Metabolomics and lipidomics in NAFLD: biomarkers and non-invasive diagnostic tests

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Abstract | Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases worldwide and is often associated with aspects of metabolic syndrome. Despite its prevalence and the importance of early diagnosis, there is a lack of robustly validated biomarkers for diagnosis, prognosis and monitoring of disease progression in response to a given treatment. In this Review, we provide an overview of the contribution of metabolomics and lipidomics in clinical studies to identify biomarkers associated with NAFLD and nonalcoholic steatohepatitis (NASH). In addition, we highlight the key metabolic pathways in NAFLD and NASH that have been identified by metabolomics and lipidomics approaches and could potentially be used as biomarkers for non-invasive diagnostic tests. Overall, the studies demonstrated alterations in amino acid metabolism and several aspects of lipid metabolism including circulating fatty acids, triglycerides, phospholipids and bile acids. Although we report several studies that identified potential biomarkers, few have been validated.

Nonalcoholic fatty liver disease (NAFLD) is often associated with metabolic comorbidities such as obesity, hyperlipidaemia, type 2 diabetes mellitus (T2DM) and metabolic syndrome[1], and it is the most common liver disease[2]. The key features of NAFLD and nonalcoholic steatohepatitis (NASH) are described in BOX 1. In Western countries, NAFLD has a prevalence of 20–40%, and NAFLD progresses to NASH in ~10–30% of affected patients[1,2]. Although NAFLD is the most prevalent liver disease worldwide, there is currently no approved pharmacotherapy or approved biomarkers to readily assess disease progression and/or regression of NASH in response to a given treatment. According to the FDA, a biomarker is "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions"[3]. Although several studies have evaluated potential biomarkers, few have been validated and even fewer have been used in clinical practice. In addition, it is unknown whether relative differences in molecular biomarker concentrations would be sufficient for diagnosis or prognosis of NASH. Moreover, the role of biomarker trajectories in individual patients is not clearly defined, and it is unknown whether monitoring these changes is essential for understanding their long-term outcome. NASH is a progressive disease that is histologically defined by the presence of hepatic fat (steatosis) with inflammation and hepatocellular ballooning, which can lead to further liver injury, advanced fibrosis, cirrhosis and hepatocellular carcinoma (HCC)[4–7]. The reference standard of assessment for treatment efficacy in NASH is liver histology[8]. Although hepatic decompensation or liver-related death are the hard clinical end points, owing to the difficulties of demonstrating drug efficacy based on these factors, 'likely' surrogate markers of these end points are accepted by regulatory bodies such as the FDA for provisional approval and are currently used to evaluate the outcome of clinical trials. These surrogate end points are histological: resolution of NASH without worsening of fibrosis, and reduction in fibrosis without worsening of NASH[9].

Although liver histology is currently the reference standard for the assessment of NASH progression and treatment efficacy, repeated liver biopsies are difficult to perform in patients with NASH because of health risk, sampling error, subjective variation in pathological interpretation, and cost[10]. Currently, surrogate clinical parameters are used to monitor response to treatment[11–13], including circulating levels of aminotransferase activities, elastography and non-invasive determination of

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Key points

- Nonalcoholic fatty liver disease (NAFLD) affects 25% of the adult world population; in about 20% of patients, it can progress to nonalcoholic steatohepatitis (NASH), which can lead to cirrhosis.
- There is an urgent need for development of clinically relevant biomarkers and non-invasive diagnostic tests for NAFLD.
- Metabolomics and lipidomics approaches have provided insightful evidence of altered metabolic pathways in NAFLD and NASH.
- There is an association between circulating amino acids and steatohepatitis, and impairment in amino acid metabolism in NAFLD is strongly correlated with insulin resistance, particularly in the muscle.
- An increase in oxidative stress results in a reduction in hepatic glutathione levels and is associated with liver damage and the progression of NAFLD to NASH.
- NASH is strongly associated with alterations in circulating fatty acids and intact lipids, which is partially due to alterations in de novo liver lipogenesis, lipolysis rate and VLDL metabolism.

steatosis. However, there is still a great demand to establish and validate other biomarkers that predict NASH progression and/or response to treatment.

Based on this demand, the Innovative Medicines Initiative Consortium 'Liver Investigation: Testing Marker Utility in Steatohepatitis' (IMI2 LITMUS) aims to develop, validate and advance towards qualification of biomarkers for testing NAFLD across a range of contexts of use. Different methodologies are considered to be part of this aim, and metabolomics is one of these approaches. The metabolomics approach is a comprehensive biochemical profiling technique, built upon decades of fundamental research in biochemistry, physiology, analytical chemistry and bioinformatics. As the human plasma lipidome is enormously diverse and complex[14], lipidomics has emerged as a related subfield of metabolomics, aiming to comprehensively characterize different molecular lipid species in biological systems as well as to investigate their function[15]. Metabolomics aims to assess metabolism and identify downstream functions of genes and proteins. The chemical and biological diversity of the metabolome is enormous[16]. This diversity requires several analytical strategies, with an extensive array of techniques, to cover a wide range of metabolites while ensuring data output quality (FIG. 1). The advantages and limitations of different analytical approaches are summarized in TABLE 1.

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Genome-scale metabolic modelling has emerged as a powerful approach that can provide valuable insights into the mechanisms of disease progression, and it has been used to attempt to identify biomarkers and underlying pathways for NAFLD–NASH[17–19]. Genome-scale metabolic models (GSMMs), as a scaffold, connect metabolic genes and their products (that is, enzymes) with metabolic pathways. Moreover, GSMMs can be constrained and contextualized with multi-'omics' datasets, thereby connecting an organism's genotype to its phenotype. Using GSMMs one can study changes in the global metabolic network topology, evaluate metabolite flux distribution, and predict uptake and secretion of specific metabolites[20].

In this Review, we evaluate the use of metabolomics and lipidomics, in clinical studies to identify biomarkers associated with NAFLD and NASH. Owing to some overlaps between NASH and other metabolic disorders such as T2DM, dyslipidaemia and insulin resistance, it is worth mentioning that some of the investigated biomarkers are associated with other aspects of metabolic disorders. Here, we focus on the metabolites that are reported to be associated with diagnosis, prognosis and progression of NAFLD and NASH. This discussion includes metabolomics studies that found any associations between these metabolites and disease pathology and clinical outcome, as well as those metabolites that are associated with disease progression or improvement following clinical intervention or treatment of NASH. Most metabolomics analyses have been conducted on plasma samples obtained after an overnight fast. We present the key metabolic pathways that have been identified in NAFLD–NASH through metabolomics approaches and that could potentially be used as biomarkers for non-invasive diagnostic tests.

Metabolomics and amino acids in NAFLD

An association between circulating amino acids and steatohepatitis has been found in several clinical studies[21–25]. An impairment in amino acid metabolism in NAFLD is linked to insulin resistance, particularly in muscle cells, in which protein metabolism and amino acid concentrations are regulated by insulin, which inhibits protein catabolism and amino acid release during fasting and promotes glucose uptake as well as protein synthesis in the postprandial state[26] (FIG. 2). Insulin resistance, commonly defined as an impaired insulin action, results in increased insulin secretion and circulating insulin concentration to achieve similar metabolic effects compared with insulin-sensitive individuals. An insulin-resistant state is often associated with increased fasting concentration of essential amino acids in circulation due to increased muscle protein catabolism despite high insulin concentrations[27,28] and impaired amino acid catabolism in both liver and adipose tissue[29]. Patients with NAFLD often exhibit an imbalance between protein synthesis and protein catabolism, leading to sarcopenia[30]. Moreover, obesity, a well-known insulin-resistant state, is associated with increased fasting amino acid levels, in particular branched-chain amino acids (BCAAs)[23,31,32]. However, there is some inconsistency in the identified patterns and levels of amino acids in patients with NAFLD–NASH.

Box 1 | **Key features of NAFLD and NASH**

Nonalcoholic fatty liver disease (NAFLD) is closely associated with obesity, dyslipidaemia and type 2 diabetes mellitus. Nonalcoholic steatohepatitis (NASH) is a progressive disease histologically defined by the presence of hepatic fat (steatosis) with inflammation and hepatocellular ballooning, and can lead to further liver injury, advanced fibrosis, cirrhosis and hepatocellular carcinoma[4–7]. A number of classification systems for histological severity of NAFLD and NASH have been proposed. The most commonly used in clinical trials are NAS (NAFLD activity score) and SAF (steatosis, activity, fibrosis). The NAS scoring system comprises four histological features that are evaluated semiquantitatively: steatosis (0–3), lobular inflammation (0–2), hepatocellular ballooning (0–2) and fibrosis (0–4)[186]. Lipotoxicity is one of the key components of NASH, which leads to toxic lipid accumulation, mitochondrial dysfunction, cell injury and chronic inflammation. Hepatic steatosis is mainly caused by an imbalance between intrahepatic triglyceride production and secretion. An excess of triglyceride content in NAFLD is associated with alterations in both adipose tissue and hepatic lipid metabolism, including increased rates of adipose tissue lipolysis, hepatic de novo lipogenesis and VLDL secretion[66,67].

Branched-chain amino acids in NAFLD. BCAAs (leucine, isoleucine and valine) are among the most studied amino acids, and, because they are essential (that is, not produced by human cells), their increased fasting concentrations in plasma reflect a catabolic state and often insulin resistance[23,33]. Kalhan et al.[21] examined the fasting-state plasma metabolomes in a group of individuals with biopsy-proven steatosis ($n = 11$) and steatohepatitis ($n = 24$) but without diabetes, and compared them with the metabolomes in a group of healthy individuals ($n = 25$) to identify biomarkers associated with steatosis and NASH. The levels of circulating BCAAs and of phenylalanine, glutamate and aspartate were higher in patients with NASH than in healthy individuals. However, BCAA levels were not significantly higher in patients with steatosis than in healthy individuals[21]. These patterns of change in levels of circulating BCAAs were also confirmed in liver biopsy samples from healthy individuals ($n = 17$), and from patients with steatosis ($n = 4$), NASH with steatosis ($n = 14$) and NASH without steatosis ($n = 23$)[22]. Human liver tissue samples were analysed using high-resolution mass spectrometry (MS)-based metabolomics to characterize BCAA liver profiles in the progressive stages of NAFLD[22]. The study showed a significant increase ($P \leq 0.05$) in the levels of BCAAs, tyrosine and phenylalanine during the transition from steatosis to NASH (in patients with NASH both with and without steatosis)[22]. Qi et al. were able to distinguish steatosis from NASH on the basis of the metabolic profiles of serum samples from 38 patients with steatosis and 21 patients with NASH using MS-based metabolomics[34]. They identified glutamate, L-glutamine and pyroglutamate from among five main metabolites able to distinguish simple steatosis from NASH, with changes of 1.80-fold, 1.81-fold and 2.26-fold, respectively[34]. They further assessed the quantitative serum levels of pyroglutamate and found higher levels in patients with NASH (7.89 mmol/l; 95% CI 5.35–10.41 mmol/l) than in patients with steatosis (3.57 mmol/l; 95% CI 0.98–7.34 mmol/l) or a healthy control group (1.48 mmol/l; 95% CI 0.48–3.97 mmol/l)[34]. Notably, the cohort sizes in both studies were small, and they used two different biofluids (plasma and serum), which might explain the differences in identified metabolites distinguishing NASH from simple steatosis. In another clinical study from the outpatient clinic of Shuguang Hospital, China, including 33 patients with NAFLD and 45 patients with NASH, the researchers performed urinary MS-based metabolomics and found elevated levels of citrulline, arginine and valine in patients with NAFLD compared with the levels in healthy individuals, and higher urinary levels of lysine, valine, citrulline, arginine, threonine, tyrosine and leucine in patients with NASH than in healthy individuals. However, when comparing urinary amino acid and related metabolites, only pyroglutamic acid could distinguish between NAFLD and NASH[35].

Kakazu et al. examined the correlation between hepatocyte lipid droplet heterogeneity and circulating BCAA levels in 80 patients with NAFLD–NASH and observed a positive association between plasma BCAA levels and maximum area of lipid droplets in the liver[24]. They further observed that a diet high in BCAAs induced heterogeneity in the lipid droplets in a mouse model of NAFLD and proposed that some BCAAs could potentially modulate the size and heterogeneity of lipid droplets in hepatocytes in patients with NAFLD–NASH. They concluded that supplementation with BCAAs could potentially be useful for advanced-stage NAFLD but not for early-stage NAFLD[24]. Indeed, a 48-week supplementation with BCAAs in patients with cirrhosis ameliorated hypoalbuminaemia, maintained skeletal muscle mass and decreased muscle fat, and improved glucose sensitivity, all factors that might contribute to improving the survival of these patients[36].

