are identifying a new level of NAFLD, NASH (14).

NASH or NAFLD can develop fibrosis, a higher sever stage, which as mentioned above can lead to cirrhosis, a phase in which a liver transplant is urgently needed, because the hepatocytes have been replaced by collagen I scar cells, otherwise carcinoma may appear (14).

This disease can differ by factors such as environment, microbiome, genetic, and epigenetic factors (15).

NAFLD is so closely associated with metabolic syndrome, T2DM and obesity, due to insulin resistance in adipose tissue. When adipose tissue enlarges and expands, it loses its power to store energy from diet. This situation causes lipolysis (12). The insulin is responsible for the regulation of lipolysis and when there is a resistance to it, uncontrolled lipolysis is produced, which triggers a release of fatty acids (originating from triglycerides), which, through the circulation of the bloodstream, gets into the liver (16).

Fatty acids can also come from *de novo* lipogenesis produced by hepatocytes from excessive carbohydrate ingestion, especially fructose. Once the free fatty acids are in the liver they can take two different paths, on the one side

there is beta-oxidation, which takes place in the mitochondria, or they can be transformed back into triglycerides which are then transported by VLDL or transformed back into free fatty acids (16). When these are in excess, two things can happen: the first, and the one that usually happens, is that, because the synthesis of free fatty acids is higher than the oxidation of fatty acids, more triglycerides are generated to counteract the excessive amount of free fatty acids. When this happens, the transport of triglycerides thanks to VLDL becomes saturated and then triglycerides accumulate in the hepatocytes, this mechanism is called steatosis (17). Due to a problem with adipocytes, from insulin resistance, they can produce lipotoxic species when they are metabolized, which lead to oxidative stress, endoplasmic reticulum stress, activation of apoptotic pathways and inflammation, which are characteristic of NASH (16). The accumulation of free fatty acids promotes TNF-alpha, which is able to activate hepatocyte apoptosis, which in turn decreases adiponectin, insulin signalling and increases inflammation (17). Adiponectin and TNF-alpha are antagonists, while one activates insulin sensitivity, the other creates insulin resistance. TNF-alpha is able to activate I-kappa-B kinase beta (IKK-b) and cJun N-terminal kinase (JNK), which phosphorylate a number of components of the insulin signalling pathway, thereby inhibiting insulin (17).

Once hepatocytes die, they have repair mechanisms that attempt to replace the dead hepatocytes by replication of mature hepatocytes, which is achieved by the mechanism that activates transforming growth factor beta (TGF-β) and hepatic stellate cells (HSC), which are the ones that end up forming fibrosis. The problem is that as many of the hepatocytes have experienced oxidative stress, they cannot replicate, therefore, depending on their replicative power and the oxidative damage they have experienced, NASH will trigger the next level (figure 3) (17).

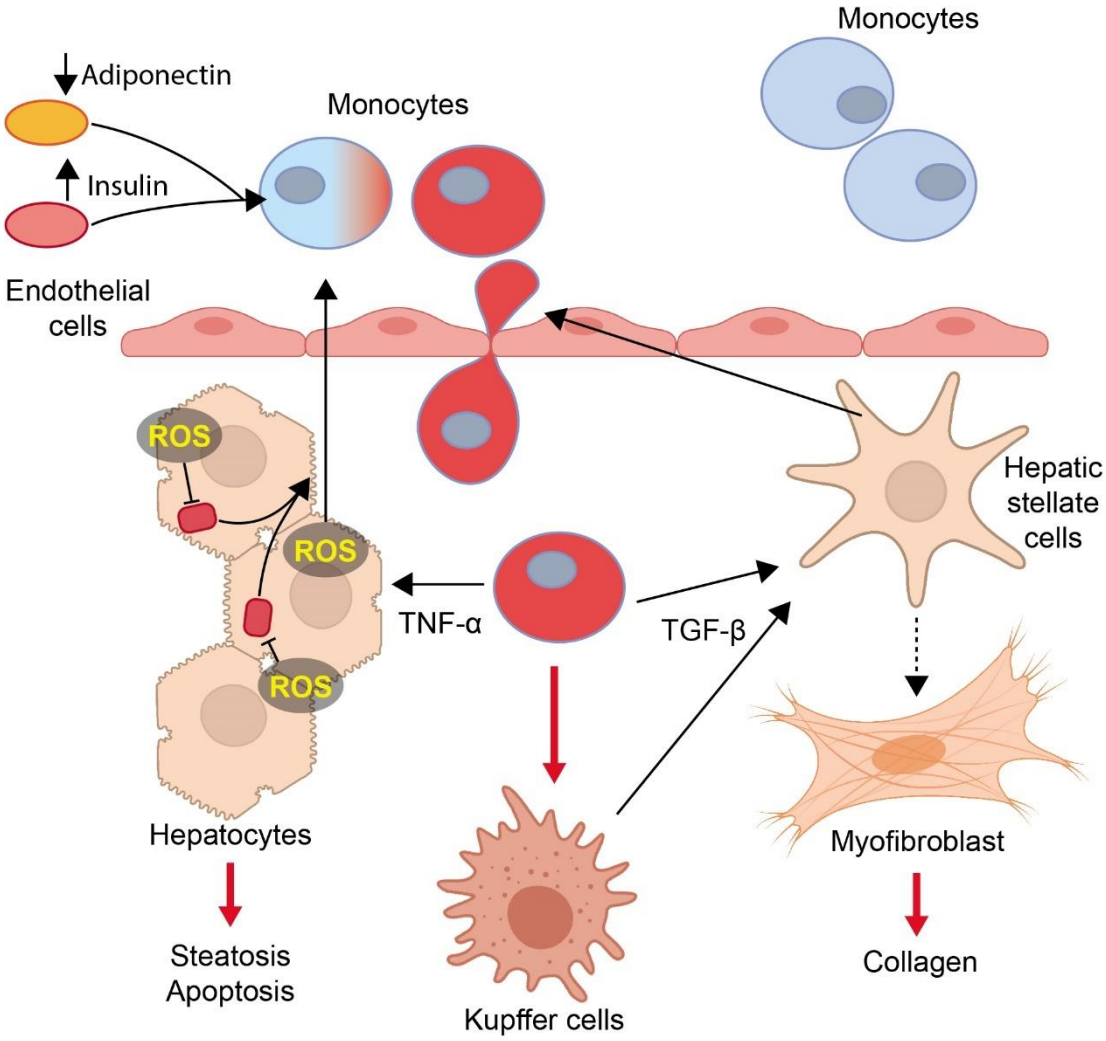

Figure 3: NAFLD's mechanisms of development

Considering the importance of lipid metabolism in NASH, a crucial component of that metabolism, carnitines, comes into focus.

Carnitine

The carnitine, or 3-hydroxy-4-(trimethylazaniumyl)butanoate, (figure 4) is a hydrophilic quaternary amine that is involved in energy metabolism, in particular the metabolism of fatty acids that occurs in the mitochondria (18).

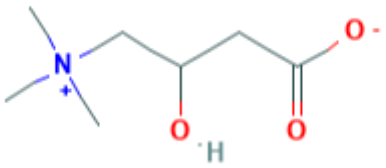

Figure 4: Two-dimensional structure of carnitine. Extracted from PubChem.

Obtention, homeostasis and function of carnitine

Carnitine can be obtained by different routes, either by ingestion, new synthesis or renal reabsorption (18).

Carnitine can be obtained from the diet. This compound, which is normally more abundant in animal and dairy products, is transported from the intestinal lumen to the enterocyte and then is released into circulation (18). Another part of it is synthesized *de novo* from the amino acids lysine and methionine, starting with a trimethyl-lysine precursor (TML), which is released from lysosomal protein degradation and ends with the hydroxylation of gamma-butyrobetaine (BB) by gamma-butyrobetaine dioxygenase (BBD) which produces L-carnitine (19). Only the brain, liver and kidney are capable of generating carnitine, as they are the only ones capable of converting BB into carnitine (20). As can be seen in figure 5, the TML precursor is hydroxylated at position 3 to give rise to 3-hydroxyTML (HTML) through the function of TML dioxygenase. This forms 4-trimethylaminobutyraldehyde (TMABA) and glycine through a reaction produced by HTML aldolase. TMABA is dehydrogenated to form BB by TMABA dehydrogenase. Once the butyrobetaine is obtained, it is hydroxylated to give carnitine as a final result, all this thanks to γ-butyrobetaine dioxygenase (20).

Finally, there is also a clear evidence that it is reabsorbed by the renal tubule by a high-affinity carnitine transporter (OCTN2) through an active transport (18). All these three combined processes enable to maintain constant levels of carnitine.

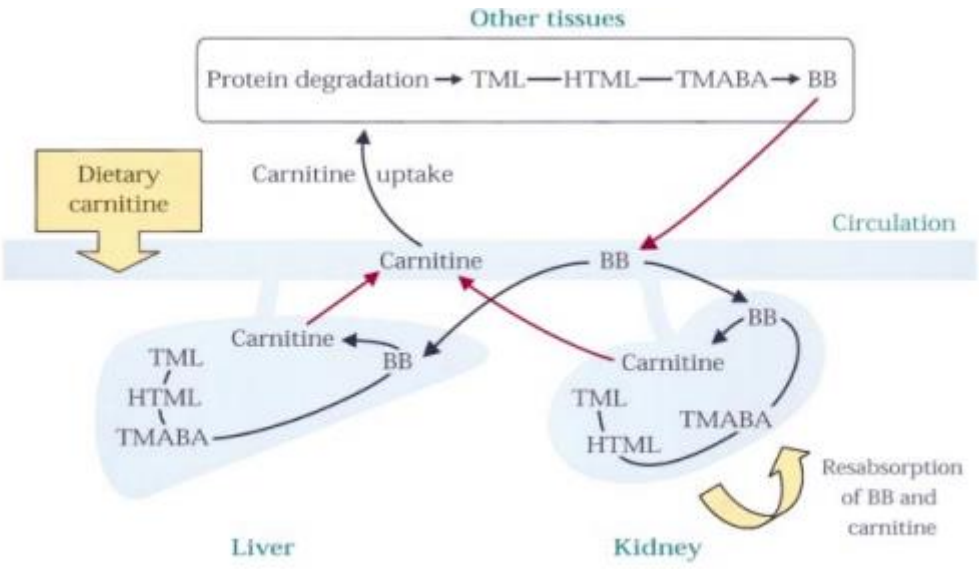

Figure 5: Carnitine synthesis process. Extracted from Vaz, Frédéric M. et al. (2002) (20)

Carnitine transport is determined by the energy requirements of the various organs. It has been shown that not all organs that require carnitines have sufficient carnitine in their pool, so there must be active transport of plasma carnitines, which is sodium ion-dependent and is inhibited by acetylcarnitine and butyrobetaine (20).

The role of carnitine is to transport long-chain fatty acids into the mitochondria so that they can then begin beta-oxidation (19). It is important to talk about long-chain fatty acids as they are the ones that need a transporter because they cannot enter into the mitochondria by themselves, as opposed to short-chain and medium-chain fatty acids (21).

Another of carnitine's main functions is to release intramitochondrial acyl-CoA and expel it from the cytoplasm into the bloodstream, in order to eliminate the excess in the mitochondria that could cause oxidative stress (21).

Fatty acid oxidation in mitochondria and peroxisome

As discussed above, fatty acids present in the liver can come from different endogenous and exogenous pathways.

The free fatty acids resulting from lipolysis in adipose tissue are transported and get into the liver tissue by a number of proteins, called fatty acid transporter protein (FATP), fatty acid binding protein (FABP) and fatty acid

translocase (FAT/CD36) (18). Once in the cytoplasm, they need to be activated for oxidation and transformed into acyl-CoA with the support of an acyl-CoA synthase (ACC) (18). This process is carried out with the binding of an ATP. With this binding, a fatty acid-AMP is formed, to which a coenzyme A (CoA) is attached. When the CoA is attached, the AMP is deleted and both the AMP and the acyl-CoA are released (18). These acyl-CoA formed need to enter in the mitochondria for beta-oxidation, but the mitochondrial membrane is impermeable to this group, which is where carnitine comes into play (18).

Carnitine binds with an ester bond to acyl-CoA and is exchanged for CoA with the assistance of carnitine palmitoyltransferase 1 (CPT1), which results in acylcarnitine (21). CPT1 is sensitive to malonyl-CoA, a product of the carboxylation of acetyl-CoA, which is a product of beta-oxidation (21). Once acylcarnitine is produced, it gets into the mitochondria through the carnitine acylcarnitine translocase (CACT) located on the inside of the external mitochondrial membrane (18). Acylcarnitine within the mitochondria is converted back to acyl-CoA using carnitine palmitoyltransferase 2 (CPT2) found in the internal mitochondrial membrane, and carnitine returns to the cytoplasm by CACT (18). Acyl-CoA may enter beta-oxidation for energy generation (21). This process is summarized in figure 6.

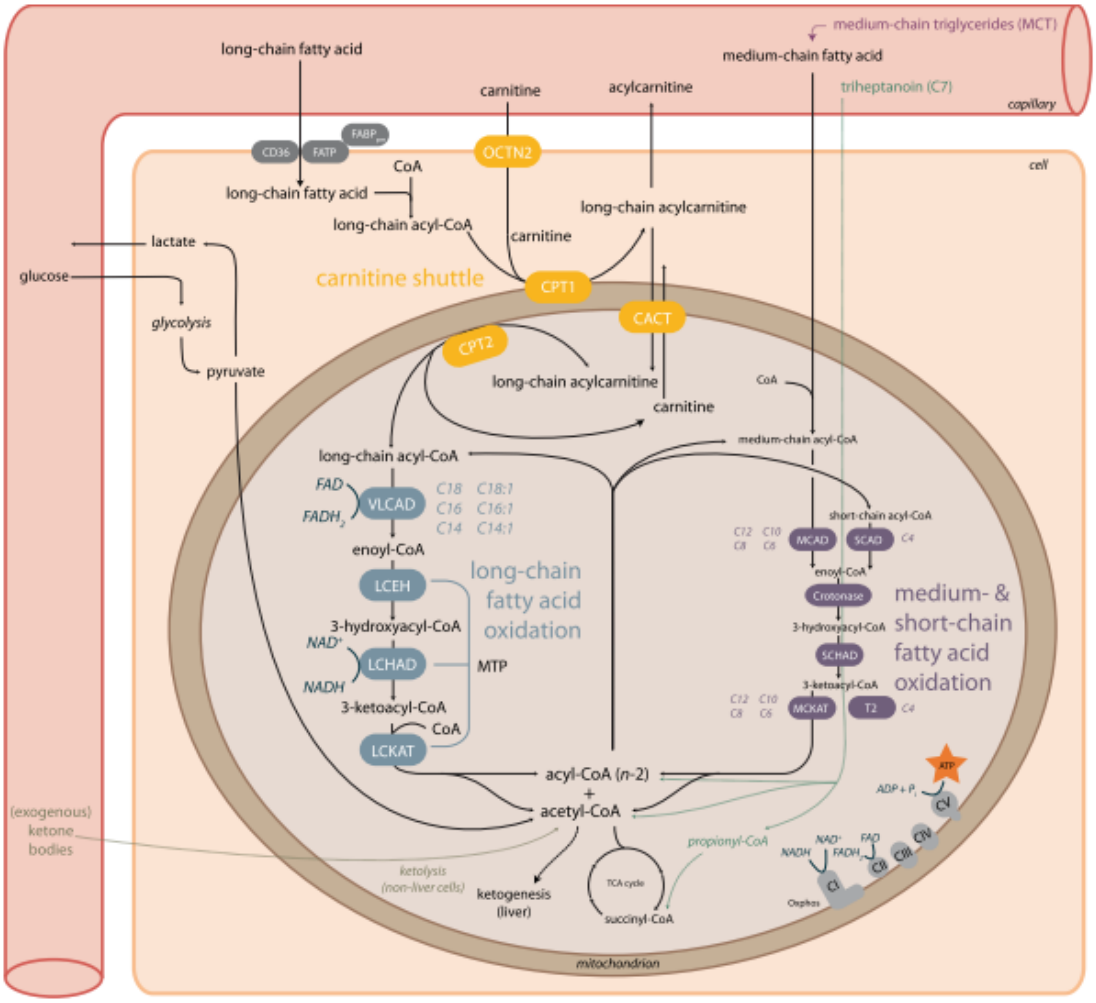

Figure 6: Oxidation of free fatty acids in mitochondria. Extracted from Knottnerus, Suzan J. G. Et al. (2018) (21)

Carnitine, which is so important in fatty acid metabolism, has a major impact on people with NAFLD or NASH. Because mitochondrial dysfunction is found in patients with these diseases. This is a knowledge gap, it is very important to investigate it as it could have several impacts and could improve or worsen the quality of life, so we should do more research on this topic.

When the inflammatory process of the hepatocytes has started, they die by programmed cell death, also known as apoptosis. This process promotes proliferation of hepatocytes as a compensatory form of hepatocyte death, which causes cell proliferation that can lead to tumor formation. Mitochondrial function is mediated by a connection between the endoplasmic reticulum and mitochondria. One of the most important proteins in this connection is mitofusin 2 (MFN2), a decrease of which causes oxidative stress and mitochondrial dysfunction (22).

