The role of PNPLA3 protein expression in obesity-associated nonalcoholic fatty liver disease (NAFLD)

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Biotechnology

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CONTENT

1. ABSTRACT

Background: NAFLD is the most prevalent type of chronic liver disease in the human history (25%). It can progress to nonalcoholic steatohepatitis (NASH) characterized by fibrosis; and cirrhosis or hepatocarcinoma, which requires a liver transplantation. It is indispensable a liver biopsy for the diagnosis of NAFLD/NASH progression, and there is not any accepted drug therapy. One of the protein codificant genes related to NAFLD is PNPLA3 (patatin-like phospholipase domain-containing 3), an enzyme involved in the lipid metabolism. The expression of this protein is associated to NAFLD, its accumulation results in a larger lipid droplet size, characteristic of NASH steatosis. There is a need to find new targets to stop NAFLD progress, and PNPLA3 could be one of them.

Objective: to compare protein expression of PNPLA3 in liver, SAT, and VAT samples of patients with severe obesity and with or without NASH.

Methods: liver, SAT and VAT samples from n=98 patients with morbid obesity candidates to receive bariatric surgery. During the procedure, tissue biopsies were performed, from which we performed histological analyses, protein extraction and western blot.

Results: we found significative differences in PNPLA3 protein expression in the liver samples of NASH patients compared to those without NASH. Also, patients with NASH showed a more prominent expression around the fibrotic areas. Adipose tissue levels of PNPLA3 protein were not significantly different between those groups, nor the distribution among the tissue. Protein expression of PNPLA3 correlated with some biochemical features, such as triglycerides and transaminases, in all three tissues. **Conclusions:** PNPLA3 protein expression was significantly increased in the hepatic tissue of patients with NASH and was present in higher quantities around areas of fibrosis. In contrast, no significant differences in PNPLA3 protein expression or localization were observed in adipose tissue between NASH and non-NASH patients.

Keywords: NAFLD, PNPLA3, obesity, inflammation

2. ACRONYMS

ABDH5: α/β-hydrolase domain-containing 5
ACE: angiotensin-converting enzyme
AINES: non-steroidal anti-inflammatory drugs
ApoB: apolipoprotein B
ARH2: histamine H2 receptor inhibitor
ATGL: adipose triglyceride lipase
CCL2,5: C-C motif chemokine ligand 2,5
CGI-58: comparative gene identification-58
ChREBP: Carbohydrate responsive-element binding protein
CKD: chronic kidney disease
CS4: ceramide synthase 4
CVD: cardiovascular disease
DAG: diacylglicerid
DMSO: dimethyl sulfoxide
EDTA: ethylenediaminetetraacetic acid
GCKR: glucokinase regulator
GLP1: glucagon-like peptide-1
HDL: high density lipoprotein
HMGCR: 3-hydroxy-3-methyl-glutaryl-CoA reductase
HSC: hepatic stellate cells
HSD17B13: hydroxysteroid 17-β dehydrogenase 13
IREα: iron responsive element alpha
LDL: low density lipoprotein
LXR: liver receptor X
LC3-I: protein 1 light chain 3
LC3-II: protein 2 light chain 3
LYPLAL1: lysophospholipase 1
MAG: monoacylglicerid
MUFA: monounsaturated fatty acid
$MgCl_2$: magnesium chloride
NAFLD : Non-alcoholic fatty liver disease
NASH: Non-alcoholic steatohepatitis
NEFA: non-esterified fatty acid
PDAC: pancreatic ductal adenocarcinoma
PERK: protein R-like reticle endoplasmatic kinase
Pik3c3: Phosphatidylinositol 3-Kinase Catalytic Subunit Type 3
p-JNK: protein and c-jun NH2-terminal kinase
PLIN1: perilipin 1
PNPLA3: patatin-like phospholipase domain-containing 3
PPP1R3B: protein phosphatase 1 regulatory subunit 3B
PtdIns3K: class III phosphatidylinositol 3-kinase
PtdIns3P: phosphatidylinositol 3-phosphate
PUFA: polyunsaturated fatty acid
ROS: reactive oxygen species
RT-PCR: reverse transcription polymerase chain reaction
RXR: retinoic receptor X
SAT: subcutaneous adipose tissue
SBREP-1c: Sterol Regulatory Element Binding Protein-1c
SGLT2: sodium-glucose cotransporter type 2
SIRT1: sirtuin 1
SNP: single nucleotide polymorphism
TAG: triacylglicerid
T2DM: type 2 diabetes Mellitus
TFG-β: tumor growth factor β
TM6SF2: transmembrane 6 superfamily member 2
ULK1: unc-51 like autophagy activating kinase 1
VAT: visceral adipose tissue
VLDL: very low density lipoprotein

3. INTRODUCTION

3.1. Obesity-associated nonalcoholic fatty liver disease: definition and prevalence.

Nonalcoholic fatty liver disease (NAFLD) occurs when there are more of 5% of hepatocytes with fat accumulation with no contribution of alcohol or viral hepatitis. NAFLD can progress from simple fat accumulation (steatosis) to necro-inflammatory forms marked by hepatocyte ballooning and inflammation. This state is defined as nonalcoholic steatohepatitis (NASH) and is the lobby for fibrosis, and ultimately cirrhosis or hepatocarcinoma, which require a liver transplantation (Cotter, 2020; Herring, 2022, Yeh, 2014). For the diagnosis is still mandatory a liver biopsy, which is an expensive and not completely safe procedure. Also, NAFLD remains without accepted drug therapy (Aravalli, 2008; Demaria, 2010; Mantovani, 2008; Eslam, 2019; Anstee, 2020).

NAFLD and NASH are some of many diseases related to obesity. Obesity is a chronic disease characterized by abnormal or excessive adipose tissue or body fat accumulation. The fundamental cause of obesity is a long-term energy imbalance, increased by external factors such as a sedentary lifestyle or hypercaloric diet and, in a few cases, internal factors such as genetics (Blüher, 2019). The body mass index (BMI) is the ratio of a person's weight to height. This measure is used to diagnose and classify obesity: BMI values of 30 to 35 kg/m^2 are characteristic of type I obesity, values between 35 to 40 kg/m^2 refer to type II obesity, and BMI greater than 40 kg/m^2 defines type III, severe or morbid obesity (Yang, 2022). Progression of NAFLD to NASH, and finally to hepatocarcinoma, is more plausible with clinically severe obesity.

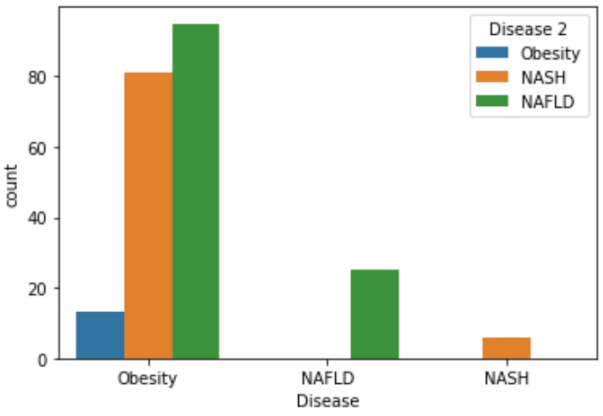

Figure 1. Prevalence of obesity, NAFLD and NASH. In count is represented the percentage of prevalence.

NAFLD is the most common type of chronic liver disease which affects more than 25% of the world's population, being the most prevalent liver diseases in human history (Yang, 2022). In the case of NASH, its worldwide prevalence is 1.5-6.45% (Armandi, 2021). On the other hand, obesity affects about 13% of the world population (World Obesity Atlas 2022). Furthermore, the worldwide prevalence of NAFLD and NASH is increased in obese population (60-95% and 81%, respectively) (Montaño-Loza, 2022).

3.2. The metabolic dysfunction in obesity and NAFLD

The hallmark of NAFLD is the accumulation of neutral lipids, typically triacylglycerides, in the lipid droplet. This results in fat accumulation in the liver (Micheloti, 2021). Obesity has many factors that contribute to NAFLD and NASH development such as dysfunction of subcutaneous white adipose tissues (scWAT), insulin resistence, inflammation, and dysbiosis of gut microbiota (Yang, 2022).

In fasting, glucose is obtained by glycogenolysis or gluconeogenesis. In the postprandial phase, the insulin concentration increases, and it inactivates them. The excess of carbohydrates from the diet can follow two pathways: the storage as glycogen, glycolysis, or the participation in lipogenesis to generate fatty acids. Insulin concentration also decreases adipose tissue lipolysis and the non-esterified fatty acid (NEFA) flux to the liver. In addition, it upregulates lipoprotein lipase, which hydrolyzes chylomicrons, and the fatty acids released are directed to storage. Some fatty acids do not go to storage and form the systemic NEFA pool, i.e., spillover NEFA. Thus, plasma NEFA comprises fatty acids from adipose tissue lipolysis and chylomicron-derived spillover in the postprandial state. Then, NEFA is transported to the liver. These fatty acids can be involved in oxidative pathways such as the Krebs cycle or suffer esterification to generate triacylglycerides (TAG). Then, TAG could be stored in cytosolic lipid droplet or secreted to VLDL (Piernatonelli, 2019; Sanchez-Torrijos, 2020)

When insulin and glucose levels are frequently elevated, insulin resistance is promoted. This condition is often observed in patients with obesity and NAFLD in both fasting and postprandial states, but especially in the latter one. This results in a dysregulation of glucose and lipid homeostasis.

The percentage of adiposity influences the mobilization of FA to the liver. Thus, an increase of plasma NEFA by an abnormal fatty acid mobilization is suggested to be related to obesity and NAFLD. Moreover, it was observed a downregulation of fatty acid trafficking genes in obese adipose tissue and a lower spillover of chylomicron-derived fatty acids in obese people. These evidences support the hypothesis in which insulin resistance is responsible of the increment of plasma NEFA in obese population.

TAG-rich lipoproteins, VLDL and chylomicrons represent the majority of plasma TAG concentration. The liver can export TAG by its incorporation into VLDL. It was demonstrated that fasting plasma TAG concentrations are higher in obesity and/or insulin

resistance or NAFLD. It is suggested that the cause is the reduction of its insulin regulation, which decreases the availability of ApoB and the activation of LDL-receptor. (Nagarajan, 2022)

3.3. Obesity-associated NAFLD concomitant diseases

3.3.1. Insulin resistance and Type 2 Diabetes (T2D)

Insulin resistence results in excess of glucose and an increase in its circulating. Thus, it increases esterification pathways promoting lipid accumulation that can store in different organs and tissues, like adipose tissue (Nagarajan, 2022).

