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Editorial Board Member of *World Journal of Hepatology*, Narina Sargsyants, MD, PhD, Deputy Director (Development, Science) in National Centre for Infectious Diseases, Head of Department of Infectious Diseases and Epidemiology at National Institute of Health, Ministry of Health Advisor in the field of Infectious Diseases, Yerevan 0033, Armenia. sknarina70@mail.ru

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Rodent models and metabolomics in non-alcoholic fatty liver disease: What can we learn?

Maria Martin-Grau, Vannina G Marrachelli, Daniel Monleon

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Maria Martin-Grau, Daniel Monleon, Department of Pathology, University of Valencia, Valencia 46010, Spain

Vannina G Marrachelli, Department of Physiology, University of Valencia, Valencia 46010, Spain

Vannina G Marrachelli, Daniel Monleon, Health Research Institute INCLIVA, Valencia 46010, Spain

Daniel Monleon, CIBER de Fragilidad y Envejecimiento Saludable (CIBERfes), Madrid 28029, Spain

Corresponding author: Daniel Monleon, PhD, Professor, Department of Pathology, University of Valencia, Avda Blasco Ibañez 15, Valencia 46010, Spain. daniel.monleon@uv.es

Abstract

Non-alcoholic fatty liver disease (NAFLD) prevalence has increased drastically in recent decades, affecting up to 25% of the world's population. NAFLD is a spectrum of different diseases that starts with asymptomatic steatosis and continues with development of an inflammatory response called steatohepatitis, which can progress to fibrosis. Several molecular and metabolic changes are required for the hepatocyte to finally vary its function; hence a "multiple hit" hypothesis seems a more accurate proposal. Previous studies and current knowledge suggest that in most cases, NAFLD initiates and progresses through most of nine hallmarks of the disease, although the triggers and mechanisms for these can vary widely. The use of animal models remains crucial for understanding the disease and for developing tools based on biological knowledge. Among certain requirements to be met, a good model must imitate certain aspects of the human NAFLD disorder, be reliable and reproducible, have low mortality, and be compatible with a simple and feasible method. Metabolism studies in these models provides a direct reflection of the workings of the cell and may be a useful approach to better understand the initiation and progression of the disease. Metabolomics seems a valid tool for studying metabolic pathways and crosstalk between organs affected in animal models of NAFLD and for the discovery and validation of relevant biomarkers with biological understanding. In this review, we provide a brief introduction to NAFLD hallmarks, the five groups of animal models available for studying NAFLD and the potential role of metabolomics in the study of experimental NAFLD.

Key Words: Non-alcoholic fatty liver disease; Liver disease; Rodent models; Metabolic profiling; Metabolomics; Biomarkers

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Core Tip: Non-alcoholic fatty liver disease (NAFLD) is a spectrum of different diseases that starts with asymptomatic steatosis, continues with steatohepatitis, and can progress to fibrosis. Current knowledge suggests that NAFLD initiates and progresses through most of nine hallmarks. Animal models remain crucial for understanding the disease and for developing tools based on biological knowledge. Metabolomics seems a valid tool for studying metabolic pathways and organ crosstalk in NAFLD. In this review, we provide a brief introduction to NAFLD hallmarks, the five groups of animal models available for studying NAFLD and the potential role of metabolomics in the study of experimental NAFLD.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) prevalence has increased drastically in the last decades, affecting up to 25% of the world's population[1]. The rise of disorders such as obesity and type 2 diabetes mellitus, as well as changes in lifestyle and diet composition, have led to a worldwide increase in the incidence of NAFLD[2-5]. Given that NAFLD reduces life expectancy by four years and triggers the appearance of different comorbidities such as cardiovascular disease, kidney damage or osteoporosis[3-5], it seems vital for specialists to establish accurate and precise guidelines or strategies to address the disease[6]. Assuming that the first stages of NAFLD are reversible[2] and to control the disease worldwide, there is a need for new non-invasive methods based on diagnostic and predictive biomarkers to help diagnose NAFLD in these early stages and avoid of the biopsy, which remains the gold standard diagnostic method[7,8]. The use of animal models remains crucial for understanding the disease[9] and for developing tools based on biological knowledge. In this review, we will provide an updated summary on NAFLD development, the importance of experimental animals uses, the rodent models currently applied, and use of metabolomics as a new methodology for improving understanding and management of NAFLD.

