“Adipose tissue insulin resistance and lipidome alterations are the characterizing factors of NASH”

Sara Guerra[1,2], Gabriele Mocciaro[1] and Amalia Gastaldelli[1,2]

[1] National Research Council (CNR), Institute of Clinical Physiology (IFC), Pisa, Italy

[2] Sant'Anna School of Advanced Studies, Pisa, Italy

ORCID Sara Guerra 0000-0002-1155-8750

ORCID Gabriele Mocciaro 0000-0001-5392-0909

ORCID Amalia Gastaldelli 0000-0003-2594-1651

Words :5317

Figures: 3

Tables: 2

References: 151

Corresponding author

Amalia Gastaldelli, PhD.,

Head of Cardiometabolic Risk Laboratory,

Institute of Clinical Physiology, Consiglio Nazionale delle Ricerche,

Via Moruzzi 1, 56100 Pisa, Italy.

Email: amalia@ifc.cnr.it

Abstract (159 words)

The prevalence of non-alcoholic fatty liver disease (NAFLD) is now 25% in the general population but increases to more than 55% in subjects with obesity and/or type 2 diabetes. Simple steatosis (NAFL) can develop into more severe, i.e., non-alcoholic steatohepatitis (NASH), cirrhosis, hepatocellular carcinoma and death.

In this review we have discussed several mechanisms that have recently been indicated as primary promoters of NAFL progression to NASH. In particular, the role of insulin resistance, mitochondrial function and lipotoxic lipids. Insulin resistance, mainly in adipose tissue, is the main driver of NAFLD due to excess release of fatty acid. We discussed several lipids that are bioactive or toxic to cells and can be used to identify individuals at risk of progression to NASH. Not only lipid, but also amino acid metabolism is impaired in NAFL/NASH, and some amino acids, such as branched-chain and aromatic amino acids, glutamate, serine, and glycine, have been linked to impaired metabolism and severity of NAFLD.

1. Diagnosis and prevalence of fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is defined as excessive triglyceride accumulation into the hepatocytes in the absence of excessive alcohol consumption and defined by the presence of steatosis in >5% of hepatocytes according to histological analysis or by >5.6% hepatic fat assessed by magnetic resonance imaging (MRI) or spectroscopy (MRS) [1]. It is estimated that the prevalence of non-alcoholic fatty liver disease (NAFLD) in the general population is about 25% [2]. The highest prevalence rate is in South America (30.5%) and in the Middle East (31.8%) population, with the lowest rate in the African continent (13.5%) [2]. These data suggest a fundamental contribution of both genetic and epigenetic backgrounds.

NAFLD can progress to a more severe form, i.e., non-alcoholic steatohepatitis (NASH), defined as the presence of steatosis, inflammation and ballooning [1] that occurs in 10-25% of the subjects with NAFLD [2]. The definitive diagnosis of NASH requires liver biopsy since there are no approved imaging or circulatory biomarkers of NASH [1].

NAFLD is a metabolic disease, so it is not surprising that its prevalence is higher in subjects overweight or obese and in presence of sedentary lifestyle, being 57% in subjects with moderate to severe obesity and 90% in patients with morbid obesity undergoing metabolic surgery [3,4].

Diabetes is a major risk factor for fatty liver disease and NAFLD is currently present in 55% and NASH in 37% of patients with T2D, respectively [5]. Prevalence of metabolic disease (obesity, T2D and NAFLD) is continuously rising. Thus, considering that the estimated prevalence of diabetes in 2045 is expected to reach 700 million subjects [6], we can expect that number of patients with NAFLD and diabetes will reach 388 millions, of whom, those with NASH would be over 260 millions.

Not only T2D is a major risk factor for NAFLD, but also NAFLD is a major risk factor for the development of T2D. A recent meta-analysis reported a 2.2-fold increased risk of diabetes in subjects with NAFLD and this risk was higher (random-effects HR 2.69) in subjects with severe liver disease [7].

Given the strong association between fatty liver disease and metabolic alterations, it has been proposed to go beyond the dichotomous definition of NAFLD and NASH and diagnose fatty liver disease on the basis of metabolic dysfunction. Thus, a new definition was proposed that

is Metabolic (dysfunction) -Associated Fatty Liver Disease (MAFLD) [8] and requires, beyond the evidence of hepatic steatosis, one of the following three criteria, namely overweight/obesity, presence of type 2 diabetes mellitus, or evidence of metabolic dysregulation [8]. However, this change from NAFLD to MAFLD is far from being accepted by the entire scientific community [9] mainly because both terms are considered 'suboptimal'. If MAFLD will help with the diagnosis, subjects with non-metabolic NAFLD (eg lean with genetic mutations) that will not fit the MAFLD criteria will not be diagnosed and will be at potential risk of future illnesses. Moreover, all clinical trial endpoints for biomarkers and new drugs are currently based on the old definition of NAFLD/NASH. Finally, the new definition allows including moderate alcohol consumption in the characterization of such patients; thus some patients may have concomitantly alcoholic fatty liver.

In the following paragraphs we discussed the current knowledge in the pathophysiology of fatty liver disease, including both metabolic and non-metabolic factors, as well as the markers of liver damage, giving attention to the alterations in lipid metabolism and production of lipotoxic lipids.

2. Mechanisms of development of NAFLD and progression of liver disease

Hepatic steatosis is the result of an imbalance between triglyceride synthesis and secretion (as very low-density lipoproteins, VLDL). Primary, but modifiable, risk factors associated with NAFLD are positive caloric intake, low physical activity and preferential accumulation of abdominal adiposity [10] (**Figure 1**), while among non-modifiable risk factors there are: older age, male sex, ethnicity (Hispanic population, followed by Caucasians and African Americans) and genetic traits [2].

2.1. *The multiple hit hypothesis*

Not all subjects with simple steatosis (NAFL) will progress to NASH (about 10-20%). The mechanisms responsible for the progression of NAFL to NASH are still unknown. Many studies are now focusing on this topic, not only to discover new therapeutic targets but also biomarkes that can substitute liver biopsy that is currently the only way to diagnose NASH.

NASH is a complex disease that involves not only metabolic alterations (responsible for steatosis) but also other mechanisms responsible for the development of inflammation and ballooning. The *two-hit* theory, which dates back to 1998, was the first to hypothesize that

the initial accumulation of lipids (first hit) was followed by a second hit that will impact the already vulnerable liver[11]. Once combined, these two hits would make the liver more susceptible to develop inflammation, apoptosis, and fibrosis[11]. This second hit should be due to mitochondrial dysfunction and oxidative stress, which allow the onset of lipid peroxidation, the activation of inflammatory pathways, unregulated hepatocyte apoptosis, and activation of hepatic stellate cells[11]. Subsequently, Tilg and Moschen suggested that alteration in the gut liver-axis, gut microbiota, (adipo)cytokines, and innate immunity might also be responsible for the second hit [12]. Thus, it was hypothesized that a *multiple-parallel* hit based on the simultaneous interaction of several factors would be responsible for the progression of NAFL to more severe forms. In particular, the *multiple-parallel* hit hypothesis highlights the link between the intestine, liver, and adipose tissue, that once dysfunctional results in the development of hepatic inflammatory processes and fibrosis and, in severe cases, hepatocellular carcinoma[12].

2.2. *Genetic factors*

In addition to the above mechanisms, genetic polymorphisms and epigenetic factors confer greater susceptibility to develop NAFL and NASH (**Figure 1**).

Several genes have been found associated to NAFLD, like Patatin-like phospholipase domain-containing protein 3 (PNPLA3 or adiponutrin) [13], transmembrane 6 superfamily member 2 (TM6SF2) [14], glucokinase regulator (GCKR) [15], membrane bound O- acyltransferase domain-containing 7 (MBOAT7) and hydroxysteroid 17β- dehydrogenase (HSD17B13), as recently reviewed in [16]. Subjects carrying the genetic mutations are at higher risk to develop NAFLD, NASH, fibrosis, severe liver disease and hepatocellular carcinoma but not comorbidities like diabetes of cardiovascular diseases [16,17].

While subjects with MAFLD are insulin resistant and at increased risk of both hepatic and nonhepatic clinical events [18], the genetic forms of NAFLD are not associated to increased IR either in muscle or in liver when compared with subjects with similar BMI without fatty liver (as summarized in **Table 1**). Subjects with either mutation for PNPLA3 gene [19,20], familial hypobetalipoproteinemia (APOB-FHBL) [21,22], acyl-CoA:diacylglycerol acyltransferase

(DGAT)[23,24], Glucokinase Regulatory Protein (GCKR) [25,26], have fatty liver but peripheral and hepatic insulin sensitivity comparable to matched subjects without mutation and/or NAFLD.

Table 1 - Genetic polymorphism related with NAFLD, IR and associated comorbidities.

Gene	Variant	Function	Increased IR	Risk of T2D	Risk of CVD	MAF	Phenotype
PNPLA3	rs738409 C>G	Lipid droplets remodelling	no	no	no	0.267	↑NAFLD, fibrosis, HCC
TM6SF2	*rs58542926*	VLDL secretion	-	-	↓	0.067	↑NAFLD, fibrosis
GCKR	*rs780094 A>G* *rs1260326 C>T*	Regulation of *de novo* lipogenesis	no	no	-	0.302 0.293	↑NAFLD, fibrosis
HSD17B13	rs72613567 T>TA	Regulation of *de novo* lipogenesis	no	Possibly ↓	Possibly ↓	0.32	↓NASH, fibrosis HCC
KLF6	*rs780094 A>G*	Regulation of *de novo* lipogenesis	no	Possibly ↓	Not known	0.068	↓NASH, fibrosis
MBOAT7	rs641738 C>T	Phospholipid metabolism and TG synthesis	no	Possibly ↓	Possibly ↓	0.408	↑NAFLD, fibrosis, HCC
DGAT2	*rs10899116 C>T* *rs1944438 C>T*	TG synthesis	no	↓	↓	0.05	↑NAFLD
APOB	*several*	VLDL secretion	no	-	↑	<0.001	↑NAFLD, fibrosis

Abbreviations: MAF: minor allele frequency; CVD, cardiovascular disease; HSD17B13, 7-β hydroxysteroid dehydrogenase 13; HT, high TAG; IR, insulin resistance; MBOAT7, Membrane Bound O-Acyltransferase Domain Containing 7; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis NT, normal TAG; PNPLA3, Patatin-like phospholipase domain-containing protein 3; T2D, type 2 diabetes.

Membrane Bound O-Acyltransferase Domain Containing 7 (MBOAT7) or the MBOAT7 rs641738 C>T variant promote NAFLD and liver damage [27]. Teo et al found no association between MBOAT7 rs641738 C>T variant and insulin resistance although hyperinsulinemia, which is an index of insulin resistance, promotes hepatic steatosis also through suppression of MBOAT7, as recently shown by our group [28].