T2D is the most common type of diabetes and is mainly induced by insulin resistence. Obesity and insulin resistence promote pancreatic islet hypertrophy and both pancreatic α- and β-cell remodeling, disarray, and apoptosis contributing directly to T2D development (Yang, 2022).

3.3.2. Metabolic syndrome

The metabolic syndrome includes abdominal obesity, hypertension, dyslipidemia, and impaired glycemia. Its prevalence increases with obesity, and so as the prevalence and severity of NAFLD. The metabolic syndrome promotes advanced hepatic fibrosis, so it is related to NASH development. Moreover, NAFLD and metabolic syndrome increases the incidence of hepatocarcinoma. Also, it induces the insulin resistance , so it is strongly related to T2DM. (Godoy-Matos, 2020; Michelotti, 2021)

3.3.3. Chronic Kidney Disease (CKD)

Lipid overload increases intrarenal fat deposition, damage in glomerular filtration rate albuminuria, insulin resistence, fibrogenesis, and gut microbiota dysbiosis. Moreover, it causes lipotoxicity. This effect increases endoplasmatic reticulum stress, producing ROS and promoting local and systemic inflammatory effects. These effects contribute to CKD (Yang, 2022; Sandino, 2022).

3.3.4. Cardiovascular disease (CVD)

Obesity is associated with many factors contributing to CVD: dylsipemia, hypertension, insulin resistence vascular endothelium dysfunction, and sleep disorders. Moreover, the overexpression of lipoproteins (e.g. high-density lipoproteins (HDLs)) alters energy metabolism, leading to endothelial dysfunction and promoting the risk of CVD (Yang, 2022; Chartrand, 2022).

3.3.5. Cancer

Obesity also is related to the progression of breast cancer, hepatocarcinoma, and PDAC. It is associated with many factors contributing to cancer progression: insulin resistance, adipose inflammation, metabolic syndrome, gut microbiota dysbiosis, vascular epithelial cell dysfunction, and tumor-promoting growth factors (e.g., FGF1). Furthermore, lipid and lipoprotein accumulation (e.g., cholesterol) induces damage in anti-tumor inflammation (Yang, 2022).

3.4. The role of PNPLA3 in NAFLD and NASH

PNPLA3 (patatin-like phospholipase domain-containing 3) is one of the nine members of patatin like phospholipase domain-containing proteins (PNPLA1-9).

Its gen is located in 22q13.31 and is highly expressed in the liver, adipose tissue, and pancreas. PNPLA3 protein structure comprises one domain, 3 motifs (GXGXXG, GXSXG, DGA/G), and one transmembrane region. PNPLA3 is defined as a single-pass type II membrane protein, and its cell location is mainly in the endoplasmic reticulum and in the lipid droplet. It has a catalytic dyad (Ser47-Asp166), unlike other lipases that have a catalytic triad (Ser-His-Asp). Although there are many approximations of PNPLA3 protein total structure, some regions are yet to be confirmed (UniProt).

3.4.1. Enzymatic activity

PNPLA3 has several enzymatic activities involved in lipid metabolism (Table 1).

*Table 1. PNPLA3's enzymatic activity and its contribution based on **Uniprot** and **BRENDA** resources.*

Enzymatic activity	Metabolism	Function
1-acylglycerol-3-phosphate O-acyltransferase	Glycerolipid and glycerophospholipid	Generate phosphatidic acid contributing to the synthesis of triglycerides and glycerophospholipids
Triglyceride lipase	Glycerolipid	Hydrolyze tag, dag, or mag and liberate fatty acids. Typically, in lipid droplet remodeling (Bruschi, 2017)
CoA-independent acylglycerol transacylase	Glycerophospholipid	May be part of acyl-chain remodeling of triglycerides. Possible contribution in lipid droplet's monolayer (Mashek, 2021)
Phospholipase A2	Phospholipid	Remove fatty acids attached to the 2-carbon position of a phospholipid

3.4.2. Regulation of PNPLA3 expression

PNPLA3 is regulated by SREBP-1c and carbohydrate intake by insulin signaling. SBREP-1c (Sterol Regulatory Element Binding Protein-1c) is a transcription factor involved in lipid biosynthesis regulation from glucose in the liver. Its expression is common in the liver and adrenal glands.

Once carbohydrates have been ingested, insulin secretion increases. In normal conditions, insulin induces SCAP to bind to insulin induced gene (*INSIG*). *INSIG* codifies for an anchoring protein with the same name that maintains SBREP-1c in the endoplasmic reticulum. Through insulin signaling, *INSIG* is dissociated from SCAP, and permits the translocation of SBREP-1c/SCAP complex from endoplasmic reticulum to Golgi. In Golgi, the complex undergoes two sequential proteolytic cleavage steps through S1P and S2P, and the nuclear transcriptionally active SREBP-1c is developed. Although generally insulin regulates its expression, SREBP-1c could be activated under endoplasmic reticulum stress conditions too, through unknown cellular pathways. (Figure 2)

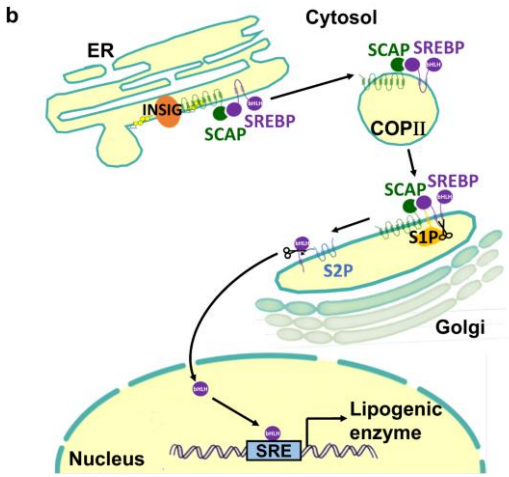

Figure 2. Regulation of SBREP-1c expression. COPII: coat protein complex II; SRE: sterol responsive element; ER: endoplasmic reticulum. (Adapted from Lee (2020)).

Then, the insulin secreted leads to the heterodimerization of LXR and RXR. The heterodimer binds to SREBP-1c, which activates the expression of PNPLA3, and the expression and activity of enzymes related to the synthesis of fatty acids such as desaturases or carboxylases. (Bruschi, 2017)

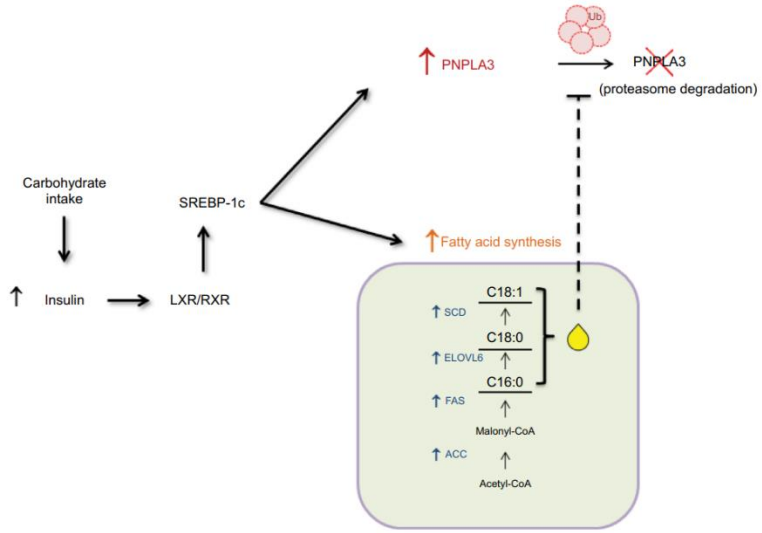

Figure 3. Regulation of PNPLA3's expression (Adapted from Bruschi, (2017)).

The scientific literature also mentions that ChREBP (Carbohydrate responsive-element binding protein) could be regulating PNPLA3 too. Carbohydrate response element binding protein (ChREBP) is a carbohydrate-signaling transcription factor that regulates glycolytic and lipogenic pathways. ChREBP expression is abundant in the liver and white and brown adipose tissues (Ortega-Prieto, 2019).

It has been observed that overexpression of ChREBP leads to increased PNPLA3 mRNA levels in mouse liver and isolated murine hepatocytes. However, this could not be replicated in hepatoma cells. In addition, it is silencing in human hepatocytes to no PNPLA3 expression although the presence of glucose. ChREBP has an indubitable role in controlling PNPLA3 mRNA levels in mouse liver and isolated murine hepatocytes, however, is missing its role in human PNPLA3 promoter. (Ortega-Prieto, 2019)

3.4.3. PNPLA3 degradation

PNPLA3 degradation is led by E3 ligase. E3 ligase proceeds to the PNPLA3 ubiquitination, marking PNPLA3 to its recognition by the proteasome. Furthermore, it was observed that in the presence of long-chain fatty acids, (especially C18:0 and C18:1) PNPLA3 stabilizes itself and its degradation its avoided. It is unknown why this happens yet.

PNPLA3 could be degraded through other pathways too, such as autophagy. However, there are not many studies about it and it needs to be fathomed (BasuRay, 2019).

3.4.4. Hepatic inflammation and scaring and PNPLA3

PNPLA3 is abundantly expressed in hepatic stellate cells (HSCs) in humans and mice. This protein can promote the secretion of proinflammatory cytokines such as CCL2, CCL5, and TFG-β. CCL2 and CCL5 lead to HSCs activation and secretion of collagen I. In late stages of NASH, this collagen generates fibrosis during liver damage. TFG-β induced expression of more proinflammatory genes in HSCs, which has an essential role in NAFLD and NASH progression (Schwartz, 2020).

3.4.5. PNPLA3 sequesters PNPLA2's cofactor: link between liver and adipose tissue

PNPLA2 or ATGL is the major triglyceride hydrolase in hepatocytes (as well as adipocytes). It is activated through its interaction with ABHD5 or CGI-58 cofactor. Tissue-specific perilipins regulate its interaction. In adipocytes, PLIN1 sequesters ABDH5, and its phosphorylation leads to its release and activation of PNPLA2. (Wang, 2019)

PNPLA2 and PNPLA3 have the same Ser-Asp catalytic dyad. They are expressed in adipose tissue and liver. However, PNPLA2 is upregulated in fasting and PNPLA3 in the postprandial phase (Wang, 2019).