Hypothesis and objectives

Obesity is characterized by an excessive accumulation of fat, which ends up producing ectopic storage of lipids in the liver, with the consequent appearance of non-alcoholic fatty liver disease (NAFL), and the progression to steatohepatitis non-alcoholic steatohepatitis (NASH). By 2021, it is already the most prevalent liver disease in human history, suffered by more than a quarter of the world's population. For these reasons, obesity and NAFLD are closely related, and share a global pandemic prevalence. Patients suffering from obesity and NASH have a poor quality of life, and the risk of premature death increases considerably. The development of NASH is silent and can progress to severe stages with liver transplantation being the only option left for these patients. Currently, a liver biopsy, a very invasive procedure, is the only method to diagnose the severity of NAFLD. As these diseases are really related with a dysregulation of lipid metabolism and mitochondrial dysfunction, carnitine and fatty acyl carnitines may be affected by them.

For these reasons our hypothesis is that NASH may modify plasma and hepatic carnitine and fatty acyl carnitine concentrations in patients with extreme obesity and NASH. To test it, the aim of our study is to determine the changes in the concentrations of carnitine-associated lipids in plasma and liver, and to study how lipid metabolism enzymes and enzymes related to mitochondrial integrity vary.

To address our objectives, we will first determine the utility of carnitines as potential non-invasive biomarkers for diagnosing obesity-associated NASH. Secondly, we will study the enzymes of lipid metabolism, and enzymes involved in mitochondrial dysfunction and integrity, to find changes associated with the progression of NAFLD.

Materials and methods

Study design

The study includes 110 obese participants (BMI>30kg/m^2) from the Hospital de Sant Joan de Reus who have undergone bariatric surgery. A liver biopsy was obtained from all of them and NASH was characterized using NAFLD Activity Score (NAS). 51 patients were classified as Non-NASH (NAS = 0-2); 49 patients were classified as NASH (NAS = 5-8) and 10 patients were classified as Uncertain NASH diagnostic (NAS = 3-4).

All these patients were over 18 years of age and both men and women.

Sampling

As mentioned above, liver biopsies were obtained while bariatric surgery was being performed by percutaneous needle punction. Patients also donated a pre-operative fasting blood sample.

Serum and plasma were collected from blood samples by centrifugation and stored at -80°C for biochemical assessments and Lipidomics.

One part of the liver biopsies was fixed in formaldehyde for 24h and then paraffin-embedded to perform histological analyses. The other part was immediately frozen at -80°C for Lipidomics and Western Blot.

Biochemical characteristics

To analyze the samples, we used an automated assay device named COBAS 800 (Roche Farma). The parameters analyzed were total cholesterol (mmol/L), HDL-cholesterol (mmol/L), LDL-cholesterol (mmol/L), triglycerides (mmol/L), glucose (mmol/L) and insulin (pmol/L). With glucose and insulin, we assessed HOMA IR, which is a mathematical model for determining insulin resistance.

Histological analyses

The paraffin-embedded samples were cut in the microtome at 2μm in order to carry out histological analysis. We stained them in two different ways: firstly, Hematoxylin and Eosin staining allowed us to observe the cellular structures of the liver, and then Masson's Trichrome staining revealed the collagen fibres that had been formed in the tissue.

The degree of NASH in patients was determined by an experimented pathologist using NAS score, which takes into account three factors: steatosis (0-3), ballooning (0-2) and lobular inflammation (0-3). Scores can range from 0 to 8 (0-2 non-NASH, 3-4 Uncertain, 5-8 NASH).

Western blot

This technique is based on the specific recognition of a protein of interest, by the use of antibodies conjugated with detection systems. The protein from a tissue homogenate must be separated by electrophoresis in the first place, and then, by the antibodies mentioned above, the detection system allows us to observe the relative amount of this protein.

Liver samples (30 mg) from patients with different severity degrees of NAFLD were homogenized in 300 μL of 0.25M sucrose containing antiphosphatases and antiproteases (Roche Farma) with sonication. Protein concentration was analyzed with Bicinchoninic Acid solution BCA method (ThermoFisher).

We carry out an electrophoresis of the samples that we have previously denatured. The electrophoresis was performed on polyacrylamide gels with sodium dodecyl sulphate in percentages of 6, 8 and 14%. After completing the electrophoresis, we transferred the proteins from the polyacrylamide gel to a nitrocellulose membrane. The membrane was then incubated with a solution of non-fat milk at 5% in Tris-buffered saline with 0.1% Tween 20 (TBS-T) that served as blocking agent. They were incubated with primary and secondary antibodies for CD36, Fatty acid synthase (FASN), acetyl CoA carboxylase (ACC) and phospho acetyl CoA carboxylase (pACC) proteins for lipid metabolism, Mitofusin 2 (MFN2) protein was studied to assess mitochondrial dynamics, and PTEN-induced kinase (PINK), PARKIN and p62

to observe mitophagy. All antibodies were diluted in BSA 5% TBS-T solution. Details on the dilution factor and the manufacturers of the antibodies are found in the table 2. Membranes were enhanced with SuperSignal West Femto chemiluminescent substrate (Pierce, Rockford, IL, USA) and images were digitally captured with ChemiDoc MP System (Bio-rad Laboratories, Hercules, CA, USA). Results were analyzed using Image Lab 2.0 software (Bio-Rad Laboratories).

Table 2: Conditions of the different reagents for each type of Western Blot.

Protein	*Primary ab*	*Primary ab dilution*	*Preparation recommendations Primary ab*	*Secondary ab*	*Secondary ab dilution*	*Transfer protocol*
ACC	Rabbit α-human/mouse Cell signaling #3662	1/1000	BSA 5% en TBS-T. Overnight	Goat α-rabbit HRP Dako ref P0448	1/1000	High
CD36	Genetex GTX100642	1/500	BSA 5% en TBS-T. Overnight	Goat α-rabbit HRP Dako ref P0448	1/1000	High/Mixed
FAH	Anti-FAH Millipore #ABN526	1/500	BSA 5% en TBS-T. Overnight	Goat α-rabbit HRP Dako ref P0448	1/5000	Mixed-Low
FASN	Rabbit α-human/mouse Cell signaling #3180	1/1000	BSA 5% en TBS-T. Overnight	Goat α-rabbit HRP Dako ref P0448	1/5000	High
MFN2	Anti-Mitofusin 2 antibody [NIAR164] ab124773	1/1000	BSA 5% en TBS-T. Overnight	Goat α-rabbit HRP Dako ref P0448	1/5000	Mixed
p62	Rabbit α-human Cell signaling #39786	1/1000	BSA 5% en TBS-T. Overnight	Goat α-rabbit HRP Dako ref P0448	1/5000	Mixed
pACC	Rabbit α-human/mouse Cell signaling #3662	1/1000	BSA 5% en TBS-T. Overnight	Goat α-rabbit HRP Dako ref P0448	1/1000	High
PARKIN	Rabbit α-human Cell signaling #2132	1/1000	BSA 5% en TBS-T. Overnight	Goat α-rabbit HRP Dako ref P0448	1/5000	Mixed
PINK	Rabbit α-human/mouse Abcam ab23707	1/1000	BSA 5% en TBS-T. Overnight	Goat α-rabbit HRP Dako ref P0448	1/5000	Mixed
Vinculin	Abcam ab73412	1/1000	BSA 5% en TBS-T. Overnight	Goat α-rabbit HRP Dako ref P0448	1/5000	High

Lipidomics

Lipidomics is a technique to quantify lipid species in different tissues. Usually, it starts with a lipid extraction procedure, followed by an analysis with liquid chromatography coupled to mass spectrometry. Depending on the objectives of the analysis, lipidomics can be targeted, non-targeted, or semi-targeted (or shotgun). The latter was the method of our choice.

Reagents

MS grade acetonitrile (CAN), methanol (MeOH) and formic acid were purchased from Sigma Aldrich (Saint Louis, MO, USA). Water (milli-Q grade) was obtained from a Milli-Q integral water purification system (Millipore Corp., Burlington, MA, USA). For analytical (AS) and internal standards (IS), carnitine mix were purchased from Cayman Chemical (Ann Arbor, MI, USA).

Lipid extraction

The method used is based on a protein precipitation using pure MeOH as extraction solvent which is capable of extract polar lipids, such as bile acids, lysophospholipids, carnitines, free fatty acids, oxylipins, among others.

For the extraction, 10 mg of liver tissue or 100 µL of plasma samples were extracted with 250 µL or 400 µL of MeOH containing IS mixture (at the final concentration detailed in Supplementary Table S1), respectively. Tissues were also disrupted on a tissue homogenizer (Precellys 24 system, Bertin Technologies, Bretonneux, France). Then, the resulting extract was centrifuged at 18000 G during 10 minutes at 4ºC and supernatants of tissues were directly analyzed by LC-MS, whereas 300 µL of plasma extracts were dried on a Savant SPD2010 SpeedVac rotatory vacuum system (Thermo Fisher, USA) and reconstituted on 100 µL of pure MeOH prior LC-MS analysis.

For calibration curves, carnitine mix (Cayman Chemical) was used as IS, and was dissolved in MeOH at the final concentration of 0.5 mM. Then, 10 serial dilutions (1:2), containing the IS mixture, were prepared and placed into glass vials for LC-MS analysis.

LC-MS/MS platform

Samples (5µL) were injected into a 1290 Infinity ultra-high-pressure liquid chromatograph (UHPLC) coupled to a to a 6550 quadrupole-time-of-flight mass spectrometer (QTOF) (Agilent Technologies, Santa Clara, CA, USA) operating on positive and negative electrospray mode over 100-1000 m/z range on two different runs. The stationary phase was an Acquity BEH C18 column (1.7 µm, 2.1 mm × 100 mm) from Waters (Milford, MA, USA) termostatized at 40 ºC, and the mobile phase consisted on a binary mixture of ACN and 0.05% aqueous formic acid. Gradient elution was as follows: 0 min, 2% B; 2 min, 50% B; 10 min, 98% B; 13 min, 98% B; 14 min, 2% B. A post run of 4 min in initial conditions was used for column conditioning. For the ESI source, the optimized parameters were as follows: gas temperature 225 ºC, drying gas flow 11 L/min, nebulizer 35 psi, sheath gas temperature 300 ºC and sheath gas flow 12 L/min. For the QTOF-MS, the capillary, nozzle and fragmentor voltages were set at 3500 V, 500 V and 380 V, respectively.

Data analysis and statistics

The identification of lipid species was performed by matching their accurate mass, isotopic distribution and tandem mass spectrum, when available, to Metlin-PCDL from Agilent containing more than 40,000 metabolites and lipids. In addition, information from Lipid Maps database (www.lipidmaps.org), chromatographic behavior of pure standards for each family and bibliographic information was used to ensure their putative identification. After putative identification of lipids, these were semi quantified using IS calibration curves for a representative compound of each lipid family using Mass Hunter Quantitative Analysis B.07.00 software (Agilent Technologies).

Statistical analyses were performed on SPSS 25 (IBM Corp., Chicago, IL, USA), MetaboAnalyst 5.0 (www.metaboanalyst.ca), GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) and RStudio (R version 4.0.2). Mann-Whitney was used to compare continuous variables, such as lipid species concentration, between two groups, Kruskall-Wallis test was used to compare continuous variables between more than two groups, and Fisher exact test was used to compare categorical variables, such as clinical

characteristics. P-values obtained in these analyses were considered significant when < 0.05. SPSS 25 was used to perform descriptive statistics of the population and MetaboAnalyst and RStudio to perform univariate and multivariate analysis of the carnitine species' concentration.

From MetaboAnalyst different analyses were performed:

- Statical Analysis: We perform both univariate analyses, such as Mann-Whitneys, Kruskall-Wallis, volcano plots, multivariate analyses, such as Principal Component Analysis (PCA) and Partial Least Squares - Discriminant Analysis (PLS-DA), and clustering analyses such as hierarchical clustered heatmaps.
- Biomarker analysis: we have generated models combining different variables, and performed receiver operating characteristic (ROC) curves of them.
- Enrichment: metabolite set enrichment analysis (MSEA).
- Pathway analysis: pathway analysis, which integrates enrichment analysis and pathway topology analysis.

Violin and bar plots were generated with GraphPad Prism.

Results

All patient characteristics are described in table 3. In it we can see that most of the patients were in the same age group. We observed more women in all groups, except for the Uncertain one, where the percentage of women was lower, but not significantly lower. In relation to the other clinical characteristics such as BMI, SAP, DAP, T2DM, HTA, DLP and Metabolic Syndrome, no significant differences were found.

Biochemical characteristics were also not significantly different. Transaminases in NASH were significantly increased with respect to Non-NASH patients.

Table 3: Clinical, biochemical and histological features of all patients

	NASH (n=49)	*Uncertain (n=10)*	*Non-NASH (n=51)*	*p-value*
Clinical characteristics				
Sex (woman, %)	63.3	30.0	74.5	
Age (years)	48(42-55)	51.5(47.5-59.2)	51(40-57)	
BMI (kg/m^2)	31.2(26.1-38.5)	43.3(41.9-47.9)	32.1(27.1-34.7)	
SAP (mmHG)	133(122.5-152.7)	143(123.2-156)	136(127.5-149.2)	
DAP (mmHG)	83(69-89)	94(79.2-102.2)	85.5(80-92)	
T2DM (%)	44.9	44.4	34	
HTA (%)	62.5	88.9	54	
DLP (%)	49	44.4	35.3	
Metabolic syndrome	81.6	88.9	64	
Biochemical variables				
Total cholesterol (mmol/L)	4.1(3.4-4.9)	3.7(3.1-4.1)	4.14 (3.3-4.9)	
HDL-cholesterol (mmol/L)	0.9(0.8-1.0)	0.9(0.8-1.0)	1.01 (0.8-1.2)	
LDL-cholesterol (mmol/L)	2.4(1.7-3.1)	1.8(1.4-2.3)	2.3 (1.7-2.7)	
Triglycerides (mmol/L)	1.7(1.3-2.2)	1.5(1.2-2.2)	1.46 (1.2-2.1)	
Glucose (mmol/L)	7.6(6.2-10.8)	6.7(5.5-8.0)	6.5 (5.3-8.7)	
Insulin (pmol/L)	104.1(58.6-146.8)	69.0(47.1-93.3)	75.7 (43.2-130.5)	
HOMA IR	5.2(2.4-6.9)	2.4(2.2-3.8)	3.2 (1.9-5.7)	
Transaminases				
ALT (µKat/L)	0.9(0.5-1.2)	0.7(0.3-1.2)	0.43 (0.3-0.6)	a
AST (µKat/L)	0.8(0.5-1.1)	0.7(0.2-1.0)	0.4 (0.3-0.5)	a
GGT (µKat/L)	0.4(0.3-0.9)	0.4(0.2-0.9)	0.3 (0.2-0.4)	a
Medication				

Metformin (%)	40.8	33.3	13.7	a
Sulfonylureas (%)	4.1	11.1	5.9	
Insulin (%)	14.3	0	10.0	
ACE-ARBS (%)	49	44.4	23.5	
Diuretic (%)	10.2	11.1	8	
Statins (%)	35.4	33.3	26.5	
Histological				
Steatosis (%)	40(10-5)	20(5.3-3)	2.8(1.6-8)	
Steatosis				
<5%	-	20.0	62.7	
5%-33%	16.3	60.0	35.3	
>33%-66%	51.0	20.0	2.0	
>66%	32.7	-	-	
	-	-	-	
Fibrosis				
F0	-	-	33.3	
F1	36.7	30.0	56.9	
F2	59.2	50.0	9.8	
F3	4.1	20.0	-	
F4	-	-	-	
	-	-	-	
Lobular inflammation				
No foci	-	-	-	
<2 foci	-	-	33.3	
2-4 foci	36.7	30.0	56.9	
>4 foci	59.2	60.0	9.8	
	4.1	10.0	-	
Ballooning				
None	-	50.0	62.7	
Few balloon cells	38.8	30.0	33.3	
Many cells/prominent	61.2	20.0	3.9	
ballooning	-	-	-	

NASH: Non-alcoholic steatohepatitis; BMI: Body mass index; systolic arterial pressure; DAP: diastolic arterial pressure; T2DM: Type 2 diabetes mellitus; HDL: high-density lipoproteins; LDL: low-density lipoproteins; HOMA-IR: homeostatic model assessment for insulin resistance; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma-glutamyl transferase; ACE-ARBS: angiotensin converting enzyme and angiotensin receptor blockers; F0: None; F1: Perisinusoidal or periportal; F2: Perisinusoidal and portal/periportal; F3: Bridging fibrosi; F4: Cirrhosis. p-values <0.05: significant differences between (a) NASH and Non-NASH.

The carnitine profile does not vary according to the degree of NASH

The different relative concentrations of acyl-carnitines in plasma showed no difference between the NASH, Non-NASH and Uncertain groups (figure 7A) despite the fact that significant carnitines appeared in Kruskall-Wallis test (figure 7B).