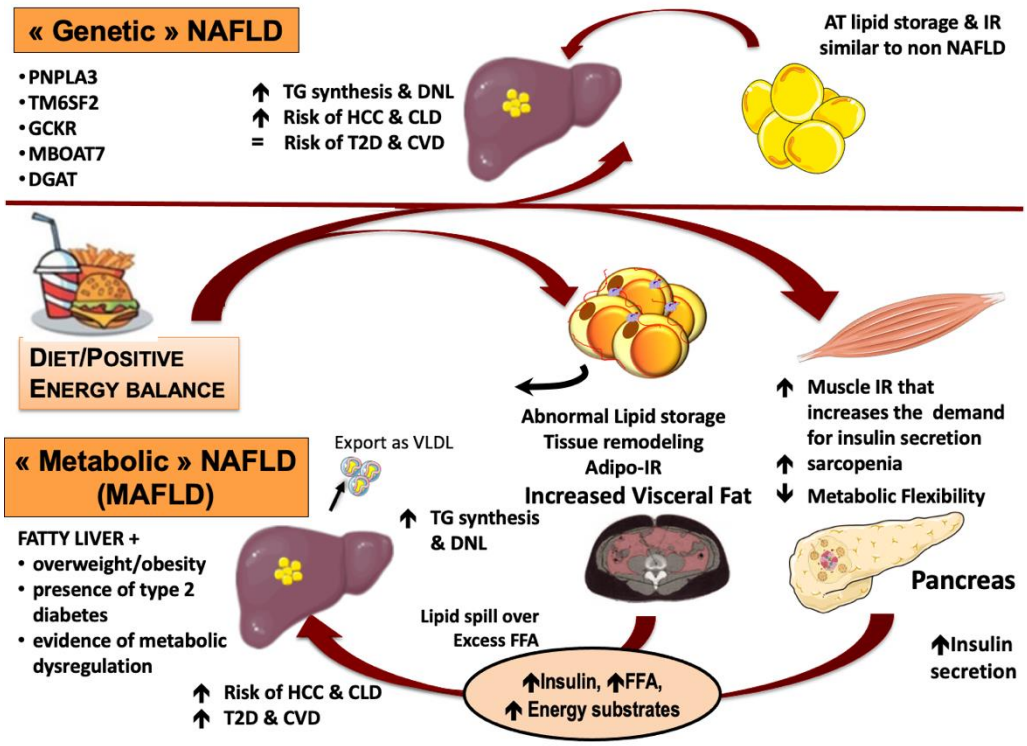

Figure 1 - **Metabolic NAFLD (MAFLD):** this is due mainly to metabolic alterations and an excess energy intake from saturated fat, sugars and refined carbohydrates. Adipose tissue insulin resistance, abdominal lipid storage and tissue remodelling favor hepatic fat accumulation, and causes lipotoxicity in other organs like muscle and pancreas. **Genetic NAFLD:** liver fat accumulates because of genetic causes. In general these patients do not have increased IR or diabetes, and often lean NAFLD patients have some of the common mutations. However, some patients may have both conditions (genetic + metabolic); in this case the risk associated with genotype is synergistically increased by total adiposity and insulin resistance. Figure modified from reference [140]

Other genes that were found associated with NAFLD, but in a protective way, are hydroxysteroid 17-β dehydrogenase 13 (HSD17B13) [29] or Krupper like factor 6 (KLF6) [30,31]. The carriers of these variants not only are protected from the development of NASH and hepatic fibrosis, but also of insulin resistance [29-31].

However, some patients may have both conditions (genetic + metabolic) and they develop insulin resistance with worsening of BMI [32]. In this case the risk associated with PNPLA3, TM6SF2 and GCKR genotype is synergistically increased by total adiposity and IR and the effect of MAFLD on disease outcomes is amplified [18,33].

2.3. *Hepatic fibrosis*

Presence of hepatic fibrosis is not a criteria for diagnosis of NASH, although severe fibrosis (F3 or cirrhosis,F4) is the strongest predictor of mortality and major clinical events in NAFLD [34,35]. Clinical events are predominantly liver-related in NAFLD patients with cirrhosis (F4),

whereas NAFLD patients with bridging fibrosis (F3) have mainly nonhepatic cancers and vascular events [35]. The rate of progression of NASH with advanced fibrosis (F3) to cirrhosis was recently estimated in the placebo arm of the trials of simtuzumab after 96 weeks of observation [36]: progression to cirrhosis occurred in 22% (48/217) of F3 patients, and liver-related clinical events occurred in 19% (50/258) of patients with cirrhosis. However, it is now recognized that liver fibrosis, which was initially thought to be an irreversible process, is dynamic and naturally reversible [37]. As for NASH, there is still no approved pharmacological treatment for liver fibrosis.

3. Insulin resistance (IR) and NAFLD

Chronic liver disease is often associated with insulin resistance and alterations in glucose metabolism and the concomitant presence of steatosis along with type 2 diabetes mellitus and/or evidence of metabolic dysregulation are used for the diagnosis of MAFLD.
Insulin resistance is defined as a defect in insulin action in different tissues and affects both glucose and fatty acid metabolism. We and others have shown that insulin resistance in NAFLD/MAFLD is present at the level of the muscle, liver and adipose tissue [38-45].

3.1. *Hepatic IR*

Hepatic insulin resistance (Hep-IR) and increased fasting glucose production are often present in subjects with NAFLD even if non-obese and without diabetes [41]. Garcia-Morzon et al evaluated several hepatic genes involved in hepatic insulin signaling and glucose metabolism in liver biopsies of non obese patients with NAFL, NASH, chronic hepatitis C (CHC) or normal liver (NL). They showed impaired hepatic insulin signaling in patients with NASH and even more in CHC patients compared to NL, but not in NAFL [46]. Among the hepatic genes with reduced expression there were the insulin receptor substrates (IRS1 and IRS2), the phosphorylated protein kinase B (pAkt), the phosphorylated forkhead box-containing protein O subfamily-1 (FoxO-1), and the phosphorylated 50 adenosine monophosphate-activated protein kinase (pAMPK). The impairment of the insulin pathway was related to fibrosis and

apoptosis but the lack of impairment in non obese NAFL indicates that hepatic fat accumulation occurs before the disruption of mechanisms responsible for insulin signalling.

Among the causes of impairment in insulin action in NAFLD there are the synthesis and accumulation of lipotoxic lipids like lipids containing saturated fatty acids (SFA), diacylglycerols (DAGs) and ceramides [47].

Figure 2 represents the various pathways of supply, uptake, synthesis, and disposal of lipids that contribute to metabolic homeostasis within the liver. High rates of DNL are associated not only with hepatic steatosis [48] but also with postprandial atherogenic dyslipidemia [49] and might explain the increased CVD risk observed in NAFLD, even in the absence of other comorbidities.

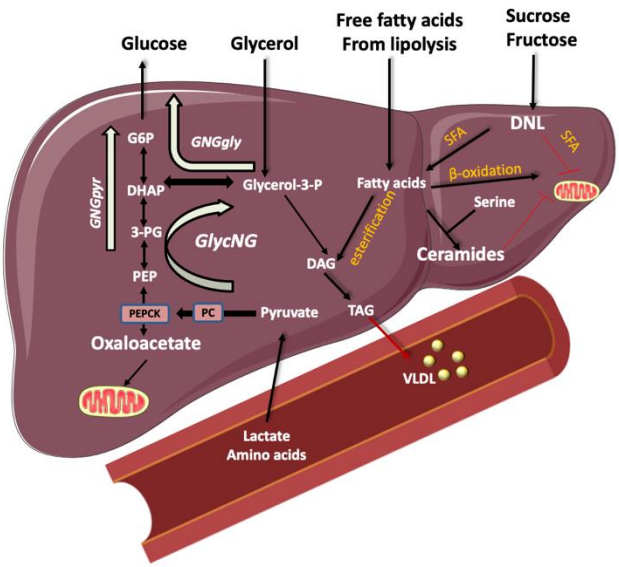

Figure 2- Overview of pathways involved in hepatic lipid accumulation. The level of intrahepatic lipids depends on the equilibrium between lipid acquisition pathways (adipose tissue lipolysis, fatty acid uptake from diet and de novo lipogenesis) and disposal pathways (β-oxidation and export as VLDL). Formation of lipotoxic compounds, such as saturated fatty acids (eg palmitic acids by DNL) and ceramides, can impact mitochondrial function and metabolism.
Abbeviations: 3-PG, 3 phosphoglycerate; DHAP, dihydroxyacetone phosphate; DNL, de novo lipogenesis; G6P, glucose-6-phosphate; GNGglyc, gluconeogenesis from glycerol 3 phosphate; GNGpyr, gluconeogenesis from pyruvate; GlycNG, glyceroneogenesis; NEFA, non-esterified fatty acids; PC, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEPCK, phosphoenol pyruvate carboxylase; SFA, saturated fatty acids; TAG, triacylglycerols; VLDL, very-low-density lipoprotein.

Excess SFA has been implicated in the development and progression from simple steatosis (NAFL) to non-alcoholic steatohepatitis (NASH); not only long term ingestion of SFA [50] but also acute dietary intake of palm oil [51] can stimulate hepatic TAG esterification and accumulation.

Palmitate is the most abundant SFA but it is highly toxic for the cell and, for this reason, it is rapidly metabolized either by esterification to DAG and then TAG or used for the synthesis of other species *in primis* dehydroceramides (DHCER) and ceramides (CER) (**Figure 2 and 3**). Palmitate is also the first product of de novo lipogenesis (DNL), of which the main substrate is dietary fructose, and can be elongated to produce other SFA (**Figure 2**). Subjects with NAFLD, especially if obese, have an increased DNL and palmitate synthesis [40].

The increased availability of FA in the liver (from either AT lipolysis and/or increased hepatic fatty acids (FA) synthesis by DNL) play a fundamental role in NAFLD [50].

Synthesis of diacylglycerols (DAGs) from fatty acid esterification is the first step during TAG synthesis. DAGs have been proposed as bioactive lipids that can induce insulin resistance through alteration of insulin signaling. In the liver, DAGs activates PKCε that impairs hepatic insulin signaling by directly inhibiting insulin receptor-ß tyrosine kinase activity in a dose-dependent manner and impairing the ability of insulin to increase insulin receptor substrate -2 (IRS2) tyrosine phosphorylation [47]; as a consequence, reduced insulin-Akt activation has an inhibitory effect on glycogen synthesis and the suppression of gluconeogenesis[52]. However, Brandon *et al*. recently challenged the role of hepatic PKCε by showing that only global deletion, but not hepatic KO of PKCε, protects mice against diet-induced glucose intolerance and insulin resistance [53].