NAFLD DISEASE

NAFLD is a spectrum of different diseases that starts with asymptomatic steatosis (NAFL) and continues with onset of an inflammatory response called steatohepatitis (NASH), which can progress to fibrosis. This hepatic fibrosis may produce cirrhosis and eventually, hepatocellular carcinoma (HCC)[2]. The first theory to explain NASH development, proposed in 1998, was known as the “two hits” hypothesis[10]. The first hit was fat storage in the hepatocytes, which would induce steatosis, the second hit being increased oxidative stress in the hepatocytes which would stimulate lipid peroxidation. It was believed this double hit was necessary to induce disease onset[10]. Currently, the “two hits” concept is considered old-fashioned by many experts. The hepatocyte needs several molecular and metabolic changes for its function to finally vary. Instead, it seems more precise to propose a “multiple hit” hypothesis[11]. This premise is intended to provide greater insight into NAFLD pathology and considers the different events that can take place in predisposed subjects during development of the disorder. Fat accumulation and synthesis of reactive oxygen species are essential events, yet other phenomena are also important and can be considered hallmarks of NAFLD initiation and progression (Figure 1).

Environmental factors

Among environmental factors, the most prominent are dietary habits, physical activity, and socio-economic aspects. Increased calories intake, and consumption of high-sugar and high-fat diets increases the risk of developing not only NAFLD but also conditions such as obesity and type 2 diabetes mellitus [4,8,12]. Hallsworth *et al*[13] was the first to show an association between sedentary behavior and physical activity levels in patients with NAFLD, finding that these patients were on average more

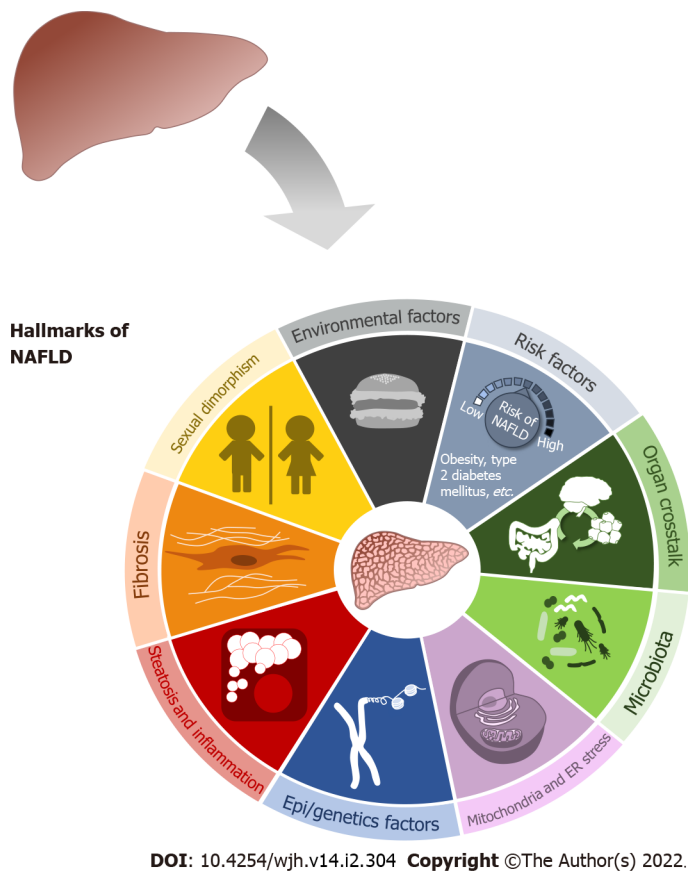


Figure 1 Hallmarks of non-alcoholic fatty liver disease. Previous studies and current knowledge suggest that in most cases, non-alcoholic fatty liver disease initiates and progresses through most of these nine hallmarks, although the triggers and mechanisms for them can be diverse. NAFLD: Non-alcoholic fatty liver disease.

sedentary, walked less and spent less time on physical activity. Furthermore, it has been demonstrated that lifestyle interventions in diet and physical activity could improve the disease prognosis[12]. Finally, regarding socio-economic aspects, the role of educational level and family economic status in development of NAFLD is still under debate[4].