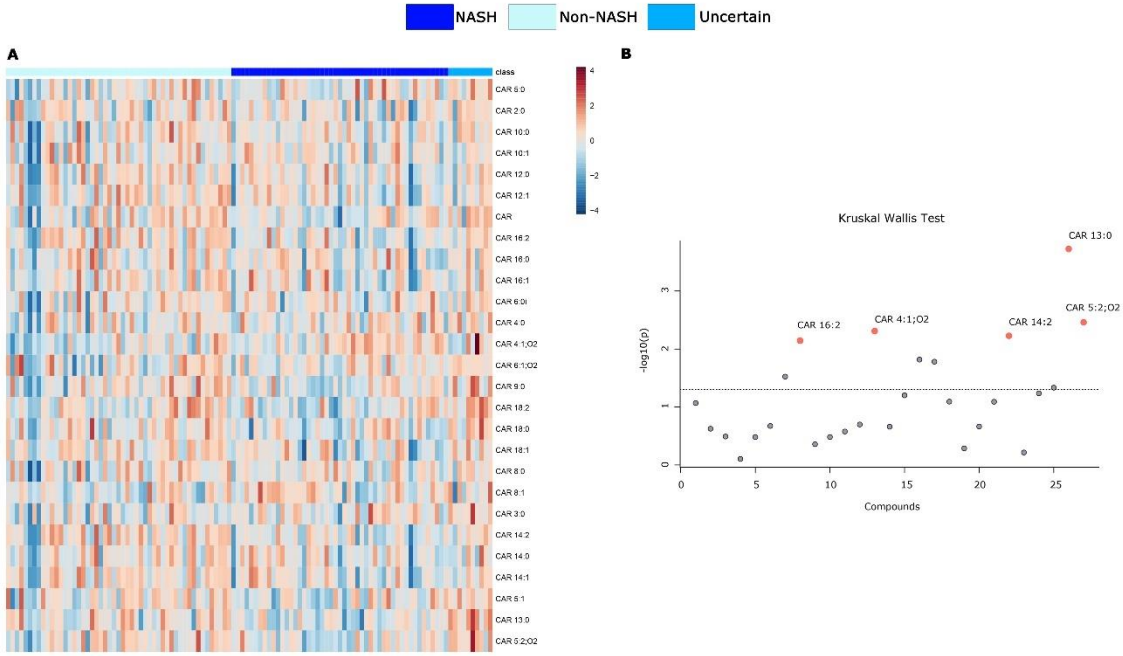

Figure 7: Heatmap (7A) and Kruskal Walllis Test (7B) of acyl-carnitines concentrations found in plasma.

NASH – Non-NASH

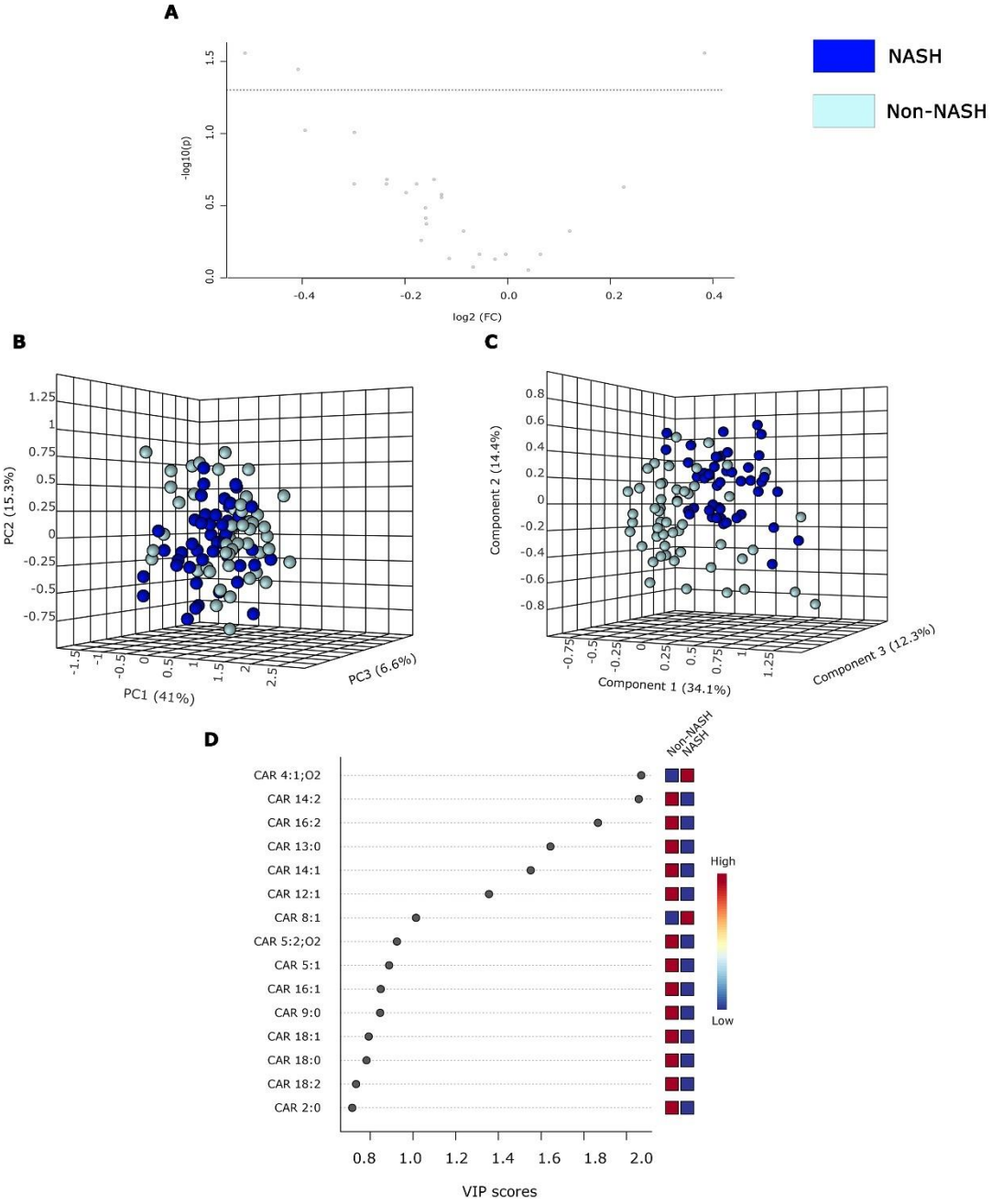

Figure 8: Volcano plot (A), PCA analysis (B), PLSDA analysis (C) and VIP score (D) of relative acyl-carnitine concentrations of NASH and Non-NASH patients.

As differences in concentration of acyl-carnitine species among groups (Figure 7B) were found, we wondered if there would be differences comparing the groups two by two. In the first place, we compared NASH and non-NASH plasma acyl-carnitines concentration, and we did not found difference in univariate (figure 8A) and multivariate analyses between them (Figure 8B-

D). Patients could not be separated by not-supervised (Principal Component Analysis, PCA) nor supervised (Partial Least Square Discriminant Analysis, PLS-DA) statistical test (figure 8B and 8C, respectively). The acyl-carnitines that had the most weight in the separation of PLS-DA were CAR 4:1;O2 and CAR 14:2 (figure 8D).

NASH – Uncertain

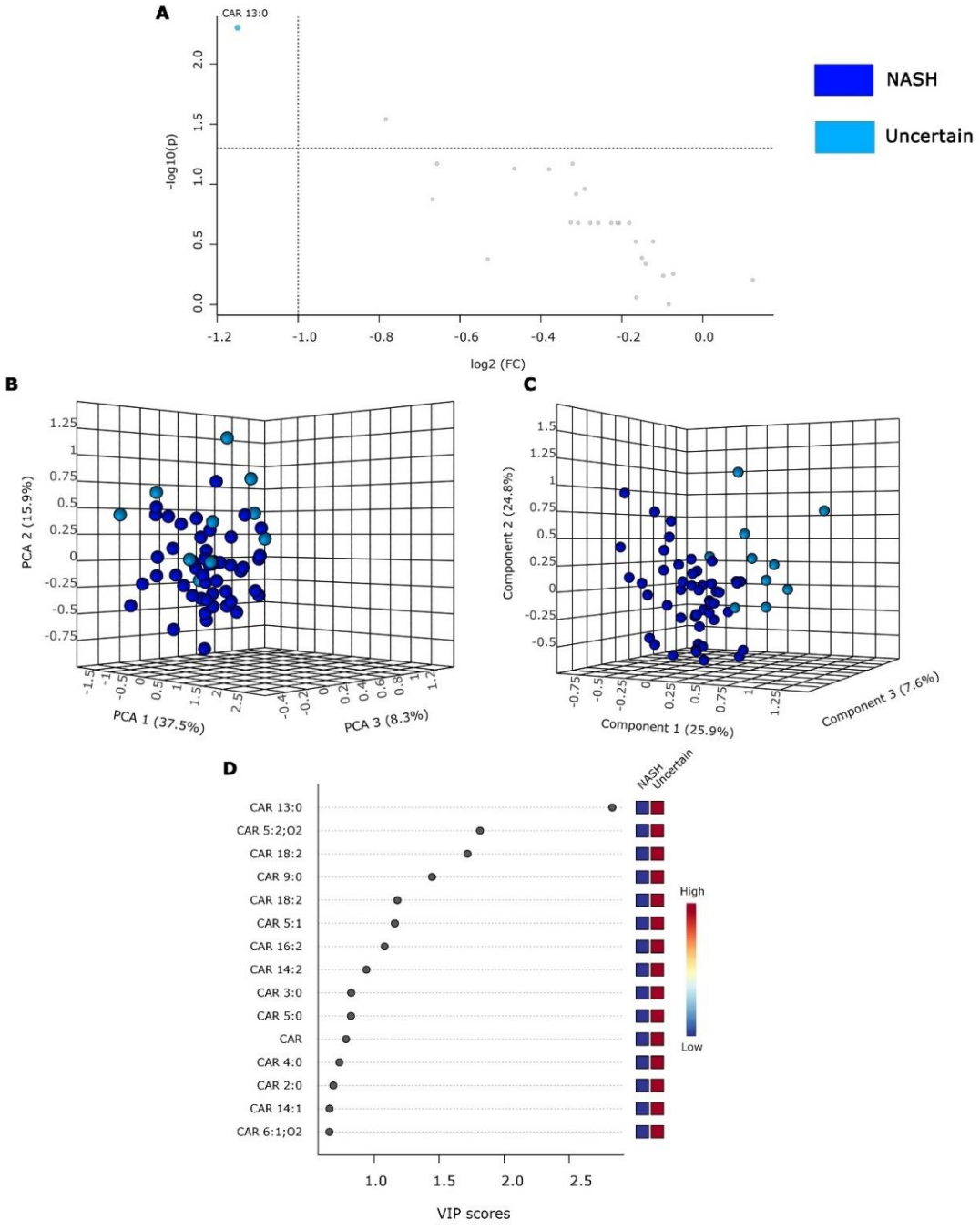

Figure 9: Volcano plot (A), PCA analysis (B), and PLSDA analysis (C) and VIP score (D) of relative acyl-carnitine concentrations of NASH and Uncertain patients.

We could see that only the concentration of acyl-carnitine CAR 13:0 was significantly decreased in NASH patients compared to Uncertain group (figure 9A). In the PCA we did not see much separation of groups (figure 9B) but in the PLSDA analysis we were able to distinguish one group from the other, although a little overlap can be seen (figure 9C). This last analysis was made by giving more importance to CAR 13:0 (figure 9D), which was found to be decreased in NASH (figure 9A).

Uncertain – Non-NASH

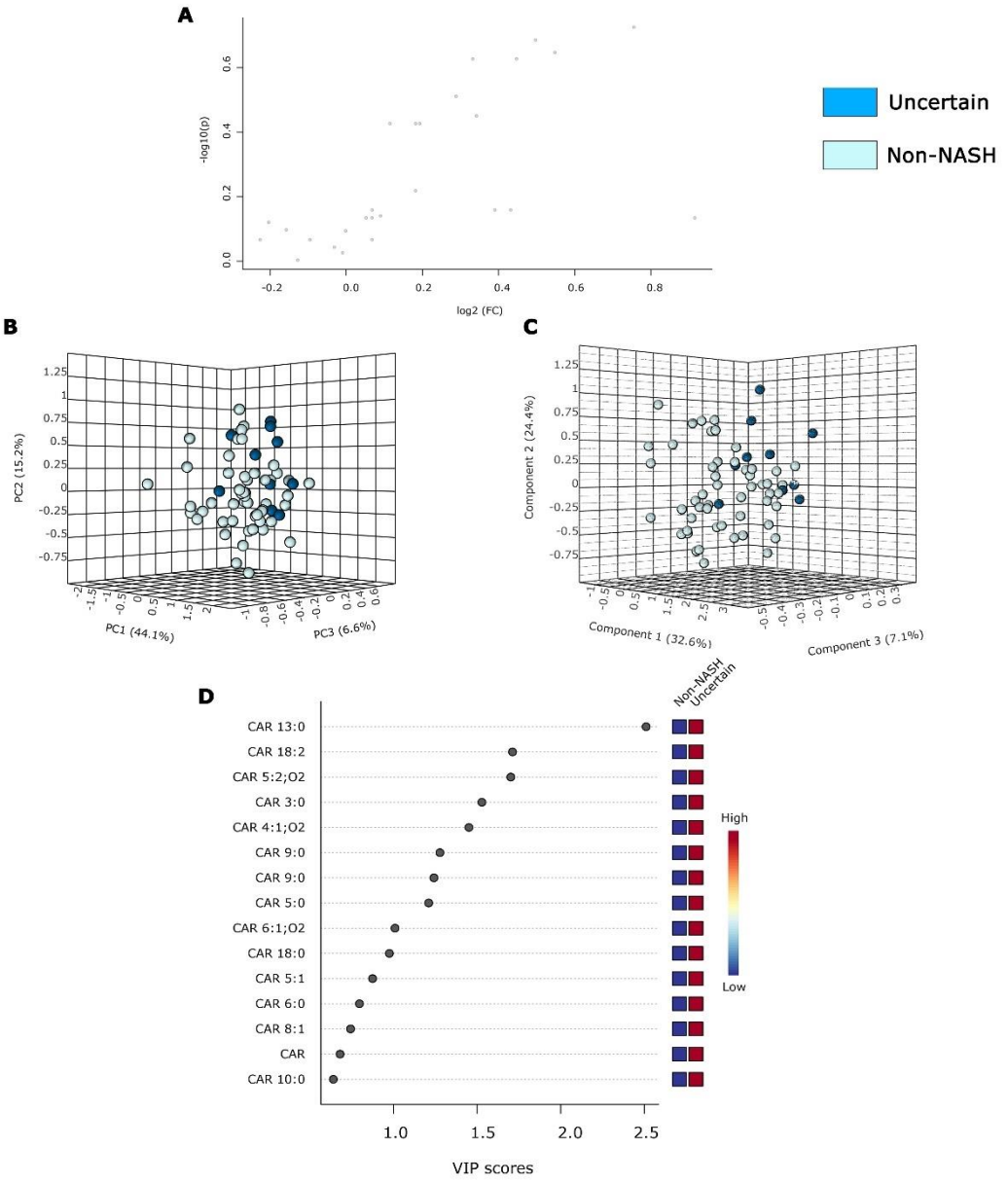

Figure 10: Volcano plot (A), PCA analysis (B), and PLSDA analysis (C) and VIP score (D) of relative carnitine concentrations of Uncertain and Non-NASH patients.

No significant differences were found in the acyl-carnitine concentrations of the Uncertain *versus* Non-NASH groups (figure 10A). Patients were not clearly separated, neither by PCA (Figure 10B) nor by PLSDA (Figure 10C). The acyl-carnitine that separated best in this case was also CAR 13:0 (figure 10D).