Ceramides (CER) are bioactive lipids potentially lipotoxic that belong to the class of sphingolipids. CER are synthesized either from the desaturation of DHCer or from sphingomyelin, from sphingosine 1-P or other complex sphingolipids (**Figure 3**).

The exact mechanism by which ceramides influence Hep-IR are unknown, but proposed mechanism relates ceramides to inhibition of Akt [54,55] that impairs FOXO1 phosphorylation and promote gluconeogenesis [56,57].

Also, dihydroceramides (DHCer), the precursors of ceramides (**Figure 3**), have been linked to hepatic insulin resistance, and their concentration is increased in subjects with NASH [58,59]. DHCer are synthesized from a backbone composed of palmitoyl-CoA and serine plus a fatty acid. DHCer is desaturated by inserting a double bond into the backbone to form CER by the enzyme dihydroceramide desaturase 1 (DES1) (**Figure 3**). The possible lipotoxic role of DHCer was recently investigated in mice without the gene *Degs1* that encodes DES1; these mice fed with an obesogenic diet had lower CER levels in serum or tissue and also did not display the lipotoxic effects previously observed in mice with high CER, i.e. increased lipid uptake and

storage, dysfunctional glucose utilization and insulin resistance [55]. These studies indicate that a high dihydroceramide to ceramide ratio (due to a reduction in DES1 activity) might be associated with a more favorable metabolic profile. Thus, not only ceramide synthesis but also degradation (by ceramidases) might play a role in the development of Hep-IR and increased risk to develop type 2 diabetes. Hepatic overexpression of acid ceramidase was associated with reduced hepatic fat accumulation, increased VLDL secretion, and improved total body glucose homeostasis and insulin sensitivity under high-fat diet feeding [54].

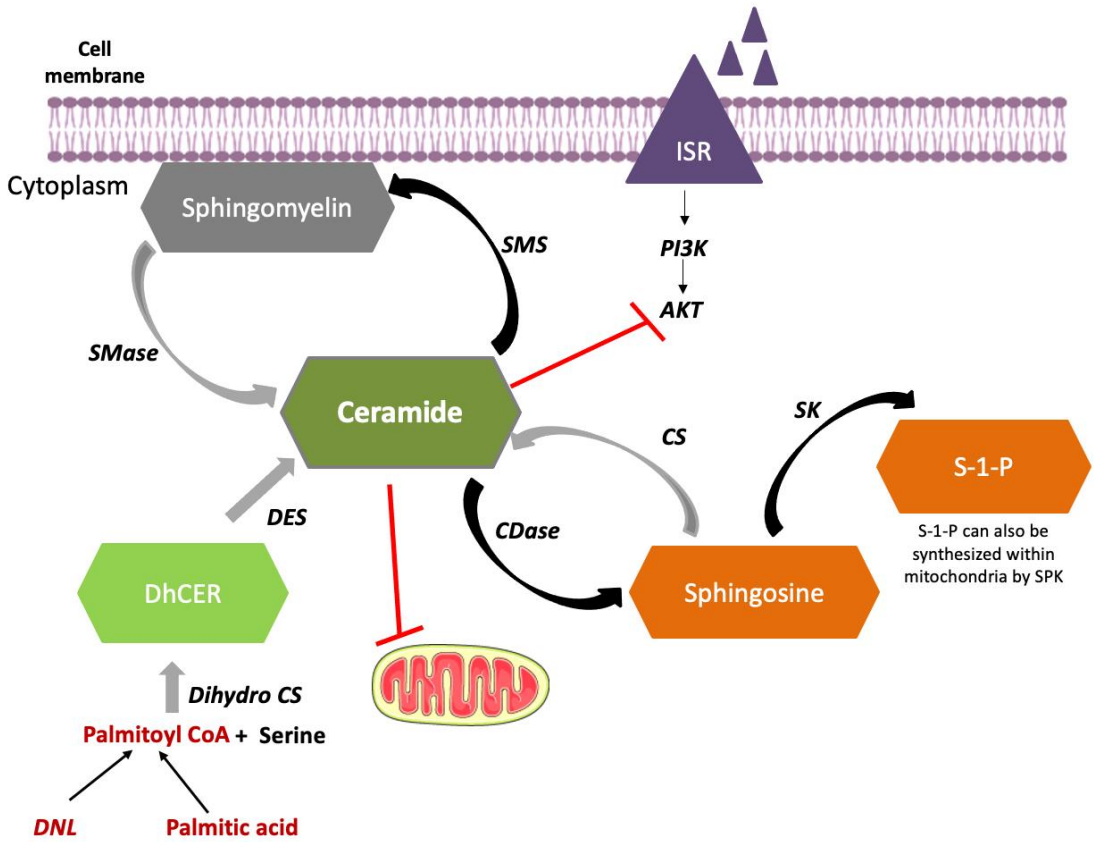

Figure 3 – Overview of pathways involved in sphingolipid synthesis. Ceramide is synthesized de novo from dihydroceramide or from other lipids like sphingomyelin and sphingosine. The synthesis of dihydroceramide involves the formation of backbone (sphinganine) composed by palmitoyl-CoA and serine to which it is then attached a fatty acid. Palmitoyl-CoA can be derived either from circulating palimitic acid or from de novo lipogenesis (DNL). Ceramide impairs both insulin signaling as well as mitochondrial function.

Hyperglycemia can also cause hepatic IR (Hep-IR), since as shown by Tripathy et al chronically induced hyperglycemia by 48 hour glucose infusion in normal glucose tolerant subjects (NGT) was sufficient to cause hepatic IR (Hep-IR) and increase substantially hepatic glucose production that was not responding to insulin infusion[60].

3.2. *Adipose tissue IR*

It is now established that it is the adipose tissue insulin resistance (AT-IR) that drives the hepatic fat accumulation and the development and progression of NAFLD to more severe forms of liver disease [39,41,61]. The main action of insulin in the adipose tissue is to stop lipolysis and stimulate the uptake and esterification of fatty acids. Insulin has a direct effect on several lipases, i.e., it inhibits adipose tissue triglyceride lipase (ATGL) [62], the first step of TG hydrolysis that results in the formation of diacylglycerol (DAG) and the release of one FA and it inhibits hormone-sensitive lipase (HSL) that hydrolysis DAG into glycerol and FA but also have the capability to hydrolize TAG and MAG [63]. Thus, insulin resistance in the adipose tissue results in higher lipolysis and free fatty acids (FFAs) serum concentrations, mainly during a fasting state but also in presence of high insulin levels (eg after a meal) [64,65].

Moreover, insulin activates adipose tissue genes involved in biosynthesis of de novo fatty acids (ACC, ACLY, FASN), glycerolipids (GPAM, LPIN1) and cholesterol (HMGCS, HMGCR, MVD) similarly in obese, never obese and subjects that lost weight after bariatric surgery [66]. The synthesis and accumulation in adipocytes of lipotoxic compounds, as diacylglycerols, cholesterol and ceramides, are associated with insulin resistance. Saturated fatty acids (SFA) in particular palmitic acid are well known lipotoxic lipids and they can stimulate the synthesis of DAGs and CERs and induce chronic inflammation and organ damage not only in the liver but also in the adipose tissue. In obese subjects the adipose tissue mRNA expression of the enzyme ceramide synthase 6 (*CERS6*) and the concentration of CER(C16:0) are increased and the *CERS6* expression is correlated with adiposity and insulin resistance [67].

The composition of fatty acids released during lipolysis strongly depends on the composition of the TG in the adipose tissue which in turn greatly depends on the average intake of fatty acids (over 2-3 years in subjects with stable weight given the low turnover rates, half-life = 1-1.5 years) and from de novo lipogenesis [68]. AT-IR is also associated with inflammation and activation of hepatic macrophages in patients with NAFLD, regardless of obesity and diabetes[42].

3.3. *Muscle IR*

Muscle IR is a characteristic feature of subjects with NAFLD[38-44] but if muscle insulin resistance or glucose intolerance are cause or consequence of fatty liver is still debated.

Bril *et al*. [43] showed that muscle insulin sensitivity, measured using the euglycemic hyperinsulinemic clamp, was decreased in subjects with NAFLD but regardless of intrahepatic triglyceride content (IHTG) measured by MRI (in NAFLD peripheral glucose uptake, Rd, was about half of Rd measured in controls with 1% IHTG). Browers et al.[44] studied glucose and lipid metabolism of non-diabetic (ND) and diabetic (T2D) subjects with/without NAFLD, well matched for gender, age (59 years) and BMI (30 kg/m2). Muscle IR was increased in in subjects with NAFLD although ND (45% more than in controls), but was unexpectedly similar to to that measured in T2D subjects with or without NAFLD.

We also have measured muscle insulin resistance and glucose tolerance during oral glucose tolerance test (OGTT) using the oral glucose insulin sensitivity index (OGIS) in non-diabetic subjects with different aetiology of fatty liver (i.e. NAFL, NASH or chronic hepatitis C (CHC)) and liver biopsy[38,69]. In patients with NAFLD we found an increase in muscle insulin resistance positively associated with the NAFLD activity score (NAS), while no association was found in CHC[38,69]. Interestingly, in NAFLD there was a strong positive association between the degree of hepatic fibrosis and peripheral IR while the worsening of steatosis was associated with more pronounced IR or glucose intolerance[38,69]. Furthermore, we observed that while insulin resistance was increased in subjects with impaired glucose tolerance (IGT) and NAFL/NASH, in patients with IGT and CHC, insulin sensitivity was similar to controls and glucose intolerance was due to dysfunctional insulin secretion[38].

As for the liver possible mechanisms that explain muscle IR in NAFLD are related to the accumulation of lipotoxic species as DAGs and CERs. Muscle DAGs activate PKCθ that impair muscle insulin signaling by reducing insulin-stimulated glucose uptake, potentially delivering excessive glucose to the liver [47]. Also CERs accumulation in muscle has been related to impaired insulin action since they antagonize insulin signaling by inhibiting transmission of signals through phosphatidylinositol-3 kinase (PI3K) and blocking activation of the anabolic enzyme Akt/PKB [70,71].

4. Is mitochondria dysfunction responsible for progression from NAFL to NASH?

The second hit hypothesis points at mitochondrial dysfunction as one of the causes of NASH and loss of hepatic insulin sensitivity [12,72]. Koliaki et al showed that only subjects with NASH, but not those with simple steatosis (NAFL), have reduced mitochondrial respiration and mitochondrial uncoupling associated with Hep-IR [73]. Mitochondrial biogenesis and activity were initially increased in NAFLD to couple with the increase in energetic substrates, indicating mitochondrial flexibility. Similar reports associated increased mitochondrial oxidative function with hepatic TAG accumulation and DNL [74,75].