Lehmann et al. applied a targeted IDQ metabolomics platform (Biocrates) to differentiate insulin-resistant and insulin-sensitive subgroups in 40 patients with NAFLD at baseline and following a 9-month lifestyle intervention, referred to as the Tuebingen lifestyle intervention programme (TULIP)[37], by measuring plasma levels of 180 metabolites[38]. They identified seven metabolites, including leucine, isoleucine and ornithine, that discriminated insulin-sensitive from insulin-resistant patients with NAFLD, with a high discriminatory power both at baseline and following lifestyle intervention (area under the receiver operating characteristic curve (AUROC) 0.77 at baseline (positive predictive value 0.75, negative predictive value 0.88, sensitivity 0.90, specificity 0.70); AUROC 0.80 at follow-up (positive predictive value 0.83, negative predictive value 0.77, sensitivity 0.75, specificity 0.85))[38]. Interestingly, they did not observe lower levels of these metabolites in insulin-sensitive patients than in insulin-resistant patients, in contrast to findings reported in patients with metabolic disorders (insulin resistance or diabetes)[29,32].

Amino acids and insulin resistance. In large-scale clinical studies, increased fasting plasma levels of BCAAs have been found to be positively associated with peripheral insulin resistance and risk of developing T2DM[29,32]. In a cohort of 2,422 individuals without diabetes in the Framingham Offspring Study with a 12-year follow-up, Wang and colleagues performed MS-based lipidomics in 189 individuals who developed diabetes and 189 propensity-matched individuals as controls; they

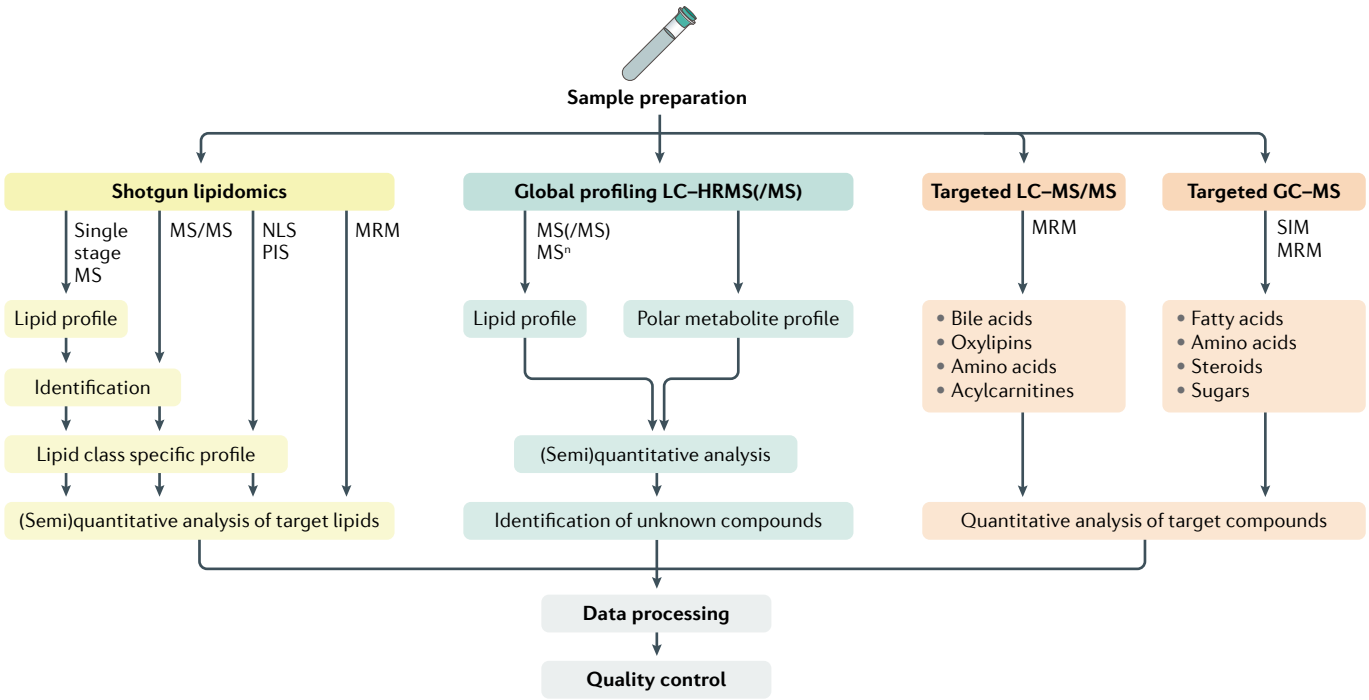


Fig. 1 | **Overview of analytical approaches and workflows as commonly applied in metabolomic analysis, including lipidomics.** For the analysis of complex lipids, most common approaches use direct infusion mass spectrometry (MS), so-called shotgun lipidomics, and comprehensive profiling using liquid chromatography (LC) coupled with high-resolution MS (HRMS, or tandem MS, also known as MS/MS). Various targeted approaches are being used for the analysis of specific groups of metabolites, such as LC–MS/MS or gas chromatography coupled with MS (GC–MS). MRM, multiple reaction monitoring; MS^n, multi-stage mass spectrometry; NLS, neutral loss scanning; PIS, precursor ion scanning; SIM, selected ion monitoring.

found that higher baseline fasting concentrations of the BCAAs leucine, isoleucine and valine and of the aromatic amino acids (AAAs) tyrosine and phenylalanine were significantly associated with the risk of developing T2DM[32]. They observed that fasting plasma levels of these amino acids were elevated at baseline when individuals were normoglycaemic, and their increase was associated with an up to fourfold increased risk of future diabetes[32]. These findings were then validated in an independent cohort. AAAs, in particular phenylalanine and its metabolite tyrosine, are frequently found to be decreased in patients with liver disease[39], whereas BCAAs are increased, mainly because of insulin resistance[29]. However, as liver disease progresses, the opposite is often observed: high AAAs and reduced BCAAs, especially in patients with chronic hepatic insufficiency[40,41], which occurs mainly because of an impairment in hepatic amino acid catabolism[40–42]. Thus, a low ratio of BCAA to AAA (known as Fischer's ratio) has been used to identify patients with more severe liver disease[41]. A simplified version of Fischer's ratio that uses only tyrosine as the denominator has been shown to be a prognostic factor for early HCC[43]. Notably, whereas BCAAs are mainly catabolized in muscle and adipose tissue[29], AAA metabolism occurs mainly in the liver[44].

Most previous studies in which upregulated BCAAs were found, however, mainly included individuals with obesity and insulin resistance, and it is therefore not easy to establish whether increased fasting concentrations of amino acids are related to NAFLD or to peripheral insulin resistance. Newgard et al. have shown that BCAAs, methionine, glutamate to glutamine ratio, and the AAAs phenylalanine and tyrosine contribute to distinguishing individuals with obesity from lean individuals[29]. A difference in amino acid concentrations in patients with NASH between those with and without obesity was also observed by Gaggini et al.[23]. Moreover, BCAA levels were strongly correlated with homeostasis model assessment of insulin resistance (HOMA-IR). Gaggini et al. addressed this question in patients with NAFLD by evaluating amino acid concentrations using quantitative gas chromatography MS (GC–MS) metabolomics and the association with fasting insulin resistance in 44 patients with biopsy-proven NAFLD without diabetes with obesity (BMI ≥30 kg/m^2, $n = 15$) and without obesity (BMI <30 kg/m^2, $n = 29$)[23]. Patients with NAFLD had increased glucose production (hepatic insulin resistance measured using tracers) and reduced glucose uptake (peripheral insulin resistance) despite high insulin concentrations compared with control individuals without NAFLD[23]. These authors also observed elevated levels of fasting glutamate, alanine, valine and isoleucine (but not leucine) in patients with NAFLD compared with the levels in individuals without NAFLD. In addition, patients with NAFLD and obesity showed increased levels of tyrosine and lysine compared with individuals without NAFLD. Interestingly, they observed that most amino acids increased with hepatic insulin resistance, whereas only tyrosine levels correlated with peripheral insulin resistance[23]. In agreement with previous reports

on associations between concentrations of amino acids and insulin resistance, they also observed that increased levels of glutamate and tyrosine and decreased levels of glycine and histidine were associated with hepatic insulin resistance[23]. A positive correlation between BCAA levels and hepatic and/or peripheral insulin resistance was also observed in a cohort of 111 healthy individuals with overweight or obesity and mild hepatic steatosis[45]. Moreover, BCAAs and tyrosine levels were positively associated with intrahepatic fat content after adjusting for age, gender, BMI and HOMA-IR[45]. In another clinical study in patients with NASH (137 patients with stage 0–4 fibrosis), plasma BCAA levels decreased with increasing liver fibrosis, and tyrosine levels increased with increasing fibrotic stage[46], consistent with the findings of previous studies[42,43]. However, reduced AAA or increased BCAA levels were previously observed in patients with other chronic severe liver diseases (that is, chronic active hepatitis, primary biliary cirrhosis or cryptogenic cirrhosis), which is possibly related to an altered insulin–glucagon ratio[29,39].

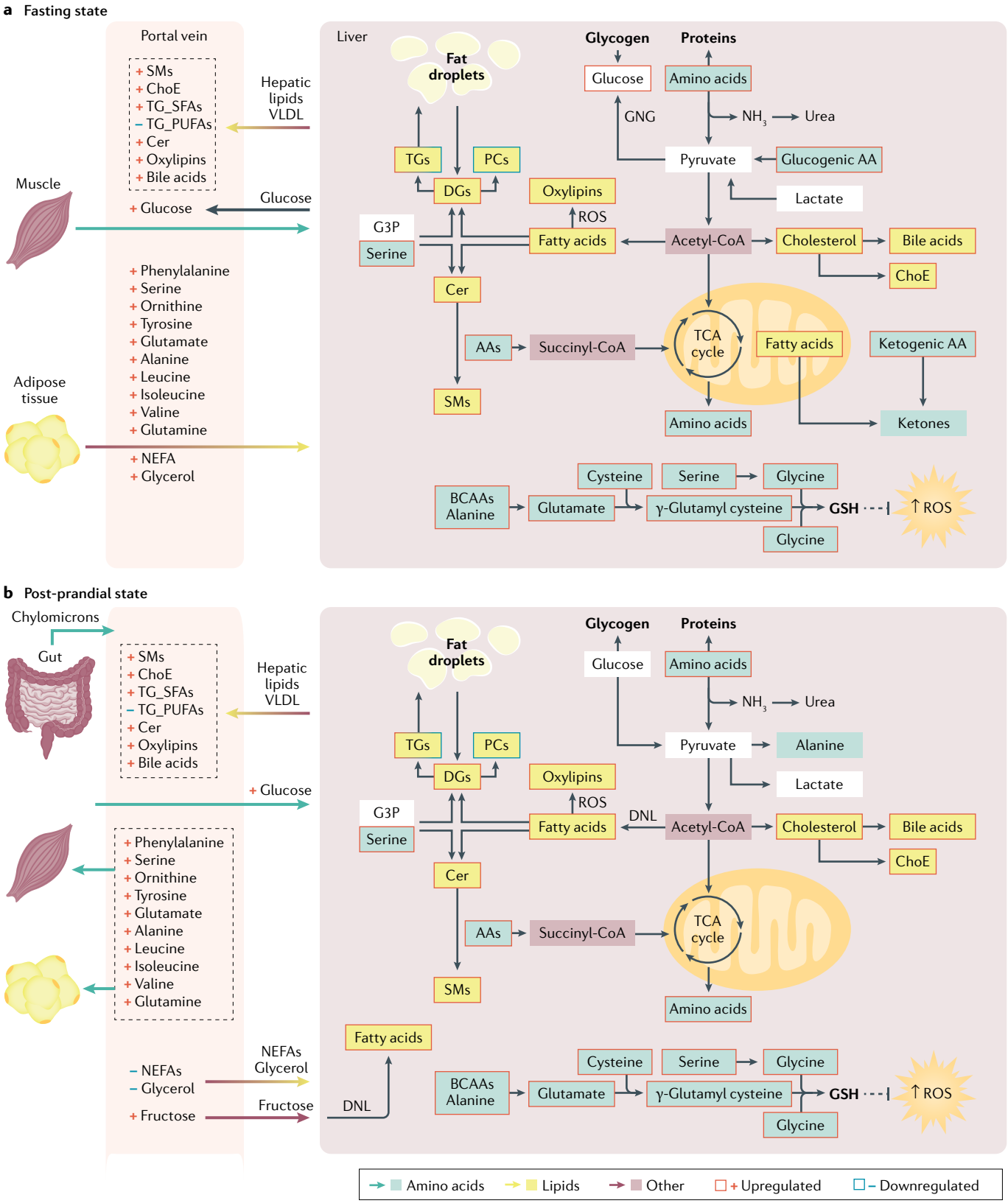
Amino acids are also used as an energy substrate and are oxidized in the tricarboxylic acid (TCA) cycle for the production of ATP (FIG. 2). Kahl et al.[47] evaluated the association between circulating levels of amino acids and hepatic phosphorus metabolism, measured in vivo using $^{31}P/^{1}H$-magnetic resonance spectroscopy (MRS), in 62 insulin-sensitive volunteers without diabetes or obesity. Plasma BCAA concentrations, particularly leucine, were positively associated with γATP, and the correlation remained after adjusting for BMI, age and sex, whereas no correlation was found between γATP and indexes of insulin resistance or steatosis, probably because in these individuals insulin resistance and intrahepatic triglycerides were low[47].