Carnitines do not have sufficient separation capacity to be biomarkers

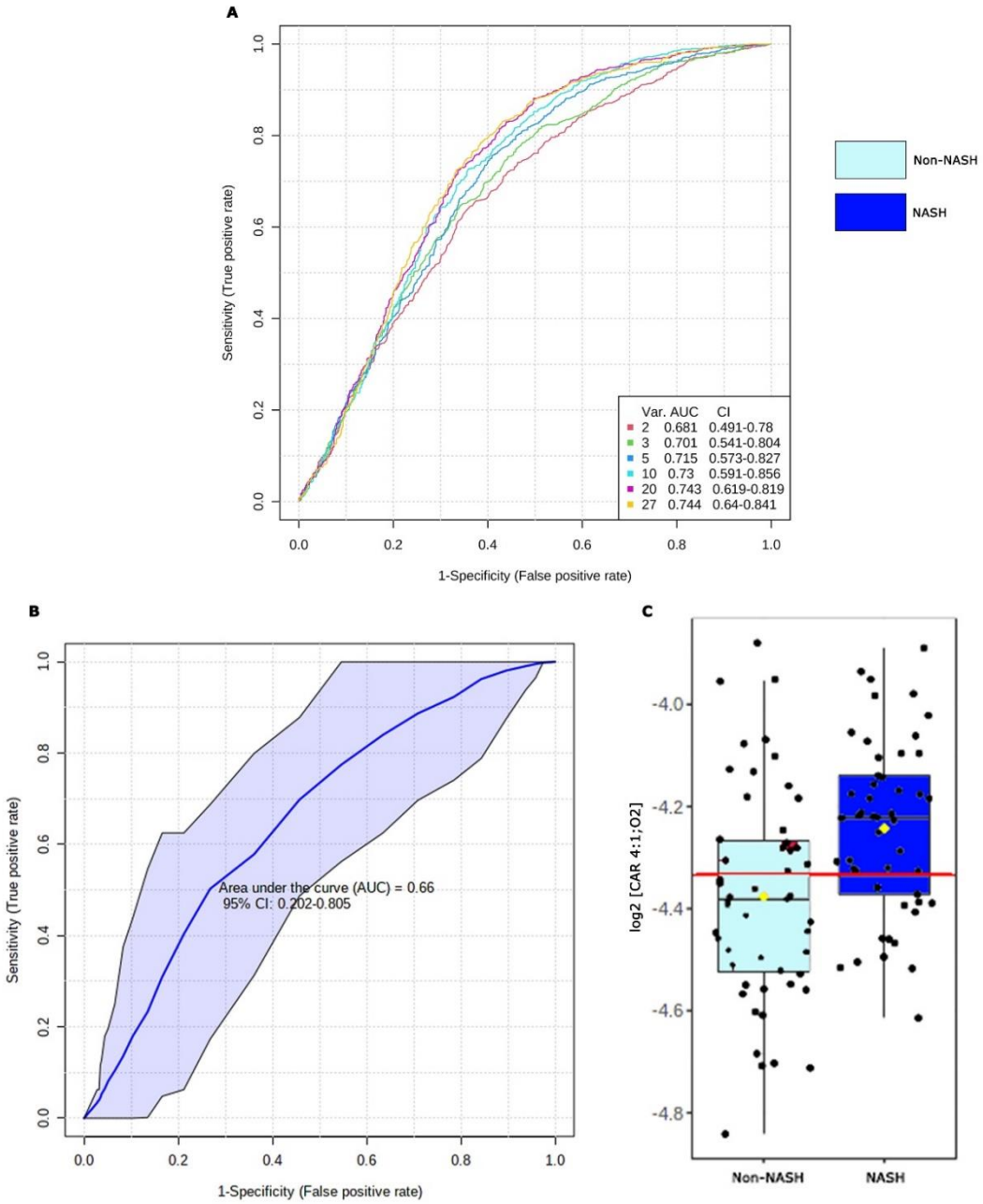

Figure 11: Receiver operating characteristics (ROC) curve of models that combined different number of variables (a), ROC of CAR 4:1;O2 (B) and the cut-off point of this acyl-carnitine (c).

We performed a Monte Carlo Cross Validation iteration with support vector machine (SVM) algorithm in order to determine whether any component or a combination of various, in this case carnitines, could be used as a potential biomarker to diagnose NASH. The best area under the curve (AUC) in receiver operating characteristics curve (ROC) was using 27 variables, meaning that the algorithm used all the acyl-carnitines we analyzed (figure 11A), and at the best we got an AUC of 0.744 (0.640-0.841). To make sure that there was no possible biomarker, we did an individual ROC curve with CAR 4:1;O2, the acyl-carnitine with best separation capacity, and we got an even lower AUC of 0.660 (0.502-0.805) (figure 11B). Cutoff point is represented in figure 11C.

MSEO indicates that the plasmatic form of carnitines does not originate in the liver

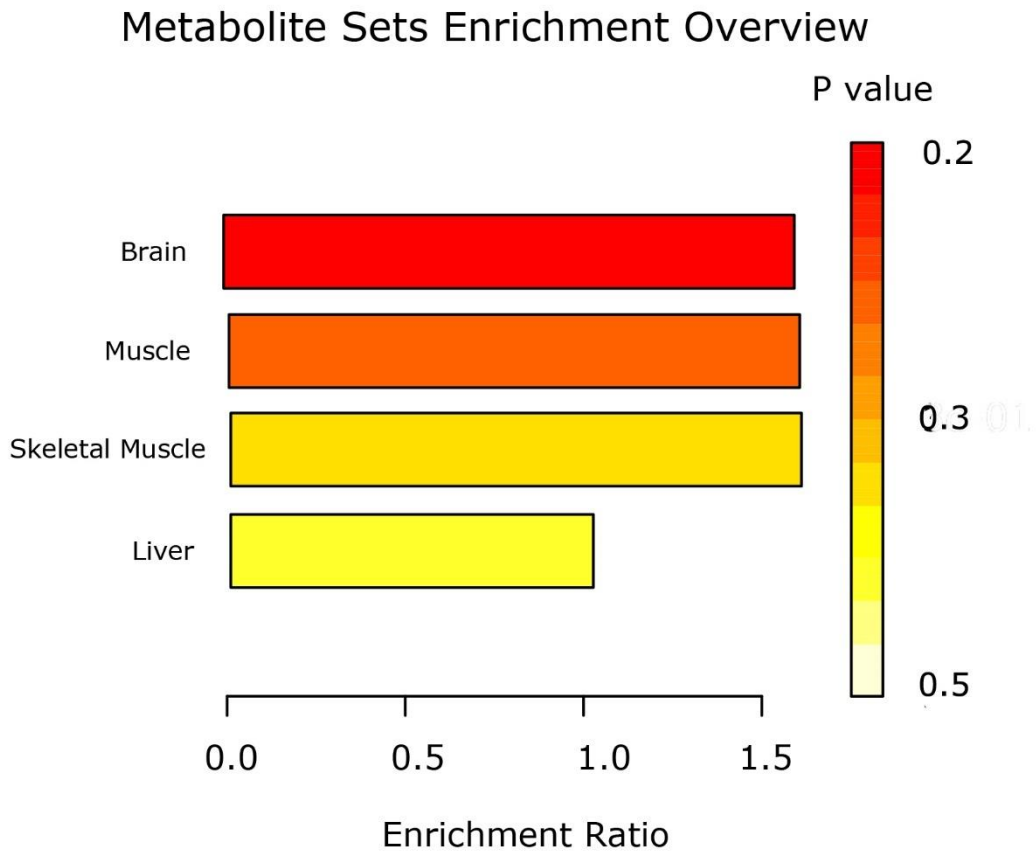

Figure 12: Metabolite sets Enrichment Overview (MSEO) of acyl-carnitines in plasma

As can be seen in figure 12, although there is not any significant organ, the acyl-carnitines from plasma may come from brain, not liver.

CAR 8:0 and CAR 5:0 characterize the carnitine profile of NASH in the liver

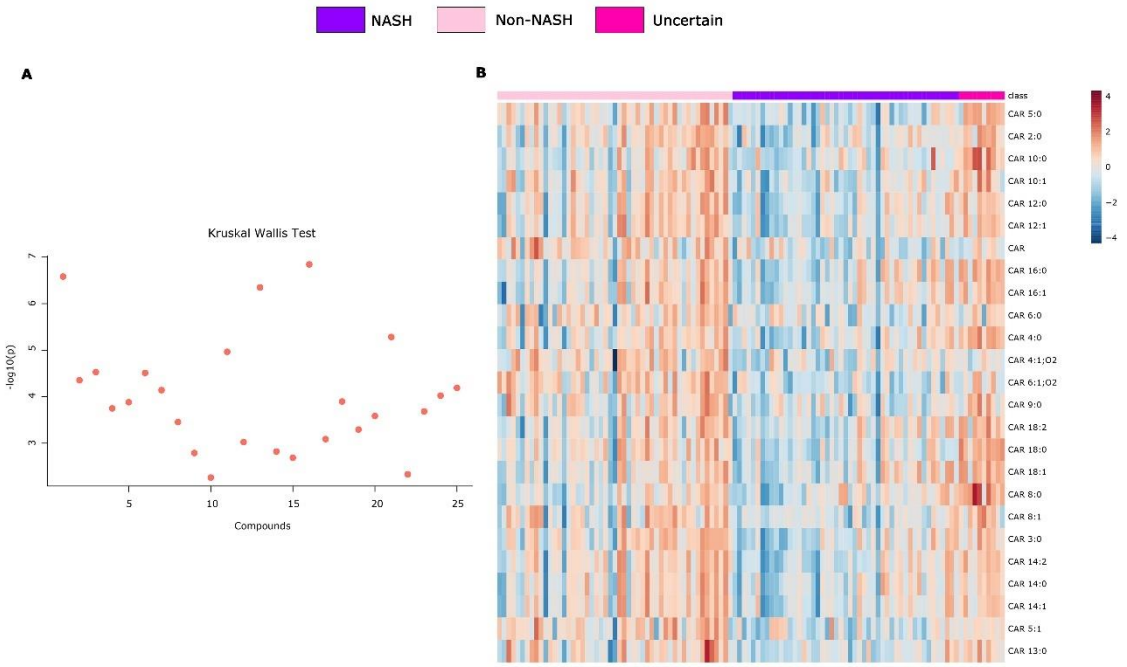

Figure 13: Kruskal Walllis Test (13A) and heatmap (13B) of acyl-carnitines concentrations found in liver.

Relative acyl-carnitine concentrations in liver were found to be much lower in NASH than in Non-NASH and Uncertain (figure 13A). All acyl-carnitines were significantly different in liver from patients with different grades of severity of NASH. A different relative concentration of these species can also be seen in figure 13B.

NASH – Non-NASH

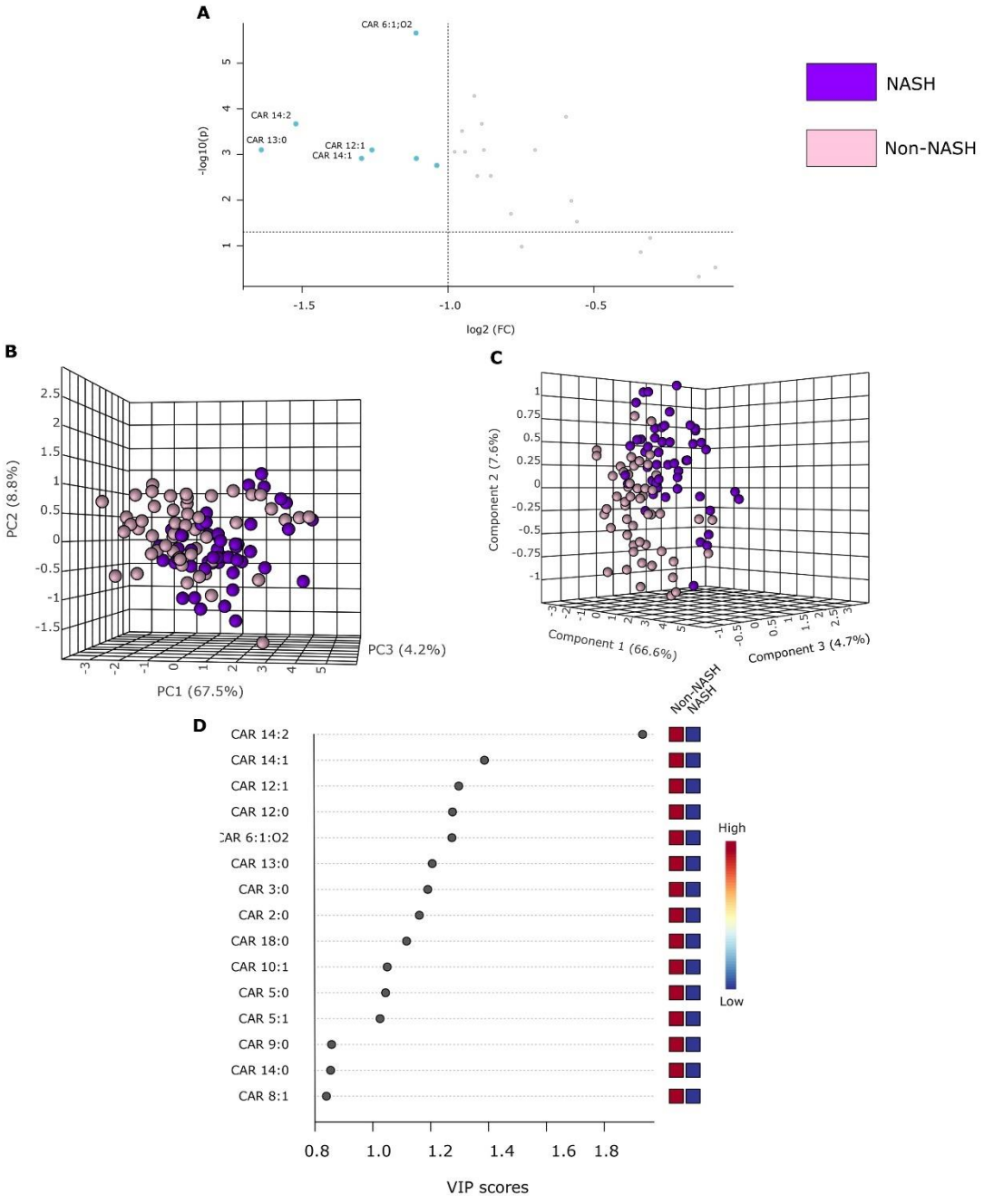

Figure 14: Volcano plot (A), PCA analysis (B), and PLSDA analysis (C) and VIP score (D) of relative acyl-carnitine concentrations of NASH and Non-NASH patients.

We wondered which differences could characterize the different stages of NALFD, so we compared the groups two by two. In the first place, we assessed the changes between acyl-carnitine concentrations in livers from

NASH and non-NASH obese patients. In liver, acyl-carnitine concentrations were decreased in NASH in relation to Non-NASH patients. The acyl-carnitines that were decreased tended to be large fatty acid compounds with the exception of CAR 6:1;O2 (figure 14A). The groups did not separate in the PCA (figure 14B). But in PLSDA we observed a better separation of the groups (figure 14C) based on CAR 14:2, which had the most separation capacity (figure 14D)

NASH – Uncertain

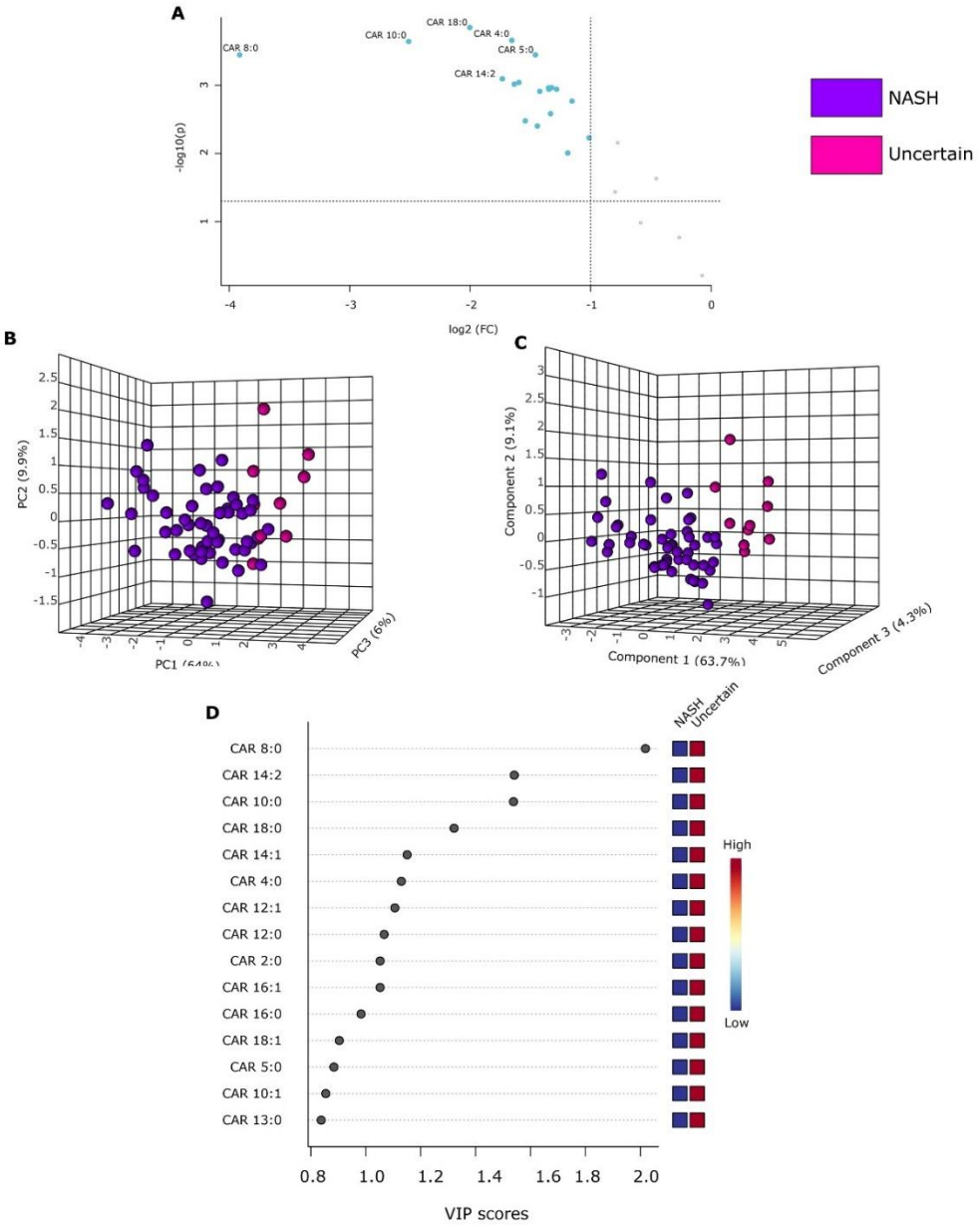

Figure 15: Volcano plot (A), PCA analysis (B), and PLSDA analysis (C) and VIP score (D) of relative acyl-carnitine concentrations of NASH and Uncertain patients.