In humans and mice, elevated body weight, hepatic steatosis and lipolysis are related to increased hepatic FA β-oxidation, TCA cycle activity [75-79] and induction of ketogenesis [76,77,80-82]. Hyperactivity of the TCA cycle appears to be an outlet for excess acetyl-CoA from β-oxidation, but this channeling may lead to ROS generation and tissue inflammation [72]. Recently, it was reported that high fat-fed animals have decreased mitochondrial respiration with succinate, but no change in FA oxidation [83]. Some reports showed that mitochondrial respiration decreases during NASH development[73,77], with reduced synthesis of ATP[84-87]. Others have shown that despite impairment in lipids or glucose metabolism, NAFL/NASH mitochondria were not dysfunctional [88,89]. Despite the differences all these reports indicate that mitochondria can adapt to excess substrates.

The remarkable mitochondrial flexibility in preventing the lipotoxic effects of excessive FFA is often insufficient to refrain from disease progression[76,77]. This flexibility is lost with more severe stages of the disease, as NASH [73]. However, if this is a consequence or cause of NASH is still unclear. Some studies report that mitochondrial function is not an early player on NAFLD progression. Decreased ATP production was observed in hepatic mitochondria of steatotic mice fed an HF diet enriched in SFA, but the disease progression was not due to mitochondrial impairment[89]. Mice fed with a “Western” diet for 24 weeks developed liver steatosis with decreased mitochondrial respiration but no changes in FA ß-oxidation and no traces of ROS, suggesting that mitochondria adapted to the excess substrate[83]. Mice fed for 16 weeks with three different hypercaloric diets HF, high sucrose, or HF-high sucrose diet

presented hepatic steatosis regardless of no changes in mitochondrial oxidative capacity, FA ß-oxidation or ROS production[90].

The discrepancy in the results may be linked to the different animal models and the different stages of the disease of human subjects. A certain mitochondrial metabolic plasticity occurs in NAFLD; however, the current data indicate that mitochondrial dysfunction occurs in severe states of liver disease progression but it does not promote its initial onset.

The dysfunctional mitochondrial activity could also be related to dysfunctional adipose tissue and excess lipolysis due to AT-IR. An et al showed that the impairment of the AT mitochondrial dicarboxylate carrier (mDIC [*SLC25A10*]) that facilitates the transport of the TCA cycle intermediates malate and succinate from the mitochondria to the cytosol, and that is predominantly expressed in the white adipose tissue, was linked to hepatic fat accumulation and NAFLD [91]. In mice with adipocyte mDIC expression either suppressed or overexpressed, lipolysis was respectively enhanced or reduced indicating a role of adipose tissue succinate in the regulation of lipolysis [91]. Moreover, mice with mDIC overexpressed showed resistance to weight gain during HF feeding and improvement in glucose tolerance and insulin sensitivity.

5. Omics markers of severity of liver disease

Several scores have been developed for the diagnosis of NAFLD and fibrosis [92], but so far, none has been validated for the diagnosis of NASH [1]. Currently, metabolomics and lipidomics have been employed to identify markers of NAFLD and of the severity of liver disease

5.1. Metabolomics

Among the metabolites, those that have been associated with severity of liver disease in several clinical studies are amino acids (AA) [93-97]. Dysfunctional amino acid metabolism in NAFLD is linked to peripheral insulin resistance (IR), i.e., in muscle since protein anabolism is stimulated by insulin while protein catabolism should be counteracted by insulin, thus regulating the release of amino acids during fasting (catabolic state) while protein synthesis is stimulated in the postprandial state when also insulin levels are increased [98,99]. Branched-chain amino acids (BCAA) leucine, valine and isoleucine are also metabolized in the adipose

tissue and in the liver and are often increased in all insulin resistant states, including NAFLD, both because of the increased muscle catabolism and because of impaired [95,100-102]. In particular, decreased adipose tissue BCAA oxidation has been associated with insulin-resistant conditions [99,103]. High fasting concentrations of BCAAs and aromatic amino acids (AAA) phenylalanine and tyrosine were found to be significantly associated with the risk of developing T2DM [102]. On the other hand, in patients with liver disease, while BCAAs are usually increased, mainly because of insulin resistance [95,101], AAA are frequently found decreased significantly in chronic hepatic insufficiency [95,104,105]; this being attributable to an impairment in hepatic amino acid catabolism since aromatic AA metabolism occurs mainly in the liver [106]. The Fisher's ratio calculated as BCAA/AAA is used to identify patients with more severe liver disease[105].

Subjects with NAFLD have an increased demand of glutathione to counteract the increased oxidative stress. The main AA involved in the synthesis and catabolism of glutathione are glutamate cysteine and glycine, but also methionine and serine since they are precursors of cysteine. In subjects with NASH, glutamate concentration increases while serine and glycine concentrations decrease [95]. The ratio of glutamate to (serine plus glycine) referred to as GSG index was found increased along with the severity of liver fibrosis in NASH [95] and in patients with HCV, especially in those that did not respond to antiviral treatment [107]. Glycine has been found positively associated with HOMA-IR [95,107,108], and its supplementations improves insulin resistance and hepatic metabolism [109,110].

Other metabolites of interest are the intermediates of the TCA cycle, particularly succinate, as described above. Its decreased transfer from mitochondria to the cytosol of adipocytes has been associated with impaired suppression of lipolysis and peripheral hepatic triglyceride accumulation while the opposite phenotype was shown when the mitochondrial transfer was enhanced [91]. In humans, reduced plasma succinate concentrations during a glucose load were found decreased in obese subjects and rescued after significant weight loss and bariatric surgery [111].

5.2. Lipidomics

The development of NAFLD is highly related to the alteration in lipid metabolism. However, not only the total amount of lipids but also their composition has been associated with

alteration in hepatic metabolism. The most relevant lipidomic studies are summarized on **Table 2**.

Lipidomic analysis of liver biopsies by high-resolution mass spectrometry has shown that subjects with NAFLD have an increased accumulation of saturated triacylglycerols (TAG) alongside other lipotoxic species like ceramides (CER) and diacylglycerols (DAGs) [58,59,112] .

In humans, circulating DAGs were found positively correlated with both HOMA-IR [59,113] and Hep-IR [47,113].

Increased levels of TAG and changes in their composition (i.e., those containing more saturated than unsaturated fatty acids) are highly associated with hepatic fat accumulation and insulin resistance [43,45,52,58,59,113]. How different FAs (e.g. saturated vs. unsaturated) are implicated on hepatic lipid accumulation and progression from NAFLD to NASH is still under debate [114]. The degree of saturation of TAG is highly dependent on both the diet and the rate of DNL. Plasma/serum TAG rich in saturated/monounsaturated fatty acids that derive from DNL and desaturation, i.e., TAG with low carbon number and low double bond content, were observed increased in NAFLD in several studies and positively correlated with IR [115-117] whereas those containing polyunsaturated fatty acids, including omega-3 and omega-6 fatty acids (PUFA, which derive from diet) were decreased in NAFLD/NASH and negatively associated with IR in healthy subjects with a wide range of insulin sensitivity [115,116,118]. These TAGs were also found predictive of type 2 diabetes development in Finnish men [119].

Moreover TAG containing the SFA palmitate, i.e., TAG(48:0) ,or stearate, ie TAG(50:0) ,or their desaturated forms palmitoleic acid and oleate, ie TAG(48:1) and TAG(50:1), were found associated both to the presence of NAFLD [117] and increased risk to develop T2D [120].

Table 2. Summary of key lipidomic studies in NAFLD

Study	Number of participants/ BMI/T2D	Diagnosis	Matrix	Main findings
Ooi et al 2021 Ref 112	50 CT BMI 45 Kg/m^2 T2D (14%) 110 NAFLD BMI 47 Kg/m^2 T2D (23%) 16 NASH BMI 50 Kg/m^2 T2D (33%)	Liver biopsy	Plasma	CER d18:0/16:0, d18:0/18:0, d18:0/20:0, d18:0/22:0, d18:0/24:0, d18:0/24:1, DAG SFA (16:0, 18:0), MUFA (18:1), PUFA (18:2), TAG SFA (16:0, 17:0, 18:0), MUFA (18:1), PUFA (18:2, 20:3, 20:4) **↑ NAFLD**
			Liver	Total dhCER, TAG, DAG, CER d18:0/18:0, d18:0/20:0, d18:0/22:0, d18:0/24:0, d18:0/24:1, LPC 26:0, PI 18:0/22:5, CE 18:0, > 20 DAG, > 40 TAG **↑ NAFLD and NASH** PC 15-MHDA/18:2, PC 15-MHDA/22:6, PC 17:1/18:2, 18:1/22:6, CE 22:5 **↓ NAFLD and NASH** Total CER, CE, THC, CER d18:1/16:0, d18:1/18:0, d18:1/20:0, d18:1/22:0, d18:1/24:0, GM3 d18:1/20:0, PC 28:0, 31:0, PC O-40:7, PS 38:4, CE 18:3, FC **↑ NAFLD** SM 37:2, d18:2/20:0, PC 17:0/18:2, 18:1/18:2, 39:5, 17:0/22:6, PC P-38:5, PE 18:1/22:6, PE P-18:1/22:4, 20:1/22:6 **↓ NAFLD** Total DHC, DHC d18:1/18:0, d18:1/22:0, d18:1/24:0, SM d18:0/16:0, PC 36:0, **↑ NASH** PC 16:1/20:4, 38:6, PC 15-MHDA/20:4, PE 16:0/20:4, 38:5, PI 38:5, **↓ NASH**

Apostolopoulou et al 2018 Ref 58	7 CT BMI 25 Kg/m^2 7 NAFLD BMI 51 Kg/m^2 7 NASH BMI 56 Kg/m^2	Liver biopsy	Liver	Total CER, LactCER 24:1, HexCER 22:0, 24:0, 24:1; dhCER 16:0, 22:0, 24:1 **↑ NASH**
			Serum	dhCER 20:0 **↑ NAFLD** Total dhCER, dhCER 16:0, 22:0, 24:1 **↑ NASH**
Puri et al 2007 Ref 118	9 CT BMI 34.5 Kg/m^2 9 NAFLD BMI 37.5 Kg/m^2 9 NASH BMI 34 Kg/m^2	Liver biopsy	Liver	TAG, DAG, total cholesterol, SFA, n6: n3 ratio **↑ NAFLD and NASH** TAG, FA 20:4n-6, TAG FA 22:6n-3 **↓ NAFLD and NASH** PC, PE **↓ NAFLD** FC, LysoPC **↑ NASH**
Puri et al 2009 Ref 132	50 CT BMI 21.2 Kg/m^2, No T2D NAFLD BMI 35.2 Kg/m2, T2D (28%) 50 NASH BMI 32.1 Kg/m^2, T2D (31%)	Liver biopsy	Plasma	TAG, DAG, FFA and CE MUFA, CE, DAG, PC, PE with 18:3 or 20:3 FA **↑ NAFLD and NASH** DAG, PC, PE, TAG SFA **↓ NAFLD** 22:6n-3/22:5n-3 ratio in PC, PE **↓ NASH**