ATGL lipase activity is reduced in NAFLD disease. Several studies focused on the availability of PNPLA3-CGI-58 interaction and its correlation with PNPLA2 in vivo and in vitro. These findings highlight that the expression of some PNPLA3 genetic variants inhibit ATGL lipase activity (Wang, 2019). PNPLA3 and PNPLA2 compete for ABHD5 in transfected cells, with PNPLA3 having a stronger affinity. Its coupling is similar to the interaction between PLIN1 or PLIN5 and CG-58 in ATGL regulation (Wang, 2019; Yang, 2019).

There are many pathways in which PNPLA2/ATGL downregulation could promote NAFLD/NASH. Lipolysis by PNPLA2/ATGL release MUFAs that participate in the activation of SIRT1. SIRT1 promotes the reduction of endoplasmic reticulum stress and inflammation by deacetylation of proteins involved in autophagy or lipophagy. In NAFLD patients, SIRT1 expression is reduced. Furthermore, it could be related to the accumulation of PNPLA3. However, further studies need to be done on this topic (Mashek, 2021).

4. HYPOTHESIS AND OBJECTIVES

Obesity-associated nonalcoholic fatty liver disease and nonalcoholic steatohepatitis is worryingly increasing their prevalence, and neither of them have an accepted treatment. In both hepatic diseases and obesity occur a dysregulation of lipid metabolism, causing an accumulation in liver and adipose tissue. PNPLA3 has a central role in lipid metabolism, and its normal expression and degradation is crucial for the physiologic functioning of liver and other organs or tissues such as adipose tissue.

Based on the suggestions about PNPLA3 being involved in the development of NASH, we hypothesize that PNPLA3 expression and/or degradation is deregulated in these patients, causing an accumulation of this protein in both hepatic and adipose tissue. Moreover, as PNPLA3 is related with hepatic inflammation and fibrosis, this accumulation could take place in the scaring zones of the hepatic tissue.

The main objective in our study is to compare protein expression of PNPLA3 in liver, SAT, and VAT samples of patients with severe obesity and with simple steatosis or NASH.

Specific objectives:

- To identify PNPLA3 protein and determine its main location in liver, SAT and VAT biopsies from obese patients with and without NASH by immunochemistry.
- To relative quantify PNPLA3 protein in liver, SAT, and VAT samples of obese patients with and without NASH by western blot.
- To assess correlations between the expression of PNPLA3 in liver, SAT, and VAT, and metabolic variables.

5. MATERIALS AND METHODS

5.1. Study design

The total participants were n = 98, all of them candidates to receive bariatric surgery. The inclusion criteria included patients with at least 18 years old and with morbid obesity (BMI>40 kg/m^2) or with obesity type II (BMI>35kg/m^2) and metabolic diseases (such as T2D, hypertension, and/or dyslipidemia). Among them, n = 58 were not diagnosed with NASH (NAS ≤ 3), and n = 40 were diagnosed (NAS ≥ 5) as NASH. The main exclusion criteria were evidence of alcohol consumption, diagnosis of any type of hepatitis or inflammatory chronic diseases or cancer.

Just before de surgery, a blood analytics was performed to assess biochemical common features in a Cobas 8000 (*Roche Diagnostics*, Basilea, Sweden). The parameters included were glucose, insulin, triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma-glutamyl transferase (GGT). Using glucose and insulin values, we calculated the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) (Matthews, 1985).

This study was approved by the Institut d'Investigació Sanitària Pere Virgili Ethics Committee (PL4NASH 112/2021). All participants signed an informed consent to be able to use the biopsies from their bariatric surgery in our study.

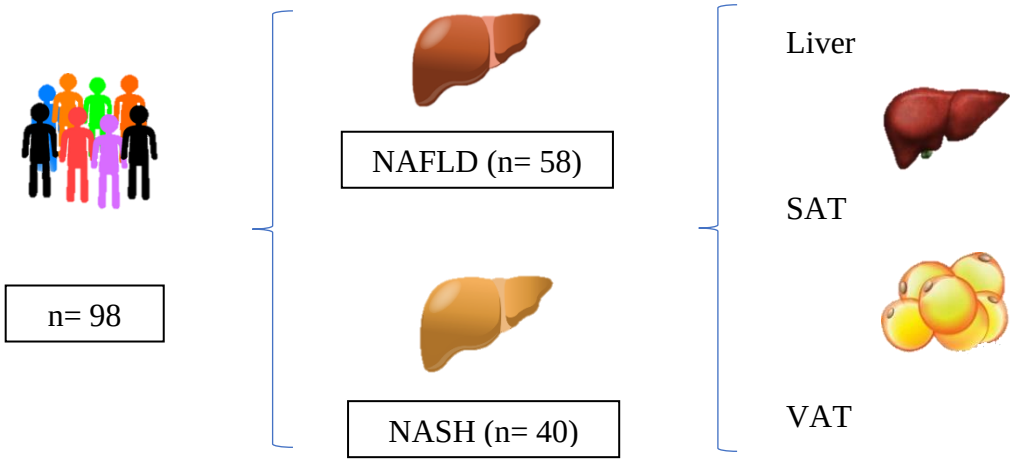

Figure 4. Schematic representation of the study design

5.2. Sampling

We obtain tissue samples (liver, SAT, and VAT) from biopsies performed during the bariatric surgery. One piece of each sample was fixed in formaldehyde for 24 hours. Then, the samples were paraffin-embedded to perform staining and immunohistochemistry. The other piece of the sample was kept at -80ºC until protein extraction.

5.3. Histological analyses

From each paraffin-embedded block, we obtained three different 2 µm sections. Two sections were used to perform Hematoxylin and Eosin, and Masson's Trichrome stainings. For liver samples, these stainings served to assess liver's severity following Kleiner et al. (2005) criteria (Kleiner, 2005). For adipose tissue samples, we used Hematoxoylin and Eosin sections to observe tissue structure, and Masson's Trichrome to investigate the distribution of fibrosis.

With the remaining section, immunohistochemistry for PNPLA3 was performed with Tris-EDTA buffer as antigen retrieval (pH=9). The concentration of the Anti-PNPLA3 antibody (67369-1-Ig, Proteintech, USA) was 1900 µg/ml and the dilution employed for the procedure was 1:1000 with BSA 2%. The secondary antibody used was Envision+System-HRP Labelled Polymer (*Dako*, Glostrup, Denmark) in a ready to use dilution. We use Mayers' hematoxylin as counterstain and we captured the micrographies with NS element software attached to Nikon E5300 optical microscope.

From the immunohistochemistry for PNPLA3, we developed a scoring method to assess this protein expression distribution in the tissue. These scores were mainly to qualify the expression of PNPLA3 near the fibrotic areas.

5.4. Protein extraction

We pulverized 50 mg of liver tissue and 200 mg of adipose tissue with liquid nitrogen. Then, we added 1 mL of Trizol (*Thermofisher Scientfic***,** Massachussets, USA**)** and we followed the manufacturer's instructions. Briefly, we used chloroform to create different phases according to the difference in density and polarity of DNA, RNA and proteins. This results in a clear aqueous upper layer (with RNA), an interphase (RNA, DNA and proteins) and a red organic lower layer (containing DNA and proteins). The lower layer contains chloroform and phenol.

We discarded the aqueous phase, and added ethanol to generate DNA precipitation and eliminate the pellet. Then, we added isopropanol to precipitate proteins and the

supernatant was discarded. In the next step guanidine hydrochloride solution was added to the precipitate to eliminate phenolic residuals. SDS 1% was employed to resuspend the precipitate. Finally, the protein extracted was quantified by BCA quantification (*Thermofisher Scientific*, Massachusetts, USA) following the manufacturer's instruction.

5.4 Western Blot

Protein extracts were denatured and run in 10% polyacrylamide gels. The Molecular Weight marker was from Proteintech (PL00002, Broad range prestained protein marker, *Proteintech*, IL, USA) and we employed β-actin as a housekeeping protein control for the relative quantification of PNPLA3.

The concentration of the antibody Anti-PNPLA3 anti-mouse (67369-1-Ig, *Proteintech*, USA) was 1900 µg/ml and Anti-β-actin anti-mouse (*Invitrogen*, USA**)** was 1mg/mL. We employed a dilution of 1:5000 and 1:2000, for liver and adipose tissue respectively.

The concentration of the secondary antibody HRP detection (Dako, Glostrup, Denmark) anti-mouse for the detection of PNPLA3 and β-actin was 1 g/l. We employed a 1:2000 and 1:8000 dilution, respectively.

Finally, we use Image Lab 6.0.1 (*Bio-Rad*, Los Angeles, California, USA) to relative quantify PNPLA3's protein expression.

5.5 Data analysis and statistics

We used RStudio with R language (version 4.2.2) to do data analysis and statistics. (Rstudio Team, 2020; Van Rossum, 2009)

To study the population characteristics, we used the *CreateTableOne* function from the TableOne package to create a table of frequencies and percentages for each categorical variable and mean and standard deviation for each continuous variable. We also stratified the table by liver severity status.

To assess the relationships between biochemical characteristics and hepatic PNPLA3 protein expression, we used the *CreateTableOne* function to create a table of frequencies and percentages for each categorical variable and mean and standard deviation for each continuous variable.

To visualize the differences in hepatic PNPLA3 protein expression between non-NASH and NASH individuals, we used the ggplot2 package to create a boxplot of PNPLA3

protein expression by NASH status. We also used the *stat_compare_means* function to add a label indicating the p-value of the statistical test.

To assess the correlations between biochemical characteristics and adipose tissue PNPLA3 protein expression, we used the mice package to impute missing values using the predictive mean matching method. We then computed Pearson's correlation coefficient between the variables using the *cor* function. To test the significance of the correlations, we used the *cor.test* function and extracted the p-values.

To identify the significant predictors of adipose tissue PNPLA3 protein expression, we performed a multiple linear regression analysis using the *lm* function. We included all variables that were significantly correlated with PNPLA3 protein expression in the model.

We tested the assumptions of normality and homoscedasticity for all statistical tests. All variables were found to have a normal distribution. P-values were considered significant when <0.05.

6. RESULTS

6.1. Features of patients

We aimed to evaluate the clinical characteristics of patients by examining the difference and similarities in their anthropometric and clinical features. To do so, we generated a table with items that should be considered in our study comparing NASH and Non-NASH patients (Table 2).