Almost all acyl-carnitine concentrations were significantly decreased in NASH in contrast to the Uncertain group (figure 15A). In both PCA and PLSDA a clear distinction of groups was seen (figure 15B and figure 15C). In PLS-DA, CAR 8:0 was the variable that had more weight to separate the patients in the 3-dimensional plot (figure 15D).

Uncertain – Non-NASH

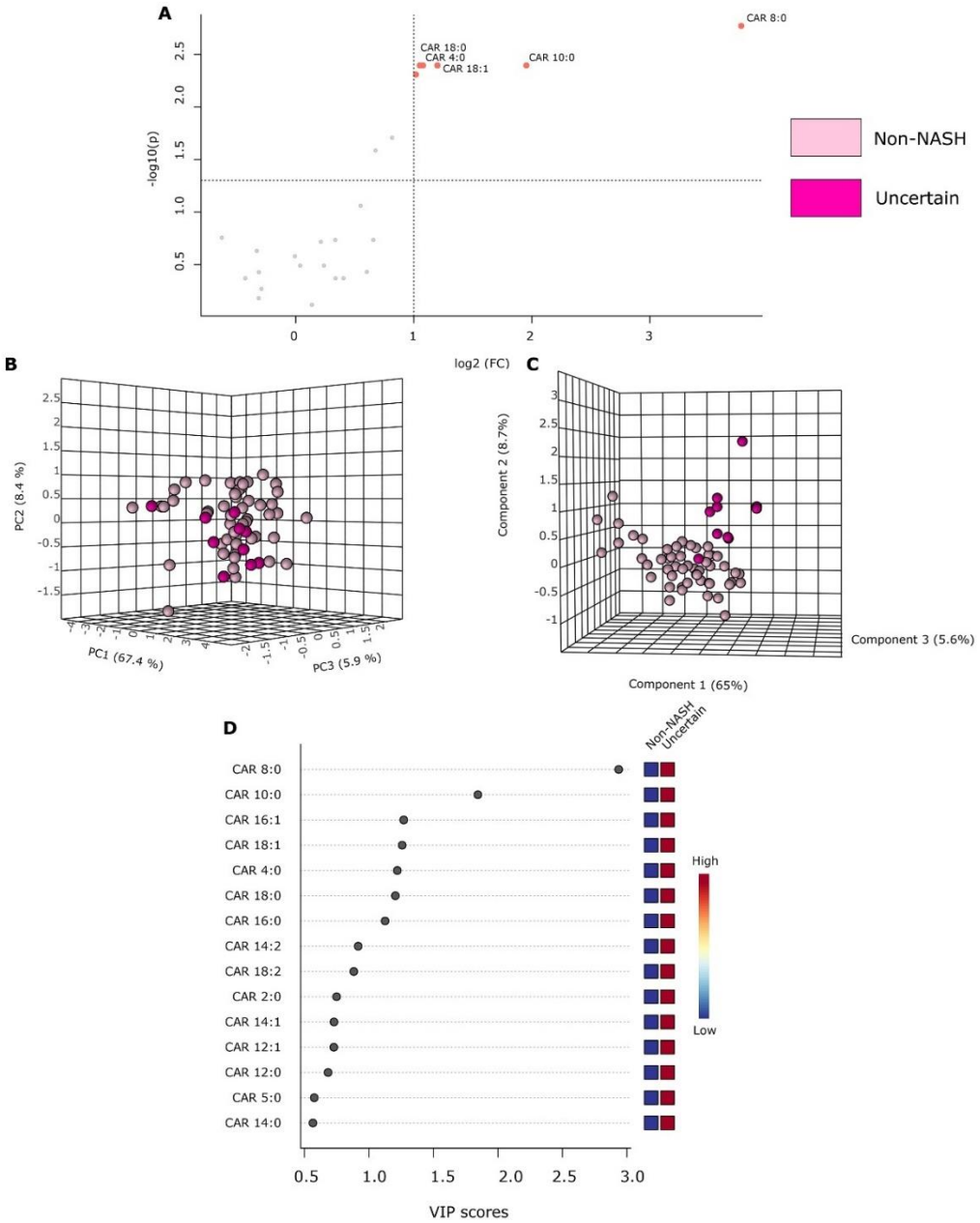

Figure 16: Volcano plot (A), PCA analysis (B), and PLSDA analysis (C) and VIP score (D) of relative acyl-carnitine concentrations of Uncertain and Non-NASH patients.

In contrast to previous liver comparisons, the relative acyl-carnitine concentration of the Uncertain group was significantly increased in relation to the Non-NASH group (figure 16A). In the PCA, the groups were completely mixed (figure 16B) but when a supervised analysis was performed, the PLSDA, we could see a distinction between the groups (figure 16C). The acyl-carnitine that separates them best was CAR 8:0 (figure 16D).

CAR 14:2, CAR 8:0 and CAR 5:0 were related to the progression of NAFLD

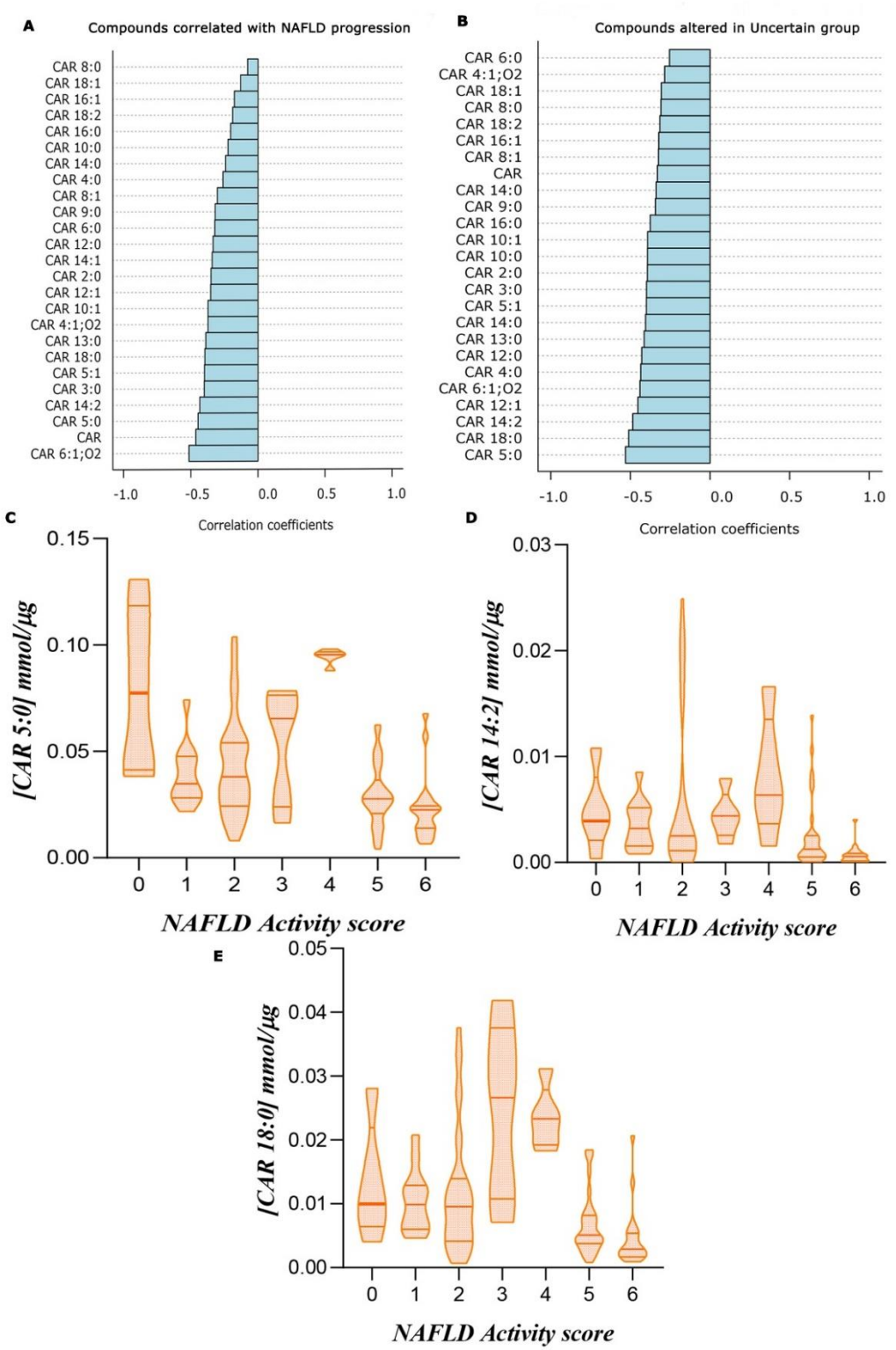

Figure 17: Pattern hunter of carnitines (A,B), compounds correlated with NAFLD progression (A), compounds altered in Uncertain group (B) and violin plots of acyl-carnitines in the NAS score (C,D,E).

When we found that acyl-carnitine concentrations were increased in the Uncertain group compared to the Non-NASH group, we looked for different patterns. If we analyzed the significance of acyl-carnitine concentrations with the NASH progression pattern, i.e. from 0 to 7, we saw almost no significance with any of the acyl-carnitines (figure 17A) whereas using another pattern, with the Uncertain group not according than the others, we did see more significant acyl-carnitines that followed this pattern, of which the most significant were CAR 14:2, CAR 8:0 and CAR 5:0 (figure 17B). In all acyl-carnitines we could see how they were found to be more diminished as NASH progresses, with the exception of the Uncertain group (figures 17C, 17D and 17E).

Relationship of the different variables in the analysis of NASH progression *versus* carnitines

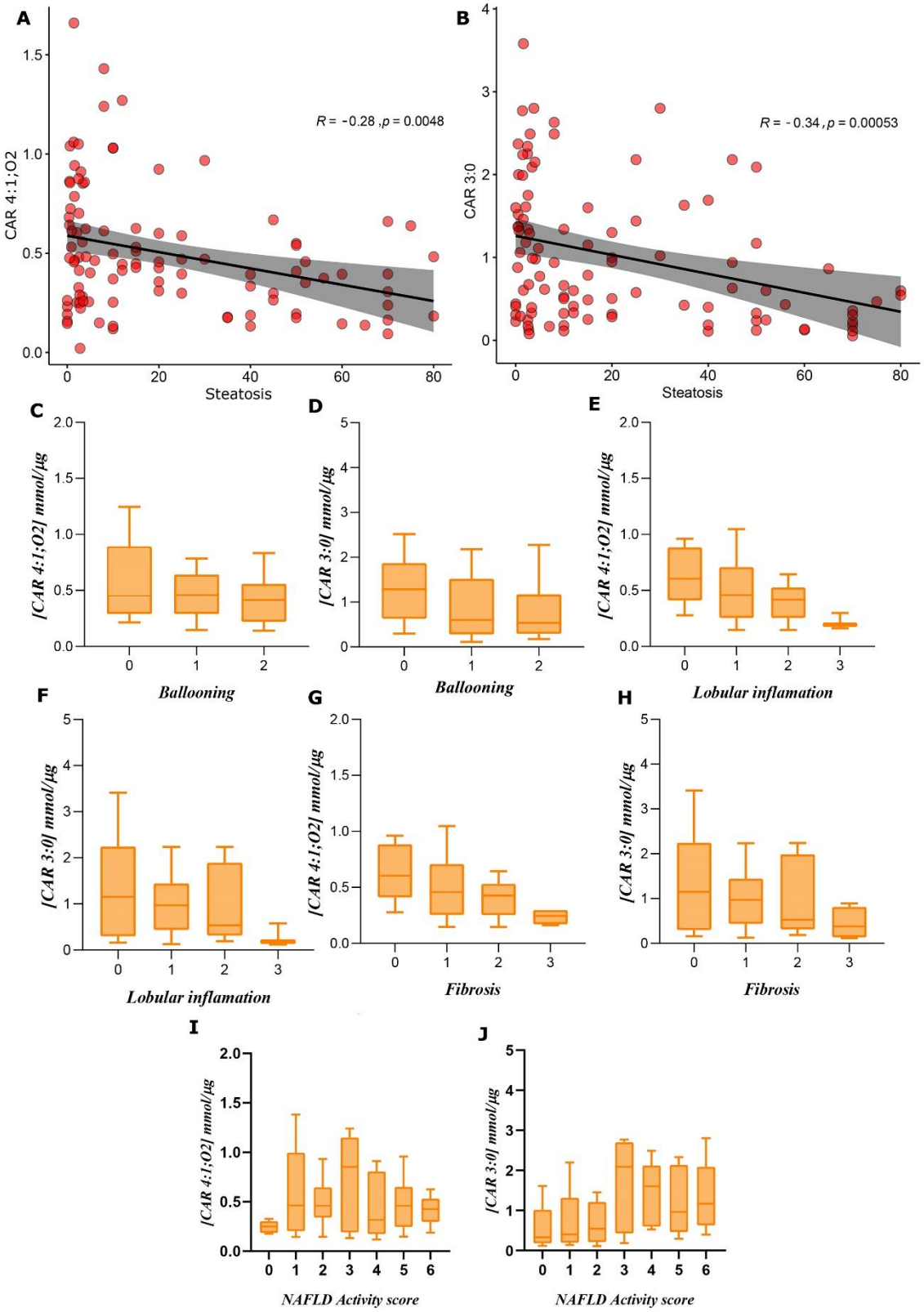

Figure 19: Correlations with the different variables of the NAFLD Activity Score.

We wondered how acyl-carnitine concentration varied along the stages of the different histological characteristics. We analyzed acyl-carnitine concentration to found the ones that correlated most along with the different stages of the histological characteristics. CAR 4:1;O2 and CAR 3:0 were the ones that correlated best with all these characteristics. These acyl-carnitines were correlated negatively with steatosis (figure 19A and 19B). A correlation was also made with Ballooning, where they were found to be decreased in the highest value of this variable (figure 19C and 19D). In fibrosis we could see how the concentration decreases as the degree of fibrosis increases (figure 19G and 19H), the same happened with lobular inflammation (figure 19E and 19F). On the other hand we looked at how important they were with the NAFLD Activity score, where we found that there was an increase in NASH groups 3 and 4 (figure 19I and 19J).

MSEO identifies the endoplasmic reticulum and mitochondria as possible organelles affected

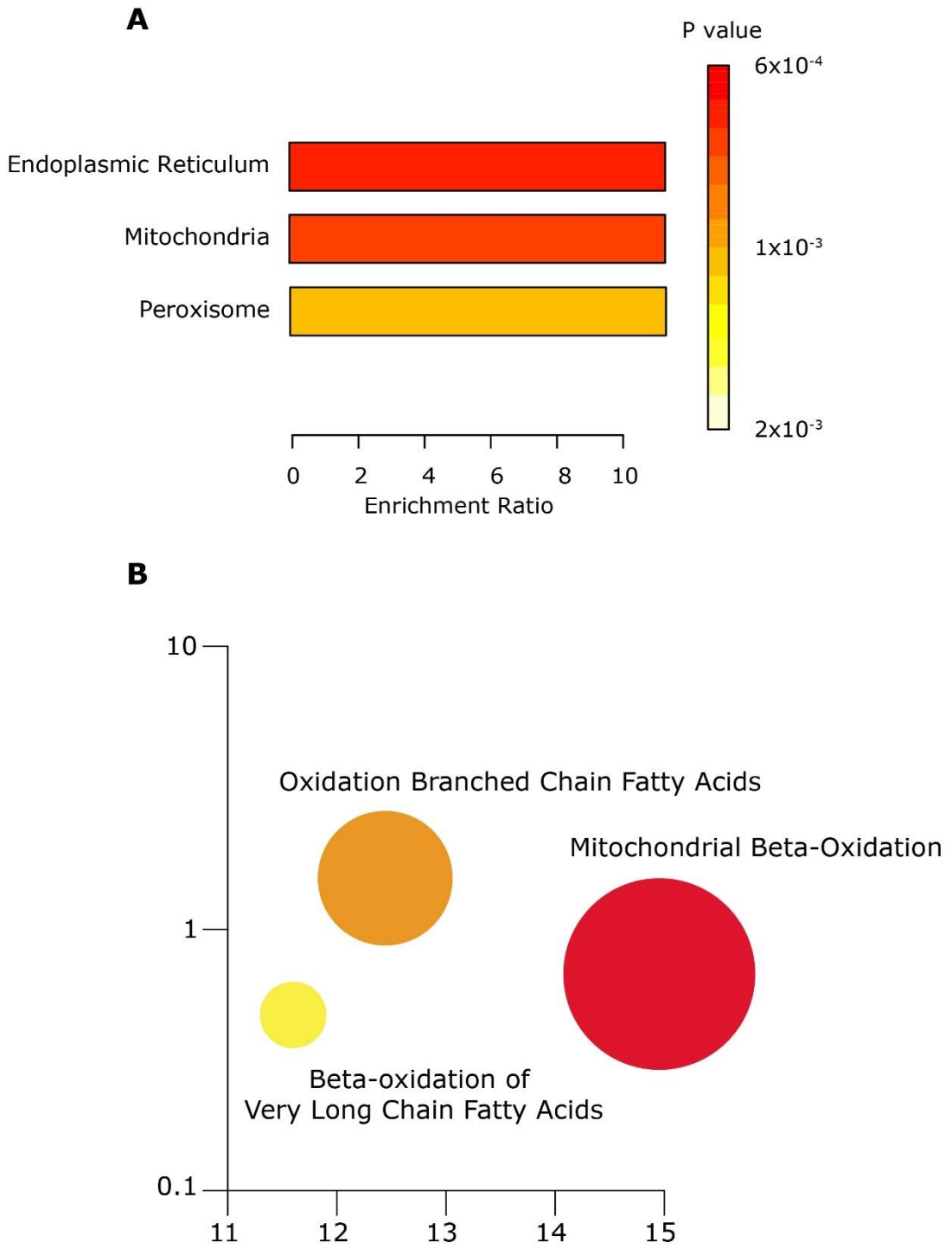

Figure 20: Metabolite sets enrichment overview (MSEO) of acyl-carnitines in liver (A) and pathway analysis (B)

The most possibly affected organelles shown in the metabolite sets enrichment overview (MSEO) were the endoplasmic reticulum and mitochondria (figure 20A). As we found that the metabolic pathways affected were fatty acid beta-oxidation (figure 20B) in mitochondria we decided to focus on lipid metabolism and mitochondrial dynamics proteins.