Barr et al 2010 Ref 133	9 CT BMI 47 Kg/m^2 No T2D 24 NAFLD BMI 44.8 Kg/m^2 No T2D 9 NASH BMI 43.2 Kg/m^2 No T2D	Liver biopsy	Serum	FFA 16:0, SM 18:0/16:0, 18:1/18:0, PC 28:0, LysoPC 20:2, 20:1, LPE P-16:0 **↑ NAFLD and NASH** FFA 18:3, 20:2n6, SM 18:1/12:0, 18:2/14:0, 18:2/16:0, 36:3, PC 18:0/22:6 **↓ NAFLD and NASH** PC (14:0/20:4, 16:0/20:3, P-18:0/20:4), LysoPC 18:1 **↑ NASH** FFA 20:4, LysoPC P-24:0, P-22:0, O-20:0 **↓ NASH**
Anjani et al 2015 Ref 131	24 CT BMI 47 Kg/m^2 No T2D 22 NASH BMI 45 Kg/m^2 T2D (86%)	Liver biopsy	Serum	PC, PE, and PG, CER (d18:0/22:0, d18:1/16:0, d18:1/18:0, d18:1/20:0, d18:1/22:0, d18:1/23:0, d18:2/20:0, d18:2/18:0, d18:2/20:0, d18:2/21:0, d18:2/22:0, d18:2/23:0); SM(36:1), PC (32:0, 32:1, 34:1, 34:3.36:1, 36:3, 36:4, 36:5, 38:3, 38:4, 38:5, 38:6, 40:4, 40:5, 40:6), PE 34:1, 34:3, 36:1, 36:2, 36:4, 36:5, 38:3, 38:4, 38:5, 38:6, 40:4, 40:5, 40:6, 40:7), LysoPC (16:0, 16:1, 20:3, 22:5), PG (36:1, 36:2, 36:3, 38:3, 38:4); PI (32:1, 34:1, 36:4, 38:4, 40:4, 40:5) **↑ NASH** CER d18:1/24:0, SM 42:3 **↓ NASH**

Gorden et al 2015 Ref 141[1]	31 CT BMI 40 Kg/m^2 17 NAFLD BMI 46 Kg/m^2 20 NASH BMI 47 Kg/m^2 20 Cirrhosis BMI 32 Kg/m^2	Liver biopsy	Plasma	TAG, CE, CER, DAG (36:2), HexCER d18:1/24:1, GlucCER d18:/24:1, d18:1/26:1, PC 36:4, 38:4, PE 38:5, 38:4, 40:6, 40:5, LysoPC 16:0, PI 36:1, 38:4, 38:3 **↑ NAFLD and NASH** HexCER d18:1/24:1, d18:1/26:1, CER m18:1/16:0, m18:1/26:1, GlucCer d18:1/26:1, 18:1/26:0, PC 32:0, PI 36:1 **↑ Cirrhosis** Total cholesterol, CE 18:2, 20:4, 20:3, TAG 52:4, 52:3, DAG 36:2, CER d18:1/18:0, 18:1/20:0, d18:1/22:0, d18:1/24:1, d18:1/24:0, d18:0/18:0, d180/24:1, m18:1/16:0, m18:1/26:1, m18:0/18:0, m18:0/20:0, m18:0/22:0, m18:0/24:0, HexCER d18:1/24:1, d18:1/26:1, GlucCer d18:1/24:1, d18:1/26:1, 18:1/26:0, SM d18:1/18:1, d18:1/18:0, d18:1/20:0, d18:1/22:0, d18:1/24:0, PC 32:0, 34:3, 36:4; 38:6, 38:5, 38:4, 38:3, 40:6, PE 36:4, 38:6, 38:5, 38:4, 40:6, 40:5, LysoPC 16:0, PI 36:1, 38:4, 38:3 **↓ Cirrhosis**
Chiappini et al 2017 Ref 123	7 CT BMI 21 Kg/m^2 39 NAFLD BMI 25 Kg/m^2 15 NASH BMI 31 Kg/m^2	Liver biopsy	Liver	SFA (14:0, 16:0, 18:0) **↑ NASH** PC, PE, PI, PS PC/PE, SM **↓ NASH**
Tiwari-Heckler et al 2018 Ref 129[1]	28 CT 25 NAFLD 42 NASH	Liver biopsy	Plasma	TAG, SM, PC **↑ NAFLD and NASH** LysoPE **↓ NAFLD and NASH**
Sanders et al 2018 Ref 117	663 CT BMI 25 Kg/m^2, No T2D 233 NAFLD BMI 31 Kg/m^2, No T2D	Ultrasound	Plasma	TAG 54:2, 48:1, 48:2, 50:1, 50:2 **↑ NAFLD** TAG 52:3, 52:4, 56:7, 56:6, 54:4, 56:8 **↓ NAFLD**

CT, control; NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; DAG: diacylglycerol; TAG: triacylglycerol; SFA: saturated fatty acids; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acids; FC: free cholesterol; CE, cholesteryl esters; FFA: free fatty acids; SM: sphingomyelin; PC: phosphatidylcholine; LysoPC, lysophosphatidylcholine; PI: phosphatidylinositol; PS: phosphatidylserine; PE: phosphatidylethanolamine; LysoPE, lyso phosphatidylethanolamine; CER: Ceramides; dhCER, dihydroceramides; DHC/Hex2Cer, dihexosylceramide; THC, trihexosylceramide; GM3; GM3 ganglioside; LactCER, lactosylceramide; GlucCER, glucosylceramide

Other lipids, such as glycerophospholipids [i.e. phosphatidylcholine (PC), phosphatidylethanolamines (PE), lysophosphatidylcholine (LPC)] and sphingolipids [CER and sphingomyelin (SM)] have also been reported to be altered in obesity and IR state and recognized to be predictors of type 2 diabetes either when increased (as several PCs) [119] or decreased as LPC 18:2 [119,121]. Recently, Oresic et al identified that a lipid triplet (serum-lipid signature comprising three molecular lipids) — the triglyceride 16:0/18:0/18:1, and the phosphatidylcholines PC 18:1/22:6 and PC O-24:1/20:4 — as a biomarker of NAFLD (AUROC 0.79, sensitivity 69.3% and specificity 74.5% when applied to the combined biomarker discovery and validation series) [122].

PC, one of the most abundant phospholipids in mammalian cells, is reduced in the liver across the NAFLD spectrum with a mechanism not fully characterized [118,123,124]. Hepatic PC are necessary for the VLDL assembly and secretion thus reduced PC levels in the liver led to impaired hepatic TG export through the VLDL [125,126]. Dietary strategies aimed at reducing hepatic PC (i.e methionine-choline deficient diet) are widely used to promote hepatic fat accumulation in murine models [127]. Of note, in humans, circulating total PC do not seem to reflect the hepatic lipidome as shown by Mannisto and colleagues [128], although specific PCs have shown to be strongly correlated [112]. Part of this might be attributable to the extensive lipidomic remodeling occurring in circulation by the lipoprotein remodeling enzymes such as phospholipid transfer protein, phospholipases A2 and lecithin:cholesterol acyltransferase, which thus far has been poorly investigated. Furthermore, circulating PC have been reported to be increased in NAFLD vs controls by some authors but not from others [129,130 131]. Puri et al reported that the percentage of plasma saturated PC were increased while polyunsaturated PC decreased in NAFLD vs controls [132]. Increased saturated plasma PC in NASH vs controls have also been reported by others [133] and Gorden et al [115] found that PC(32:0), i.e. a phosphatidylcholine that contains 2 palmitic acids, was increased 1.39 fold in NASH vs simple steatosis. This suggests that the fatty acyl chain saturation levels might be a better marker of NAFLD than total PC.

Hepatic and serum concentrations of sphingolipids (including ceramides and sphingomyelins) are often found increased in metabolic diseases and also in NASH compared to simple steatosis [58,115]. In the liver CER accumulation is proportional to hepatic TAG and associated with IR [58,59]. There is a good correlation between hepatic and serum ceramides since most of

the plasma/serum CER are secreted by the liver as demonstrated by Watt et al that looked at circulating ceramides after fatty acid infusion [134]. Gorden et al [115] performed lipidomics of plasma and liver tissues of patients with steatosis, NASH, or cirrhosis vs control subjects without liver disease. Whereas hepatic ceramide concentrations were not different, plasma DHCer 18:0 and 24:1 and CER 18:0, 20:0, 22:0, 24:0, 24:1, and in steatotic patients were similar to control subjects but higher in the NASH and lower in the cirrhosis group compared to controls. The studies conducted by Chaurasia et al [55] indicate that a reduced dihydroceramide to ceramide ratio (an index of increased in DES1 activity) might be associated with a more unfavorable metabolic profile.

The leading cause of death in NAFLD patients is CVD, although the mechanism is not entirely understood [135]. A CER-based risk score to predict CVD in primary and secondary prevention has been proposed and validated in different cohorts [136,137]. The score is referred to as CERT1 and it is based on the levels of specific CER (CER (d18:1/16:0), CER (d18:1/18:0), CER (d18:1/24:1)) and their respective ratios to CER (d18:1/24:0). Patients are stratified into quartiles according to a score ranging from 0 to 12 points. The quartiles are defined as low, moderate, increased, and high risk, showing linear increase in CVD risk across the quartiles independently of other known risk factors such as LDL cholesterol levels [138]. Recently, Hilvo and colleagues proposed a novel lipidomic CVD risk stratification score based upon the CERT1, defined as CERT2 (PMID: 31209498). The latter included only one CER to CER ratio (CER (d18:1/24:1)/ CER (d18:1/24:0)), two CER to PC ratios ((CER (d18:1/16:0)/(PC 16:0/22:5), (CER (d18:1/18:0)/ (PC 14:0/22:6)) and PC (16:0/16:0) [139]. Like CERT1, CERT2 was also based on a point-based scale (ranging from 0 to 12) for quartile stratification but provided a better risk estimation for CVD. Whether these scores can provide a stratification tool to predict CVD in NAFLD patients has not been reported yet and it would be an interesting venue to investigate.

There is not always agreement among different studies due in part to technical differences since there is not a standardized method, and also because of different inclusion criteria of the study population, including presence or absence of pharmacological treatments, differences in HDL-C, LDL-C, and blood pressure levels, blood sampling in fasting state or not, small sample sizes, and lack of validation cohorts. It is therefore crucial to standardize lipidomic studies to have a clearer picture of the circulating lipidome in NAFLD, obesity and IR.