Table 2. Features of patients.

	Non-NASH (n = 58)	**NASH** (n = 40)	**p-value**
Anthropometrics			
Sex (Woman (n (%))	44 (75.9)	29 (72.5)	0.889
Age (years)	48.72 ± 11.57	49.20 ± 11.73	0.843
BMI (kg/m^2)	44.26 ± 6.74	46.18 ± 6.19	0.190
Heart rate (beats per minute)	69.47 ± 10.33	77.20 ± 14.81	0.094
Systolic blood pressure (mmHg)	127.57 ± 25.21	127.10 ± 18.32	0.936
Diastolic blood pressure (mmHg)	75.00 ± 10.87	73.69 ± 11.25	0.657
Hip perimeter (cm)	131.43 ± 15.51	137.91 ± 10.78	0.099
Waist perimeter (cm)	125.38 ± 15.19	131.74 ± 16.10	0.141
Concomitant diseases			
Without diagnosed comorbidities (n (%))	9 (15.5)	1 (2.5)	0.080
T2D (n (%))	15 (25.9)	11 (27.5)	1.000
Hypertension (n (%))	27 (46.6)	25 (62.5)	0.177
Dyslipidemia (n (%))	11 (19.0)	17 (42.5)	**0.021**
Metabolic syndrome (n (%))	20 (34.5)	13 (32.5)	1.000
Prescribed drugs			
Angiotensin II blockers (n (%))	1 (1.7)	9 (22.5)	**0.003**
Anilids (n (%))	2 (3.4)	5 (12.5)	0.190
Beta blockers (n (%))	2 (3.4)	1 (2.5)	1.000
Biguanides (n (%))	4 (6.9)	9 (22.5)	0.053
Calcium channel blockers (n (%))	-	5 (12.5)	**0.022**
Fibrates (n (%))	-	1 (2.5)	0.851
Insulin (n (%))	4 (6.9)	2 (5.0)	1.000

BMI: body mass index; T2D: type 2 diabetes.

The first part of the analysis consisted in studying the anthropometric measures. There were no significant differences between NASH and non-NASH patients in terms of sex, age, BMI, systolic and diastolic blood pressure, and waist perimeter. Although not considered significant ($p<0.1$), hip perimeter and heart rate tended to be higher in the NASH group.

Regarding concomitant diseases, the non-NASH group had a higher proportion of patients without diagnosed comorbidities, but the difference was not statistically significant. There were no significant differences between the two groups in terms of T2D, hypertension, and metabolic syndrome. However, the NASH group had a significantly higher prevalence of dyslipidemia compared to the non-NASH group.

Prescribed drugs did not follow the trends observed in the prevalence of concomitant diseases, as the use of antihypertensive drugs such as angiotensin II blockers and calcium channel blockers were significantly different between NASH and non-NASH patients. In contrast, the use of fibrates, which are usually prescribed for dyslipidemia, were not significantly different between the studied groups of patients.

6.2. PNPLA3 expression location varied with NASH diagnosis in hepatic tissue

While we did not observe PNPLA3 expression within the fibrotic scars in the liver, we did detect expression in the surrounding area. This expression differed significantly between NASH and non-NASH patients (p=0.04). Specifically, 50% of patients with NASH exhibited PNPLA3 expression around fibrosis, compared to 21.6% of non-NASH patients.

We observed a non-significant trend (p=0.06) towards greater expression of PNPLA3 in areas with steatosis in patients with NASH. Specifically, 66.7% of patients with NASH displayed this expression around cells with steatosis, compared to 42.9% of non-NASH patients (Figure 5).

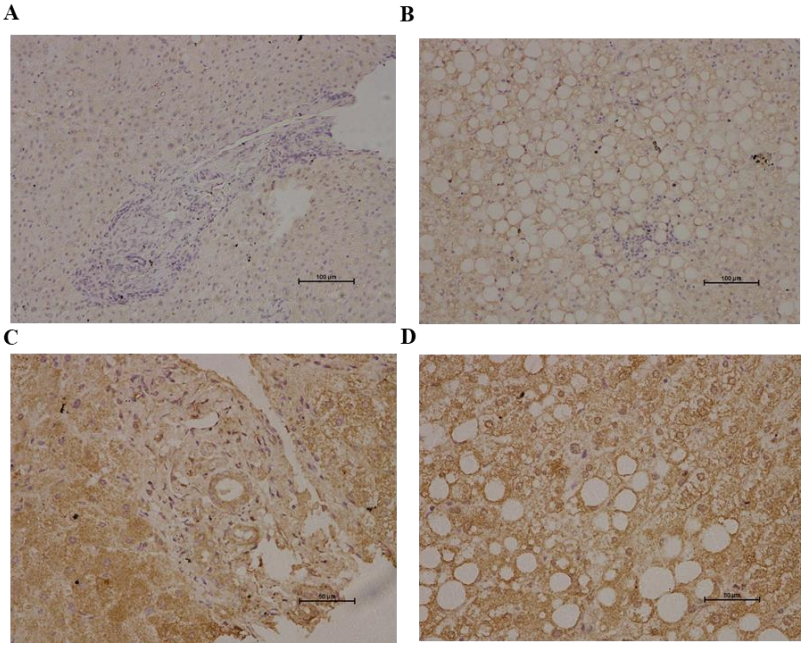

Figure 5. Immunohistochemistry for PNPLA3 in liver samples. A: no steatosis detected. PNPLA3's expression is not localized in vascular zones; B: PNPLA3's expression and steatosis; C and D: PNPLA3's expression around fibrosis and steatosis.

6.3. PNPLA3 co-localized with fibrosis in visceral adipose tissue, independently from NASH

In subcutaneous and visceral adipose tissue, we found that the perilobular and pericellular regions expressed PNPLA3 in both NASH and non-NASH patients. Furthermore, we consistently observed PNPLA3 expression in the lumen of blood vessels, which co-localized with erythrocytes, also independently of the liver's severity.

We found that not all samples of subcutaneous adipose tissue had PNPLA3 expression in the same location as fibrosis, but this variability in localization was not linked to a NASH diagnosis (p=0.166). On the other hand, all examined samples of visceral adipose tissue, both from NASH and non-NASH patients, co-localized PNPLA3 expression with fibrosis (Figure 6).

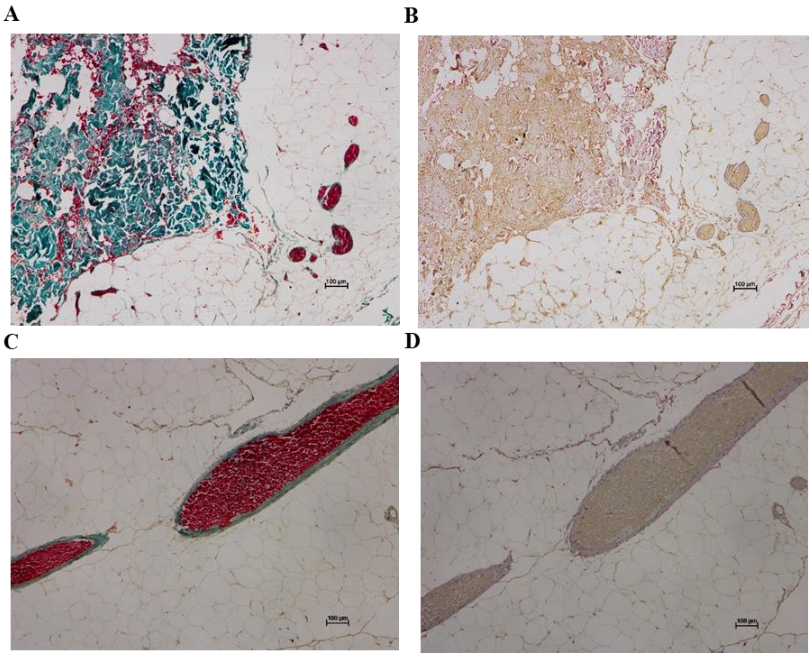

Figure 6. Histological analysis of SAT (A and B), VAT (C and D). A and C are Masson's Trichrome, and B and D are immunohistochemistry for PNPLA3. Vascular zones are in red, PNPLA3's expression zones are in brown and fibrotic zones in blue. A and B: PNPLA3's expression in vascular zones, and no fibrosis detected in PNPLA3's expression zone. C and D: PNPLA3's expression in vascular zones, and fibrosis around PNPLA3's expression.

6.4. Hepatic PNPLA3 protein expression was significantly increased in NASH patients

We performed a Western Blot to compare the relative amount of PNPLA3 in non-NASH and NASH patients. In liver, we observed more PNPLA3 relative amount in NASH patients compared to those without NASH (Figure 7A). This was confirmed after intensity band quantification, as the difference was significant ($p<0.001$) (Figure 7B).

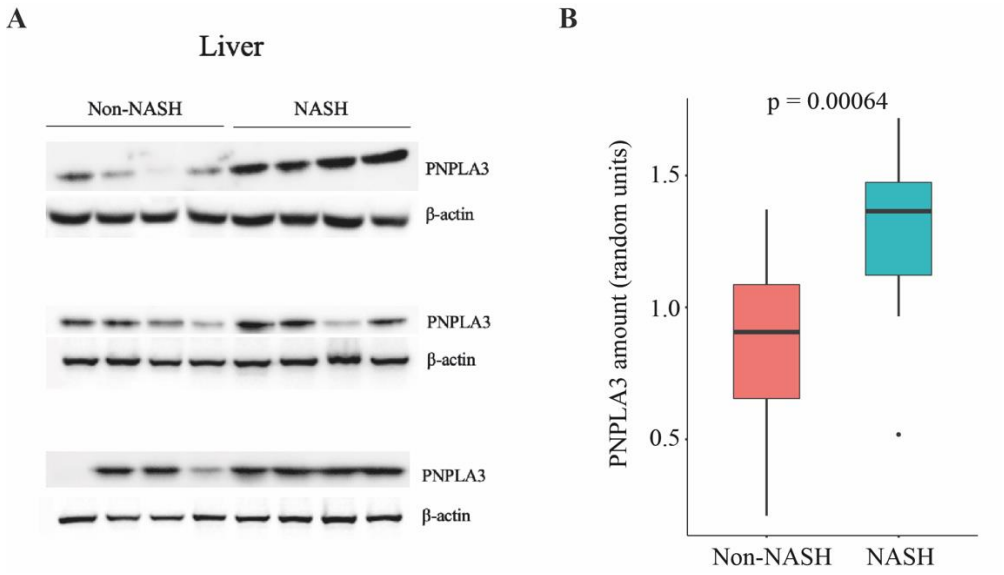

Figure 7. PNPLA3's expression in NAFLD patients with simple steatosis and NASH. It is shown a significative difference in liver, where NASH patients have more PNPLA3's expression compared to non-NASH patients.