As mentioned above, there is inconsistency in the levels and patterns of reported circulating amino acids in patients with NAFLD–NASH, which is difficult to explain on the basis of currently available information. However, we can speculate about several factors that could potentially explain this discrepancy, including small sample sizes of patients with NAFLD, and differences in analytical methodology, applied quantitative methods, and characterization of patients with NAFLD–NASH, especially with respect to BMI, insulin resistance and degree of fibrosis. This further emphasizes the

Table 1 | **Analytical methodologies for metabolomics**

Method	Description	Advantages	Limitations
Direct infusion (shotgun) MS	No pre-separation of metabolites prior to the detection, direct infusion to the MS detector; used with both low-resolution and high-resolution MS; typically used for lipidomics and several hundreds of lipids are detected; relative quantification	Relatively fast, robust relative quantification for compounds with internal standards; possibility to detect unknown compounds with HRMS	Matrix effects can interfere with the quantification; less specific as there is no possibility to separate isomeric compounds with identical mass
Non-target LC–HRMS	Metabolites are separated by LC prior to their detection; the high-resolution MS is used for detection, allowing detection of unknown metabolites; typically 400–2,000 compounds are detected; possibility for combined relative or quantitative analysis of selected target compounds; used for both lipids and aqueous metabolites	More compounds can be detected with higher sensitivity owing to pre-separation by LC; separation of isomeric compounds as well as unknown compounds	Large amount of data; full quantification of all compounds is not possible owing to the number of compounds detected, including the unknown compounds; identification of unknown compounds can be tedious; matrix effects can interfere with the quantification; owing to wide range of compounds, optimization for each individual compound is not possible
Targeted LC–QqQMS	Preselected target compounds are analysed, typically 10–100 compounds with optimized method; pre-separation of compounds by LC, followed by tandem MS with low-resolution MS; used for both lipid analysis (e.g. bile acids, oxylipins) and for polar metabolites	Good sensitivity and selectivity; wide linear range; best approach for quantitative analysis; suitable also for clinical applications	Only information of preselected compounds
Targeted GC–MS	Preselected target compounds are analysed, typically 20–150 compounds with optimized method; pre-separation of compounds by GC; MS gives fragmentation spectra for the analytes	GC gives very good selectivity and sensitivity; identification of the compounds more straightforward than in LC-based approaches owing to large commercial spectral libraries and robust spectrum in GC–MS	Suitable only for sufficiently volatile compounds; more tedious sample preparation; typically derivatization is needed; in metabolomics, used mainly for fatty acid analysis, amino acid analysis; suitable also for simple sugars, carboxylic acids and specific steroids

GC, gas chromatography; HRMS, high-resolution mass spectrometry; LC, liquid chromatography; LC–QqQMS, triple quadrupole mass spectrometry; MS, mass spectrometry.



need for deep metabolic phenotyping studies in large cohorts of well-characterized patients with NAFLD and NASH using standardized quantitative assays to assess levels and patterns of circulating amino acids. As previously stated, peripheral insulin resistance is very common in patients with NAFLD. However, insulin resistance is also present in the liver and adipose tissue as evidenced by the impaired suppression of hepatic glucose production and lipolysis, respectively[47–49]. Glucose oxidation processes have been suggested to be

◀ Fig. 2 | **Overview of the key metabolic pathways in fasting and postprandial states involved in the pathogenesis of NAFLD. a** | During fasting, the liver is the main source of glucose, which is produced by glycogenolysis or gluconeogenesis for amino acids (released by the muscle), lactate and/or glycerol (released together with fatty acids by the adipose tissue during lipolysis). Glucogenic amino acids include alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, methionine, proline, serine and valine. Leucine and lysine are ketogenic, whereas phenylalanine, isoleucine, threonine, tryptophan and tyrosine are both glucogenic and ketogenic. Amino acids are also involved in the synthesis of ceramides and sphingomyelins (SMs). Fatty acids that are taken up by the liver are either oxidized to produce energy and ketone bodies (for example, β-hydroxybutyrate and acetoacetate) or to be re-esterified to diglycerides (DGs) and triglycerides (TGs). Newly synthesized triglycerides can be stored as lipid droplets or be secreted as VLDL. Glutamate, glycine, cysteine and serine are used for the synthesis of glutathione (GSH) in response to oxidative stress. **b** | In the postprandial state, insulin decreases hepatic glucose production and stimulates glucose uptake and glycogen synthesis. De novo lipogenesis (DNL) is stimulated by carbohydrate (mainly fructose) and insulin. Dietary fats are absorbed as chylomicrons. Primary bile acids (synthesized in the liver from cholesterol) are secreted in response to the meal to facilitate the digestion of fats. Dietary amino acids and fatty acids are taken up by the liver but also by muscle (amino acids for protein synthesis) and adipose tissue (fatty acids for triglycerides). AAs, amino acids; BCAAs, branched-chain amino acids; Cer, ceramides; ChoE, cholesterol esters; CoA, coenzyme A; G3P, glyceraldehyde 3-phosphate; GNG, gluconeogenesis; NAFLD, nonalcoholic fatty liver disease; NEFA, non-esterified fatty acid; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SFA, saturated fatty acid; TCA, tricarboxylic acid.

accelerated in the development of NASH, as the pentose phosphate pathway was found to be upregulated in a methionine-deficient and choline-deficient diet-induced NASH mouse model[50]. Pathway analysis performed using urine samples from 108 individuals (30 individuals as controls, 33 patients with NAFLD or steatosis and normal liver function, and 45 patients with NASH and abnormal liver function) revealed that in addition to pathways of energy metabolism and amino acid metabolism, the pentose phosphate pathway might also be involved in the progression from NAFLD to NASH[35]. In fact, HbA_{1c} was increased in patients with NAFLD or NASH compared with healthy individuals[35].

Amino acids and glutathione metabolism. Several studies have suggested that an increase in oxidative stress in the liver is associated with liver damage and the progression of NAFLD to NASH. The increase in oxidative stress results in consumption of the major intracellular antioxidant, glutathione, and consequently a reduction in hepatic glutathione levels[7]. The amino acids involved in the synthesis and catabolism of glutathione are glutamate, cysteine and glycine, but methionine and serine, being precursors of cysteine, are also involved.

Zhou et al. performed MS-based metabolomics on 318 patients who had undergone a liver biopsy because of suspected NASH. They found increased levels of circulating glutamate and isoleucine, and decreased levels of glycine and serine, in patients with NASH[51]. They also developed a MS-based model score for NASH and compared its diagnostic performance with scores based on routinely available data and the patatin-like phospholipase domain-containing 3 (PNPLA3) rs738408 genotype. They used backward stepwise logistic regression analyses of variables from clinical data and metabolomics to develop the NASH ClinLipMet score. The best scoring system was achieved using five metabolites, consisting of three amino acids (glutamate, isoleucine and glycine) and two lipids (lysophosphatidylcholine LPC 16:0 and phosphatidylethanolamine PE 40:6), and clinical data such as aspartate aminotransferase (AST), fasting insulin and *PNPLA3* genotype. This scoring system identified patients with NASH with an AUROC of 0.866 (95% CI 0.820–0.913). The NASH ClinLipMet score identified patients with NASH with higher accuracy than the clinical or metabolic profiling data alone[51].

Dasarathy and colleagues assessed the kinetics and rate of glycine oxidation and ureagenesis in healthy individuals ($n = 8$) and in patients with NASH ($n = 6$) at the basal level and in response to an intravenous infusion of intralipid which leads to increased plasma free fatty acids (FFAs)[52]. Although there were no statistically significant differences in the concentrations of glycine, cysteine, urea and oxidation of glycine in patients with NASH compared with the concentrations in healthy individuals at basal levels, plasma glutathione levels increased in patients with NASH in response to intralipid infusion and had a higher contribution to serine to biosynthesis of cystathionine. The researchers concluded that as plasma glutathione is mostly derived from the liver, this increase might be due to increased glutathione synthesis as a result of the elevated oxidative stress caused by higher fatty acid oxidation in patients with NASH[52]. Methionine is the source of cysteine, the precursor of glutathione[53]. Using stable isotope tracer infusion in 15 patients with NASH and 19 healthy age-matched individuals as controls, Kalhan et al. measured the kinetics of methionine and its transmethylation and transsulfuration, finding lower rates of methylation of homocysteine and transmethylation of methionine in patients with NASH, whereas the rate of transsulfuration of methionine was similar to that in the control group[53]. They also confirmed their previous findings that there are no significant differences in fasting plasma concentrations of total cysteine, homocysteine and glutathione between healthy individuals and patients with NASH[52,53]. However, in response to enteral protein load, they found a statistically significant decrease in total cysteine and increase in total homocysteine in the plasma of healthy individuals; a statistically significant increase in plasma homocysteine concentration after the meal, but no change in plasma total cysteine concentration, was observed in the NASH group, while plasma levels of glutathione were similar in the two groups[53]. The researchers speculated that the activity of methionine adenosyltransferase (MAT), the enzyme responsible for *S*-adenosylmethionine (SAMe) synthesis, was reduced in patients with NASH owing to the oxidative stress caused by increased fatty acid oxidation, resulting in a lower rate of transmethylation reactions[53]. SAMe is the source of essentially all methyl transfer reactions in cells, which regulate key biological processes such as VLDL synthesis and export, gene expression and redox homeostasis[54,55]. Thus an impairment in SAMe synthesis favours NAFLD. Increased plasma levels of homocysteine, cysteine and cysteinylglycine were also found in 64 children with biopsy-proven NAFLD compared with the levels in age-matched healthy children. In addition, circulating glutathione levels were decreased in children with NAFLD compared with the control group[56].

Two human genes encode MAT: *MAT1A* is expressed in normal differentiated liver and *MAT2A* is expressed in all extrahepatic tissues as well as in fetal liver and HCC cells[57]. *Mat1a*-knockout mice have chronically low hepatic SAMe levels and impaired synthesis and release of VLDL, which leads to the intrahepatic accumulation of lipids and oxidized fatty acids, oxidative stress, and abnormal hepatic and serum lipid signatures, which trigger the spontaneous development of steatosis and its progression to NASH, fibrosis and HCC[58]. A unique serum lipid profile that distinguished between *Mat1a*-knockout and wild-type mice was assessed using serum samples from a large cohort of 535 patients with biopsy-proven NAFLD (66% with steatosis ($n = 353$) and 34% with NASH ($n = 182$)). Nearly half of the patients showed a phenocopy that matched this *Mat1a*-knockout lipidomic signature[58]. Integration of other omics data might improve this novel subtyping approach in patients with NAFLD, enabling further interpretation of the complex biochemical processes and the heterogeneity of the disease, opening the door to the development of precision NASH treatments[54].

Betaine (*N*,*N*,*N*-trimethylglycine) is a product of choline oxidation and provides an alternative pathway for methylation of homocysteine[53]. Circulating betaine levels were decreased in patients with NAFLD and negatively correlated with histological severity including inflammation, ballooning and fibrosis[59]; these results are interesting because, although the mechanisms are still to be elucidated, in animal studies betaine supplementation seems to protect against high-fat-diet-induced NAFLD[60].

Mardinoglu et al. used a combination of GSMM and MS-based metabolomics in 86 individuals with varying degrees of hepatic steatosis to elucidate the molecular mechanisms involved in the progression of NAFLD[18]. They observed lower fasting plasma levels of glycine, serine, betaine and *N*-acetylglycine in individuals with a high degree of steatosis than in those with a low degree of steatosis, as well as a negative correlation between the plasma levels of these metabolites and hepatic steatosis. However, they did not observe any correlation between hepatic steatosis and the plasma levels of cysteine and glutamine, which are also required for the synthesis of glutathione[18]. Furthermore, they assessed the effects of short-term dietary supplementation with serine (each patient received one oral dose of ~20 g of L-serine (200 mg/kg/day) for 14 days) on hepatic steatosis and liver functions in six patients with a high degree of hepatic steatosis[18]. After supplementation with serine they observed statistically significant decreases in plasma levels of alanine aminotransferase (ALT), AST, alkaline phosphatase and hepatic steatosis (decreases between 1% and 23%), but not in plasma levels of triglycerides[18].