6. Conclusions

In this review we have discussed several mechanisms that have been recently indicated as primary drivers of progression from NAFL to NASH. In particular, the important role of adipose tissue insulin resistance and lipotoxic species like DAGs and Ceramides and the shift in lipid composition towards an increase in SFA (either because of diet and/or increased DNL) that has been shown in several studies to be associated with more severe forms of NAFLD and insulin resistance. Not only lipids, but also amino acid metabolism is altered in NAFLD/NASH and some amino acids, like BCAA, Aromatic AA or GSG index might be used as markers of altered hepatic metabolism and severity of NAFLD. Although the genetic mutations are highly associated with NAFLD and increase the risk of progression to NASH cirrhosis, these are not associated to insulin resistance but rather to a defect in hepatic lipid secretion that results in steatosis.

Acknowledgments and Funding: AG acknowledge the financial support from the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie Grant Agreement No. 722619 (FOIE GRAS project) and Grant Agreement No. 734719 (mtFOIE GRAS project), and S. G.. is recipient of an Early Stage Researcher grant under the FOIE GRAS project

Conflict of Interest statement/Financial Disclosure

The authors declare no conflict of interest for this manuscript.

References

1. European Association for the Study of the Liver, European Association for the Study of Diabetes, European Association for the Study of Obesity. EASL-EASD-EASO Clinical Practice Guidelines for the management of non-alcoholic fatty liver disease. *J Hepatol.* 2016;64(6):1388-1402.
2. Younossi Z, Tacke F, Arrese M, et al. Global Perspectives on Nonalcoholic Fatty Liver Disease and Nonalcoholic Steatohepatitis. *Hepatology.* 2019;69(6):2672-2682.
3. Bedossa P, Tordjman J, Aron-Wisnewsky J, et al. Systematic review of bariatric surgery liver biopsies clarifies the natural history of liver disease in patients with severe obesity. *Gut.* 2017;66(9):1688-1696.
4. Souto KP, Meinhardt NG, Ramos MJ, Ulbrich-Kulkzynski JM, Stein AT, Damin DC. Nonalcoholic fatty liver disease in patients with different baseline glucose status undergoing bariatric surgery: analysis of intraoperative liver biopsies and literature review. *Surg Obes Relat Dis.* 2018;14(1):66-73.
5. Younossi ZM, Golabi P, de Avila L, et al. The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. *J Hepatol.* 2019;71(4):793-801.
6. Williams R, Karuranga S, Malanda B, et al. Global and regional estimates and projections of diabetes-related health expenditure: Results from the International Diabetes Federation Diabetes Atlas, 9th edition. *Diabetes research and clinical practice.* 2020;162:108072.
7. Mantovani A, Petracca G, Beatrice G, Tilg H, Byrne CD, Targher G. Non-alcoholic fatty liver disease and risk of incident diabetes mellitus: an updated meta-analysis of 501 022 adult individuals. *Gut.* 2021;70(5):962-969.
8. Eslam M, Newsome PN, Sarin SK, et al. A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. *J Hepatol.* 2020;73(1):202-209.
9. Younossi ZM, Rinella ME, Sanyal AJ, et al. From NAFLD to MAFLD: Implications of a Premature Change in Terminology. *Hepatology.* 2021;73(3):1194-1198.
10. Gastaldelli A, Cusi K. From NASH to diabetes and from diabetes to NASH: Mechanisms and treatment options. *JHEP Reports.* 2019;1(4):312-328.
11. Day CP, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology.* 1998;114(4):842-845.
12. Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology.* 2010;52(5):1836-1846.
13. Romeo S, Kozlitina J, Xing C, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet.* 2008;40(12):1461-1465.
14. Liu YL, Reeves HL, Burt AD, et al. TM6SF2 rs58542926 influences hepatic fibrosis progression in patients with non-alcoholic fatty liver disease. *Nat Commun.* 2014;5:4309.
15. Tan HL, Zain SM, Mohamed R, et al. Association of glucokinase regulatory gene polymorphisms with risk and severity of non-alcoholic fatty liver disease: an interaction study with adiponutrin gene. *Journal of gastroenterology.* 2014;49(6):1056-1064.
16. Eslam M, George J. Genetic contributions to NAFLD: leveraging shared genetics to uncover systems biology. *Nature reviews Gastroenterology & hepatology.* 2020;17(1):40-52.
17. Brouwers M, Simons N, Stehouwer CDA, Isaacs A. Non-alcoholic fatty liver disease and cardiovascular disease: assessing the evidence for causality. *Diabetologia.* 2020;63(2):253-260.
18. Liu Z, Suo C, Shi O, et al. The Health Impact of MAFLD, a Novel Disease Cluster of NAFLD, Is Amplified by the Integrated Effect of Fatty Liver Disease-Related Genetic Variants. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association.* 2020.
19. Kantartzis K, Peter A, Machicao F, et al. Dissociation between fatty liver and insulin resistance in humans carrying a variant of the patatin-like phospholipase 3 gene. *Diabetes.* 2009;58(11):2616-2623.
20. Sevastianova K, Kotronen A, Gastaldelli A, et al. Genetic variation in PNPLA3 (adiponutrin) confers sensitivity to weight loss-induced decrease in liver fat in humans. *The American journal of clinical nutrition.* 2011;94(1):104-111.
21. Amaro A, Fabbrini E, Kars M, et al. Dissociation between intrahepatic triglyceride content and insulin resistance in familial hypobetalipoproteinemia. *Gastroenterology.* 2010;139(1):149-153.
22. Visser ME, Lammers NM, Nederveen AJ, et al. Hepatic steatosis does not cause insulin resistance in people with familial hypobetalipoproteinaemia. *Diabetologia.* 2011;54(8):2113-2121.
23. Kantartzis K, Machicao F, Machann J, et al. The DGAT2 gene is a candidate for the dissociation between fatty liver and insulin resistance in humans. *Clin Sci (Lond).* 2009;116(6):531-537.
24. Monetti M, Levin MC, Watt MJ, et al. Dissociation of hepatic steatosis and insulin resistance in mice overexpressing DGAT in the liver. *Cell metabolism.* 2007;6(1):69-78.

25. Perez-Martinez P, Delgado-Lista J, Garcia-Rios A, et al. Glucokinase regulatory protein genetic variant interacts with omega-3 PUFA to influence insulin resistance and inflammation in metabolic syndrome. *PLoS One.* 2011;6(6):e20555.
26. Vaxillaire M, Cavalcanti-Proenca C, Dechaume A, et al. The common P446L polymorphism in GCKR inversely modulates fasting glucose and triglyceride levels and reduces type 2 diabetes risk in the DESIR prospective general French population. *Diabetes.* 2008;57(8):2253-2257.
27. Teo K, Abeysekera KWM, Adams L, et al. rs641738C>T near MBOAT7 is associated with liver fat, ALT and fibrosis in NAFLD: A meta-analysis. *J Hepatol.* 2021;74(1):20-30.
28. Meroni M, Dongiovanni P, Longo M, et al. Mboat7 down-regulation by hyper-insulinemia induces fat accumulation in hepatocytes. *EBioMedicine.* 2020;52:102658.
29. Luukkonen PK, Tukiainen T, Juuti A, et al. Hydroxysteroid 17-beta dehydrogenase 13 variant increases phospholipids and protects against fibrosis in nonalcoholic fatty liver disease. *JCI insight.* 2020;5(5).
30. Bechmann LP, Gastaldelli A, Vetter D, et al. Glucokinase links Kruppel-like factor 6 to the regulation of hepatic insulin sensitivity in nonalcoholic fatty liver disease. *Hepatology.* 2012;55(4):1083-1093.
31. Miele L, Beale G, Patman G, et al. The Kruppel-like factor 6 genotype is associated with fibrosis in nonalcoholic fatty liver disease. *Gastroenterology.* 2008;135(1):282-291 e281.
32. Stender S, Kozlitina J, Nordestgaard BG, Tybjaerg-Hansen A, Hobbs HH, Cohen JC. Adiposity amplifies the genetic risk of fatty liver disease conferred by multiple loci. *Nat Genet.* 2017;49(6):842-847.
33. Carlsson B, Linden D, Brolen G, et al. Review article: the emerging role of genetics in precision medicine for patients with non-alcoholic steatohepatitis. *Aliment Pharmacol Ther.* 2020;51(12):1305-1320.
34. Ekstedt M, Hagstrom H, Nasr P, et al. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. *Hepatology.* 2015;61(5):1547-1554.
35. Vilar-Gomez E, Calzadilla-Bertot L, Wai-Sun Wong V, et al. Fibrosis Severity as a Determinant of Cause-Specific Mortality in Patients With Advanced Nonalcoholic Fatty Liver Disease: A Multi-National Cohort Study. *Gastroenterology.* 2018;155(2):443-457 e417.
36. Sanyal AJ, Harrison SA, Ratziu V, et al. The Natural History of Advanced Fibrosis Due to Nonalcoholic Steatohepatitis: Data From the Simtuzumab Trials. *Hepatology.* 2019;70(6):1913-1927.
37. Schuppan D, Surabattula R, Wang XY. Determinants of fibrosis progression and regression in NASH. *J Hepatol.* 2018;68(2):238-250.
38. Svegliati-Baroni G, Gaggini M, Carli F, et al. Mechanisms for increased risk of diabetes in chronic liver diseases. *Liver international : official journal of the International Association for the Study of the Liver.* 2020;40(10):2489-2499.
39. Lomonaco R, Ortiz-Lopez C, Orsak B, et al. Effect of adipose tissue insulin resistance on metabolic parameters and liver histology in obese patients with nonalcoholic fatty liver disease. *Hepatology.* 2012;55(5):1389-1397.
40. Smith GI, Shankaran M, Yoshino M, et al. Insulin resistance drives hepatic de novo lipogenesis in nonalcoholic fatty liver disease. *J Clin Invest.* 2020;130(3):1453-1460.
41. Bugianesi E, Gastaldelli A, Vanni E, et al. Insulin resistance in non-diabetic patients with non-alcoholic fatty liver disease: sites and mechanisms. *Diabetologia.* 2005;48(4):634-642.
42. Rosso C, Kazankov K, Younes R, et al. Crosstalk between adipose tissue insulin resistance and liver macrophages in non-alcoholic fatty liver disease. *J Hepatol.* 2019;71(5):1012-1021.
43. Bril F, Barb D, Portillo-Sanchez P, et al. Metabolic and histological implications of intrahepatic triglyceride content in nonalcoholic fatty liver disease. *Hepatology.* 2017;65(4):1132-1144.
44. Brouwers B, Schrauwen-Hinderling VB, Jelenik T, et al. Metabolic disturbances of non-alcoholic fatty liver resemble the alterations typical for type 2 diabetes. *Clin Sci.* 2017;131(15):1905-1917.
45. Gastaldelli A, Cusi K, Pettiti M, et al. Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. *Gastroenterology.* 2007;133(2):496-506.
46. Garcia-Monzon C, Lo Iacono O, Mayoral R, et al. Hepatic insulin resistance is associated with increased apoptosis and fibrogenesis in nonalcoholic steatohepatitis and chronic hepatitis C. *J Hepatol.* 2011;54(1):142-152.
47. Samuel VT, Shulman GI. The pathogenesis of insulin resistance: integrating signaling pathways and substrate flux. *J Clin Invest.* 2016;126(1):12-22.
48. Lambert JE, Ramos-Roman MA, Browning JD, Parks EJ. Increased de novo lipogenesis is a distinct characteristic of individuals with nonalcoholic fatty liver disease. *Gastroenterology.* 2014;146(3):726-735.
49. Matikainen N, Adiels M, Soderlund S, et al. Hepatic lipogenesis and a marker of hepatic lipid oxidation, predict postprandial responses of triglyceride-rich lipoproteins. *Obesity.* 2014;22(8):1854-1859.