6.5. Significant correlation between biochemical characteristics and PNPLA3 hepatic amount

Table 3. Correlation between biochemical characteristics and PNPLA3 hepatic amount among NASH and non-NASH patients.

	Non-NASH (n = 58)	**NASH** (n = 40)	**p-value**
Glucose (mmol/L)	6.96 ± 2.14	8.55 ± 3.04	**0.008**
Insulin (pmol/L)	105.62 ± 236.39	95.57 ± 52.77	0.845
HOMA-IR	5.81 ± 16.50	5.30 ± 3.31	0.888
Triglycerides (mmol/L)	1.55 ± 0.64	1.83 ± 0.84	0.118
Total cholesterol (mmol/L)	4.40 ± 1.10	4.33 ± 1.14	0.792
HDL cholesterol (mmol/L)	1.06 ± 0.35	0.90 ± 0.19	**0.052**
LDL cholesterol (mmol/L)	2.66 ± 0.91	2.52 ± 0.99	0.581
ALT (µKat/L)	0.49 ± 0.23	0.97 ± 0.38	**<0.001**
AST (µKat/L)	0.50 ± 0.20	0.98 ± 0.32	**<0.001**
GGT (µKat/L)	0.40 ± 0.40	0.82 ± 0.99	**0.013**

ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: gamma glutamyl-transferase; HDL: high-density lipoprotein; HOMA-IR: homeostatic model assessment for insulin resistance; LDL: low-density lipoprotein.

Biochemical characteristics were assessed, and when comparing patients with and without NASH, we found significant differences in variables such as ALT, AST, GGT, glucose and triglycerides. No significant differences were found in insulin, HOMA-IR, cholesterol, or LDL-C (table 3).

We conducted a study to investigate whether there were any correlations between these variables and hepatic PNPLA3 protein expression. We found that total cholesterol, HDL-cholesterol, LDL-cholesterol, ALT, AST, and GGT were significantly correlated with PNPLA3 protein expression in the liver (Figure 8). However, no significant correlations were observed for other variables such as insulin, HOMA-IR, or triglycerides.

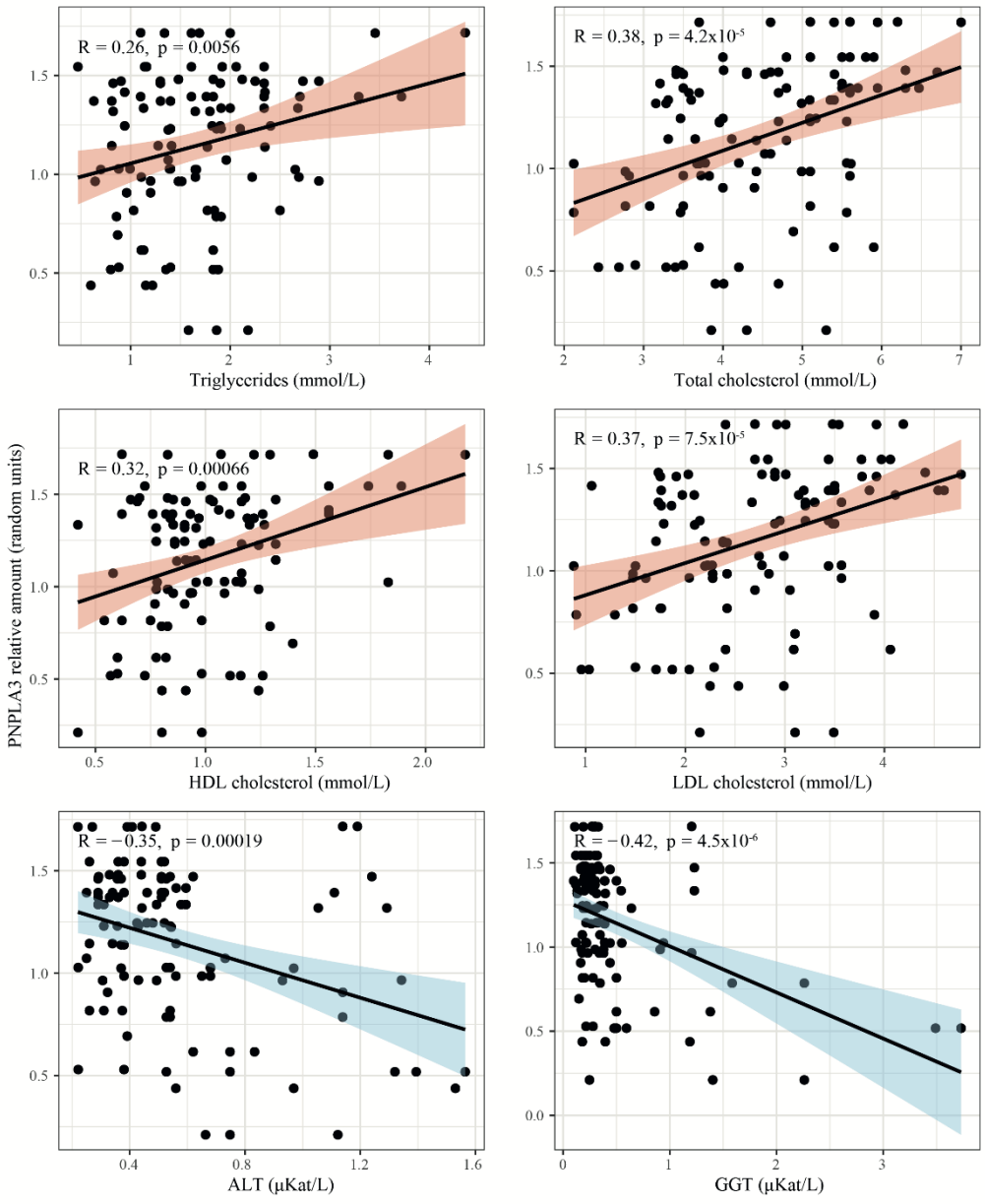

Figure 8. Correlations between PNPLA3 relative amount in liver and triglycerides, cholesterol (total, HDL and LDL), ALT and GGT, and fitter logistic regression model. It is observed a significative correlation in all of the biochemical features ($p<0.05$)

6.6. PNPLA3 hepatic expression was not related to fibrosis nor steatosis

We conducted an analysis to investigate whether there was a relationship between PNPLA3 hepatic expression and either steatosis or fibrosis grade. Our results showed a significant correlation with steatosis, although the significant p-value of the correlation may be misleading. As shown in Figure 9A, the plotted data points did not appear to show a strong relationship between the variables, potentially indicating overfitting in the model. Moreover, we did not observe a significant association between PNPLA3 hepatic expression and fibrosis (Figure 9B).

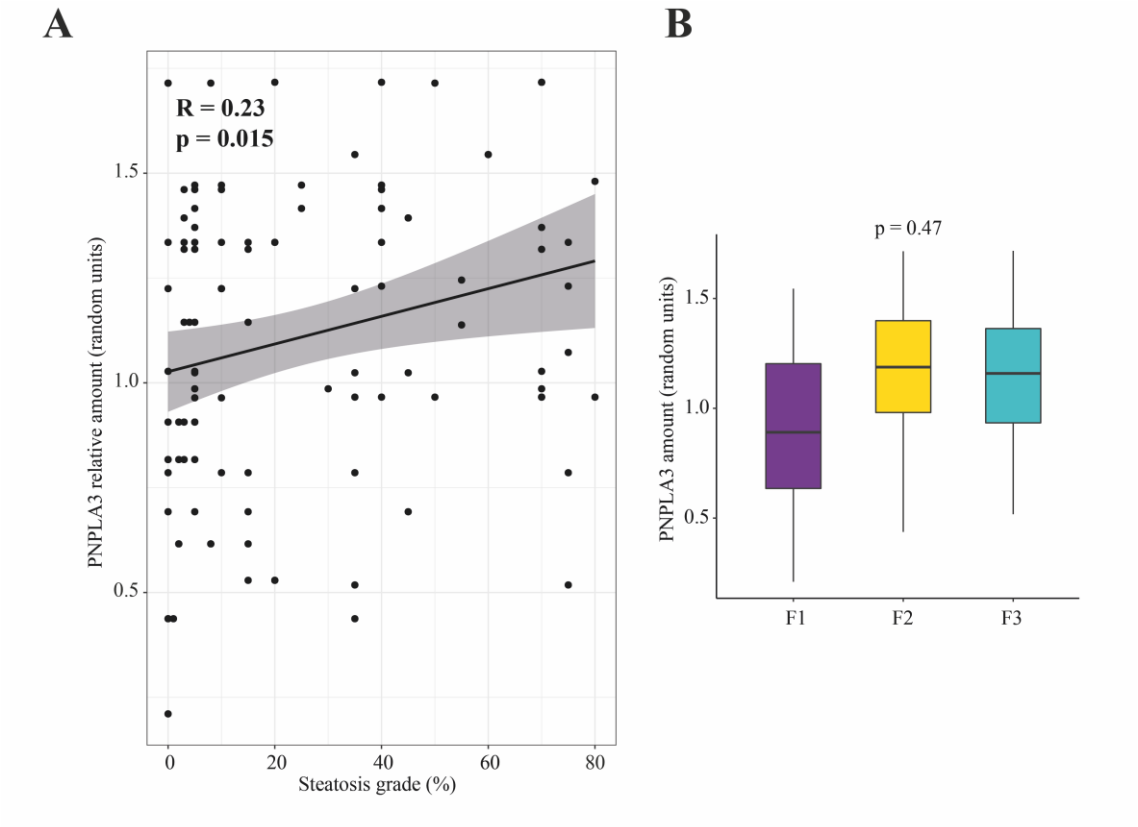

Figure 9. PNPLA3 relative amount was not significantly related to steatosis nor fibrosis. A: Correlation plot between PNPLA3's relative amount and steatosis grade. B: Box plot between PNPLA3's relative amount and the different fibrosis stages (F1: perisinusoidal or periportal fibrosis; F2: perisinusoidal and periportal fibrosis; F3: bridging fibrosis).