Intracellular factors

At the cellular level, important events such as mitochondrial dysfunction[14], endoplasmic reticulum (ER) stress[15,16], and activation of the inflammasome[17] contribute to fat accumulation in cells (steatosis) and inflammation. Genetic variants and epigenetic factors must also be taken into account in NAFLD progression[11,18]. A decade ago, PNPLA3 I148M was the first genetic variant reported to be associated with NAFLD. Currently, 13 genetic variants have been linked to increased risk of NAFLD or NASH, with the exception of the variant UCP2 866, which reduces the risk of NASH[18]. Some of these variants, such as TM6SF2, PNPLA3, NCAN, and PPP1R3B, have been linked to inherited NAFLD[8].

Extracellular factors

As a complete organ, the liver includes many non-parenchymal cells besides hepatocytes which contribute to the proper functioning of the organ. Among these are liver sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs) and several immune cells, such as Kupffer cells[19,20]. Most of these cell types are essential to maintain homeostasis in the liver at the extracellular level, and alteration in their function has been associated with the NAFLD progression. LSECs maintain portal pressure and inhibit HSCs and Kupffer cells activation. During the first reversible stage of NAFLD, LSECs lose their functions, and, in turn, induce inflammation and fibrosis[21,22]. HSCs contribute to initiation and progression of liver fibrosis[19,23,24], one of the hallmarks of NAFLD evolution. Immune cells can be activated during liver disease creating a pro-inflammatory environment in the organ which contributes to NASH, fibrosis, cirrhosis, and HCC progression[19,25].

Organ crosstalk

NAFLD illness is not limited to hepatic disease: the NAFLD liver interacts with other organs, creating an organ crosstalk[26], which provides further support for the “multiple hits” hypothesis. As a first example, adipose tissue (AT) dysfunction is related to NAFLD disease[11,26]. Excess fat consumption

produces hypertrophy in adipocytes. AT can release several hormones or cytokines called adipokines which generate a pro-inflammatory environment[27]. This inflammatory state occurs first in the AT, then in the liver[28]. Furthermore, noncoding RNA[29] and extracellular vesicles[26,30] from the AT are linked to development of NAFLD and cell-to-cell communication. In the context of NAFLD, the gut-liver axis refers to the relationship between gut integrity, gut microbiota, and the liver[11,26,31]. Both organs are directly connected by the portal vein. In general, the gut presents different kinds of barriers and mechanisms to maintain its integrity. One function of these barriers is to control the passage of substances into the portal vein and the liver[31,32]. Further evidence suggests that the intestinal barriers are altered, and intestinal permeability is increased in NAFLD disease. Taking advantage of this altered permeability, bacteria can translocate more easily into the blood, enter the portal vein and finally reach the liver[32]. Increased gut permeability and bacterial translocation are associated with liver cirrhosis[31]. The gut microbiota is also altered in NAFLD due to intestinal microbial dysbiosis[33]. It has been shown that bacteria phyla are modified under high-fat diet-induced liver steatosis in rodent models[34] and human studies of NAFLD, NASH, and cirrhosis[35]. Variations in bacteria composition lead to altered concentration of some metabolites. This phenomenon, added to reduced permeability, triggers the arrival of molecules such as lipopolysaccharides in the systemic circulation and activation of Toll-like receptor in cells. Moreover, metabolism of trimethylamine which can be oxidized in the liver ultimately forming trimethylamine N-oxide, has been linked to NAFLD progression and cardiovascular disease[33]. Additionally, the liver has been associated with the brain[26]. The arcuate nucleus of the hypothalamus regulates satiety. In 2005, De Souza *et al*[36] proved that a high-fat diet caused several proinflammatory-related changes in mRNA expression in the hypothalamus of Wistar. Furthermore, cirrhotic patients can develop hepatic encephalopathy, a neurological comorbidity associated with NAFLD disease[37,38]. Finally, the kidney and the liver have also been linked. The study of Musso *et al* [39] in 2014 revealed that NAFLD severity was correlated with severity of chronic kidney disease (CKD). Many pathways are shared between NAFLD and CKD, so progression of NAFLD will contribute to CKD progression and vice versa[40].