50. Luukkonen PK, Sadevirta S, Zhou Y, et al. Saturated Fat Is More Metabolically Harmful for the Human Liver Than Unsaturated Fat or Simple Sugars. *Diabetes Care.* 2018;41(8):1732-1739.
51. Hernandez EA, Kahl S, Seelig A, et al. Acute dietary fat intake initiates alterations in energy metabolism and insulin resistance. *J Clin Invest.* 2017;127(2):695-708.
52. Petersen MC, Shulman GI. Roles of Diacylglycerols and Ceramides in Hepatic Insulin Resistance. *Trends in pharmacological sciences.* 2017;38(7):649-665.
53. Brandon AE, Liao BM, Diakanastasis B, et al. Protein Kinase C Epsilon Deletion in Adipose Tissue, but Not in Liver, Improves Glucose Tolerance. *Cell Metab.* 2019;29(1):183-191 e187.
54. Xia JY, Holland WL, Kusminski CM, et al. Targeted Induction of Ceramide Degradation Leads to Improved Systemic Metabolism and Reduced Hepatic Steatosis. *Cell Metab.* 2015;22(2):266-278.
55. Chaurasia B, Tippetts TS, Mayoral Monibas R, et al. Targeting a ceramide double bond improves insulin resistance and hepatic steatosis. *Science.* 2019;365(6451):386-392.
56. Sajan MP, Ivey RA, Lee MC, Farese RV. Hepatic insulin resistance in ob/ob mice involves increases in ceramide, aPKC activity, and selective impairment of Akt-dependent FoxO1 phosphorylation. *J Lipid Res.* 2015;56(1):70-80.
57. Accili D. Insulin Action Research and the Future of Diabetes Treatment: The 2017 Banting Medal for Scientific Achievement Lecture. *Diabetes.* 2018;67(9):1701-1709.
58. Apostolopoulou M, Gordillo R, Koliaki C, et al. Specific Hepatic Sphingolipids Relate to Insulin Resistance, Oxidative Stress, and Inflammation in Nonalcoholic Steatohepatitis. *Diabetes Care.* 2018;41(6):1235-1243.
59. Luukkonen PK, Zhou Y, Sadevirta S, et al. Hepatic ceramides dissociate steatosis and insulin resistance in patients with non-alcoholic fatty liver disease. *J Hepatol.* 2016;64(5):1167-1175.
60. Tripathy D, Merovci A, Basu R, Abdul-Ghani M, DeFronzo RA. Mild Physiologic Hyperglycemia Induces Hepatic Insulin Resistance in Healthy Normal Glucose-Tolerant Participants. *J Clin Endocrinol Metab.* 2019;104(7):2842-2850.
61. Musso G, Cassader M, De Michieli F, Rosina F, Orlandi F, Gambino R. Nonalcoholic steatohepatitis versus steatosis: adipose tissue insulin resistance and dysfunctional response to fat ingestion predict liver injury and altered glucose and lipoprotein metabolism. *Hepatology.* 2012;56(3):933-942.
62. Chakrabarti P, Kim JY, Singh M, et al. Insulin inhibits lipolysis in adipocytes via the evolutionarily conserved mTORC1-Egr1-ATGL-mediated pathway. *Mol Cell Biol.* 2013;33(18):3659-3666.
63. Recazens E, Mouisel E, Langin D. Hormone-sensitive lipase: sixty years later. *Progress in lipid research.* 2020;82:101084.
64. Gastaldelli A, Gaggini M, DeFronzo RA. Role of Adipose Tissue Insulin Resistance in the Natural History of Type 2 Diabetes: Results From the San Antonio Metabolism Study. *Diabetes.* 2017;66(4):815-822.
65. Groop LC, Bonadonna RC, DelPrato S, et al. Glucose and free fatty acid metabolism in non-insulin-dependent diabetes mellitus. Evidence for multiple sites of insulin resistance. *J Clin Invest.* 1989;84(1):205-213.
66. Mileti E, Kwok KH, Andersson DP, et al. Human white adipose tissue displays selective insulin resistance in the obese state. *Diabetes.* 2021.
67. Turpin SM, Nicholls HT, Willmes DM, et al. Obesity-induced CerS6-dependent C16:0 ceramide production promotes weight gain and glucose intolerance. *Cell Metab.* 2014;20(4):678-686.
68. Hodson L, Skeaff CM, Fielding BA. Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Progress in lipid research.* 2008;47(5):348-380.
69. Rosso C, Mezzabotta L, Gaggini M, et al. Peripheral insulin resistance predicts liver damage in nondiabetic subjects with nonalcoholic fatty liver disease. *Hepatology.* 2016;63(1):107-116.
70. Stratford S, Hoehn KL, Liu F, Summers SA. Regulation of insulin action by ceramide: dual mechanisms linking ceramide accumulation to the inhibition of Akt/protein kinase B. *J Biol Chem.* 2004;279(35):36608-36615.
71. Powell DJ, Hajduch E, Kular G, Hundal HS. Ceramide disables 3-phosphoinositide binding to the pleckstrin homology domain of protein kinase B (PKB)/Akt by a PKCzeta-dependent mechanism. *Mol Cell Biol.* 2003;23(21):7794-7808.
72. Satapati S, Kucejova B, Duarte JA, et al. Mitochondrial metabolism mediates oxidative stress and inflammation in fatty liver. *J Clin Invest.* 2015;125(12):4447-4462.
73. Koliaki C, Szendroedi J, Kaul K, et al. Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. *Cell Metab.* 2015;21(5):739-746.

74. Miele L, Grieco A, Armuzzi A, et al. Hepatic mitochondrial beta-oxidation in patients with nonalcoholic steatohepatitis assessed by 13C-octanoate breath test. *The American journal of gastroenterology.* 2003;98(10):2335-2336.
75. Sunny NE, Parks EJ, Browning JD, Burgess SC. Excessive hepatic mitochondrial TCA cycle and gluconeogenesis in humans with nonalcoholic fatty liver disease. *Cell Metab.* 2011;14(6):804-810.
76. Patterson RE, Kalavalapalli S, Williams CM, et al. Lipotoxicity in steatohepatitis occurs despite an increase in tricarboxylic acid cycle activity. *Am J Physiol Endocrinol Metab.* 2016;310(7):E484-494.
77. Satapati S, Sunny NE, Kucejova B, et al. Elevated TCA cycle function in the pathology of diet-induced hepatic insulin resistance and fatty liver. *J Lipid Res.* 2012;53(6):1080-1092.
78. Iozzo P, Bucci M, Roivainen A, et al. Fatty acid metabolism in the liver, measured by positron emission tomography, is increased in obese individuals. *Gastroenterology.* 2010;139(3):846-856, 856 e841-846.
79. Jelenik T, Kaul K, Sequaris G, et al. Mechanisms of Insulin Resistance in Primary and Secondary Nonalcoholic Fatty Liver. *Diabetes.* 2017;66(8):2241-2253.
80. Sunny NE, Satapati S, Fu X, et al. Progressive adaptation of hepatic ketogenesis in mice fed a high-fat diet. *Am J Physiol Endocrinol Metab.* 2010;298(6):E1226-1235.
81. Cotter DG, Ercal B, Huang X, et al. Ketogenesis prevents diet-induced fatty liver injury and hyperglycemia. *J Clin Invest.* 2014;124(12):5175-5190.
82. Mannisto VT, Simonen M, Hyysalo J, et al. Ketone body production is differentially altered in steatosis and non-alcoholic steatohepatitis in obese humans. *Liver international : official journal of the International Association for the Study of the Liver.* 2015;35(7):1853-1861.
83. Stankova P, Kucera O, Peterova E, et al. Adaptation of Mitochondrial Substrate Flux in a Mouse Model of Nonalcoholic Fatty Liver Disease. *Int J Mol Sci.* 2020;21(3).
84. Schmid AI, Szendroedi J, Chmelik M, Krssak M, Moser E, Roden M. Liver ATP synthesis is lower and relates to insulin sensitivity in patients with type 2 diabetes. *Diabetes Care.* 2011;34(2):448-453.
85. Abdelmalek MF, Lazo M, Horska A, et al. Higher dietary fructose is associated with impaired hepatic adenosine triphosphate homeostasis in obese individuals with type 2 diabetes. *Hepatology.* 2012;56(3):952-960.
86. Szendroedi J, Chmelik M, Schmid AI, et al. Abnormal hepatic energy homeostasis in type 2 diabetes. *Hepatology.* 2009;50(4):1079-1086.
87. Fritsch M, Koliaki C, Livingstone R, et al. Time course of postprandial hepatic phosphorus metabolites in lean, obese, and type 2 diabetes patients. *Am J Clin Nutr.* 2015;102(5):1051-1058.
88. Lund MT, Kristensen M, Hansen M, et al. Hepatic mitochondrial oxidative phosphorylation is normal in obese patients with and without type 2 diabetes. *J Physiol.* 2016;594(15):4351-4358.
89. Einer C, Hohenester S, Wimmer R, et al. Mitochondrial adaptation in steatotic mice. *Mitochondrion.* 2018;40:1-12.
90. Simoes ICM, Karkucinska-Wieckowska A, Janikiewicz J, et al. Western Diet Causes Obesity-Induced Nonalcoholic Fatty Liver Disease Development by Differentially Compromising the Autophagic Response. *Antioxidants (Basel).* 2020;9(10).
91. An YA, Chen S, Deng Y, et al. The mitochondrial dicarboxylate carrier prevents hepatic lipotoxicity by inhibiting white adipocyte lipolysis. *J Hepatol.* 2021.
92. European Association for the Study of the Liver, List of panel members, Berzigotti A, et al. Easl Clinical Practice Guidelines (Cpgs) On Non-Invasive Tests For Evaluation Of Liver Disease Severity And Prognosis- 2020 Update. *J Hepatol.* 2021.
93. Kalhan SC, Guo L, Edmison J, et al. Plasma metabolomic profile in nonalcoholic fatty liver disease. *Metabolism.* 2011;60(3):404-413.
94. Lake AD, Novak P, Shipkova P, et al. Branched chain amino acid metabolism profiles in progressive human nonalcoholic fatty liver disease. *Amino Acids.* 2015;47(3):603-615.
95. Gaggini M, Carli F, Rosso C, et al. Altered amino acid concentrations in NAFLD: Impact of obesity and insulin resistance. *Hepatology.* 2018;67(1):145-158.
96. Kakazu E, Sano A, Morosawa T, et al. Branched chain amino acids are associated with the heterogeneity of the area of lipid droplets in hepatocytes of patients with non-alcoholic fatty liver disease. *Hepatol Res.* 2019;49(8):860-871.
97. Barr J, Caballeria J, Martinez-Arranz I, et al. Obesity-dependent metabolic signatures associated with nonalcoholic fatty liver disease progression. *J Proteome Res.* 2012;11(4):2521-2532.
98. Biolo G, Gastaldelli A, Zhang XJ, Wolfe RR. Protein synthesis and breakdown in skin and muscle: a leg model of amino acid kinetics. *Am J Physiol.* 1994;267(3 Pt 1):E467-474.