6.7. Adipose tissue PNPLA3 protein expression was not different between patients with and without NASH

We conducted a western blot analysis to determine if there were any differences in PNPLA3 expression in adipose tissue between individuals with and without NASH (Figure 10). While there seemed to be a trend towards lower PNPLA3 expression in

adipose tissue from NASH patients, the differences did not reach statistical significance when we quantified the bands (Figure 11).

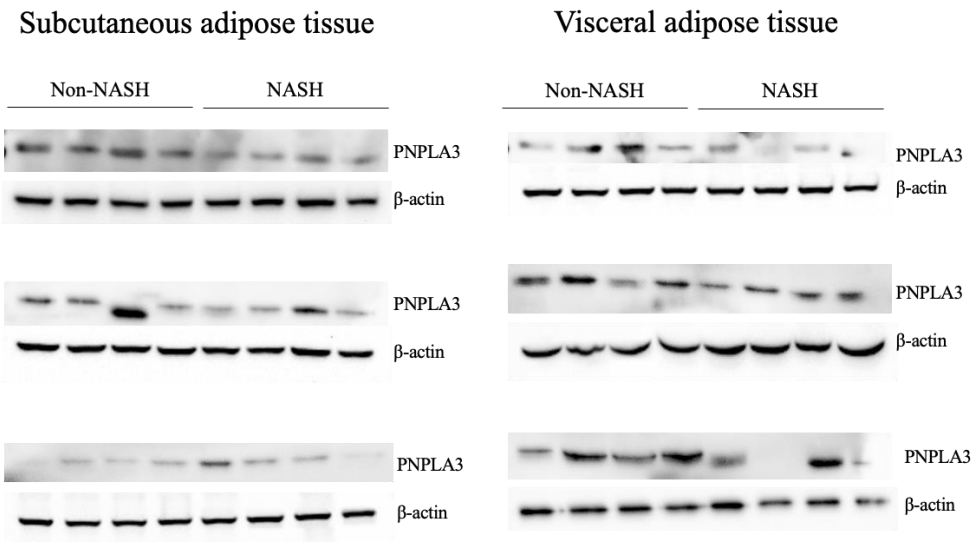

Figure 10. Western blot analyses of adipose tissue samples from patients with and without NASH.

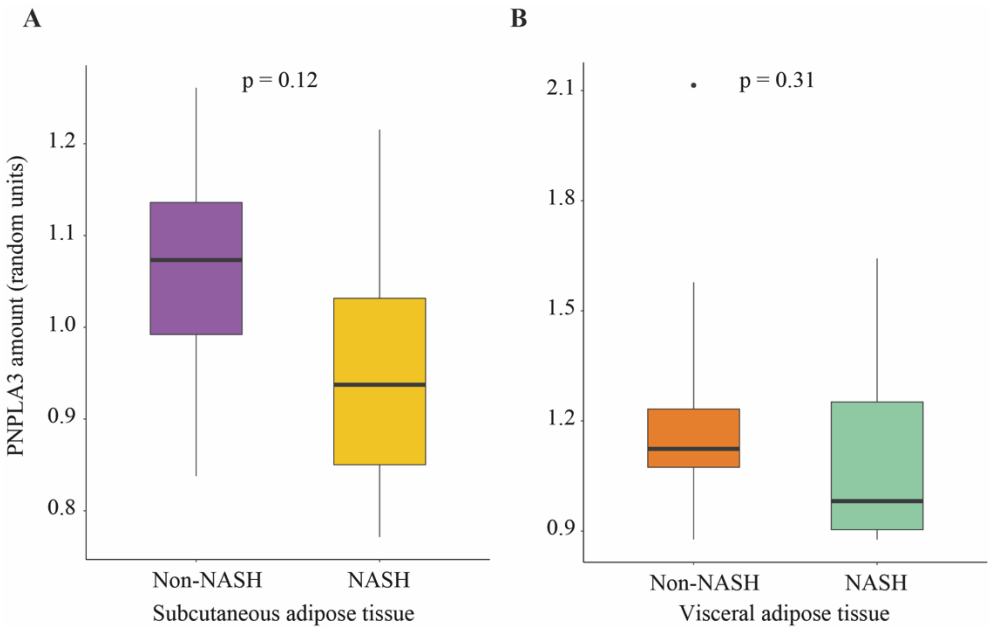

Figure 11. PNPLA3 protein relative amount in adipose tissues was not significantly different between NASH and non-NASH patients. A: subcutaneous adipose tissue, B: visceral adipose tissue.

6.8. Adipose tissue PNPLA3 protein expression correlated with circulating cholesterol and transaminases

Adipose tissue PNPLA3 protein expression was found to be significantly correlated with several biochemical markers. In subcutaneous adipose tissue, PNPLA3 expression correlated with HDL cholesterol, ALT, and GGT (Figure 12A). In visceral adipose tissue, PNPLA3 expression correlated with triglycerides and total cholesterol (Figure 12B).

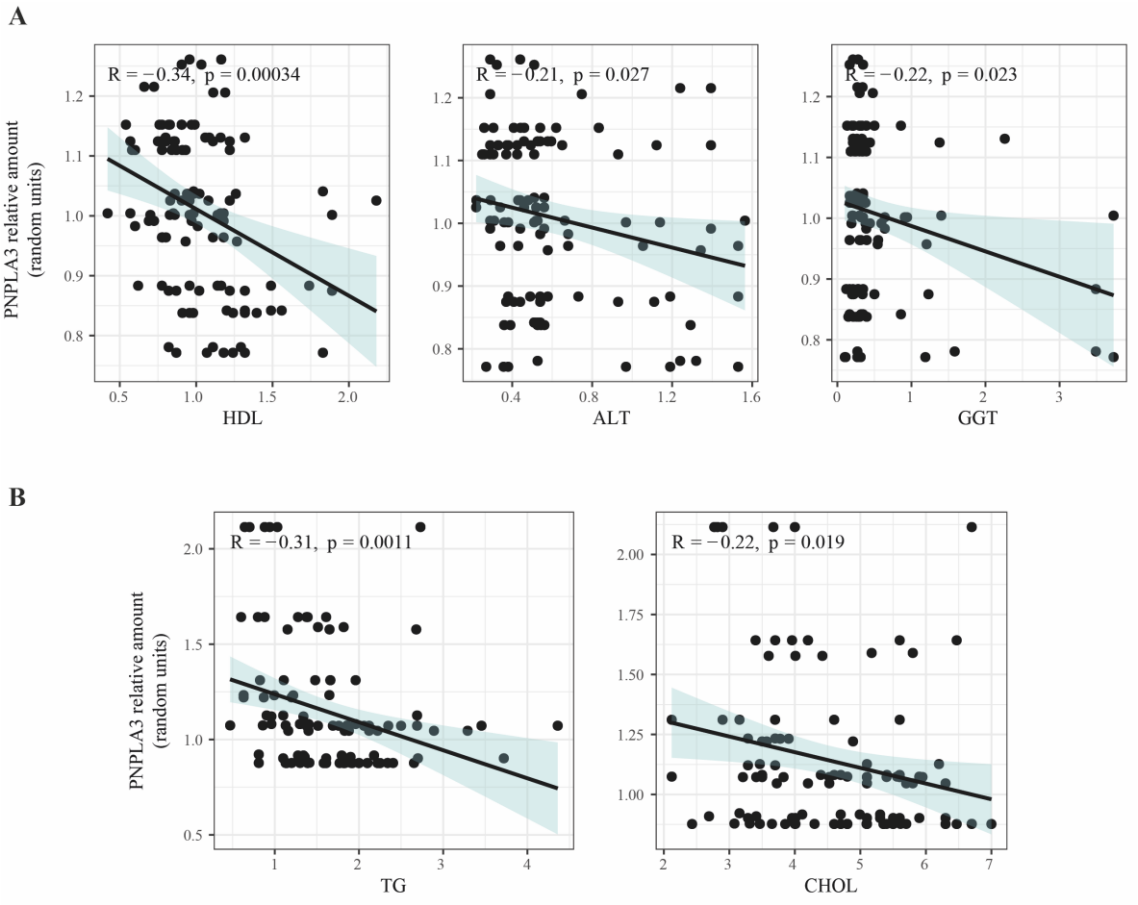

Figure 12. Significant correlations between adipose tissues PNPLA3 protein expression and biochemical variables. A: correlation between subcutaneous adipose tissue PNPLA3 amount and high-density lipoprotein (HDL) cholesterol, alanine aminotransferase (ALT), and gamma glutamyl-transferase (GGT); B: correlation between visceral adipose tissue PNPLA3 amount and triglycerides (TG) and total cholesterol (CHOL).

7. DISCUSSION

The main objective in our study is to compare protein expression of PNPLA3. We studied patients with severe obesity and with or without NASH, so it is convenient to compare the effects of these diseases in our patients in first place.

The characteristic fat accumulation in NAFLD results in lipotoxicity. Lipotoxicity induces mitochondrial production of ROS and inflammation, promoting the development of NASH (10). The increment of hip perimeter is due to fat accumulation, so it is common to see NASH patients with larger hip perimeter. Furthermore, inflammation is a typical characteristic of the development of obesity-associated NAFLD concomitant diseases (Chartrand, 2022; Sandino, 2022; Yang, 2022). So, it is habitual that NASH patients present more comorbidities than non-NASH patients such as dyslipidemia, hypertension, metabolic syndrome, T2DM, among others. Besides, there is a strong relationship with NAFLD and insulin resistance. The insulin resistance promotes lipid accumulation that can store in different organs and tissues as the adipose tissue (Godoy-Matos, 2020; Mashek, 2021; Nagarajan, 2022). As the lipotoxicity is increased in NASH, is common to have more deregulated insulin metabolism. Also, the imbalance of the lipid metabolism is typical of dyslipidemia. Furthermore, dyslipidemia and hypertension are included in the definition of metabolic syndrome (Godoy-Matos, 2020), which in turn promotes hepatic advanced fibrosis (Michelotti, 2021). Despite this, in our study we just observed significative differences in dyslipidemia, probably because of the small number of patients involved.