Glutamate and glutamine. Glutamate has a central role in hepatic amino acid metabolism, as it is one of the major substrates for the synthesis of glutathione but also because it is involved in the transdeamination and/or catabolism of most amino acids, which gives rise to glutamate[23]. Glutamine and glutamate are also involved in nitrogen metabolism and ammonia production. Glutamine is quantitatively the most common nitrogen transporter between tissues. Glutamine metabolism is altered in liver disease, and glutaminolysis TCA cycle intermediates increase with fibrosis severity in patients with biopsy-proven NAFLD even after accounting for potential confounders such as age, sex and severity of the metabolic syndrome[21,23,61]. Glutamate is also involved in the synthesis of γ-glutamyl dipeptides that are produced by the conjugation of glutamate with other amino acids in the γ-glutamyl cycle. These compounds were found to be increased in patients with NAFLD–NASH compared with levels in healthy individuals[21]. Similar results were observed by Soga et al., who measured γ-glutamyl dipeptides by capillary electrophoresis time-of-flight MS metabolomics in 248 serum samples from healthy individuals as controls ($n = 53$) and patients with various liver injuries including simple steatosis ($n = 9$) and NASH ($n = 11$)[62]. They found increases in the levels of γ-glutamyl dipeptides in patients with liver injuries, including NAFLD and NASH. In addition, they found that the glutamyl dipeptide levels decreased during liver disease progression (and were lower in patients with NASH than in those with simple steatosis), indicating that an increase in oxidative stress is required for metabolic reprogramming of hepatic stellate cells and liver fibrogenesis, at least in mice[61].

Another study showed that carbon tetrachloride-treated mice with liver fibrosis have decreased glutamine and increased glutamate levels in both serum and liver tissue. Moreover, the glutamate to glutamine ratio in the liver was positively correlated with the severity of myofibroblast accumulation and fibrosis and with the glutamate to glutamine ratio in the serum[61]. Glutamate, the glutamate to glutamine ratio and α-ketoglutarate are associated with the decline in glutathione production[18,21,23,62,63]. In another study, MS-based metabolomics analysis of plasma samples was performed in patients with NASH who responded to vitamin E treatment ($n = 16$) and those who did not ($n = 15$)[64] to identify metabolic profiles that are reflective of histological improvement at the end of treatment. Lower circulating levels of γ-glutamylated amino acids including γ-glutamyl leucine, γ-glutamyl isoleucine and γ-glutamyl valine were associated with histological improvement in response to vitamin E, and the authors speculated that this association was indicative of decreased glutathione turnover and improved systemic oxidative stress[64].

Gaggini et al. introduced a new index using amino acids (glutamic acid, glycine and serine) that are involved in glutathione synthesis (GSG index = glutamate/(serine + glycine)) and examined the association between the GSG index and alterations in liver histology in patients with biopsy-proven NASH compared with individuals without NAFLD[23]. The researchers observed a strong association between the GSG index and liver enzymes including ALT and AST but especially γ-glutamyltransferase. Compared with using BCAAs and AAAs, they were able to better discriminate NAFLD–NASH according to the degree of fibrosis using the GSG index in liver biopsy samples[23]. In a study in a cohort of 78 adolescents investigating the use of the GSG index, an index of >0.36 (upper tertile) was found to be associated with a twofold

higher prevalence of NAFLD than a lower index, despite similar adiposity, abdominal fat distribution and liver insulin resistance[65].

Lipidomics in NAFLD–NASH

Our understanding of lipid metabolism and the metabolic pathways that contribute to lipotoxicity in NASH has improved considerably owing to advances in lipidomics. Hepatic steatosis, which is an important component of NAFLD and NASH, is mainly caused by an imbalance between intrahepatic triglyceride production and secretion. Hepatic steatosis arises when hepatic de novo lipogenesis (DNL) and fatty acid uptake from the circulation saturate the capacity of the liver to oxidize fatty acids and secrete triglycerides in the form of VLDL[66–68] (FIG. 2). Assessing fatty acid flux is a well-established method to understand lipid metabolism, which includes profiling non-esterified fatty acids (NEFAs) and esterified fatty acids into several lipid classes, including glycerolipids, phospholipids, sphingolipids and cholesterol esters.

Fatty acids and oxidized fatty acids. The main structural components of complex lipids are fatty acids that are introduced via the diet, released by adipose tissue during lipolysis, or synthesized via DNL in the liver (FIG. 2). Donnelly et al. measured triglyceride composition in the liver of patients with NAFLD, and demonstrated that most of the fatty acids in triglycerides arise from NEFAs released by adipose tissue (59%), whereas only 15% arise from the diet and 26% from DNL[69]. This result is in agreement with those of other studies, showing that adipose tissue insulin resistance that results in high lipolysis and NEFA release in the circulation is the most important metabolic defect in patients with NAFLD, regardless of the presence of obesity[70], and is associated with increased liver fibrosis[71–73]. Improvement in adipose tissue insulin resistance, for example after treatment with pioglitazone, is associated with a reduction in liver fibrosis in patients with NASH[73,74].

The first step of DNL is the synthesis of palmitic acid (C16:0), which is a saturated fatty acid (SFA). Palmitic acid can be incorporated into other lipids (for example, triglycerides, diacylglycerols, ceramides and phospholipids), elongated to stearic acid (C18:0) or desaturated to palmitoleic acid (C16:1n-7). Stearoyl-coenzyme A desaturase 1, one of the key enzymes and rate-limiting steps in DNL, desaturates stearic and palmitic acids into oleic acid (C18:1n-9) and palmitoleic acid, respectively[75], which are the most abundant fatty acids found in phospholipids, cholesterol esters and triglycerides as well as VLDL particles[76,77]. Puri et al. performed lipidomics analysis on liver biopsy samples from patients with NAFLD, NASH and healthy individuals ($n = 9$ per group)[78]. Profiling of NEFAs showed no significant difference in the levels of stearic acid, oleic acid, palmitic acid and palmitoleic acid between patients with NAFLD–NASH and the control group, although the total levels of hepatic SFA and polyunsaturated fatty acids (PUFAs) increased significantly in patients with NAFLD and NASH compared with the control group. The authors also observed a significant decrease in the levels of γ-linolenic acid (C18:3n-6) and arachidonic acid (C20:4n-6) in patients with NASH[78]. Kalhan et al. observed a significant reduction in several NEFAs, including eicosapentaenoate (C20:5n-3), docosahexaenoate (C22:6n-3), 10-undecenoate (C11:1n-1) and arachidonic acid in patients with NASH compared with the control group[21]. By contrast, only caprate (C10:0) and 10-undecenoate were significantly lower in patients with steatosis than in healthy individuals. Only linolenic acid (C18:3n-3) and undecanoic acid (C11:0) were significantly higher in patients with steatosis than in those with NASH[21].

The increment in the dietary intake of ω-6 PUFA in the past few decades has led to an increase in the ω-6 to ω3 ratio, which predisposes to NAFLD development[79]. A reduction in the ω-6 to ω-3 ratio was found to be beneficial for steatosis in animal models[80,81]. Consumption of dietary ω-6 fatty acids also contribute to NASH development, inducing mitochondrial dysfunction by disrupting mitochondrial respiratory chain and cell death, in mice[82]. Consistent with this, Puri et al. found an increased hepatic ω-6 to ω-3 ratio ($P < 0.05$) in patients with NASH ($n = 9$) compared with patients with steatosis ($n = 9$) and the control group ($n = 9$)[78]. ω-6 and ω-3 fatty acids can be oxidized via lipoxygenases (LOXs) or cyclooxygenases (COXs) or through non-enzymatic pathways to generate oxylipins[83].

The circulating levels of oxidized arachidonic acid were evaluated in 50 lean healthy individuals, and in 25 patients with overweight or obesity with steatosis, and in 50 patients with overweight or obesity with NASH[84]. Interestingly, no difference in the levels of COX products of arachidonic acid (mainly prostaglandins and thromboxane A_2) was observed, whereas the levels of LOX metabolites 5*S*-hydroxyeicosatetraenoic acid (5-HETE), 8-HETE and 15-HETE progressively increased from healthy individuals to patients with NAFLD to patients with NASH, and 11-HETE was significantly increased in patients with NASH only[84]. Increases in the levels of 5-HETE, 9-HETE, 11-HETE, 12-HETE and 15-HETE in patients with NASH have also been reported, although the significant changes were limited to patients with morbid obesity (BMI >30 kg/m^2)[25]. Arachidonic acid-derived oxidized products generated from 5-LOX, 12-LOX and 15-LOX pathways have also been assessed as non-invasive NASH biomarkers. Although the study was performed in a small cohort (ten patients with biopsy-proven NAFL and nine patients with biopsy-proven NASH), the results also revealed higher levels of these compounds in patients with NAFL than in patients with NASH[85]. The researchers highlighted 11,12-dihydroxyeicosatrienoic acid (11,12-diHETrE) as the best candidate for non-invasive discrimination between NAFL and NASH, as it showed an AUROC of 1. In addition, 13,14-dihydro-15-ket oprostaglandin D2 and 20-carboxy arachidonic acid were suggested as good biomarkers for discriminating NAFL and NASH (AUROC 0.93 and 0.96, respectively)[85]. In 2020, Caussy et al. also found that plasma levels of 11,12-diHETE, tetranor-12-HETE, adrenic acid and 14,15-diHETE were positively associated with fibrosis stage in a cohort of 427 patients with biopsy-proven

NAFLD[86]. The researchers also performed a longitudinal study of circulating eicosanoid levels in 63 patients with NASH and significant fibrosis (stage 2 or 3) in a phase II clinical trial of 24 weeks. They identified a combination of eicosanoids and PUFAs that predicted liver fibrosis improvement (one or more stages) with an AUROC of 0.74 (95% CI 0.62–0.87)[86].

Feldstein et al. found that the non-enzymatically derived oxylipins 9-hydroxyoctadecadienoic acid (9-HODE), 13-HODE, 9-oxo-octadecadienoic acid (9-oxoODE) and 13-oxoODE were significantly elevated in patients with NASH ($n = 37$) compared with patients with steatosis ($n = 23$) or individuals with normal liver biopsy ($n = 13$)[87]. On the basis of these results, the researchers generated a risk score for NASH (oxNASH), including the 13-HODE to linoleic acid ratio, age, BMI and AST, which was able to detect NASH with an AUROC of 0.83 (95% CI 0.73–0.93) in the training cohort ($n = 73$ patients) and 0.74 (95% CI 0.60–0.88) in the validation cohort ($n = 49$ patients)[87]. A study performed in adolescents with obesity also revealed an association between 9-HODE, 13-HODE, 9-oxo-ODE and 13-oxo-ODE and caspase-cleaved cytokeratin 18 fragment (CK-18), a biomarker of liver injury, in patients with steatosis, independent of age, sex, *z*-score BMI or ethnicity[88]. The authors also hypothesized that the association between these compounds and liver injury might be driven by the *PNPLA3* rs738409 single-nucleotide polymorphism, and that they might have a harmful effect on insulin secretion, as patients with impaired glucose tolerance showed higher levels of these oxidized fatty acids[88].

Changes in the levels of circulating oxylipins were also evaluated in a double-blinded, randomized, placebo-controlled trial for evaluation of pentoxifylline in patients with NASH ($n = 47$, 21 treated with pentoxifylline and 26 receiving a placebo)[89]. The effects of the drug included a significant reduction in the circulating levels of several oxidized fatty acids and, remarkably, a significant correlation between improved histological fibrosis scores and reduced HODEs and oxoODEs, as well as a correlation between improvement in lobular inflammation and decreased levels of HETEs[89]. There is also some evidence of a potential role for specialized pre-resolving mediators in the resolution of NASH and fibrosis[90].

Despite the valuable results of these studies, most were performed in small cohorts and were not validated. Therefore, large, multicentre clinical trials are needed to evaluate the precision and robustness of the use of biomarkers based on oxidized fatty acids as non-invasive biomarkers of NASH. Nevertheless, one of the challenges of using oxylipins as diagnostic markers is their stability in biological samples (they can be produced rapidly). Sampling and sample handling could have a major effect on the reported concentrations of these lipids, thereby potentially limiting their clinical use in diagnostic assays.

Triglycerides, phospholipids and sphingolipids. Elevation in peripheral fatty acid flux and DNL was demonstrated in patients with NAFLD[69]. An increase in the levels of molecular species of triglycerides containing fatty acids with a low carbon number and a double bond (that is, shorter-chain saturated and monounsaturated fatty acids) was observed in patients with NAFLD in several studies[68,77,91,92]. Interestingly, this pattern of changes in triglycerides has also been found in metabolic disorders, especially those associated with insulin resistance such as obesity[93,94]. Moreover, decreased levels of triglycerides containing PUFAs, including ω-3 and ω-6 fatty acids, have also been found in patients with NAFL and NASH[78,92]. However, the ω-6 to ω-3 ratio in triglycerides was elevated in patients with NASH and NAFL compared with the ratio in the control group[78].