Western Blot showed altered lipid metabolism and mitochondrial dynamics' proteins in NAFLD progression

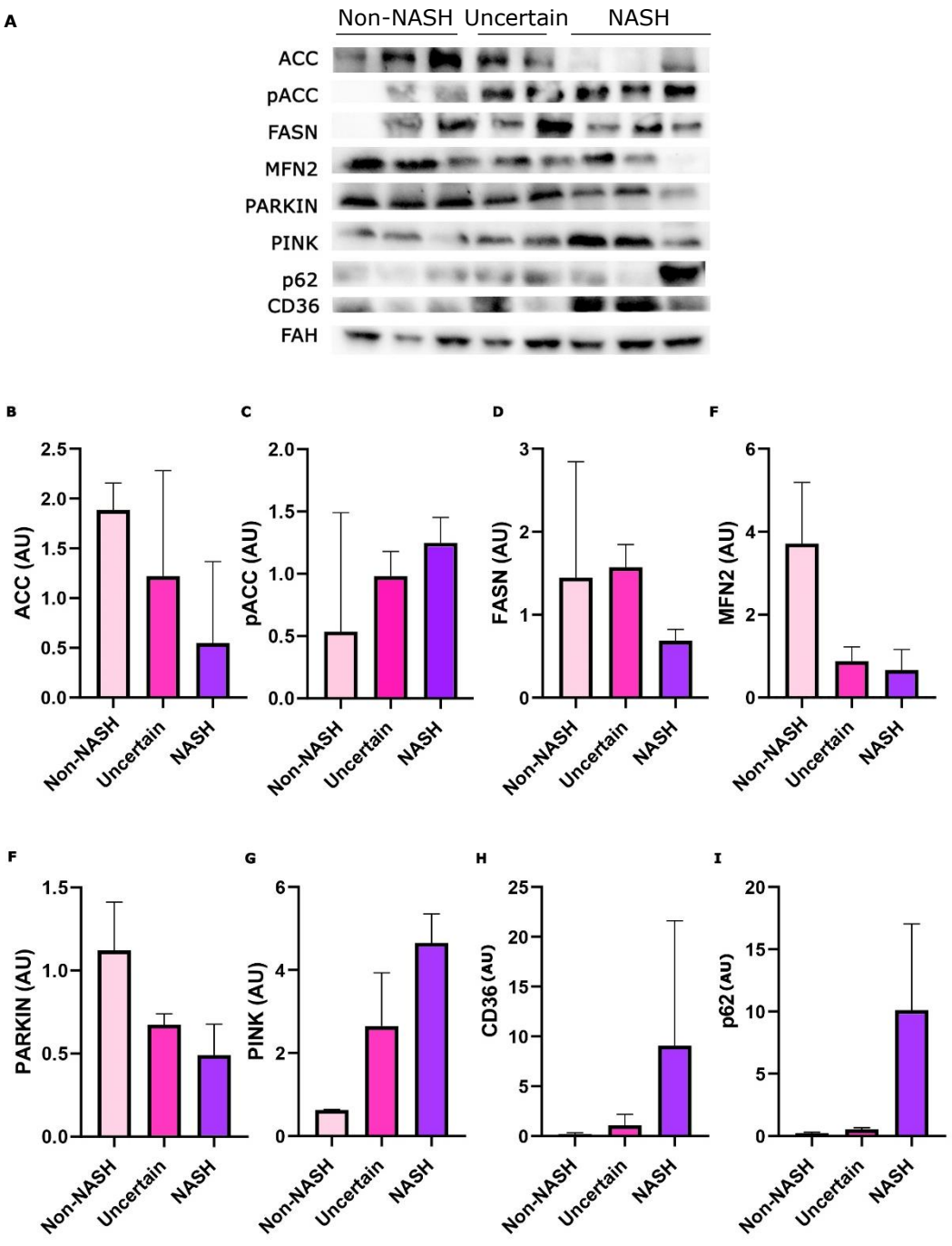

Figure 21: Western blot (A) and the different quantifications of protein of interest's bands (B,C,D,E,F,G,H,I)

As we saw in the MSEA that the most affected pathways could be the ones related to lipid metabolism and organelles such as mitochondria, we analyzed by western blot the relative amount of some proteins: Fatty acid synthase (FASN), acetyl CoA carboxylase (ACC) and phospho acetyl CoA carboxylase (pACC) for fatty acid synthesis. Mitofusin 2 (MFN2) is a protein that plays a role in mitochondrial dynamics. PTEN-induced kinase (PINK) and PARKIN are proteins that participate in mitophagy (figure 21A). We could see that proteins from lipid metabolism were significantly altered as NAFLD progressed to NASH. MFN2 was less present in NASH patients, as well as PARKIN. PINK was relatively more present in NASH patients. The cluster of differentiation 36 (CD36) is responsible for capturing fatty acids, and the ubiquitin-binding protein (p62), with which the cells carry out autophagy (figure 21B,C,D,E,F,G,H,I).

Discussion

Obesity is a global problem nowadays, and its prevalence is gradually increasing. Since 30 last years it has been increasing over the decades, making the impact on the economy and on public health even higher. Obesity has been associated with different metabolic diseases such as T2DM, MS, and DLP, among many others, as well as being related to NAFLD and NASH. The fact that obesity is so closely related with other diseases increases global morbidity and mortality (23).

Fatty acyl carnitines are transporters of fatty acids into the mitochondria for beta-oxidation. As mitochondria may be malfunctioning in patients with NASH, we have analyzed the different carnitine species, both in plasma and liver of different severity degree of NAFLD obese patients, in order to determine their role on NASH.

Transaminases are not specific and are therefore insufficient to determine the severity associated with NASH, so the diagnosis must be performed through liver biopsy (24). In the studied patients, ALT and AST transaminases were both significantly increased, almost twofold, in NASH patients compared to Non-NASH. Numerous studies have demonstrated an increase in transaminases, however none of them can use them as biomarkers (24). On the other hand, an increase in GGT and ALT transaminase has been associated with endothelial dysfunction, which would explain a close association of NASH with heart disease, among other diseases (25).

Although there is no specific medication to combat NASH, many patients who also presented T2DM took metformin, which has a beneficial effect on both T2DM and NAFLD (26). Therefore, we found that a high percentage of NASH patients took metformin as opposed to Non-NASH patients, even though they suffered from T2DM.

Increased steatosis has been seen in patients with NASH but in order to determine the degree of NAFLD it should be combined with other parameters. Therefore, to determine the degree of NAFLD an expert hepatologist assessed NAS, that considers two additional parameters: ballooning and lobular inflammation. On the other hand, fibrosis cannot be considered a reliable

feature to determine NASH progression even though it is used for assessing its stage (27).

Although in our study we did not find any specie that was significantly different between the groups, Kalhan et al. have informed that 14 different carnitine species were significantly elevated in contrast to control samples (28). This may be because carnitines are released into the plasma in order to prevent the toxicity produced by acyl CoA at high concentrations in the mitochondria (28). Studies suggest that the short- and medium-chain acylcarnitines in plasma are from the liver, while the long-chain acylcarnitines may come from other tissue (29).

One study found that patients with cellular hepatocarcinoma (HCC) and steatohepatitis had increased serum acylcarnitine concentrations in contrast to patients without cellular hepatocarcinoma but with steatohepatitis (30). A possible answer to this problem could be a dysregulation of CPT2, caused by the suppression of fatty acid oxidation, which is a characteristic of liver cancer cells (31)(32). Our results were not consistent with the above, as we found a decrease in plasma levels, but this decrease could be because NASH patients in our study did not have as developed disease and as much fibrosis as people with HCC.

Carnitines may be potential effective biomarkers for different diseases such as T2DM, heart failure or cancer, as well as many other metabolic diseases (33) but cannot be used as biomarkers to diagnose NASH. As we can see from the ROCs generated, any carnitine species cannot be used as a biomarker, as there are too many false positives and false negatives.

An increase of acylcarnitines in the brain, which can be used as a substrate for fatty acid oxidation, would result in a large amount of acetyl CoA, which seems to be favorably related to ageing, so the plasma origin of carnitines is not from the liver, but from the brain (34).

A low carnitine level in liver has been shown in patients with liver disease, resulting in an accumulation of fatty acids in the liver, as low carnitine levels mean that fatty acids cannot be translocated into the mitochondria and tend to accumulate (19) This low carnitine level can come from low levels of CPT1 or low levels of OCTN2, its transporter, being a condition called primary

carnitine deficiency (PCD) (19). Another problem with this lack of carnitine and the accumulation of fatty acids in the liver would be the oxidative stress caused by the increase of carnitine level (35). Many disorders are related to the transport and metabolism of carnitines, so they may be decreased in NASH (36).

L-carnitine is a good therapeutic treatment because it is able to increase the expression of Beta-oxidation genes, fatty acid transport and reduce oxidative stress and inflammation (37), as well as having an antioxidant effect (38). In our study, the Uncertain group, which severity is between the NASH and Non-NASH patients, had more carnitines' concentration than the Non-NASH group. We believe that this could be a response to fight the oxidative stress that is starting to build up, as well as to try to save the mitochondria.

Carnitines not only transport fatty acids into the mitochondria, but have also been linked to the endoplasmic reticulum for the provision of acyl CoA in the luminal endoplasmic reticulum (39) They are also important in peroxisomal metabolism, because peroxisomes degrade fatty acids that have been transported as acylcarnitines from the mitochondria, and acyltransferases are peroxisomal enzymes (40).

As we saw that the signalling pathways most affected were those related with mitochondrial fatty acid beta-oxidation, we analyzed the mitochondrial proteins that mostly affect this pathway, and proteins that could inform us about the state of mitochondria and endoplasmatic reticulum.

We observed that relative presence of ACC increased along with NAFLD severity progression. ACC carboxylates Acetyl CoA to malonyl CoA and this can be used as a regulator as it inhibits CPT1 to inactivate fatty acid oxidation in mitochondria (41).

FASN is used for fatty acid synthesis, i.e. lipogenesis (42) and its activity has been related to the accumulation of fat in the liver (43).

pACC inactivates FASN and thus decreases malonyl CoA, which, when decreased, stimulates fatty acid oxidation (43). As pACC stimulates fatty acid oxidation, it is decreased in NASH, as in this pathology, beta-oxidation is impaired, consistent with our findings.

MFN2 is involved in outer mitochondrial membrane (OMM) fusion (44). It is also related to endoplasmic reticulum stress that induces a binding between the ER and mitochondria (44). Mitochondrial fusions and fissions make the mitochondrion function properly, so if we decrease MFN2, the mitochondrion will not be able to function appropriately (45)(22).

MFN2 is also closely related to both PINK and PARKIN. PINK phosphorylates MFN2 and PARKIN phosphorylates ubiquitin. PINK translocates PARKIN into the mitochondria when the mitochondria are damaged (46). A decrease in MFN2 results in mitochondrial dysfunction that should activate mitophagy, but instead induces abnormal mitochondrial proliferation. This is where the role of PARKIN and PINK comes in, which activate phosphorylation and ubiquitination of MFN2 and are responsible for activating mitophagy (46). We found that PARKIN was decreased in NASH and PINK increased. As there was little MFN2, there was also a decreased amount of PARKIN. Surprisingly, PINK was increased, probably due to the fact that depending on the mitochondrial damage, some mechanisms or others are used to activate mitophagy. SQSTM/p62 could be used to induce it, independently of PINK and PARKIN (47).

Our study has been an observational study where we have found some limitations which can be solved by further research. One of the limitations we have identified is that of the Uncertain group, which is made up of a relatively small number of patients, and as it is not made up of an adequate number of patients, these selected patients could have different characteristics, which could be out of the ranges that we expected to find. Another limitation is that, as a clinical study, we cannot confirm that patients follow the established medication and indications. Obesity is more prevalent in women than in men, therefore the gender difference could not be demonstrated in this study, as there is a higher percentage of women than men in the research. Finally, the western blot technique is semi-quantitative, we should check the mRNA to be able to determine the results.

In the future, the group could investigate the sex difference in NAFLD progression and the effect of obesity in non-obese patients, with an increase in the n of studies that could be carried out.

On the other hand, using the results obtained in the western blot, we could focus on mitochondria and its autophagy. These studies could be performed by generating KO mice, KO cells, or cells in different conditions, in order to isolate the different proteins of interest, and see what would happen without them. We could also produce primary cultivations, for the same purpose.

Finally, we could increase the omics analysis, metabolomics to be able to see the differences at the level of the Krebs cycle and transcriptomics to be able to investigate the mRNA of the western blot proteins.

Conclusion

According to the results obtained, the conclusions are as follows:

The plasma carnitine profile is of no relevance either in the disease or as a possible biomarker.

The carnitine profile in the liver does show a distinction in the progression of NAFLD severity.

As the progression of NAFLD increases, carnitines decrease, with the exception of people with a non-definite NASH diagnostic, as they appear increased.

In NASH livers, mitophagy and apoptosis are activated and fatty acid beta-oxidation is suppressed.

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References

1. Nimptsch K, Konigorski S, Pischon T. Diagnosis of obesity and use of obesity biomarkers in science and clinical medicine. Metabolism. 2019;92:61-70.

2. Caballero B. Humans against Obesity: Who Will Win? 2019;

3. De Lorenzo A, Soldati L, Sarlo F, Calvani M, Di Lorenzo N, Di Renzo L. New obesity classification criteria as a tool for bariatric surgery indication. World J Gastroenterol. 2016;22(2):681-703.

4. Apovian CM. Obesity: Definition, Comorbidities, Causes, and Burden. 2014.

5. Chooi YC, Ding C, Magkos F. The epidemiology of obesity. Metabolism. 2019;92:6-10.

6. Health Effects of Overweight and Obesity in 195 Countries over 25 Years | Enhanced Reader [Internet]. [citat 16 maig 2021]. Disponible a: chrome-extension://dagcmkpagjlhakfdhnbomgmjdpkdklff/enhanced-reader.html?openApp&pdf=https%3A%2F%2Fwww.nejm.org%2Fdoi%2Fpdf%2F10.1056%2FNEJMoa1614362%3FarticleTools%3Dtrue

7. Gastaldelli A, Miyazaki Y, Pettiti M, Matsuda M, Mahankali S, Santini E, et al. Metabolic effects of visceral fat accumulation in type 2 diabetes. J Clin Endocrinol Metab. 2002;87(11):5098-103.

8. Jarolimova J, Tagoni J, Stern TA. Obesity: Its epidemiology, comorbidities, and management. Prim Care Companion J Clin Psychiatry. 2013;15(5).

9. Woolf SH, Schoomaker H. Life Expectancy and Mortality Rates in the United States, 1959-2017. JAMA - J Am Med Assoc. 2019;322(20):1996-2016.

10. Maurice J, Manousou P. Non-alcoholic fatty liver disease. Clin Med J R Coll Physicians London. 1 juny 2018;18(3):245-50.

11. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease—Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology. 2016;64(1):73-84.

12. Polyzos SA, Kountouras J, Mantzoros CS. Obesity and nonalcoholic fatty liver disease: From pathophysiology to therapeutics. Metabolism. 2019;92:82-97.

13. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41(6):1313-21.

14. Cobbina E, Akhlaghi F. Non-alcoholic fatty liver disease (NAFLD)–pathogenesis, classification, and effect on drug metabolizing enzymes and transporters. Vol. 49, Drug Metabolism Reviews. Taylor and Francis Ltd; 2017. p. 197-211.

15. Younossi Z, Anstee QM, Marietti M, Hardy T, Henry L, Eslam M, et al. Global burden of NAFLD and NASH: Trends, predictions, risk factors and prevention. Nat Rev Gastroenterol Hepatol. 2018;15(1):11-20.

16. Friedman SL, Neuschwander-Tetri BA, Rinella M, Sanyal AJ. Mechanisms of NAFLD development and therapeutic strategies. Vol. 24, Nature Medicine. Nature Publishing Group; 2018. p. 908-22.