Sexual dimorphism

NAFLD affects more men than women[41,42], due to the protective role of estrogens against disease development[43-46]. Nonetheless, in women of a certain age and under certain risk factors, incidence is higher than in men and they experience a more aggressive disease course. These risk factors are: (1) Earlier age of menarche; (2) Polycystic ovary syndrome; (3) Gestational diabetes; and (4) Menopause[46-49]. Interestingly, sex differences extend beyond incidence rates: NAFLD appears to develop in distinct ways in males and females[50-54]. However, further studies on about molecular processes are needed for enhanced insight into sexual dimorphism in NAFLD[55].

RODENT MODELS IN NAFLD

NAFLD is a complex disease which affects many hepatic parameters, as well as functions of other organs. With current methodologies, it is virtually impossible to study the “multiple hits” hypothesis of NAFLD as a whole in humans, because this requires access to multiple tissues, biofluids, and controlled environments. Animal models therefore remain essential for studying initiation and progression of NAFLD, and present various advantages over clinical research: (1) The possibility to obtain multiple samples and carry out longitudinal studies; (2) Shorter time to disease onset; (3) The possibility of controlling the variables of our model; and (4) Use of genetically modified animals to study a specific gene or metabolic pathway alteration. Compared to *in vitro* studies, animal models can be used to study the whole liver and organ crosstalk between the liver and other organs[56].

Nevertheless, a perfect animal model[9,57] providing information on all potential triggers and causes of NAFLD is elusive. Therefore, it is vital to know the stage of the disease to be studied and which model reproduces the physiopathological characteristics we want to study. Focusing on model selection, among key common characteristics, a good model must imitate certain aspects of the human NAFLD disorder, be reliable and reproducible, have low mortality, and be compatible with simple and viable methods[9]. Development of obesity or insulin resistance, AT inflammation, alterations of intestinal physiology, and a specific liver phenotype (Table 1) are traits that mimic human NAFLD[58,59]. Several animal models can be used to study metabolic diseases, including NAFLD, but rodents are the most commonly used. Rodent models are preferred because they easily develop obesity, type 2 diabetes mellitus, and NAFLD[60]. In mice, the ideal model genetic background is the strain C57BL/6, and specifically the substrain C57BL/6J, as C57BL/6J mice are more insulin resistant than C57BL/6N mice[61], which allows for better isolation of the NAFLD process from other metabolic alterations. For rat models, Wistar or Sprague Dawley rats are usually chosen, although other models besides rats and mice, such as New Zealand white rabbits, Guinea pigs, or Tree shrews, have also been used[60]. Rabbits, and many non-rodent models like pigs, have the important advantage of longer pre-pubertal stages, which allow them to mimic the subclinical NAFLD situation in children with greater precision than would be possible with mice or rats[59,62]. Also, pigs are anatomically and metabolically more similar