Given the relevance of triglyceride content to the development of NAFLD and NASH, several studies were performed to assess the potential of using these lipids as biomarkers for the non-invasive diagnosis of NAFLD. BMI-dependent lipidomic profiles associated with NAFLD and NASH were described in plasma samples from a cohort of 467 patients (90 with normal liver, 246 with steatosis and 131 with NASH)[25]. Several lipid classes, particularly sphingolipids, were reported to be differentially altered in NASH and steatosis depending on the BMI subgroup. However, apart from these obesity-dependent changes, the differences in glycerolipid levels were found to be similar across all the BMI subgroups: triglyceride concentrations were higher in patients with steatosis than in patients with normal liver, whereas the abundance of several triglyceride species was decreased in patients with NASH compared with those with steatosis[25]. On the basis of these findings in a cohort of 467 patients, Mayo et al. reported a test consisting of 28 triglyceride species split into two panels: 11 triglyceride species were able to distinguish between NAFLD and normal liver, although without discriminatory power between NASH and steatosis; and 20 triglyceride species were able to discriminate between NASH and steatosis, although not between normal liver and NASH[95]. The test is a two-step BMI-dependent logistic regression algorithm that discriminates firstly between normal liver and NAFLD, and secondly between steatosis and NASH. Mayo et al. also included an independent and multicentre cohort of 192 patients (7 with normal liver, 109 with steatosis and 76 with NASH, based on liver biopsy) and demonstrated that the test was able to discriminate between normal liver and NAFLD (AUROC 0.90 ± 0.02 in the original cohort, 0.88 ± 0.05 in the validation cohort) and between steatosis and NASH (AUROC 0.95 ± 0.01 in the original cohort, 0.79 ± 0.04 in the validation cohort)[95]. Notably, the performance of the test remained constant in the range of 25–60 kg/m^2, although the error rate in NASH diagnosis was dependent on glucose concentration: the test performed better in patients with glucose levels <136 mg/dl (AUROC 0.81 ± 0.04)[95]. Bril et al. also evaluated this test in 220 patients with type 2 diabetes mellitus for the discrimination between steatosis and NASH and found an AUROC of 0.79 (95% CI 0.68–0.90) in patients with glycated haemoglobin <53 mmol/mol and AUROC 0.87 (95% CI 0.76–0.97) in patients with HOMA-IR <3 (REF.[96]). However, the test performed less well in patients with high insulin resistance or poor glycaemic control[96].

Serum levels of intact lipids, including phospholipids, sphingolipids and triglycerides, were measured using LC–MS-based lipidomics in 679 well-characterized patients with NAFLD to identify blood markers for prediction of NAFLD and liver fat content[97]. Liver fat content was also measured using ^{1}H-MRS in 54% of the participants ($n = 369$) and using a liver biopsy in the remaining 46%. The authors divided the lipid data into nine lipid clusters and observed an overall significant increase in the levels of triglycerides containing fatty acids with a low carbon number and double bond count as well as generally lower concentrations of lysophospholipids and ether lipids in patients with NAFLD compared with the concentrations in the healthy individuals as controls[97]. Higher liver fat content was also associated with increased levels of triglycerides containing saturated or monounsaturated fatty acids and decreased concentrations of PUFA-containing phospholipids and ether lipids. In addition, they identified 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 0.74 and 0.71 in the discovery and validation series; AUROC 0.79, sensitivity 69.3% and specificity 74.5% in the combined biomarker discovery and validation series)[97]. Notably, triglycerides with low double bond and carbon number, which were found to be increased in patients with NAFLD, were previously reported to be associated with T2DM risk and insulin resistance[77,98,99] as well as with DNL[68]. Notably, the blood samples in this multicentre study were collected differently across the centres. Of the blood samples used, 78% were citrate plasma, 16% were heparin plasma and 7% were serum samples. Nevertheless, based on systematic studies, sample type did not have a major effect on measured lipid levels[100].

Gorden et al. performed lipidomics analysis on liver biopsy samples and plasma samples from 88 individuals classified based on liver histology as normal ($n = 31$), steatotic ($n = 17$), NASH ($n = 20$) or cirrhotic ($n = 20$) to identify circulating lipid biomarkers that correlated with NAFLD disease progression[92]. In the liver, they observed significant differences in the levels of most cholesterol esters and triglycerides as well as several sphingolipids and phospholipids across disease states. In plasma, differences across histological categories were observed for major phospholipids and sphingolipids, including several 1-deoxy and dihydro species of sphingomyelins and ceramides. Using linear discriminant analysis, the researchers found 20 plasma metabolites as a metabolic signature of progression in NAFLD that could discriminate NASH and steatosis[92]. Interestingly, the majority of these metabolites were sphingolipids and phospholipids and they did not detect any triglycerides or diacylglycerols associated with NAFLD progression[92]. Elevation of some serum sphingolipids in patients with NASH ($n = 16$) compared with the levels in patients with NAFL ($n = 8$) was also observed in another study using lipidomics analysis[101]. Despite the valuable insights generated, the study was done in a very small cohort and the exclusion list for patient selection was limited.

Among sphingolipids, ceramides have received considerable attention in NASH research. Ceramides are biologically active lipids involved in mitochondrial dysfunction, cellular proliferation and oxidative stress, and they contribute to a pro-inflammatory environment and have been implicated in the development of NAFLD and hepatic insulin resistance[102–104]. Dihydroceramides, which are considered markers of de novo ceramide synthesis, are converted into ceramides by the enzyme dihydroceramide desaturase 1 (DES1), which inserts a conserved double bond into the backbone of dihydroceramides[105]. Dihydroceramides have been linked to insulin resistance, and their hepatic concentration is increased in patients with NASH[106,107]. A high dihydroceramide to ceramide ratio, which indicates a reduction in DES1 activity and hepatic ceramide levels, has also been linked to an improvement in hepatic steatosis and systemic insulin resistance in murine models[108,109]. It has been reported that the liver is the major contributor to the circulating levels of these species (in addition to a substantial contribution from adipocytes)[109]; thus, plasma ceramide levels could potentially reflect the changes in the hepatic levels.

Lipidomics has also proven to be a powerful tool for assessing heterogeneity of NAFLD and identifying the underlying mechanisms. Luukkonen et al. compared obesity-associated NAFLD and insulin resistance ('metabolic' NAFLD) with NAFLD associated with a common I148M variant in *PNPLA3* at rs738409 ('genetic' NAFLD) using lipidomic analysis in liver biopsy samples from 125 individuals[106]. The study found that 'metabolic' NAFLD is specifically associated with increased concentrations of triglycerides of low carbon number and low double bond count as well as with lipotoxic ceramides and diacylglycerols in the liver, while no such changes were observed in genetic NAFLD[106]; these observations provide a potential link between obesity, NAFLD and the development of insulin resistance and T2DM. A distinct lipid signature, comprising a relative decrease in triglycerides, was also found in the serum of patients with a I148M variant in *PNPLA3* compared with the levels in non-carriers[110]. A follow-up study identified a mechanism by which the *PNPLA3* I148M variant contributed to steatosis, namely via increased hepatic retention of PUFAs[111]. In contrast to PNPLA3, lipidomic study of liver biopsy samples has shown that the E167K variant in the gene encoding transmembrane 6 superfamily member 2 (TM6SF2), which is also known to be associated with NAFLD, contributes to steatosis by increasing the synthesis of PUFAs, whereas the synthesis of complex lipids containing PUFAs is impaired[112].

A deficiency in polyunsaturated phosphatidylcholines, the main phospholipids of the outer layer of VLDL, leads to impaired VLDL formation and export and, therefore, patients with NAFLD carrying the *TM6SF2* E167K variant have lower levels of the most abundant circulating triglycerides, such as TG(16:0/18:2/18:1) and TG(16:0/18:1/18:1)[113]. The variant rs641738 (C>T) of the gene encoding membrane-bound *O*-acyltransferase domain-containing 7 (MBOAT7) has also been associated with increased risk of development and progression of NAFLD in patients[114]. MBOAT7 is

a lysophosphatidylinositol (LPI) transferase that preferentially incorporates arachidonic acid into the second acyl chain of LPIs[115]; therefore, a decreased arachidonoylphosphatidylinositol to total phosphatidylinositol ratio has been found in the plasma of patients carrying the MBOAT7 variant[114]. Interestingly, hepatic expression of *MBOAT7* is reduced in patients with NASH and obesity, independently of genetic background[116,117], leading to an impaired phosphatidylinositol remodelling that generates an imbalance in phosphatidylinositol-related lipid mediators[117] and promotes the conversion of saturated LPIs to triglycerides[116,118]. Fondevila et al. also found increased circulating levels of LPIs in patients with NASH compared with the levels in patients with NAFL, which was associated with a higher severity of inflammation and increased hepatocellular ballooning[119]. On the other hand, carriers of the protective variant (rs72613567:TA) in the gene encoding hydroxysteroid 17β-dehydrogenase 13 (HSD17B13) have increased levels of phospholipids in the liver and decreased fibrosis[120]. Similarly, carriers of the protective variant (rs2642438) in the gene encoding mitochondrial amidoxime-reducing component 1 (MARC1) have elevated hepatic polyunsaturated phosphatidylcholines compared with non-carriers[121].

Taken together, lipidomic studies in NAFLD have shown that lipid profiles are not specific to steatosis per se, but can instead distinguish subtypes of NAFLD.

Bile acids. There has been growing interest in the potential role of bile acids in the development of NASH. Bile acids are synthesized in the liver from cholesterol through two pathways, either through the classic pathway via cytochrome 7α hydroxylase (CYP7A1) enzyme or through an acidic pathway via mitochondrial cytochrome 27α hydroxylase (CYP27A). Bile acid homeostasis is maintained through multiple negative feedback loops for bile acid synthesis and a tightly regulated enterohepatic circulation of bile acids. Bile acids have several important functions as they not only assist lipid digestion and absorption once they are released into the intestine, but also act as signalling molecules controlling glucose, lipid, and energy homeostasis through farnesoid X receptor (FXR) and the G protein-coupled bile acid receptor TGR5 (REF.[122]). FXR also has a regulatory role in triglyceride metabolism[123–125]. In the liver, FXR activation results in downregulation of CYP7A1, which in addition to inhibition of the classic bile acid synthetic pathway also reduces the expression of several genes mediating FFA synthesis, thereby attenuating DNL[126–128], as demonstrated both in rodent models and in human hepatocytes[129–131]. Several studies have shown increases in the levels of bile acids in liver tissue[132,133], plasma[132,134,135] and faeces[134] of patients with NASH. A positive association between circulating bile acids (taurocholic acid, taurochenodeoxycholic acid and glycocholic acid) and HOMA-IR has also been observed in individuals with obesity[136].

Most studies have shown dysregulation of bile acid metabolism in patients with NASH, including elevated levels of primary conjugated bile acids, decreased levels of specific secondary bile acids and alteration of excreted bile acids[129,132,133,135] (FIG. 3). Mechanistically, it has been indicated that CYP7A1 expression is dysregulated in patients with NAFLD, with most studies reporting upregulated expression[129,135,137–139], although there are also studies showing no association between CYP7A1 expression and NASH[140]. Overall, there is agreement across the results that bile acids are elevated in the circulation and in the liver of patients with NAFLD–NASH. Alterations have also been observed in expression of bile acid transporters in humans, such as the canalicular bile acid transporter (bile salt excretory pump (BSEP)) and the sodium taurocholate co-transporting polypeptide (NTCP). Although the studies have been somewhat inconsistent, BSEP expression has been shown to be decreased in patients with steatosis or NASH compared with expression in healthy individuals[135,138,141], thus indicating decreased bile secretion. On the other hand, *NTCP* mRNA expression levels have been shown to be statistically significantly upregulated in patients with NAFLD compared with the levels in healthy individuals[129,135,139], thus suggesting increased uptake of bile acids by the liver. It should be noted that alteration of BSEP expression might be partially influenced by the BMI of individuals.