Regarding the prescribed drugs, we did not find any correlation between them and the results of the comorbidities. Still, the significative difference in angiotensin II blockers and calcium channel blockers could be due to the tendency in NASH patients to have more hypertension. Moreover, there is a close significative difference in biguanides and NASH patients. This may be due to the irregular insulin metabolism already mentioned, even though, there is no significative difference in the insulin prescribed drug intake.

There is evidence to suggest that the protein PNPLA3, which is involved in lipid metabolism, may play a role in the development and progression of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH). As we also have seen, higher levels of PNPLA3 in the liver have been correlated with the presence of steatosis, or fat accumulation, in the liver, as well as with the severity of fibrosis in patients with NASH. Additionally, PNPLA3 has been shown to regulate the hydrolysis and synthesis

of triglycerides in the liver, which may contribute to the accumulation of fat on the liver in NAFLD and NASH patients. (Bruschi, 2017; Uniprot)

In the presence of an excess of glucose in NAFLD and/or NASH patients, glucose tends to contribute to lipogenesis (Nagarajan, 2022), promoting the lipotoxicity of the disease. (Nagarajan, 2022) Then, it is not unusual to find more glucose levels in NASH patients. Also, it is normal to see ALT, AST and GGT increased in NASH patients, as the increased levels of these enzymes are detected in more injured livers. (Moreno-Torres, 2022)

The glucose digestion brings cholesterol biosynthesis substrate as acetyl-CoA, promoting cholesterol biosynthesis. Besides, the excess of glucose induces LDLR degradation via ChREBP, reducing the cholesterol uptake. Also, cholesterol absorption from intestine and bile is promoted too, through NPC1L1 enterocyte expression. Furthermore, in endoplasmic stress conditions SREBP2 is activated through unknown cell pathways and promote HMGCR expression, an enzyme required for cholesterol biosynthesis. Then, the increased lipotoxicity and the stress can cause the close significative differences in HDL levels shown in NASH patients, even though there aren't significative differences in total and LDL cholesterol (Xiao, 2022).

There are some genetic variants present in NAFLD population that influencing glucose homeostasis (GCKR, PPP1R3B) and lipid metabolism (TM6SF2, HSD17B13, LYPLAL1), among the latter one, PNPLA3. SNP rs738409 is robustly associated to NAFLD (Anstee, 2020).

rs738409 is a SNP that consists in a change from C to A, T or G. C>G polymorphism resulting in a missense mutation, which in turn leads to a change from Ile to Met in the 148 amino acid (Uniprot). PNPLA3-I148M phenotype consists in a larger lipid droplet size (BasuRay, 2019; Negoita, 2019). Numerous studies have clinical evidence of the association of PNPLA3 I148M and NAFLD and its progression to NASH. The risk to develop steatosis is higher in homozygous individuals with this PNPLA3 variant. PNPLA3 I148M can be involved in a different enzymatic activity, promote fibrosis, increase inflammation, and participate in regulatory pathways culminating in a decrease of lipolysis (BasuRay, 2019; Schwartz, 2020; Pingitore, 2019).

It is thought that the rs738409 genetic variant may cause PNPLA3 to undergo a conformational change that makes it inaccessible to E3 ligase enzymes, which are involved in ubiquitination. Alternatively, PNPLA3-I148M may be easily deubiquitylated,

or have its ubiquitin removed, and stabilized on lipid droplets in the liver. Other proteins on the surface of these droplets may bind to and inhibit the degradation of PNPLA3-I148M (Negoita, 2019).

The genetic variant PNPLA3 I148M has an increased 1-acylglycerol-3-phosphate O-acyltransferase activity, which leads to an increase in the synthesis of triglycerides and diacylglycerides. It also has a reduced triglyceride hydrolase activity towards substrates with polyunsaturated fatty acids (PUFAs). This leads to an accumulation of PUFA-containing diacylglycerides and a reduction in PUFA-containing phosphatidylcholine, which is a phospholipid found in the lipid droplets. The accumulation of saturated fatty acids, particularly palmitic acid, can initiate p-JNK signaling, leading to the production of ROS and inflammation. These changes may contribute to the development of lipotoxicity, which is characteristic of NAFLD and NASH (Mashek, 2021; Uniprot).

Moreover, the excess of glucose contributes to PNPLA3 accumulation, characteristic in NAFLD and NASH disease. It is suggested that the accumulation is increased in PNPLA3 I148M variant. The excess of glucose leads to downregulate AMPK. Then, mTORC1 is activated by other components of its regulation as the protein Rheb due to the insulin resistance. mTORC1 inhibit complexes related to the phagophore elongation and curvature as ULK1 and PtdIns3K complex, resulting in the lack of the autophagosome in PNPLA3 degradation (Condon, 2019; Dossou, 2019; Negoita, 2019).

The lipotoxicity in NAFLD and/or NASH occurs in the liver, because of the fatty acids' mobilization from the adipose tissue lipolysis to the liver. To eliminate the excess of fatty acids, the metabolism rearranges itself to process the fatty acids, performing alternative pathways as the lipid synthesis. PNPLA3 can process fatty acids by its acyltransferase activity. Also, PNPLA3 I148M has this enzymatic activity increased (Uniprot), which suggests a correlation of the rs738409 and the accumulation of PNPLA3 in the liver of NASH patients. With its acyltransferase activity, PNPLA3 promotes the triglycerides and diacylglycerides synthesis. So, it can be explained the positive correlation of PNPLA3 relative amount and triglycerides. As normal PNPLA3 accumulation tends to contribute to lipotoxicity via its acyltransferase activity (Basuray, 2019; Negoita, 2019; Uniprot), it is expected a positive correlation of PNPLA3 relative amount and total, HDL, and LDL cholesterol levels too (Li, 2021). In addition, the increase in the amount of triglycerides and diacylglycerides contributes to the characteristic larger size of the lipid droplets in hepatic steatosis (BasuRay, 2019; Negoita, 2019). However, the results indicate the

opposite. The results showed a negative correlation of the PNPLA3 amount and ALT and GGT level, so as steatosis and fibrosis. These results suggested that PNPLA3 is not determinant to NASH development. What is undeniable, however, is that PNPLA3 can contribute to its development by generating more endoplasmic stress (Mashek, 2021; Schwartz, 2020; Wang, 2019; Yang, 2019).

Alternatively, PNPLA3 has also been found to be expressed in adipose tissue and to play a role in the accumulation of fat in this tissue. PNPLA3 has been shown to be involved in the regulation of fat storage and energy metabolism. Elevated levels of PNPLA3 in adipose tissue have been associated with increased risk for obesity and related metabolic disorders, such as type 2 diabetes and nonalcoholic fatty liver disease (NAFLD). Some studies have suggested that PNPLA3 may contribute to the development of obesity by disrupting the balance between lipid storage and breakdown in adipose tissue, leading to excess fat accumulation. In our study, we did not find a significant difference in the protein expression of PNPLA3 between NASH and non-NASH patients, though. This could be due to an effective degradation, which does not fit with the hypothesis of PNPLA3 I148M lack of degradation in the liver. The effect of this genetic variant is mostly unknown in the adipose tissue.

In the adipose tissue, PNPLA3 is not so expressed as in liver. Also, as the lipotoxicity is focused on the liver, PNPLA3 is required mostly in liver. This could explain the non-significative differences of PNPLA3's protein expression in adipose tissue. Then, in the analyses of correlations, as the biochemicals features increases (HDL, ALT, GGT, triglycerides, total cholesterol) with lipotoxicity, PNPLA3 decreases because its own mobilization to liver (Mancina, 2020; Saeed, 2017).

This work has some limitations that should be clarified. First, the study was accomplished with a small number of patients. Moreover, we only observed the PNPLA3 protein expression. To complete the study, it should be analyzed the PNPLA3's mRNA expression. Also, it would be interesting to see PNPLA3 mRNA and protein expression due to the genotype to see the influence of rs738409. Furthermore, in the literature it is suggested a sexual dimorphism in PNPLA3 expression that would be interesting to evidence too.

8. CONCLUSIONS

In this study, we analyzed PNPLA3 protein expression in liver and adipose tissue samples from patients with obesity-associated nonalcoholic steatohepatitis (NASH). PNPLA3, also known as phospholipase domain-containing 3, is a protein that has been previously linked to the development and progression of NAFLD and NASH. We aimed to determine whether PNPLA3 expression in hepatic and adipose tissues was related with the presence of NASH in patients with morbid obesity. Our results suggest the following points:

- PNPLA3 was expressed more significantly around fibrotic areas in hepatic tissue from NASH patients. The localization of the expression of this protein did not vary between the adipose tissue from NASH and non-NASH patients.
- PNPLA3 protein relative amount was significantly increased in hepatic tissue from patients with NASH, compared to the non-NASH ones. Although there weren't significant differences, PNPLA3 protein relative amount tended to be decreased in adipose tissue from patients with NASH, compared with non-NASH ones.
- PNPLA3 protein relative amount in all tissues significantly correlated with some biochemical features of patients: triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, ALT, and GGT with hepatic PNPLA3 protein expression; HDL cholesterol, ALT, and GGT with subcutaneous adipose tissue PNPLA3 protein expression; and triglycerides and total cholesterol with visceral adipose tissue PNPLA3 protein expression, compared to patients without NASH.

These findings suggest that PNPLA3 may play a role in the pathogenesis of NASH, specifically in the development of liver fibrosis. Further studies are needed to confirm these observations and to determine the mechanisms by which PNPLA3 contributes to the progression of NASH.