Table 1 Summary of existing rodent models of non-alcoholic fatty liver disease

Rodent models	Obesity	Insulin resistance	Steatosis	NASH	Fibrosis	HCC
Dietary						
Deficient diet						
MCD	No	Hepatic IR	Yes	Yes	Yes	No
CDAA	No	No	Yes	Yes	Yes	Yes
High-amount diet						
HFD	Yes	Yes	Yes	Yes	Yes	No
HFHS	Yes	Yes	Yes	Yes	Yes	No
High fructose diet	No	Yes	Yes	No	No	No
HFHC	Yes	Yes	Yes	Yes	Yes	No
Atherogenic diet (cholesterol + cholate)	No	Hepatic IR	Yes	Yes	Yes	No
Cafeteria diet or Western diet	Yes	Yes	Yes	Yes	No	-
ALIOS	Yes	Yes	Yes	Yes	Yes	Yes
AMLN	Yes	Yes	Yes	Yes	Yes	No
DIAMOND	Yes	Yes	Yes	Yes	Yes	Yes
Genetic						
<i>ob/ob</i>	Yes	Yes	Yes	No	No	No
<i>db/db</i>	Yes	Yes	Yes	No	No	No
KK-Ay	Yes	Yes	Yes	No	No	No
<i>foz/foz</i>	Yes	Yes	Yes	No	No	No
<i>fa/fa</i>	Yes	Yes	Yes	No	No	No
PTEN knockout	No	No	Yes	Yes	Yes	Yes
PPAR- α knockout	No	No	Yes	No	No	No
SREBP-1c transgenic	No	Yes	Yes	No	No	No
Chemicals						
Tetracycline	No	No	Yes	Yes	Yes	-
CCl ₄	No	No	Yes	Yes	Yes	Yes
TAA	-	-	Yes	Yes	Yes	Yes
STZ	-	-	-	Yes	-	-
DMN	-	-	No	Yes	Yes	Yes
DEN	No	-	Yes	Yes	Yes	Yes
Porphyrinogenic agents (DDC or GF)	-	-	Yes	Yes	-	-
MSG	Yes	Yes	Yes	Yes	No	Yes
Tunicamycin	-	-	Yes	Yes	-	-
Surgical						
CBDL	-	-	Yes	Yes	Yes	-
Combined models						
<i>ob/ob</i> + MCD diet	Yes	-	Yes	Yes	No	No
<i>db/db</i> + MCD diet	Yes	Yes	Yes	Yes	Yes	No
HFD + thermoneutral housing at 30 °C	-	-	-	Yes	Yes	-

HFD + CCl ₄	No	-	Yes	Yes	Yes	Yes
HFD + DEN	Yes	-	Yes	Yes	Yes	Yes
CDA + CCl ₄	No	-	Yes	Yes	Yes	Yes
STAM model	No	-	Yes	Yes	Yes	Yes

ALIOS: American lifestyle-induced obesity syndrome model (high-fat + trans-fat + fructose); AMLN: Amylin liver NASH model; CBDL: Common bile duct ligation; CCl₄: Carbon tetrachloride; CDA: Choline-deficient, L-amino defined diet; DDC: 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DEN: Diethylnitrosamine; DIAMOND: Diet-induced animal model of non-alcoholic fatty liver disease mice; DMN: Dimethylnitrosamine; GF: Griseofulvin; HCC: Hepatocellular carcinoma; HFD: High-fat diet; HFHC: High-fat high-cholesterol diet; HFHS: High-fat high-sugars diet (mainly fructose or sucrose); MCD: Methionine and choline deficient diet; MSG: Monosodium glutamate; NASH: Non-alcoholic steatohepatitis; STZ: Streptozotocin; STAM: Stelic animal model of NASH (STZ + HFD); TAA: Thioacetamide.

to humans than rodent models. Nonetheless, these non-murine species have some drawbacks, such as they involve more complicated and less generally established genetic approaches, and housing larger animals can be more difficult from a logistic and economic point of view[59]. Models of smaller size and shorter lifetimes than mice and rats have also been explored. For example, use of zebrafish as a NAFLD model is recently increasing an inexpensive model in which NAFLD develops quickly[63].

Despite the wide variety of models, rodents are still the preferred species for experimental NAFLD research because of their small size, ease of maintenance, short life span, and available genetic resources. The current rodent models used in NAFLD can be stratified into five main groups, depending on the disease inducer: dietary, genetic, chemical, surgical, and combined (mix of different models). Pathological characteristics of these rodent models are summarized in (Table 1).