Currently, only a few studies have assessed hepatic bile acids in patients with NASH. In one study, bile acid composition was determined by gas chromatography in liver tissue from patients with NASH ($n = 15$) and healthy individuals ($n = 8$). Total bile acid concentrations were significantly higher in patients with NASH (107.6 ± 18.2 nmol/g, wet weight) than in the control group (67.1 ± 6.5 nmol/g; $P < 0.05$)[133]. Individual bile acids including cholic, chenodeoxycholic and deoxycholic acids were also significantly increased in patients with NASH compared with the control group (by 43%, 64% and 92%, respectively)[133]. In addition, there was a significant association between cholic acid and histological markers of liver injury such as inflammation in patients with NASH ($r = 0.54$, $P < 0.05$). Conversely, deoxycholic acid levels were negatively associated with inflammation in patients with NASH ($r = -0.65$, $P < 0.01$). Chenodeoxycholic acid was also significantly associated with both portal and lobular fibrosis in patients with NASH ($r = 0.49$, $P < 0.05$)[133]. Lake et al. evaluated liver biopsy samples from seven healthy individuals, four patients with steatosis, 14 patients with NASH with fatty liver (>5% fat deposition) and 23 patients with NASH without fatty liver (<5% fat deposition) and found that in patients with NASH, taurine-conjugated bile acids were increased, whereas cholic acid and glycodeoxycholic acid were decreased, suggesting a potential shift during NASH towards the alternative pathway of bile acid synthesis, which is mediated by increased mRNA and protein expression of CYP7B1 (REF.[140]). In another study in 20 individuals without diabetes and 22 patients with diabetes, of whom 77.7% had NAFLD with a NAFLD activity score (NAS) of ≥2, total hepatic bile acids were significantly downregulated ($P = 0.0017$) in the group with diabetes compared with the control group[142]. The significant decrease was mainly due to the conjugated bile acids. In a Chinese cohort ($n = 134$ individuals), analysis of

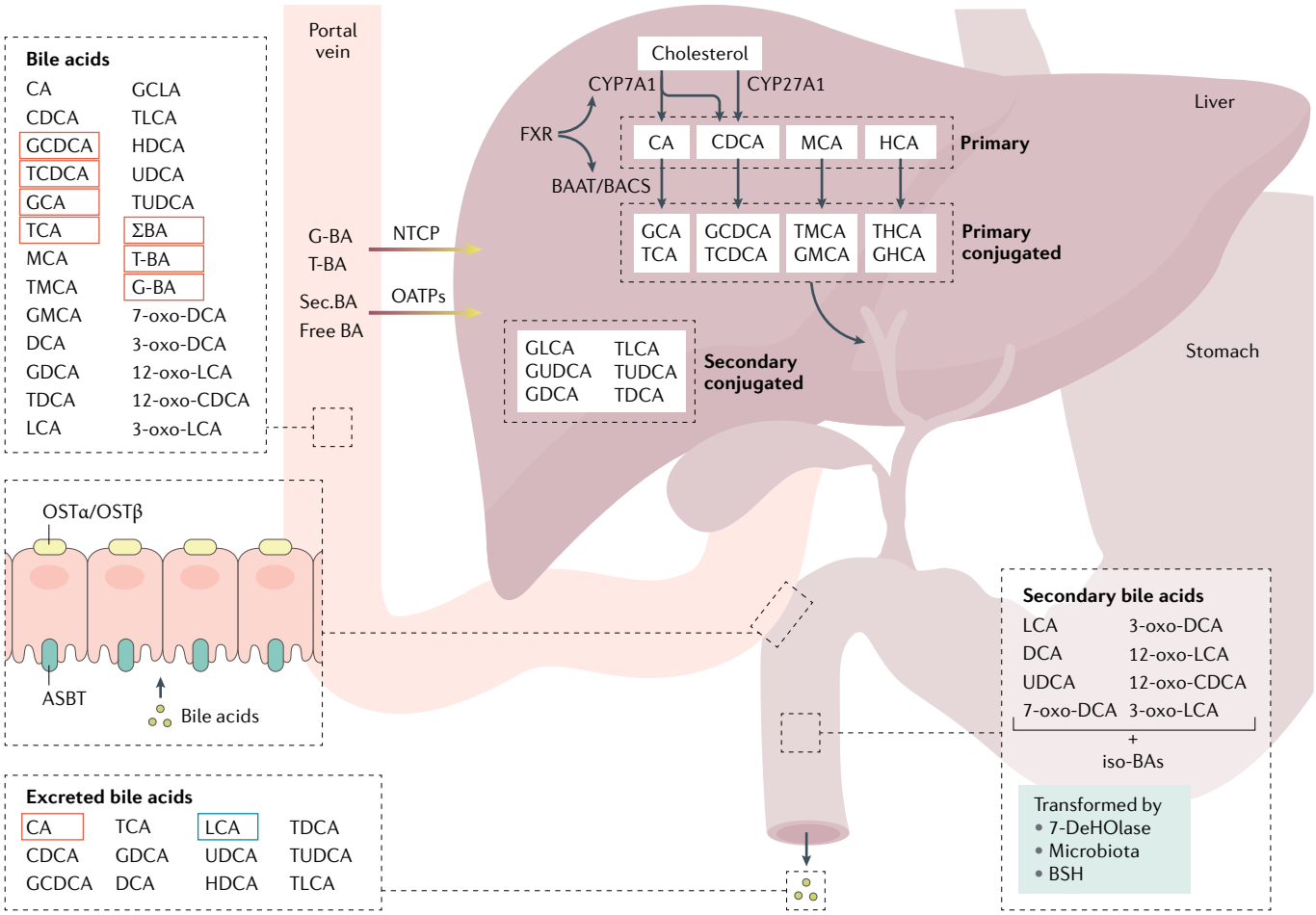


Fig. 3 | **Overview of the key bile acid pathways involved in NAFLD.** Bile acids marked with red boxes indicate upregulation and blue boxes indicate downregulation. Bile acids are synthesized from cholesterol via two pathways. The major, classic pathway is initiated and rate-limited by cytochrome P450 family 7 subfamily A member 1 (CYP7A1) to synthesize CA and CDCA. The alternative pathway is initiated and catalysed by CYP27A1 to synthesize CDCA. CDCA can be further converted into HCA and MCA in the liver. Then, the bile acids are conjugated to the amino acids taurine or glycine before being released into the intestine, with hepatocyte nuclear factor 4α (HNF4α) having a central role in bile acid conjugation by direct regulation of very long-chain acyl-CoA synthetase (VLACSR) and BAAT. In the distal ileum and colon, the bile acids undergo a variety of bacterial transformations including deconjugation, dehydroxylation and epimerization by the gut microbiota to produce a variety of secondary bile acids. Bile acids are recovered into portal blood through a combination of passive absorption in the proximal small intestine, active transport via apical sodium-dependent bile salt transporter (ASBT) in the distal ileum, and passive absorption in the colon and via organic solute transporter α/β (OSTα/β). The majority (90–95%) of bile acids secreted into the small intestine are actively reabsorbed in the terminal ileum and circulate back to the liver while about 5% are excreted via faeces. BA, bile acid; BAAT, bile acid CoA:amino acid *N*-acetyltransferase; BACS, bile acid-coenzyme A synthase; BSH, bile salt hydrolase; CA, cholic acid; CDCA, chenodeoxycholic acid; CYP27A, cytochrome 27α hydroxylase; DCA, deoxycholic acid; FXR, farnesoid X receptor; G-BA, glycine-conjugated bile acid; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GCLA, glycolithocholic acid; GDCA, glycodeoxycholic acid; GHCA, glycohyocholic acid; GLCA, glycolithocholic acid; GMCA, glycomuricholic acid; GUDCA, glycoursodeoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; MCA, muricholic acid; NAFLD, nonalcoholic fatty liver disease; NTCP, Na^+-taurocholate co-transporting peptide; OATP, organic anion transporting polypeptide; OSTα/β, organic solute transporter subunits α/β heterodimer; T-BA, taurine-conjugated bile acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid; THCA, taurohyocholic acid; TLCA, taurolithocholic acid; TMCA, tauromuricholic acid; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

hepatic and circulating bile acids revealed increased levels of circulating and hepatic conjugated chenodeoxycholic acid in patients with NASH as well as an elevated ratio of conjugated chenodeoxycholic acid to muricholic acid in patients with NAFLD compared with the levels in healthy individuals[143]. Moreover, the same study showed an association between the altered bile acid profile and the severity of liver lesions.

There are more studies related to circulating levels of bile acids in patients with NASH. Kalhan and colleagues observed a significant increase in fasting plasma concentration of bile acids including glycocholic, taurocholic and taurochenodeoxycholic acid in patients with NASH compared with healthy individuals[132]. In another study, bile acids were measured in 39 patients with NAFL, 59 patients with NASH and ten healthy individuals, and an increase in serum levels of bile acids and in bile acid transport in NASH were found[139]. Adiponectin was also found to be inversely correlated with serum bile acids and hepatocellular death, related to a potential effect of adiponectin on bile acid homeostasis-related genes, especially *CYP7A1* (REF.[139]). Several studies have

shown that systemic total bile acid concentrations are elevated in patients with T2DM[144,145], but many of the studies did not account for obesity and the insulin sensitivity status of the patients versus controls, which might have caused challenges in interpreting the findings. In a cohort of 86 individuals (24 controls, 25 with NAFL and 37 with NASH), the total levels of plasma primary bile acids increased in a stepwise manner from the controls to patients with NAFL and to those with NASH, and the changes in the bile acid profile were most severe in those with NASH and were independent of the presence of diabetes in patients with NAFL[135]. Legry et al. studied bile acid metabolism in a cohort of drug-naive patients with obesity with NASH ($n = 32$) and controls (no NASH) matched for BMI and insulin resistance ($n = 26$)[138]. The alterations in bile acid metabolism were associated with insulin resistance, rather than with liver necroinflammation itself. These findings highlight the complex interactions between insulin resistance and bile acid metabolism in NAFLD and NASH. Other studies have found elevated levels of specific bile acids in patients with NAFLD and NASH[129,146].

Ferslew et al. quantified bile acids in 15 healthy individuals and 7 patients with NASH during fasting as well as following a high-fat meal (at 30, 60, 90 and 120 min) using MS-based metabolomics[147]. They observed an increase in the fasting levels of both primary and secondary bile acids in patients with NASH. Total fasting serum bile acid concentrations (total concentrations of unconjugated bile acids, and bile acids conjugated with glycine, taurine and sulfate) in patients with NASH were increased 2.2-fold to 2.4-fold compared with the concentrations in healthy individuals, which was due predominantly to higher concentrations of taurine-conjugated and glycine-conjugated bile acids. Taurine-conjugated bile acids increased 5.6-fold, whereas glycine-conjugated bile acids increased only by 3.2-fold[147]. Increases in serum bile acids were also observed in both healthy individuals and patients with NASH following a challenge with a standardized high-fat breakfast, although patients with NASH demonstrated a greater postprandial increase in all bile acid groups except lithocholic acid and 6α-hydroxylated bile acids. In addition, postprandial levels of chenodeoxycholic acid, deoxycholic acid and ursodeoxycholic acid were significantly increased in patients with NASH compared with the levels in healthy individuals ($P < 0.05$). The researchers concluded that the observed distinct shift in levels of fasting and postprandial circulating bile acids, especially hydrophobic bile acids, might influence NAFLD progression and could have implications in the diagnosis and staging of NASH[147].

In summary, most studies have found increased levels of circulatory total bile acids in patients with NASH and steatosis, mainly due to upregulation of conjugated bile acids. Conflicting results have been reported on the levels of secondary bile acids in NAFLD. Several studies have indicated that it is the ratios between specific bile acids, that is, the composition of the bile acid pool, that have a crucial role in NAFLD–NASH rather than the concentrations of total or individual bile acids. The results also indicate that insulin resistance is a contributing factor in the dysregulation of circulating bile acids. In liver, only a limited number of studies have been performed, with contradictory results. Thus, there is currently no clear consensus for the hepatic bile acid profile in patients with NAFLD.

Gut microbial metabolites in NAFLD–NASH. The gut microbiota interacts with the liver via the so-called gut–liver axis, which describes the bidirectional relationship between the gut, its microbiota, and the liver. Dysfunction of the gut–liver axis can markedly contribute to the development and progression of NAFLD[148]. Metabolites derived from the intestinal microbiota, such as short-chain fatty acids (SCFAs), bile acids and AAAs and their derivatives, have emerged as important factors modulating the pathological process of NAFLD, as previously discussed[148]. The gut microbiota can also modulate the endocannabinoid system as well as choline metabolism, which is needed for the synthesis of VLDL particles and transport of lipids out of the liver. In addition, lipopolysaccharides are associated with the gut–liver axis as they activate the production of pro-inflammatory cytokines in liver macrophages, causing inflammation of hepatocytes[149,150]. Overall, the mechanisms underlying the hepatic responses to the bioactive substances derived from gut bacteria have been associated with the regulation of lipid metabolism, immune signalling response and redox homeostasis[151].

Specifically, NAFLD has been associated with intestinal bacterial overgrowth and increased intestinal permeability, faecal secondary bile acids, and intestinal SCFAs, BCAAs and AAAs, such as tryptophan[152–156]. Patients with NAFLD have also been shown to have higher levels of both SCFA-producing bacteria and SCFAs[156,157]. SCFAs have multiple roles. They are involved in the regulation of inflammation, cell proliferation and mucus secretion. In hepatocytes, they are substrates for gluconeogenesis and lipid synthesis, and they promote the accumulation of hepatic FFAs by inhibiting the activity of adenosine monophosphate-activated protein kinase[157].

As discussed earlier, the gut microbiota modulates the bile acid pool, and the composition of the gut microbiome determines the deconjugation, dehydroxylation and oxidation of secondary bile acids[158]. Moreover, bile acids can modulate the gut microbial composition owing to their antimicrobial properties, either directly or by indirect effects through FXR-induced antimicrobial peptides as shown in murine models and in ileal explants[159–161]. Moreover, they can also modify the gut barrier function, for example by regulating tight junctions and their actions[162]. Bile acids influence FXR-mediated signalling in the intestine and the liver and, therefore, have a substantial effect on overall metabolism.