17. Jou J, Choi SS, Diehl AM. Mechanisms of disease progression in nonalcoholic fatty liver disease. Semin Liver Dis. 2008;28(4):370-9.

18. Longo N, Frigeni M, Pasquali M. Carnitine transport and fatty acid oxidation. Biochim Biophys Acta - Mol Cell Res. 1 octubre 2016;1863(10):2422-35.

19. Savic D, Hodson L, Neubauer S, Pavlides M. The importance of the fatty acid transporter l-carnitine in non-alcoholic fatty liver disease (Nafld). Vol. 12, Nutrients. MDPI AG; 2020. p. 1-17.

20. Vaz FM, Wanders RJA. Carnitine biosynthesis in mammals. Biochem J. 2002;361(3):417-29.

21. Knottnerus SJG, Bleeker JC, Wüst RCI, Ferdinandusse S, IJlst L, Wijburg

FA, et al. Disorders of mitochondrial long-chain fatty acid oxidation and the carnitine shuttle. Vol. 19, Reviews in Endocrine and Metabolic Disorders. Springer New York LLC; 2018. p. 93-106.

22. Hernández-Alvarez MI, Sebastián D, Vives S, Ivanova S, Bartoccioni P, Kakimoto P, et al. Deficient Endoplasmic Reticulum-Mitochondrial Phosphatidylserine Transfer Causes Liver Disease. Cell. 2019;177(4):881-895.e17.

23. Upadhyay J, Farr O, Perakakis N, Ghaly W, Mantzoros C. Obesity as a Disease. Med Clin North Am. 2018;102(1):13-33.

24. Kashyap SR, Diab DL, Baker AR, Yerian L, Bajaj H, Gray-Mcguire C, et al. Triglyceride levels and not adipokine concentrations are closely related to severity of nonalcoholic fatty liver disease in an obesity surgery cohort. Obesity. 2009;17(9):1696-701.

25. Arinc H, Sarli B, Baktir AO, Saglam H, Demirci E, Dogan Y, et al. Serum gamma glutamyl transferase and alanine transaminase concentrations predict endothelial dysfunction in patients with non-alcoholic steatohepatitis. Ups J Med Sci. 2013;118(4):228-34.

26. Akshintala D, Chugh R, Amer F, Cusi K. Nonalcoholic Fatty Liver Disease: The Overlooked Complication of Type 2 Diabetes. Endotext. MDText.com, Inc.; 2000.

27. Bei Y. 乳鼠心肌提取 HHS Public Access. Physiol Behav. 2017;176(3):139-48.

28. Kalhan SC, Guo L, Edmison J, Dasarathy S, McCullough AJ, Hanson RW, et al. Plasma metabolomic profile in nonalcoholic fatty liver disease. Metabolism. març 2011;60(3):404-13.

29. Makrecka-Kuka M, Sevostjanovs E, Vilks K, Volska K, Antone U, Kuka J, et al. Plasma acylcarnitine concentrations reflect the acylcarnitine profile in cardiac tissues. Sci Rep. 2017;7(1):1-11.

30. Takaya H, Namisaki T, Kitade M, Shimozato N, Kaji K, Tsuji Y, et al. Acylcarnitine: Useful biomarker for early diagnosis of hepatocellular carcinoma in non-steatohepatitis patients. World J Gastrointest Oncol.

2019;11(10):887-97.

31. Fujiwara N, Nakagawa H, Enooku K, Kudo Y, Hayata Y, Nakatsuka T, et al. HHS Public Access. 2019;67(8):1493-504.

32. Farma F De. By Peroxisome Proliferator-Activated Receptor Through a Partially Conserved Peroxisome Proliferator-Responsive Element. Biochem j. 2003;729:721-9.

33. McCann MR, De la Rosa MVG, Rosania GR, Stringer KA. L-carnitine and acylcarnitines: Mitochondrial biomarkers for precision medicine. Metabolites. 2021;11(1):1-21.

34. Currais A, Huang L, Goldberg J, Petrascheck M, Ates G, Pinto-Duarte A, et al. Elevating acetyl-CoA levels reduces aspects of brain aging. Elife. 2019;8:1-21.

35. Carnitina, Acilcarnitinas y Beta-Oxidación [Internet]. [citat 14 març 2021]. Disponible a: https://www.lipidmaps.org/resources/lipidweb/lipidweb_html/lipids/simple/carnitin/index.htm

36. Almannai M, Alfadhel M, El-Hattab AW. Carnitine inborn errors of metabolism. Molecules. 2019;24(18).

37. Ishikawa H, Takaki A, Tsuzaki R, Yasunaka T, Koike K, Shimomura Y, et al. L-carnitine prevents progression of non-alcoholic steatohepatitis in a mouse model with upregulation of mitochondrial pathway. PLoS One. 2014;9(7).

38. Polimeni L, del Ben M, Baratta F, Perri L, Albanese F, Pastori D, et al. Oxidative stress: New insights on the association of nonalcoholic fatty liver disease and atherosclerosis. World J Hepatol. 2015;7(10):1325-36.

39. Gooding JM, Shayeghi M, Saggerson ED. Membrane transport of fatty acylcarnitine and free L-carnitine by rat liver microsomes. Eur J Biochem. 2004;271(5):954-61.

40. Houten SM, Wanders RJA, Ranea-robles P, Sciences G, Diseases M, Gastroenterology A. HHS Public Access. 2021;1-15.

41. Cho. 乳鼠心肌提取 HHS Public Access. Physiol Behav. 2016;176(1):100–106.

42. Chen Q, Wang T, Li J, Wang S, Qiu F, Yu H, et al. Effects of natural products on fructose-induced nonalcoholic fatty liver disease (NAFLD). Nutrients. 2017;9(2).

43. Tang K, Deng Y, Zheng C, Nie H, Pan M, Chen R, et al. Prevention of Nonalcoholic Hepatic Steatosis by Shenling Baizhu Powder: Involvement of Adiponectin-Induced Inhibition of Hepatic SREBP-1c. Oxid Med Cell Longev. 2020;2020.

44. Filadi R, Pendin Di, Pizzo P. Mitofusin 2: From functions to disease. Cell Death Dis. 2018;9(3).

45. Chandhok G, Lazarou M, Neumann B. Structure, function, and regulation of mitofusin-2 in health and disease. Biol Rev. 2018;93(2):933-49.

46. Wager MGT and JFS. 基因的改变NIH Public Access. Bone. 2011;23(1):1-7.

47. Yamada T, Dawson TM, Yanagawa T, Iijima M, Sesaki H. SQSTM1/p62 promotes mitochondrial ubiquitination independently of PINK1 and PRKN/parkin in mitophagy. Autophagy. 2019;15(11):2012-8.

Supplementary material

Supplementary Table S1. Internal standards and final concentration used in lipid extraction.

Internal Standard	*Final concentration (mM)*
Acetylcarnitine-d3	0.25
Butyrylcarnitine-d3	0.5
Free carnitine-d9	1
Isovalerylcarnitine-d9	0.5
Myristoylcarnitine-d9	0.5
Octanoylcarnitine-d3	0.5
Palmitoylcarnitine-d3	0.1
Propionylcarnitine-d3	0.5

Supplementary Table S2. Carnitines concentration in plasma and p-value of differents groups.

	NASH	*Uncertain*	*Non-NASH*	*P-Value (NASH-Non-NASH*	*P-Value (NASH-Uncertain)*	*P-Value (Non-NASH-Uncertain)*
Valeryl-carnitine (CAR 5:0)	$3.3\cdot10^{-8}$($2.7\cdot10^{-8}$-$4.3\cdot10^{-8}$)	$4.1\cdot10^{-8}$($3.5\cdot10^{-8}$-$5.2\cdot10^{-8}$)	$3.2\cdot10^{-8}$($2.6\cdot10^{-8}$-$4.3\cdot10^{-8}$)	$8.93\cdot10^{-01}$	$2.91\cdot10^{-02}$	$4.27\cdot10^{-02}$
Acetyl-carnitine (CAR 2:0)	$2.0\cdot10^{-5}$($1.6\cdot10^{-5}$-$2.5\cdot10^{-5}$)	$2.4\cdot10^{-5}$($2.1\cdot10^{-5}$-$2.7\cdot10^{-5}$)	$2.2\cdot10^{-5}$($1.5\cdot10^{-5}$-$2.7\cdot10^{-5}$)	$2.63\cdot10^{-01}$	$9.36\cdot10^{-02}$	$4.83\cdot10^{-01}$
Decanoyl-carnitine (CAR 10:0)	$6.0\cdot10^{-8}$($5.2\cdot10^{-8}$-$7.4\cdot10^{-8}$)	$8.2\cdot10^{-8}$($5.7\cdot10^{-8}$-$9.2\cdot10^{-8}$)	$6.9\cdot10^{-8}$($4.5\cdot10^{-8}$-$9.6\cdot10^{-8}$)	$4.10\cdot10^{-01}$	$1.30\cdot10^{-01}$	$3.97\cdot10^{-01}$
Decenoyl-carnitine (CAR 10:1)	$3.0\cdot10^{-8}$($2.6\cdot10^{-8}$-$3.8\cdot10^{-8}$)	$3.0\cdot10^{-8}$(2.4·10-8-$3.4\cdot10^{-8}$)	$2.8\cdot10^{-8}$($2.3\cdot10^{-8}$-$4.1\cdot10^{-8}$)	$5.53\cdot10^{-01}$	$5.72\cdot10^{-01}$	$9.84\cdot10^{-01}$
Dodecanoyl-carnitine (CAR 12:0)	$1.9\cdot10^{-8}$($1.4\cdot10^{-8}$-$2.3\cdot10^{-8}$)	$2.4\cdot10^{-8}$($1.4\cdot10^{-8}$-$2.7\cdot10^{-8}$)	$2.1\cdot10^{-8}$($1.5\cdot10^{-8}$-$2.8\cdot10^{-8}$)	$1.77\cdot10^{-01}$	$2.93\cdot10^{-01}$	$9.07\cdot10^{-01}$
Dodecenoyl-carnitine (CAR 12:1)	$1.5\cdot10^{-8}$($1.1\cdot10^{-8}$-$2.1\cdot10^{-8}$)	$2.0\cdot10^{-8}$($1.1\cdot10^{-8}$-$2.0\cdot10^{-8}$)	$1.8\cdot10^{-8}$($1.1\cdot10^{-8}$-$2.6\cdot10^{-8}$)	$7.93\cdot10^{-02}$	$4.67\cdot10^{-01}$	$7.19\cdot10^{-01}$
Free carnitine (CAR)	$2.9\cdot10^{-5}$($2.6\cdot10^{-5}$-$3.4\cdot10^{-5}$)	$4.1\cdot10^{-5}$($3.2\cdot10^{-5}$-$4.3\cdot10^{-5}$)	$3.2\cdot10^{-5}$(2.6·10-5-$3.9\cdot10^{-5}$)	$1.29\cdot10^{-01}$	$9.71\cdot10^{-03}$	$1.29\cdot10^{-01}$
Hexadecadienyl-carnitine (CAR 16:2)	$4.4\cdot10^{-9}$($3.0\cdot10^{-9}$-$5.8\cdot10^{-9}$)	$6.0\cdot10^{-9}$($4.7\cdot10^{-9}$-$7.2\cdot10^{-9}$)	$6.5\cdot10^{-9}$($3.8\cdot10^{-9}$-$8.4\cdot10^{-9}$)	$3.91\cdot10^{-03}$	$3.39\cdot10^{-02}$	$7.55\cdot10^{-01}$

Hexadecanoyl-carnitine (CAR 16:0)	$3.8\cdot10^{-8}$($3.2\cdot10^{-8}$-$4.3\cdot10^{-8}$)	$4.1\cdot10^{-8}$($3.6\cdot10^{-8}$-$4.3\cdot10^{-8}$)	$3.7\cdot10^{-8}$($3.2\cdot10^{-8}$-$4.4\cdot10^{-8}$)	$5.81\cdot10^{-01}$	$2.03\cdot10^{-01}$	$3.50\cdot10^{-01}$
Hexadecenyl-carnitine (CAR 16:1)	$9.1\cdot10^{-9}$($7.3\cdot10^{-9}$-$1.3\cdot10^{-8}$)	$1.0\cdot10^{-8}$($7.0\cdot10^{-9}$-$1.3\cdot10^{-8}$)	$1.0\cdot10^{-8}$($8.7\cdot10^{-9}$-$1.4\cdot10^{-8}$)	$1.40\cdot10^{-01}$	$5.05\cdot10^{-01}$	$8.30\cdot10^{-01}$
Hexanoyl-carnitine (CAR 6:0)	$1.7\cdot10^{-8}$($1.5\cdot10^{-8}$-$2.3\cdot10^{-8}$)	$2.4\cdot10^{-8}$($1.6\cdot10^{-8}$-$2.6\cdot10^{-8}$)	$2.0\cdot10^{-8}$($1.6\cdot10^{-8}$-$2.4\cdot10^{-8}$)	$6.84\cdot10^{-01}$	$1.25\cdot10^{-01}$	$1.39\cdot10^{-01}$
Butyryl-carnitine (CAR 4:0)	$9.5\cdot10^{-8}$($6.9\cdot10^{-8}$-$1.2\cdot10^{-7}$)	$1.2\cdot10^{-7}$($9.0\cdot10^{-8}$-$1.6\cdot10^{-7}$)	$8.9\cdot10^{-8}$($6.8\cdot10^{-8}$-$1.1\cdot10^{-7}$)	$5.93\cdot10^{-01}$	$1.10\cdot10^{-01}$	$8.65\cdot10^{-02}$
Succinyl-carnitine (CAR 4:1;O2)	$6.0\cdot10^{-7}$($4.2\cdot10^{-7}$-$7.6\cdot10^{-7}$)	$4.1\cdot10^{-7}$($3.3\cdot10^{-7}$-$9.0\cdot10^{-7}$)	$4.2\cdot10^{-7}$($3.0\cdot10^{-7}$-$5.4\cdot10^{-7}$)	$1.06\cdot10^{-03}$	$3.12\cdot10^{-01}$	$4.59\cdot10^{-01}$
Adipoyl-carnitine (CAR 6:1;O2)	$1.7\cdot10^{-8}$($1.4\cdot10^{-8}$-$2.3\cdot10^{-8}$)	$2.1\cdot10^{-8}$($2.0\cdot10^{-8}$-$2.2\cdot10^{-8}$)	$1.8\cdot10^{-8}$($1.1\cdot10^{-8}$-$2.6\cdot10^{-8}$)	$8.07\cdot10^{-01}$	$8.23\cdot10^{-02}$	$1.10\cdot10^{-01}$
Nonanoyl-carnitine (CAR 9:0)	$1.9\cdot10^{-9}$($1.4\cdot10^{-9}$-$2.6\cdot10^{-9}$)	$2.6\cdot10^{-9}$($1.8\cdot10^{-9}$-$5.1\cdot10^{-9}$)	$2.3\cdot10^{-9}$($1.7\cdot10^{-9}$-$2.9\cdot10^{-9}$)	$7.81\cdot10^{-02}$	$4.33\cdot10^{-02}$	$3.50\cdot10^{-01}$
Octadecadienyl-carnitine (CAR 18:2)	$8.7\cdot10^{-9}$($6.4\cdot10^{-9}$-$1.1\cdot10^{-8}$)	$1.4\cdot10^{-8}$($8.5\cdot10^{-9}$-$1.9\cdot10^{-8}$)	$9.8\cdot10^{-9}$($6.6\cdot10^{-9}$-$1.2\cdot10^{-8}$)	$2.26\cdot10^{-01}$	$8.38\cdot10^{-03}$	$1.49\cdot10^{-02}$
Octadecanoyl-carnitine (CAR 18:0)	$8.3\cdot10^{-9}$($7.0\cdot10^{-9}$-$1.1\cdot10^{-8}$)	$1.2\cdot10^{-8}$($9.8\cdot10^{-9}$-$1.4\cdot10^{-8}$)	$9.3\cdot10^{-9}$($7.8\cdot10^{-9}$-$1.2\cdot10^{-8}$)	$6.51\cdot10^{-02}$	$1.37\cdot10^{-02}$	$6.42\cdot10^{-02}$
Octadecenyl-carnitine (CAR 18:1)	$9.5\cdot10^{-8}$($7.9\cdot10^{-8}$-$1.1\cdot10^{-7}$)	$9.9\cdot10^{-8}$($9.6\cdot10^{-8}$-$1.4\cdot10^{-7}$)	$1.0\cdot10^{-7}$($8.9\cdot10^{-8}$-$1.2\cdot10^{-7}$)	$4.67\cdot10^{-02}$	$1.15\cdot10^{-01}$	$7.41\cdot10^{-01}$
Octanoyl-carnitine (CAR 8:0)	$3.8\cdot10^{-8}$($3.0\cdot10^{-8}$-$4.8\cdot10^{-8}$)	$4.7\cdot10^{-8}$($3.4\cdot10^{-8}$-$5.7\cdot10^{-8}$)	$3.9\cdot10^{-8}$($2.6\cdot10^{-8}$-$5.7\cdot10^{-8}$)	$6.52\cdot10^{-01}$	$2.03\cdot10^{-01}$	$4.71\cdot10^{-01}$
Octenoyl-carnitine (CAR 8:1)	$2.1\cdot10^{-8}$($1.5\cdot10^{-8}$-$2.9\cdot10^{-8}$)	$2.1\cdot10^{-8}$($1.3\cdot10^{-8}$-$4.0\cdot10^{-8}$)	$1.7\cdot10^{-8}$($1.3\cdot10^{-8}$-$2.7\cdot10^{-8}$)	$9.59\cdot10^{-02}$	$8.40\cdot10^{-01}$	$3.30\cdot10^{-01}$
Propionyl-carnitine (CAR 3:0)	$2.8\cdot10^{-7}$($2.3\cdot10^{-7}$-$3.6\cdot10^{-7}$)	$3.1\cdot10^{-7}$($2.9\cdot10^{-7}$-$4.2\cdot10^{-7}$)	$2.6\cdot10^{-7}$($2.1\cdot10^{-7}$-$3.3\cdot10^{-7}$)	$3.19\cdot10^{-01}$	$7.88\cdot10^{-02}$	$3.71\cdot10^{-02}$
Tetradecadienyl-carnitine (CAR 14:2)	$1.5\cdot10^{-8}$($1.1\cdot10^{-8}$-$2.1\cdot10^{-8}$)	$1.9\cdot10^{-8}$($1.4\cdot10^{-8}$-$2.4\cdot10^{-8}$)	$2.1\cdot10^{-8}$($1.4\cdot10^{-8}$-$2.9\cdot10^{-8}$)	$1.98\cdot10^{-03}$	$1.06\cdot10^{-01}$	$5.20\cdot10^{-01}$
Tetradecanoyl-carnitine (CAR 14:0)	$6.4\cdot10^{-9}$($5.6\cdot10^{-9}$-$8.0\cdot10^{-9}$)	$6.5\cdot10^{-9}$($5.0\cdot10^{-9}$-$8.6\cdot10^{-9}$)	$7.0\cdot10^{-9}$($5.7\cdot10^{-9}$-$8.7\cdot10^{-9}$)	$3.41\cdot10^{-01}$	$9.84\cdot10^{-01}$	$6.13\cdot10^{-01}$
Tetradecenoyl-carnitine (CAR 14:1)	$4.2\cdot10^{-8}$($2.8\cdot10^{-8}$-$5.4\cdot10^{-8}$)	$4.7\cdot10^{-8}$($3.3\cdot10^{-8}$-$6.0\cdot10^{-8}$)	$5.1\cdot10^{-8}$($3.6\cdot10^{-8}$-$6.5\cdot10^{-8}$)	$1.77\cdot10^{-02}$	$3.63\cdot10^{-01}$	$5.85\cdot10^{-01}$
Tiglyl-carnitine (CAR 5:1)	$4.8\cdot10^{-9}$($3.5\cdot10^{-9}$-$6.5\cdot10^{-9}$)	$6.0\cdot10^{-9}$($5.1\cdot10^{-9}$-$8.2\cdot10^{-9}$)	$5.6\cdot10^{-9}$($3.8\cdot10^{-9}$-$7.2\cdot10^{-9}$)	$1.14\cdot10^{-01}$	$1.62\cdot10^{-02}$	$2.42\cdot10^{-01}$