Dietary models

Dietary models, which can be classified as deficient or high amount diets, are an excellent option for studying NAFLD disease. Deficient diets are not generally found in humans, as they are based on absence of essential elements. However, in animals, methionine and choline-deficient diet (MCD) or choline-deficient, L-amino defined diet are effective in generating liver damage[64,65]. The diets most closely resembling humans experience are the high amount calorie diets with an excessively high amount of specific nutrients, mainly fats and sugars[9,57,59,66]. Different diets can be defined by the high concentration of nutrients or how they are combined. Among these are high-fat diets, high-cholesterol and cholate diets (atherogenic diet), high-fat high-cholesterol diets, high-sugar diets based on fructose or sucrose, and high-fat high-sugar diets. Their effects on NAFLD development are shown in (Table 1). Lastly, there are different animal models of NAFLD based on diets that promote NASH in a short period: (1) American lifestyle-induced obesity syndrome model (ALIOS model); (2) Amylin liver NASH model (AMLN model); and (3) Diet-induced animal model of NAFLD mice (DIAMOND model) [57]. The ALIOS model is based on a high-fat diet (45% fats, 2% trans fats), drinking water with fructose and glucose, and a sedentary behavior (cages without wire racks), promoting obesity[67]. The AMLN model is based on a high-fat (40% fats, 18% trans fats), high-fructose (22%) and cholesterol (2%) diet[68, 69]. ALIOS and AMLN are very similar, but with different fat percentages, and in the AMLN model fructose is given in pellet form rather than in drinking water[68]. A variant of the AMLN model called the Gubra amylin NASH (GAN) diet is currently used, with the same composition, but trans-fat-free diet and with increased saturated fatty acids[70]. The DIAMOND model is based on a high-fat (42%), high-carbohydrate and cholesterol (0.1%) diet but with an added high-fructose and glucose solution [71]. All these models are modified Western or Cafeteria diets (combination of fat and sugars) presenting more or less the same composition but in different proportions[63,72].

Genetic models

Genetic models allow us to study genetic and pathophysiological consequences of alterations in certain genes potentially involved in NAFLD development. These models are based on mechanistic hypotheses and have the main limitation that every specific mutation in a single gene is not usually found in humans[9]. Nevertheless, they provide two major advantages over other models: first, the means to study disease mechanisms in NAFLD, and second, the opportunity to improve our knowledge of a specific mechanism in the disease models[73]. Nowadays, genetic engineering tools have facilitated generation of transgenic animals and knockouts, either by commercial houses or in academic laboratories[62,73-75]. There are many genetic models for NAFLD, each one based on different pathways affected in the disease[76]. The genetic models most commonly used in the study of NAFLD are reported in (Table 1).

Chemical models

The most widespread chemical models for studying NAFLD are those based on liver damage through tetracycline, carbon tetrachloride (CCl₄), thioacetamide (TAA), and streptozotocin[9,65]. These models

can produce significant liver damage depending on the experimental exposure time (days, weeks, or months) and the dose delivered, but in the focus is generally, on producing liver steatosis and fibrosis [63,65,74,77]. Treatment with the chemicals diethylnitrosamine (DEN) or dimethylnitrosamine (DMN) is typically used to induce HCC and the approach may be too aggressive for studying NAFLD alone [56,60,74]. The porphyrinogenic agents (3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) and griseofulvin (GF) and the chemical monosodium glutamate (MSG) are less often used but can also induce steatosis and NASH [78]. The chemical Tunicamycin produces ER stress in the hepatocytes which can in turn induce steatosis [79,80]. Overall, chemical models represent a faster and more dramatic way to study liver damage, but the disease initiation and progression bears less resemblance to human NAFLD than diet or genetic models.

Surgical models

Hepatobiliary system surgery can induce NAFLD in experimental models. The most common surgical model is Bile Duct Ligation (BDL), which is used to produce fibrosis, cirrhosis and as a consequence, liver failure in rodents [65,74,81]. BDL can be performed in mice and rats [65], but this model is difficult to implement in mice, as several surgical complications can arise [56]. Surgical models are the least used models of NAFLD because of their complexity and lack of similarity to human NAFLD.