Changes in intestinal microbiota composition can also lead to alterations in bioavailability of choline and increased portal influx of trimethylamine, both of which have been associated with hepatic steatosis and damage in mice[141]. Choline deficiency is associated with a decreased methylation capacity, perturbed phosphatidylcholine synthesis, and impaired VLDL assembly and secretion, which can then initiate the accumulation of triglycerides in the liver[163,164].

Other microbial metabolites that have been identified as potential inducers of steatosis and hepatic inflammation in humans include AAAs such as phenylacetic acid, imidazole propionate and 3-(4-hydroxyphenyl)lactate[165,166]. Other derivatives of the amino acid tryptophan, such as indolic compounds (indole and indole-3-acetate), have been indicated to have a protective role by preserving liver integrity in mice[167].

Fluxomics in NAFLD–NASH

While metabolomics and lipidomics describe a static situation, the measurement of metabolic fluxes, so-called fluxomics, enables a dynamic picture of the situation providing a better understanding of phenotype. In metabolic diseases, such as NAFLD, the metabolic remodelling is due to changes in metabolic fluxes[168]. Fluxomic analysis requires the use of stable isotope MS measurement of tracer enrichments and labelled metabolic products, and mathematical models of tracer kinetics in plasma samples (FIG. 4).

Human metabolic flux analysis using stable isotope tracers labelled with ^{13}C and/or ^{2}H have been used since the 1970s to study glucose metabolism (that is, to measure endogenous glucose production, glucose disposal, gluconeogenesis, glycogenolysis, and hepatic and peripheral insulin resistance) as well as lipid metabolism (that is, lipolysis, DNL, VLDL secretion and clearance, and triglyceride synthesis). ^{15}N isotopes have been used to study amino acid metabolism (that is, protein synthesis and catabolism, and urea turnover)[169].

Using fluxomic analysis, it has been demonstrated that patients with biopsy-proven NAFLD have increased insulin resistance at the level of the liver, muscle and adipose tissue[70] and that gluconeogenesis is increased independently of the amount of liver fat[48,170]. Moreover, metabolic diseases such as NAFLD and T2DM are also associated with increased DNL[66,69,171] and glyceroneogenesis[172,173], whereas whether VLDL secretion is impaired or reduced is still under debate[66,174].

Lately, fluxomic analysis has also been used in animal and cell studies, enabling a more comprehensive understanding of intracellular fluxes, including alterations in mitochondrial activity and the TCA cycle[116,175,176].

Conclusions and future directions

This Review evaluate the contribution of metabolomics and lipidomics in clinical studies aimed at identifying biomarkers associated with the pathophysiology of NAFLD and NASH. Considering the prevalence of NAFLD, the importance of monitoring metabolism during the early stages of disease pathogenesis, and the lack of robustly validated diagnostic and prognostic biomarkers for NAFLD, there is an urgent need for advances towards the qualification of biomarkers for NAFLD in clinical practice. Although metabolomics and lipidomics approaches have provided insightful evidence into altered metabolic pathways in NAFLD and NASH, we note that very few reported biomarkers have been validated and used in clinical practice (TABLE 2). Overall, the studies demonstrated alterations

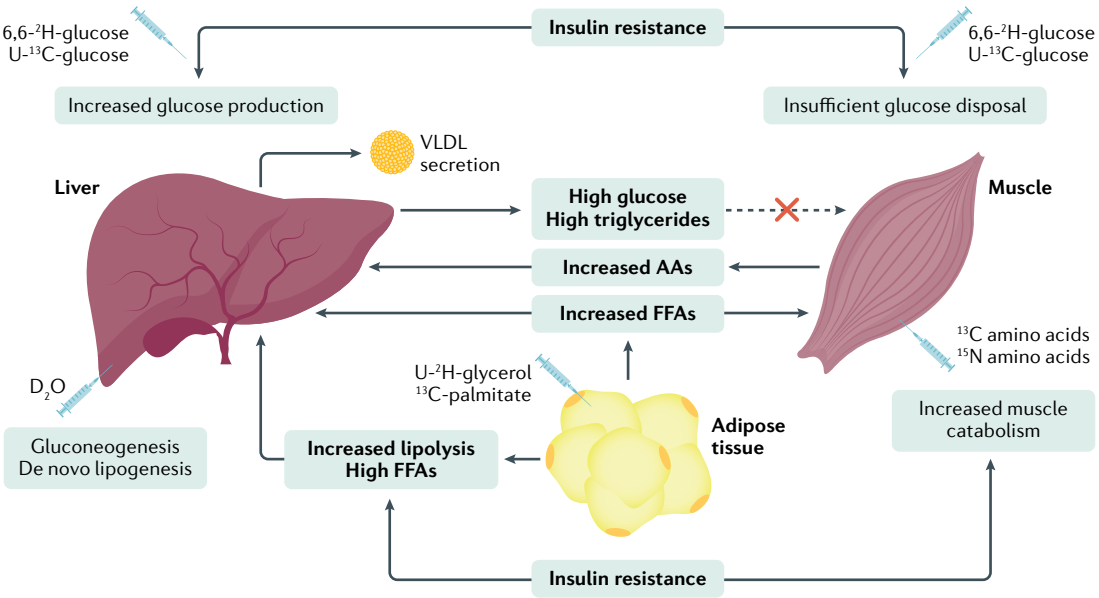


Fig. 4 | **Fluxomics in the insulin-resistant state in NAFLD.** Insulin resistance is associated with the development and progression of nonalcoholic fatty liver disease (NAFLD). In an insulin-resistant state, insulin fails to stimulate glucose metabolism in several organs, including muscle, liver and adipose tissue. This results in increased endogenous glucose production (EGP) and insufficient glucose disposal. Insulin resistance in adipose tissue results in increased lipolysis and circulating fatty acids, which in turn can contribute to the worsening of insulin resistance and ectopic fat accumulation. In addition, insulin resistance results in excess muscle catabolism and increased circulating amino acids. Using fluxomics and stable isotope (^{13}C, ^{2}H and ^{15}N) tracers, it is possible to measure metabolic fluxes in vivo in humans, including EGP, lipolysis, VLDL secretion and protein catabolism. Potential biomarkers of NAFLD include triglycerides and free fatty acids (FFAs) (due to adipose tissue lipolysis), amino acids (AAs) (released during fasting mainly by muscle protein catabolism), and hepatic lipids, such as triglycerides, ceramides and phosphatidylcholines. Adapted from REF.[185], Springer Nature.

Table 2 | **Associations between metabolite changes and NAFLD**

Biomarkers or test	Study description	Study size	Analytical method	Type of association with indication (upregulation or downregulation)	Ref.
Amino acids					
Amino acid profile	Analysis of BCAAs and other hydrophobic amino acids in insulin-resistant and insulin-sensitive individuals	1,761 individuals	LC–MS	Plasma glutamate concentration was strongly associated with insulin resistance and circulating TG; low glutamine and high glutamate concentrations were associated with incidence of diabetes	64
Amino acid profile	Association between amino acid profile and fibrosis stage of NASH	137 individuals	LC–MS	Plasma BCAA levels and BCAA to Tyr ratio were negatively associated with fibrosis stage, and Tyr levels increased with increasing fibrotic staging; Tyr levels were also correlated with HOMA-IR	46
Amino acid profile	Association between amino acid profile and liver histology in patients with NASH	64 individuals	GC–MS	Increased plasma amino acid concentrations were observed mainly in patients with obesity and NAFLD; association between GSG index (glutamate/(serine + glycine)) and ALT, AST and especially GGT; GSG index discriminates mild and severe fibrosis	23
Methionine	Measurement of methionine metabolism, turnover and transsulfuration in NASH	34 individuals	GC–MS	Attenuated activity of methionine adenosyltransferase and lower rate of synthesis of SAMe in NASH	53
Amino acid profile	Analysis of liver from individuals with simple steatosis or with NASH with or without fatty liver; integration of metabolomics and transcriptomics	58 individuals	LC–MS and transcriptomics	Increased BCAA and tyrosine, and increased BCAT1 activity in liver of patients with NASH	22
Glycine, serine and urea	Glycine oxidation and urea synthesis; contribution of glycine carbon to serine in NASH	14 individuals	GC–MS	Increased synthesis of serine from glycine in NASH	52
Amino acids and acylcarnitines					
Plasma amino acid profile and acylcarnitines	Effect of obesity on amino acid profile	141 individuals	GC–MS	Increased BCAAs, tyrosine, phenylalanine, alanine, glutamate–glutamine and decreased glycine in individuals with obesity	29
Metabolome or lipidome					
Bile acids, glutathione lipid, amino acid metabolism	Prediction of steatosis and NASH	60 individuals	LC–MS and GC–MS	Higher levels of BCAAs, phenylalanine, tyrosine and glutamate, but lower plasma GSH in NASH; alterations in levels of FFA: C20:5n-3, C22:6n-3, C11:1n-1 and C20:4n-6 reduced in NASH compared with controls; C10:0 and C11:1n-1 decreased in steatosis compared with controls; C18:3n-3 and C11:0 increased in steatosis compared with NASH	21
265 metabolites	Metabolomics analysis of NAFLD and effect of lifestyle intervention	40 individuals	LC–MS and GC–MS	BCAAs leucine and isoleucine, ornithine, acylcarnitines and LPC discriminate between insulin-sensitive and insulin-resistant patients with NAFLD	38
GSMM	Investigation of the metabolic differences in liver between individuals with varying degrees of steatosis	86 individuals	GSMMs	Fasting plasma levels of glycine and serine showed significantly negative correlations with hepatic steatosis	18
Metabolomic profile	Integration of global transcriptomic data, from human liver biopsy samples, and metabolic flux data, measured across the human splanchnic vascular bed, within a GSMM of human metabolism	16 individuals	GC–MS	Increased fasting hepatic import and degradation of glycine, serine, tyrosine, cysteine and alanine, with net release of glutamate, fatty acids, TG and glucose in NAFLD	19
Lipidomic profiling	Study of NAFLD progression by lipidomics in hepatic tissue	27 individuals	Folch method and GC–MS	Decrease in arachidonic acid in FFA, TG and phosphatidylcholines in NASH; eicosapentaenoic acid and docosahexanoic acid decreased in TG in NASH; the n-6 to n-3 FFA ratio increased in NASH	78

Table 2 (cont.) | **Associations between metabolite changes and NAFLD**

Biomarkers or test	Study description	Study size	Analytical method	Type of association with indication (upregulation or downregulation)	Ref.
Metabolome or lipidome (cont.)					
Plasma lipid and eicosanoid profiling	Circulating lipidomic profiles associated with NAFLD	125 individuals	LC–MS and GC–MS	Increased total plasma monounsaturated fatty acids; progressive increase in LOX metabolites 5-HETE, 8-HETE, and 15-HETE from normal liver to NAFLD to NASH; increased levels of 11-HETE in NASH	84
Oxidized lipids	Evaluation of the circulating profile of lipid oxidation products in patients with NAFLD	122 individuals	LC–MS	Increased levels of 9-HODE, 13-HODE, 9-oxoODE and 13-oxoODE in NASH compared with steatosis or normal liver	87
Lipidomics and amino acids analysis	Discrimination between normal liver and NAFLD, and discrimination between steatosis and NASH	467 individuals	LC–MS	BMI-dependent lipidomic profiles associated with NAFLD and NASH; increased levels of TG in NAFLD and decreased PUFAs in NASH compared with steatosis; several lipid classes, especially sphingolipids or HETEs, differentially altered depending on the BMI subgroup in NASH compared with steatosis	25
Lipidomic profiling	Blood biomarkers for prediction of liver fat content and NAFLD	679 individuals	LC–MS	Increased levels of TG species with low carbon number and double-bond content and decreased levels of LPC and ether phospholipids in NAFLD	97
Lipidomic profiling	Correlation of lipid biomarkers with NAFLD progression	88 individuals	LC–MS	Significant differences in TG, cholesteryl ester, sphingolipids and phospholipids across disease states	92
Lipidomics and amino acids analysis	Identification of NASH subtypes	535 individuals	LC–MS	Determination of NAFLD subtype classification and determination of NASH biomarkers per subtype	58
Bile acids					
Bile acid profiling	Characterization of bile acid profiles in liver tissue of patients with steatohepatitis	37 individuals	GC–MS	Chenodeoxycholic acid was significantly correlated with hepatic fibrosis in NASH	133
Bile acid profiling	Bile acid synthesis alteration in progression from steatosis to NASH	48 individuals	LC–MS	Increased levels of taurine-conjugated bile acids, and decreased levels of glycodeoxycholic acid in NASH	140
Bile acid profiling	Assessment of bile acid alterations associated with NASH	58 individuals	LC–MS	Alterations in bile acids strongly correlated with insulin resistance in NASH	138
Bile acid profiling of serum and urine	Study of bile acid profiling in patients with NASH in fasting or postprandial conditions	24 individuals	LC–MS	Increase in bile acid levels, including cytotoxic secondary and the more hydrophobic species in NASH	147

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCAAs, branched-chain amino acids; BCAT1, branched-chain amino acid transaminase 1; FFA, free fatty acid, GC, gas chromatography; GGT, γ-glutamyltransferase; GSG, glutathione synthesis; GSH, glutathione; GSMMs, genome-scale metabolic models; HETE, hydroxyeicosatetraenoic acids; HODE, hydroxy-octadecadienoic acid; HOMA-IR, homeostatic model assessment for insulin resistance; LC, liquid chromatography; LOX, lipoxygenase; LPC, lysoglycerophosphatidylcholine; MS, mass spectrometry; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; oxoODE, oxo-octadecadienoic acid; PUFA, polyunsaturated fatty acid; SAMe, S-adenosyl methionine; TG, triglyceride; Tyr, tyrosine.

in amino acid metabolism and several aspects of lipid metabolism (TABLE 3).