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10. REFERENCES

Anstee, Q. M., Darlay, R., Cockell, S., Meroni, M., Govaere, O., Tiniakos, D., Burt, A. D., Bedossa, P., Palmer, J., Liu, Y. L., Aithal, G. P., Allison, M., Yki-Järvinen, H., Vacca, M., Dufour, J. F., Invernizzi, P., Prati, D., Ekstedt, M., Kechagias, S., … Daly, A. K. (2020). Genome-wide association study of non-alcoholic fatty liver and steatohepatitis in a histologically characterised cohort. *Journal of Hepatology*, *73*(3), 505–515. https://doi.org/10.1016/j.jhep.2020.04.003

Aravalli RN, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. *Hepatology*. 2008;48(6):2047-2063. doi:10.1002/hep.22580

Armandi, A., & Bugianesi, E. (2021). Natural history of NASH. *Liver International*, *41*(S1), 78–82. https://doi.org/10.1111/liv.14910

BasuRay, S. (2019). PNPLA3-I148M: a problem of plenty in non-alcoholic fatty liver disease. In *Adipocyte* (Vol. 8, Issue 1, pp. 201–208). Taylor and Francis Inc. https://doi.org/10.1080/21623945.2019.1607423

Blüher, M. (2019). Obesity: global epidemiology and pathogenesis. In *Nature Reviews Endocrinology* (Vol. 15, Issue 5, pp. 288–298). Nature Publishing Group. https://doi.org/10.1038/s41574-019-0176-8

Bruschi, F. V., Tardelli, M., Claudel, T., & Trauner, M. (2017). PNPLA3 expression and its impact on the liver: current perspectives. *Hepatic Medicine: Evidence and Research*, *Volume 9*, 55–66. https://doi.org/10.2147/hmer.s125718

Chartrand, D. J., Murphy-Després, A., Alméras, N., Lemieux, I., Larose, E., & Després, J. P. (2022). Overweight, Obesity, and CVD Risk: a Focus on Visceral/Ectopic Fat. In *Current Atherosclerosis Reports* (Vol. 24, Issue 4, pp. 185–195). Springer. https://doi.org/10.1007/s11883-022-00996-x

Condon, K. J., & Sabatini, D. M. (2019). Nutrient regulation of mTORC1 at a glance. *Journal of Cell Science*, *132*(21). https://doi.org/10.1242/JCS.222570

Cotter TG, Rinella M. Nonalcoholic Fatty Liver Disease 2020: The State of the disease. *Gastroenterology*. 2020;158(7):1851-1864. doi:10.1053/j.gastro.2020.01.052

Demaria S, Pikarsky E, Karin M, et al. Cancer and inflammation: promise for biologic therapy. *J Immunother*. 2010;33(4):335-351. doi:10.1097/CJI.0b013e3181d32e74

Dossou, A. S., & Basu, A. (2019). The emerging roles of mTORC1 in macromanaging autophagy. In *Cancers* (Vol. 11, Issue 10). MDPI AG. https://doi.org/10.3390/cancers11101422

Eslam, M., & George, J. (2019). Genetic Insights for Drug Development in NAFLD. In *Trends in Pharmacological Sciences* (Vol. 40, Issue 7, pp. 506–516). Elsevier Ltd. https://doi.org/10.1016/j.tips.2019.05.00

Godoy-Matos, A. F., Silva Júnior, W. S., & Valerio, C. M. (2020). NAFLD as a continuum: From obesity to metabolic syndrome and diabetes. In *Diabetology and Metabolic Syndrome* (Vol. 12, Issue 1). BioMed Central. https://doi.org/10.1186/s13098-020-00570-y

Herring, W. L., Gould, I. G., Wittrup-Jensen, V., Ertle, J., Kuti, E., & Wolowacz, S. (2022). Evaluation of emerging NASH therapies: the impact of treatment efficacy profiles on long-term health outcomes. *Journal of Comparative Effectiveness Research*. https://doi.org/10.2217/cer-2021-0194

Kleiner, D. E., Brunt, E. M., van Natta, M., Behling, C., Contos, M. J., Cummings, O. W., Ferrell, L. D., Liu, Y. C., Torbenson, M. S., Unalp-Arida, A., Yeh, M., McCullough, A. J., & Sanyal, A. J. (2005). Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*, *41*(6), 1313–1321. https://doi.org/10.1002/hep.20701

Lee, S. H., Lee, J. H., & Im, S. S. (2020). The cellular function of SCAP in metabolic signaling. In *Experimental and Molecular Medicine* (Vol. 52, Issue 5, pp. 724–729). Springer Nature. https://doi.org/10.1038/s12276-020-0430-0

Li H, Yu XH, Ou X, Ouyang XP, Tang CK. Hepatic cholesterol transport and its role in non-alcoholic fatty liver disease and atherosclerosis. *Prog Lipid Res*. 2021;83:101109. doi:10.1016/j.plipres.2021.101109

Mancina RM, Spagnuolo R. Cross talk between liver and adipose tissue: A new role for PNPLA3?. *Liver Int*. 2020;40(9):2074-2075. doi:10.1111/liv.14561

Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature*. 2008;454(7203):436-444. doi:10.1038/nature07205

Mashek, D. G. (2021). Hepatic lipid droplets: A balancing act between energy storage and metabolic dysfunction in NAFLD. In *Molecular Metabolism* (Vol. 50). Elsevier. https://doi.org/10.1016/j.molmet.2020.101115

Matthews, D. R., Hosker, J. R., Rudenski, A. S., Naylor, B. A., Treacher, D. F., & Turner, R. C. (1985). Homeostasis model assessment: insulin resistance and fl-cell function from fasting plasma glucose and insulin concentrations in man. In *Diabetologia* (Vol. 28).

Michelotti, A., de Scordilli, M., Palmero, L., Guardascione, M., Masala, M., Roncato, R., Foltran, L., Ongaro, E., & Puglisi, F. (2021). NAFLD-related hepatocarcinoma: The malignant side of metabolic syndrome. In *Cells* (Vol. 10, Issue 8). MDPI. https://doi.org/10.3390/cells10082034

Montaño-Loza AJ.(2022) Evaluation of fibrosis in patients with nonalcoholic fatty liver disease. In *Rev Gastroenterol Mex (Engl Ed)* (Vol. 87, Issue 1, pp. 1-3). PMID: 34774458. https://doi.org/10.1016/j.rgmxen.2021.10.004

Moreno-Torres, M., Quintás, G., & Castell, J. v. (2022). The Potential Role of Metabolomics in Drug-Induced Liver Injury (DILI) Assessment. In *Metabolites* (Vol. 12, Issue 6). MDPI. https://doi.org/10.3390/metabo12060564

Nagarajan, S. R., Cross, E., Sanna, F., & Hodson, L. (2022). Dysregulation of hepatic metabolism with obesity: factors influencing glucose and lipid metabolism. In *Proceedings of the Nutrition Society* (Vol. 81, Issue 1, pp. 1–11). Cambridge University Press.https://doi.org/10.1017/S0029665121003761

Negoita, F., Blomdahl, J., Wasserstrom, S., Winberg, M. E., Osmark, P., Larsson, S., Stenkula, K. G., Ekstedt, M., Kechagias, S., Holm, C., & Jones, H. A. (2019). PNPLA3 variant M148 causes resistance to starvation-mediated lipid droplet autophagy in human hepatocytes. *Journal of Cellular Biochemistry*, *120*(1), 343–356. https://doi.org/10.1002/jcb.27378

Ortega-Prieto, P., & Postic, C. (2019). Carbohydrate sensing through the transcription factor ChREBP. *Frontiers in Genetics*, *10*(JUN). https://doi.org/10.3389/fgene.2019.00472

Pierantonelli I, Svegliati-Baroni G. Nonalcoholic Fatty Liver Disease: Basic Pathogenetic Mechanisms in the Progression From NAFLD to NASH. *Transplantation*. 2019;103(1):e1-e13. doi:10.1097/TP.0000000000002480

Pingitore, P., & Romeo, S. (2019). The role of PNPLA3 in health and disease. In *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids* (Vol. 1864, Issue 6, pp. 900–906). Elsevier B.V. https://doi.org/10.1016/j.bbalip.2018.06.018

RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/.

Saeed A, Dullaart RPF, Schreuder TCMA, Blokzijl H, Faber KN. Disturbed Vitamin A Metabolism in Non-Alcoholic Fatty Liver Disease (NAFLD). *Nutrients*. 2017;10(1):29. Published 2017 Dec 29. doi:10.3390/nu10010029

Sanchez-Torrijos, Y., Ampuero, J. (2020). The Spectrum of NAFLD: From the Organ to the System. In: Romero-Gomez, M. (eds) NAFLD and NASH. Springer, Cham. https://doi.org/10.1007/978-3-030-37173-9_1

Sandino, J., Martín-Taboada, M., Medina-Gómez, G., Vila-Bedmar, R., & Morales, E. (2022). Novel Insights in the Physiopathology and Management of Obesity-Related Kidney Disease. *Nutrients, 14*(19), 3937. https://doi.org/10.3390/nu14193937

Schwartz, B. E., Rajagopal, V., Smith, C., Cohick, E., Whissell, G., Gamboa, M., Pai, R., Sigova, A., Grossman, I., Bumcrot, D., Sasidharan, K., Romeo, S., Sehgal, A., & Pingitore, P. (2020). Discovery and Targeting of the Signaling Controls of PNPLA3 to Effectively Reduce Transcription, Expression, and Function in Pre-Clinical NAFLD/NASH Settings. *Cells*, *9*(10). https://doi.org/10.3390/cells9102247

UniProt Available online: https://www.uniprot.org/uniprotkb/Q9NST1/entry (accessed on 15 October 2022).

Van Rossum, G., & Drake, F. L. (2009). *Python 3 Reference Manual*. Scotts Valley, CA: CreateSpace.

Wang, Y., Kory, N., Basuray, S., Cohen, J. C., & Hobbs, H. H. (2019). PNPLA3, CGI-58, and Inhibition of Hepatic Triglyceride Hydrolysis in Mice. *Hepatology*, *69*(6). https://doi.org/10.1002/hep.30583/suppinfo

World Obesity Atlas 2022 Available online: https://www.worldobesity.org/resources/resource-library/world-obesity-atlas-2022 (accessed on 7 November 2022).

Xiao, X., Luo, Y., & Peng, D. (2022). Updated Understanding of the Crosstalk Between Glucose/Insulin and Cholesterol Metabolism. In *Frontiers in Cardiovascular Medicine* (Vol. 9). Frontiers Media S.A. https://doi.org/10.3389/fcvm.2022.879355

Yang, A., Mottillo, E. P., Mladenovic-Lucas, L., Zhou, L., & Granneman, J. G. (2019). Dynamic interactions of ABHD5 with PNPLA3 regulate triacylglycerol metabolism in brown adipocytes. *Nature Metabolism*, *1*(5), 560–569. https://doi.org/10.1038/s42255-019-0066-3

Yang, M., Liu, S., & Zhang, C. (2022). The Related Metabolic Diseases and Treatments of Obesity. In *Healthcare (Switzerland)* (Vol. 10, Issue 9). MDPI. https://doi.org/10.3390/healthcare10091616

Yeh MM, Brunt EM. Pathological features of fatty liver. *Gastroenterology*. 2014;147(4):754-764. doi:10.1053/j.gastro.2014.07.056