Tridecanoyl-carnitine (CAR 13:0)	$6.7\cdot10^{-9}$($4.8\cdot10^{-9}$-$8.8\cdot10^{-9}$)	$1.5\cdot10^{-8}$($8.9\cdot10^{-9}$-$1.9\cdot10^{-8}$)	$8.0\cdot10^{-9}$($6.3\cdot10^{-9}$-$1.1\cdot10^{-8}$)	$1.40\cdot10^{-02}$	$1.71\cdot10^{-04}$	$6.77\cdot10^{-03}$
Glutaconyl-carnitine (CAR 5:2;O2)	$2.1\cdot10^{-9}$($1.8\cdot10^{-9}$-$2.9\cdot10^{-9}$)	$3.7\cdot10^{-9}$($2.8\cdot10^{-9}$-$4.3\cdot10^{-9}$)	$2.6\cdot10^{-9}$($2.0\cdot10^{-9}$-$3.3\cdot10^{-9}$)	$5.39\cdot10^{-02}$	$1.99\cdot10^{-03}$	$2.44\cdot10^{-02}$

Supplementary Table S3. Concentration of carnitines in liver and p-value of differents groups

	NASH	*Uncertain*	*Non-NASH*	*P-Value (NASH-Non-NASH*	*P-Value (NASH-Uncertain)*	*P-Value (Non-NASH-Uncertain)*
Valeryl-carnitine (CAR 5:0)	$2.4\cdot10^{-2}$($1.5\cdot10^{-2}$-$3.1\cdot10^{-2}$)	$8.3\cdot10^{-2}$($5.7\cdot10^{-2}$-$9.6\cdot10^{-2}$)	$4.2\cdot10^{-2}$($3.0\cdot10^{-2}$-$5.9\cdot10^{-2}$)	$4.04\cdot10^{-06}$	$6.88\cdot10^{-05}$	$3.06\cdot10^{-02}$
Acetyl-carnitine (CAR 2:0)	$1.9\cdot10^{+0}$($7.9\cdot10^{-1}$-$2.5\cdot10^{+0}$)	$5.3\cdot10^{+0}$($2.7\cdot10^{+0}$-$1.0\cdot10^{+1}$)	$3.4\cdot10^{+0}$($1.7\cdot10^{+0}$-$5.3\cdot10^{+0}$)	$4.09\cdot10^{-04}$	$2.98\cdot10^{-04}$	$8.65\cdot10^{-02}$
Decanoyl-carnitine (CAR 10:0)	$2.7\cdot10^{-4}$($1.6\cdot10^{-4}$-$5.9\cdot10^{-4}$)	$1.9\cdot10^{-3}$($7.8\cdot10^{-4}$-$6.1\cdot10^{-3}$)	$4.9\cdot10^{-4}$($2.2\cdot10^{-4}$-$8.4\cdot10^{-4}$)	$2.35\cdot10^{-02}$	$2.63\cdot10^{-05}$	$5.25\cdot10^{-04}$
Decenoyl-carnitine (CAR 10:1)	$7.1\cdot10^{-4}$($4.0\cdot10^{-4}$-$1.2\cdot10^{-3}$)	$1.7\cdot10^{-3}$($1.0\cdot10^{-3}$-$3.6\cdot10^{-3}$)	$1.3\cdot10^{-3}$($7.4\cdot10^{-4}$-$2.2\cdot10^{-3}$)	$3.89\cdot10^{-04}$	$2.06\cdot10^{-03}$	$2.59\cdot10^{-01}$
Dodecanoyl-carnitine (CAR 12:0)	$6.1\cdot10^{-4}$($3.2\cdot10^{-4}$-$1.4\cdot10^{-3}$)	$2.2\cdot10^{-3}$($1.1\cdot10^{-3}$-$3.4\cdot10^{-3}$)	$1.4\cdot10^{-3}$($7.1\cdot10^{-4}$-$3.2\cdot10^{-3}$)	$6.67\cdot10^{-04}$	$5.11\cdot10^{-04}$	$2.12\cdot10^{-01}$
Dodecenoyl-carnitine (CAR 12:1)	$3.0\cdot10^{-4}$($1.2\cdot10^{-4}$-$4.7\cdot10^{-4}$)	$1.0\cdot10^{-3}$($6.2\cdot10^{-4}$-$1.7\cdot10^{-3}$)	$6.4\cdot10^{-4}$($3.1\cdot10^{-4}$-$1.0\cdot10^{-3}$)	$3.15\cdot10^{-04}$	$2.45\cdot10^{-04}$	$8.12\cdot10^{-02}$
Free carnitine (CAR)	$1.0\cdot10^{+1}$($6.9\cdot10^{+0}$-$1.3\cdot10^{+1}$)	$1.1\cdot10^{+1}$($9.9\cdot10^{+0}$-$1.7\cdot10^{+1}$)	$1.4\cdot10^{+1}$($1.2\cdot10^{+1}$-$2.2\cdot10^{+1}$)	$1.77\cdot10^{-05}$	$1.51\cdot10^{-01}$	$1.34\cdot10^{-01}$
Hexadecanoyl-carnitine (CAR 16:0)	$1.7\cdot10^{-2}$($8.1\cdot10^{-3}$-$3.9\cdot10^{-2}$)	$6.5\cdot10^{-2}$($4.9\cdot10^{-2}$-$9.1\cdot10^{-2}$)	$2.5\cdot10^{-2}$($1.5\cdot10^{-2}$-$3.7\cdot10^{-2}$)	$5.66\cdot10^{-02}$	$3.77\cdot10^{-04}$	$1.14\cdot10^{-03}$
Hexadecenyl-carnitine (CAR 16:1)	$4.6\cdot10^{-3}$($2.3\cdot10^{-3}$-$8.2\cdot10^{-3}$)	$1.8\cdot10^{-2}$($1.0\cdot10^{-2}$-$2.5\cdot10^{-2}$)	$6.8\cdot10^{-3}$($2.7\cdot10^{-3}$-$1.3\cdot10^{-2}$)	$9.18\cdot10^{-02}$	$6.39\cdot10^{-04}$	$8.06\cdot10^{-03}$
Hexanoyl-carnitine (CAR 6:0)	$5.6\cdot10^{-3}$($3.9\cdot10^{-3}$-$1.1\cdot10^{-2}$)	$1.2\cdot10^{-2}$($5.8\cdot10^{-3}$-$1.5\cdot10^{-2}$)	$1.2\cdot10^{-2}$($5.6\cdot10^{-3}$-$2.3\cdot10^{-2}$)	$1.89\cdot10^{-03}$	$9.36\cdot10^{-02}$	$6.26\cdot10^{-01}$
Butyryl-carnitine (CAR 4:0)	$3.3\cdot10^{-2}$($1.9\cdot10^{-2}$-$5.6\cdot10^{-2}$)	$1.2\cdot10^{-1}$($8.4\cdot10^{-2}$-$1.5\cdot10^{-1}$)	$5.7\cdot10^{-2}$($2.9\cdot10^{-2}$-$7.6\cdot10^{-2}$)	$7.54\cdot10^{-03}$	$1.68\cdot10^{-05}$	$6.99\cdot10^{-04}$
Succinyl-carnitine (CAR 4:1;O2)	$3.7\cdot10^{-1}$($1.8\cdot10^{-1}$-$5.3\cdot10^{-1}$)	$3.8\cdot10^{-1}$($3.0\cdot10^{-1}$-$4.8\cdot10^{-1}$)	$5.5\cdot10^{-1}$(3.3·10^-1-$8.6\cdot10^{-1}$)	$2.83\cdot10^{-04}$	$5.99\cdot10^{-01}$	$6.85\cdot10^{-02}$
Adipoyl-carnitine (CAR 6:1;O2)	2.2·10-2(1.5·10-2-3.2·10-2)	$3.0\cdot10^{-2}$($2.3\cdot10^{-2}$-$8.2\cdot10^{-2}$)	$5.0\cdot10^{-2}$($3.3\cdot10^{-2}$-$7.4\cdot10^{-2}$)	$8.28\cdot10^{-08}$	$3.06\cdot10^{-02}$	$2.84\cdot10^{-01}$
Nonanoyl-carnitine (CAR 9:0)	2.1·10-4(1.3·10-4-3.4·10-4)	$3.9\cdot10^{-4}$($2.6\cdot10^{-4}$-$8.7\cdot10^{-4}$)	$3.6\cdot10^{-4}$(1.7·10^-4-$5.8\cdot10^{-4}$)	$1.98\cdot10^{-03}$	$7.65\cdot10^{-03}$	$3.70\cdot10^{-01}$

Octadecadienyl-carnitine (CAR 18:2)	$2.0\cdot10^{-2}$($1.2\cdot10^{-2}$-$3.2\cdot10^{-2}$)	$5.1\cdot10^{-2}$($3.2\cdot10^{-2}$-$6.7\cdot10^{-2}$)	$2.5\cdot10^{-2}$($1.7\cdot10^{-2}$-$3.9\cdot10^{-2}$)	$1.25\cdot10^{-01}$	$9.22\cdot10^{-04}$	$5.34\cdot10^{-03}$
Octadecanoyl-carnitine (CAR 18:0)	$4.5\cdot10^{-3}$($2.7\cdot10^{-3}$-$7.4\cdot10^{-3}$)	$2.4\cdot10^{-2}$($1.7\cdot10^{-2}$-$3.2\cdot10^{-2}$)	$9.6\cdot10^{-3}$($5.6\cdot10^{-3}$-$1.4\cdot10^{-2}$)	$7.23\cdot10^{-05}$	$5.44\cdot10^{-06}$	$7.51\cdot10^{-04}$
Octadecenyl-carnitine (CAR 18:1)	$3.0\cdot10^{-2}$($1.6\cdot10^{-2}$-$5.8\cdot10^{-2}$)	$1.0\cdot10^{-1}$($6.5\cdot10^{-2}$-$1.4\cdot10^{-1}$)	$3.8\cdot10^{-2}$($1.9\cdot10^{-2}$-$5.8\cdot10^{-2}$)	$2.87\cdot10^{-01}$	$5.51\cdot10^{-04}$	$4.88\cdot10^{-04}$
Octanoyl-carnitine (CAR 8:0)	$3.5\cdot10^{-4}$($1.4\cdot10^{-4}$-$7.6\cdot10^{-4}$)	$2.5\cdot10^{-3}$($1.6\cdot10^{-3}$-$1.2\cdot10^{-2}$)	$4.2\cdot10^{-4}$($1.7\cdot10^{-4}$-$7.6\cdot10^{-4}$)	$4.71\cdot10^{-01}$	$6.30\cdot10^{-05}$	$6.50\cdot10^{-05}$
Octenoyl-carnitine (CAR 8:1)	$4.4\cdot10^{-3}$($2.9\cdot10^{-3}$-$6.0\cdot10^{-3}$)	$9.8\cdot10^{-3}$($5.6\cdot10^{-3}$-$1.8\cdot10^{-2}$)	$8.5\cdot10^{-3}$($3.5\cdot10^{-3}$-$1.3\cdot10^{-2}$)	$1.07\cdot10^{-03}$	$2.60\cdot10^{-03}$	$3.60\cdot10^{-01}$
Propionyl-carnitine (CAR 3:0)	$4.3\cdot10^{-1}$($2.4\cdot10^{-1}$-$9.4\cdot10^{-1}$)	1.1·10+0($6.4\cdot10^{-1}$-$1.9\cdot10^{+0}$)	$1.3\cdot10^{+0}$($5.7\cdot10^{-1}$-$2.0\cdot10^{+0}$)	$2.68\cdot10^{-04}$	$4.12\cdot10^{-03}$	$7.55\cdot10^{-01}$
Tetradecadienyl-carnitine (CAR 14:2)	$8.3\cdot10^{-4}$($3.1\cdot10^{-4}$-$2.1\cdot10^{-3}$)	$5.3\cdot10^{-3}$($3.0\cdot10^{-3}$-$8.5\cdot10^{-3}$)	$2.8\cdot10^{-3}$($1.2\cdot10^{-3}$-$5.4\cdot10^{-3}$)	$4.27\cdot10^{-05}$	$1.86\cdot10^{-04}$	9.77·10-02
Tetradecanoyl-carnitine (CAR 14:0)	$3.1\cdot10^{-3}$($1.4\cdot10^{-3}$-$6.8\cdot10^{-3}$)	$9.3\cdot10^{-3}$($5.6\cdot10^{-3}$-$1.1\cdot10^{-2}$)	$5.8\cdot10^{-3}$($2.9\cdot10^{-3}$-$9.7\cdot10^{-3}$)	$1.51\cdot10^{-02}$	$5.30\cdot10^{-03}$	$1.49\cdot10^{-01}$
Tetradecenoyl-carnitine (CAR 14:1)	$4.2\cdot10^{-3}$($1.7\cdot10^{-3}$-$7.0\cdot10^{-3}$)	$1.5\cdot10^{-2}$($9.6\cdot10^{-3}$-$2.3\cdot10^{-2}$)	$9.1\cdot10^{-3}$($5.0\cdot10^{-3}$-$2.2\cdot10^{-2}$)	$6.18\cdot10^{-04}$	$1.51\cdot10^{-03}$	$2.12\cdot10^{-01}$
Tiglyl-carnitine (CAR 5:1)	$1.4\cdot10^{-3}$($8.8\cdot10^{-4}$-$2.6\cdot10^{-3}$)	$2.8\cdot10^{-3}$($1.9\cdot10^{-3}$-$3.0\cdot10^{-3}$)	$2.7\cdot10^{-3}$($2.0\cdot10^{-3}$-$4.3\cdot10^{-3}$)	$4.02\cdot10^{-05}$	$1.91\cdot10^{-02}$	$3.50\cdot10^{-01}$
Tridecanoyl-carnitine (CAR 13:0)	$7.8\cdot10^{-4}$($4.5\cdot10^{-4}$-$1.2\cdot10^{-3}$)	$1.6\cdot10^{-3}$($1.2\cdot10^{-3}$-$3.7\cdot10^{-3}$)	$1.6\cdot10^{-3}$($8.0\cdot10^{-4}$-$3.1\cdot10^{-3}$)	$2.16\cdot10^{-04}$	$4.05\cdot10^{-04}$	$4.83\cdot10^{-01}$