Impairment in amino acid metabolism in NAFLD is linked to insulin resistance and results in higher fasting concentrations of essential amino acids, as has also been observed in obesity. In patients with chronic liver disease, AAAs, in particular phenylalanine and its metabolite tyrosine, are frequently found to be decreased whereas BCAAs, especially leucine, isoleucine, valine and glutamate–glutamine are increased, mainly because of insulin resistance. However, as liver disease progresses, the opposite is often observed, with high AAAs and reduced BCAAs, especially in patients with chronic hepatic insufficiency. In addition, a reduction in hepatic glutathione levels and elevation of methionine levels has been associated with liver damage and the progression of NAFLD to NASH. However, there is some inconsistency in the identified patterns and levels of reported amino acids in patients with NAFLD–NASH. This inconsistency is, in part, due to application of different metabolomics approaches, but also to the characterization of the clinical cohort (for example, different stage of NAFLD or different insulin resistance state). Our assessment further emphasizes the importance of implementing validated methods as well as identifying large well-characterized clinical cohorts.

Another important observation is related to alterations in lipid metabolism. NASH is strongly associated with alterations in circulating fatty acid flux and intact lipids such as triglycerides and phospholipids, which is partially due to alterations in liver DNL, lipolysis rate and VLDL metabolism. Elevation in peripheral fatty acid

Table 3 | **Non-invasive predictive panels based on metabolomics for NAFLD and NASH diagnosis**

Metabolic class	Panel	Metabolites included	Analytical method	Panel description	Study size	AUROC	Ref.
Oxidized fatty acids	oxNASH	Ratio 13-HODE to linoleic acid, age, BMI and AST	LC–MS	Risk score for the diagnosis of NASH	Estimation cohort ($n = 73$)	0.83 (95% CI 0.73–0.93)	87
					Validation cohort ($n = 49$)	0.74 (95% CI 0.60–0.98)	
	NA	11,12-diHETrE	LC–MS	Differentiation between NAFL and NASH	29 individuals	1	85
	NA	dhk PGD2 and 20-COOH AA	LC–MS	Differentiation between NAFL and NASH	29 individuals	1 (panel); if considered individually: dhk PGD2: 0.93 (95% CI 0.82–1.00) 20-COOH AA: 0.96 (95% CI 0.86–1.00)	
Oxidized fatty acids and PUFAs	NA	5-HETE, 7,17-DiHDPA, adrenic acid, arachidonic acid, eicosapentaenoic acid, 16-HDoHE and 9-HODE	LC–MS	Prediction of liver fibrosis improvement (≥1 stage)	63 patients with NASH with stage 2 or 3 fibrosis; longitudinal study: baseline and 24 weeks	0.74 (95% CI 0.62–0.87)	86
TGs	OWLiver	28 TGs and BMI	LC–MS	Discrimination between normal liver and NAFLD and between NAFL and NASH	Discovery cohort ($n = 467$)	NAFLD vs normal liver: 0.90 ± 0.02 NASH vs NAFL: 0.90 ± 0.01	95
					Validation cohort ($n = 192$)	NAFLD vs normal liver: 0.88 ± 0.05 NASH vs NAFL: 0.79 ± 0.04	
					Validation cohort in patients with T2DM ($n = 220$)	NASH vs NAFL: Patients with HbA_{1c} <53 mmol/mol: 0.79 (95% CI 0.68–0.90) Patients with HOMA-IR <3: 0.87 (95% CI 0.76–0.97)	96
TGs and phospholipids	Lipid triplet	TG(16:0/18:0/18:1), PC(18:1/22:6) and PC(O-24:1/20:4)	LC–MS	Prediction of NAFLD	679 individuals	Discovery series ($n = 287$): 0.74 (95% CI 0.69–0.80) Validation series ($n = 392$): 0.71 (95% CI 0.67–0.77) Discovery and validation series combined: 0.79 (95% CI 0.75–0.82)	97
Phospholipids, acylcarnitines and amino acids	NA	LPC C16:0, ornithine, leucine, isoleucine, and C3:0, C16:0, and C18:0 carnitines	LC–MS and GC–MS	Differentiation between insulin-resistant and insulin-sensitive patients with NAFLD	Discovery cohort of patients with NAFLD ($n = 40$) Individuals without NAFLD ($n = 17$)	Discovery cohort patients with NAFLD: Baseline: 0.77 ($P = 0.00023$) Follow-up after a 9-month lifestyle intervention: 0.80 ($P = 0.000019$) Individuals without NAFLD: no differences between the insulin-sensitive and insulin-resistant individuals	38
Phospholipids and amino acids	NASH ClinLipMet score	AST, fasting insulin, PNPLA3 genotype, glutamate, isoleucine glycine, LPC(16:0), and PC(40:6)	LC–MS and GC–MS	Prediction of NASH	318 individuals	0.866 (95% CI 0.82–0.91)	51

20-COOH AA, 20-carboxy arachidonic acid; AST, aspartate aminotransferase; AUROC, area under the receiver operating characteristic curve; dhk PGD2, 13,14-dihydro-15-ketoprostaglandin D_2; DiHDPA, dihydroxydocosapentaenoic acid; diHETrE, dihydroxyeicosatrienoic acid; GC, gas chromatography; HDoHE, hydroxydocosahexaenoic acid; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HOMA-IR, homeostatic model assessment for insulin resistance; LC, liquid chromatography; LPC, lysoglycerophosphatidylcholine; MS, mass spectrometry; NA, not applicable; NAFL, nonalcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PC, phosphatidylcholine; PNPLA3, patatin-like phospholipase domain-containing protein 3; PUFA, polyunsaturated fatty acid; T2DM, type 2 diabetes mellitus; TG, triglyceride.

flux and triglycerides containing fatty acids with low carbon number and double bonds as well as reduction in the levels of triglycerides containing PUFAs, including ω-3 and ω-6 fatty acids, have been observed in NAFLD. Interestingly, this pattern of changes in triglycerides has also been reported in metabolic disorders, especially those associated with insulin resistance. Moreover, several studies have shown an increase in total bile acids in both liver and plasma of patients with NASH. In particular, a statistically significant increase in fasting plasma concentration of bile acids including glycocholic, taurocholic and taurochenodeoxycholic acid in patients with NASH has been observed. Although there are several reports on potential biomarkers, very few have been validated and used in the clinic, which is not unexpected, as different methods were applied in different populations.

From an analytical perspective, the method of metabolite or lipid identification and quantification in particular might have a major effect on biomarker selection. In addition, some of the findings are based on relative differences in molecular biomarker concentrations, and it is not known whether this would be sufficient for diagnosis or prognosis of NASH.

For diagnostic applications, as well as for the reproducibility of biomarker studies in general, it would be important to accurately quantify the metabolites, that is, by using appropriate internal standards, calibration and quality control, and using standard reference materials such as NIST-SRM 1950 (REF.[177]). Although quantification might not be practically possible in comprehensive screening studies covering hundreds or even thousands of metabolites, steps can already be taken to improve the accuracy of quantification, as, for example, recommended in the lipidomics community[177]. Subsequent validation studies would need to further focus on metabolite quantification, as this is the best way to assure the reliability of diagnostic signatures. Across different analytical methods, relative concentrations of different metabolites measured might change if not accurately quantified. Additionally, for each biomarker, it would be essential to assess analytical variation with respect to normal, within-person variation. Moreover, the role of biomarker trajectories in individual patients was not assessed in most of the studies, which could be essential for understanding the long-term outcome of patients.

In order to derive robust metabolic signatures of NAFLD–NASH, the reported biomarkers will need to be consolidated and validated. From an analytical perspective, this can be achieved, for example by harmonization of analytical approaches, including the use of similar methods of quantitation. While the discovery of metabolomics platforms with comprehensive metabolite coverage is important for the identification of putative disease biomarkers, further developments and validation of these biomarkers will require methods that can achieve accurate quantification of selected metabolites, thus assuring better interlaboratory and interstudy reproducibility. Current efforts to improve metabolomics and lipidomics workflows[177,178] are expected to improve our ability to effectively bridge the biomarker discovery stage, validation studies and translation to the clinic.

Another area that is likely to lead to substantial advances in NASH biomarkers is investigation of multiomics biomarkers[179–182]. The gut microbiome is also a rich source of circulating metabolite biomarkers, as discussed in this Review and elsewhere[148,151,152,167]. Genome-scale metabolic reconstructions are now available for large numbers of gut microbial species[183]. Application of genome-scale metabolic modelling on shotgun metagenomics data from stool samples can predict which metabolites are being produced in specific diseases or physiological conditions[184]. Such predictions can be validated by stool and serum metabolomics. Adding different layers of omics data is thus likely to lead to both improved sensitivity as well as specificity of NAFLD–NASH biomarkers. However, clinical validation and translation of multimodal diagnostic tools might prove more challenging than only a single layer, both technologically as well as from the health economics perspective. Nevertheless, adding multiple layers of data will enrich our knowledge about the complex pathophysiology of NAFLD–NASH and is expected to facilitate our search for biomarker candidates with the greatest sensitivity and specificity.

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Author contributions
M.M. researched data for the article, made a substantial contribution to discussion of content, wrote the first draft, and reviewed/edited the manuscript before submission. A.G. and T.H. made a substantial contribution to discussion of content, wrote the article, and reviewed/edited the manuscript before submission. E.A. researched data for the article and wrote the article. C.A. and M.G. researched data for the article, and reviewed/edited the manuscript before submission. J.B., Q.M.A., O.M., P.O. and J.M.M. reviewed/edited the manuscript before submission. J.-F.D. made a substantial contribution to discussion of content, and reviewed/edited the manuscript before submission. M.O. researched data for the article, made a substantial contribution to discussion of content, contributed to writing and reviewed/edited the manuscript before submission.

Competing interests
J.B. was an employee and shareholder of Pfizer. E.A., C.A. and P.O. are employees of OWL Metabolomics. A.G. is a consultant for Eli Lilly, Inventiva, Genentech, Menarini, Gilead, Novo Nordisk, AstraZeneca and Boehringer outside the submitted work. J.-F.D. is on advisory committees for Abbvie, Bayer, BMS, Falk, Genfit, Genkyotex, Gilead Science, HepaRegenix, Intercept, Lilly, Merck and Novartis; speaking and teaching at Bayer, BMS, Intercept, Genfit, Gilead Science and Novartis. Q.M.A. has active research collaborations (including research collaborations supported through the EU IMI2 LITMUS Consortium*) with Abbvie, Antaros Medical*, Allergan/Tobira*, AstraZeneca*, BMS*, Boehringer Ingelheim International GMBH*, Echosens*, Ellegaard Gottingen Minipigs AS*, Eli Lilly & Company Ltd.*, Exalenz Bioscience Ltd.*, Genfit SA*, Glympse Bio, GlaxoSmithKline, HistoIndex*, Intercept Pharma Europe Ltd.*, iXscient Ltd.*, Nordic Bioscience*, Novartis Pharma AG*, Novo Nordisk A/S*, One Way Liver SL*, Perspectum Diagnostics*, Pfizer Ltd.*, Resoundant*, Sanofi-Aventis Deutschland GMBH*, SomaLogic Inc.* and Takeda Pharmaceuticals International SA*, acts as a consultant for 89Bio, Abbott Laboratories, Acuitas Medical, Allergan/Tobira, Altimmune, AstraZeneca, Axcella, Blade, BMS, BNN Cardio, Celgene, Cirius, CymaBay, EcoR1, E3Bio, Eli Lilly & Company Ltd., Galmed, Genentech, Genfit SA, Gilead, Grunthal, HistoIndex, Indalo, Imperial Innovations, Intercept Pharma Europe Ltd., Inventiva, IQVIA, Janssen, Madrigal, MedImmune, Metacrine, NewGene, NGMBio, North Sea Therapeutics, Novartis, Novo Nordisk A/S, Pfizer Ltd., Poxel, ProSciento, Raptor Pharma, Servier, Terns, Viking Therapeutics and Speaker for Abbott Laboratories, Allergan/Tobira, BMS, Clinical Care Options, Falk, Fishawack, Genfit SA, Gilead, Integritas Communications, Kenes and MedScape, and has received royalties from Elsevier Ltd (Davidson's Principles & Practice of Medicine textbook). All other authors declare no competing interests.

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