99. Neinast MD, Jang C, Hui S, et al. Quantitative Analysis of the Whole-Body Metabolic Fate of Branched-Chain Amino Acids. *Cell Metab.* 2019;29(2):417-429 e414.
100. Luzi L, Castellino P, DeFronzo RA. Insulin and hyperaminoacidemia regulate by a different mechanism leucine turnover and oxidation in obesity. *Am J Physiol.* 1996;270(2 Pt 1):E273-281.
101. Newgard CB, An J, Bain JR, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab.* 2009;9(4):311-326.
102. Wang TJ, Larson MG, Vasan RS, et al. Metabolite profiles and the risk of developing diabetes. *Nature medicine.* 2011;17(4):448-453.
103. Herman MA, She P, Peroni OD, Lynch CJ, Kahn BB. Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels. *J Biol Chem.* 2010;285(15):11348-11356.
104. Morgan MY, Marshall AW, Milsom JP, Sherlock S. Plasma amino-acid patterns in liver disease. *Gut.* 1982;23(5):362-370.
105. Fischer JE, Rosen HM, Ebeid AM, James JH, Keane JM, Soeters PB. The effect of normalization of plasma amino acids on hepatic encephalopathy in man. *Surgery.* 1976;80(1):77-91.
106. Matthews DE. An overview of phenylalanine and tyrosine kinetics in humans. *J Nutr.* 2007;137(6 Suppl 1):1549S-1555S; discussion 1573S-1575S.
107. Gaggini M, Carli F, Rosso C, et al. Altered Metabolic Profile and Adipocyte Insulin Resistance Mark Severe Liver Fibrosis in Patients with Chronic Liver Disease. *Int J Mol Sci.* 2019;20(24).
108. Mardinoglu A, Bjornson E, Zhang C, et al. Personal model-assisted identification of NAD(+) and glutathione metabolism as intervention target in NAFLD. *Mol Syst Biol.* 2017;13(3):916.
109. Rom O, Liu Y, Liu Z, et al. Glycine-based treatment ameliorates NAFLD by modulating fatty acid oxidation, glutathione synthesis, and the gut microbiome. *Sci Transl Med.* 2020;12(572).
110. White PJ, Lapworth AL, McGarrah RW, et al. Muscle-Liver Trafficking of BCAA-Derived Nitrogen Underlies Obesity-Related Glycine Depletion. *Cell Rep.* 2020;33(6):108375.
111. Astiarraga B, Martinez L, Ceperuelo-Mallafre V, et al. Impaired Succinate Response to a Mixed Meal in Obesity and Type 2 Diabetes Is Normalized After Metabolic Surgery. *Diabetes Care.* 2020;43(10):2581-2587.
112. Ooi GJ, Meikle PJ, Huynh K, et al. Hepatic lipidomic remodeling in severe obesity manifests with steatosis and does not evolve with non-alcoholic steatohepatitis. *J Hepatol.* 2021;75(3):524-535.
113. Magkos F, Su X, Bradley D, et al. Intrahepatic diacylglycerol content is associated with hepatic insulin resistance in obese subjects. *Gastroenterology.* 2012;142(7):1444-1446 e1442.
114. Alkhouri N, Dixon LJ, Feldstein AE. Lipotoxicity in nonalcoholic fatty liver disease: not all lipids are created equal. *Expert Rev Gastroenterol Hepatol.* 2009;3(4):445-451.
115. Gorden DL, Myers DS, Ivanova PT, et al. Biomarkers of NAFLD progression: a lipidomics approach to an epidemic. *J Lipid Res.* 2015;56(3):722-736.
116. Kotronen A, Velagapudi VR, Yetukuri L, et al. Serum saturated fatty acids containing triacylglycerols are better markers of insulin resistance than total serum triacylglycerol concentrations. *Diabetologia.* 2009;52(4):684-690.
117. Sanders FWB, Acharjee A, Walker C, et al. Hepatic steatosis risk is partly driven by increased de novo lipogenesis following carbohydrate consumption. *Genome Biol.* 2018;19(1):79.
118. Puri P, Baillie RA, Wiest MM, et al. A lipidomic analysis of nonalcoholic fatty liver disease. *Hepatology.* 2007;46(4):1081-1090.
119. Suvitaival T, Bondia-Pons I, Yetukuri L, et al. Lipidome as a predictive tool in progression to type 2 diabetes in Finnish men. *Metabolism.* 2018;78:1-12.
120. Rhee EP, Cheng S, Larson MG, et al. Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. *J Clin Invest.* 2011;121(4):1402-1411.
121. Gall WE, Beebe K, Lawton KA, et al. alpha-hydroxybutyrate is an early biomarker of insulin resistance and glucose intolerance in a nondiabetic population. *PLoS One.* 2010;5(5):e10883.
122. Oresic M, Hyotylainen T, Kotronen A, et al. Prediction of non-alcoholic fatty-liver disease and liver fat content by serum molecular lipids. *Diabetologia.* 2013;56(10):2266-2274.
123. Chiappini F, Coilly A, Kadar H, et al. Metabolism dysregulation induces a specific lipid signature of nonalcoholic steatohepatitis in patients. *Sci Rep.* 2017;7:46658.
124. Hall Z, Chiarugi D, Charidemou E, et al. Lipid Remodeling in Hepatocyte Proliferation and Hepatocellular Carcinoma. *Hepatology.* 2021;73(3):1028-1044.

125. Jacobs RL, Devlin C, Tabas I, Vance DE. Targeted deletion of hepatic CTP:phosphocholine cytidylyltransferase alpha in mice decreases plasma high density and very low density lipoproteins. *J Biol Chem.* 2004;279(45):47402-47410.
126. Buchman AL, Dubin MD, Moukarzel AA, et al. Choline deficiency: a cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation. *Hepatology.* 1995;22(5):1399-1403.
127. Im YR, Hunter H, de Gracia Hahn D, et al. A Systematic Review of Animal Models of NAFLD Finds High-Fat, High-Fructose Diets Most Closely Resemble Human NAFLD. *Hepatology.* 2021.
128. Mannisto V, Kaminska D, Karja V, et al. Total liver phosphatidylcholine content associates with non-alcoholic steatohepatitis and glycine N-methyltransferase expression. *Liver international : official journal of the International Association for the Study of the Liver.* 2019;39(10):1895-1905.
129. Tiwari-Heckler S, Gan-Schreier H, Stremmel W, Chamulitrat W, Pathil A. Circulating Phospholipid Patterns in NAFLD Patients Associated with a Combination of Metabolic Risk Factors. *Nutrients.* 2018;10(5).
130. Zhou Y, Oresic M, Leivonen M, et al. Noninvasive Detection of Nonalcoholic Steatohepatitis Using Clinical Markers and Circulating Levels of Lipids and Metabolites. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association.* 2016;14(10):1463-1472 e1466.
131. Anjani K, Lhomme M, Sokolovska N, et al. Circulating phospholipid profiling identifies portal contribution to NASH signature in obesity. *J Hepatol.* 2015;62(4):905-912.
132. Puri P, Wiest MM, Cheung O, et al. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology.* 2009;50(6):1827-1838.
133. Barr J, Vazquez-Chantada M, Alonso C, et al. Liquid chromatography-mass spectrometry-based parallel metabolic profiling of human and mouse model serum reveals putative biomarkers associated with the progression of nonalcoholic fatty liver disease. *J Proteome Res.* 2010;9(9):4501-4512.
134. Watt MJ, Barnett AC, Bruce CR, Schenk S, Horowitz JF, Hoy AJ. Regulation of plasma ceramide levels with fatty acid oversupply: evidence that the liver detects and secretes de novo synthesised ceramide. *Diabetologia.* 2012;55(10):2741-2746.
135. Targher G, Byrne CD, Lonardo A, Zoppini G, Barbui C. Non-alcoholic fatty liver disease and risk of incident cardiovascular disease: A meta-analysis. *J Hepatol.* 2016;65(3):589-600.
136. Laaksonen R, Ekroos K, Sysi-Aho M, et al. Plasma ceramides predict cardiovascular death in patients with stable coronary artery disease and acute coronary syndromes beyond LDL-cholesterol. *European heart journal.* 2016;37(25):1967-1976.
137. Havulinna AS, Sysi-Aho M, Hilvo M, et al. Circulating Ceramides Predict Cardiovascular Outcomes in the Population-Based FINRISK 2002 Cohort. *Arteriosclerosis, thrombosis, and vascular biology.* 2016;36(12):2424-2430.
138. Hilvo M, Vasile VC, Donato LJ, Hurme R, Laaksonen R. Ceramides and Ceramide Scores: Clinical Applications for Cardiometabolic Risk Stratification. *Front Endocrinol (Lausanne).* 2020;11:570628.
139. Hilvo M, Meikle PJ, Pedersen ER, et al. Development and validation of a ceramide- and phospholipid-based cardiovascular risk estimation score for coronary artery disease patients. *European heart journal.* 2020;41(3):371-380.
140. Gastaldelli A. Insulin resistance and reduced metabolic flexibility: cause or consequence of NAFLD? *Clin Sci.* 2017;131(22):2701-2704.
141. Gorden DL, Myers DS, Ivanova PT, et al. Biomarkers of NAFLD progression: a lipidomics approach to an epidemic. *J Lipid Res.* 2015;56(3):722-736.