Combined models

Genetic models do not usually develop NASH, fibrosis, or HCC spontaneously, so they are often supplemented with diet to achieve worse liver damage [9,57,62]. This is also the case with chemical models, in which the dose for inducing liver damage is often too aggressive but combining a low dose with some NAFLD-inducing diet modifications can help producing a model that progresses at a slower pace, which allows detection of the different stages of NAFLD progression [60,65,66,77]. These combined models genetic plus diet or chemical plus diet, are also a common option for studying NAFLD [76].

METABOLOMICS IN NAFLD RODENT MODELS

Currently, liver function is routinely controlled by blood analysis in which clinicians test for transaminases, albumin, platelets, bilirubin and clotting factors. Patients presenting abnormal levels of these parameters, especially transaminases, and whose medical history reveals risk factors for diabetes, obesity or metabolic syndrome, undergoes a non-invasive imaging method, mainly ultrasonography and elastography, to confirm the presence of steatosis and fibrosis in the liver. If the result is positive, the NAFLD fibrosis score and FIB-4 index scores can be applied. Depending on the score, patients are classified as at low, medium or high risk of fibrosis. The goal of these imaging methods is to detect whether fibrosis is present, due to the different follow-up required in patients with fibrosis. An invasive imaging method, biopsy, is performed on those with a high risk of fibrosis or with an unclear diagnosis under non-invasive imaging methods [8,82-84]. Nowadays, biopsy remains the gold-standard for diagnosis of hepatic steatosis, NASH and fibrosis, as histology confirms tissue damage [7,8]. Biopsy has a relatively high incidence of false negatives, since the fragment finally analyzed only represents about 1/50000 of the organ and analysis may vary between pathologists [7]. Moreover, non-invasive imaging methods also present disadvantages. Steatosis can only be detected at over 30% and these methods cannot determine whether NASH is present [85,86]. We are still far from achieving the main objective: NAFLD prevention and a rapid diagnosis. New non-invasive diagnostic methods are needed, and one alternative could be use of metabolomics in the search for new biomarkers.

Personalized medicine has become a fundamental strategy in the future of healthcare. The possibility of tailor-made treatments for patient groups will help streamline healthcare costs and enhance efficacy and safety of interventions. The transition to a personalized medicine model has been facilitated by recent advances in "omics" technologies that are allowing the degree of personalization in the diagnosis and treatment of different diseases to be increased to levels unimaginable just a few years ago [87]. Metabolomics is an emerging research area and can be considered, at a biochemical level, as the end of the "omic" cascade since changes in the metabolome constitute the organism's last response to genetic, chemical and environmental alterations [63].

Small biochemicals are the end products of all the regulatory processes present in a cell, tissue, or organism, including transcriptional and translational regulation and posttranslational modifications. Consequently, metabolic changes are among the best reporters of the organism's response to a disease process. The application of metabolomics to the study of metabolic diseases may increase our understanding of the pathophysiological processes involved, and thus help us to identify potential biomarkers. The identification and quantification of these low molecular weight molecules define the metabolic phenotype of these diseases and studying the metabolic changes that occur in response to different pathophysiological processes may help establish the mechanisms underlying the disease.

Metabolites can be measured in several body fluids or tissues, although plasma and urine are the most frequently used samples in metabolic research, they are readily available and have clinical relevance as a source of potential biomarkers. Almost all cells in the body communicate with plasma,

either directly or through different tissues and biological fluids, releasing at least part of their intracellular content. By contrast, urine is produced by renal filtration of plasma and is widely considered to be among the most important samples for diagnosis as it contains not only many plasma components but also the catabolic products of different metabolic pathways.

Metabolic fingerprinting and metabolic profiling are two different approaches to the study of metabolites in biological samples. Metabolic fingerprinting does not aim to identify the entire set of metabolites but rather to compare patterns or fingerprints of metabolites that change in response to a disease state, pharmacological therapies, or environmental alterations. This approach can be used as a diagnostic tool to evaluate the disease state by comparing healthy controls and disease subjects. Nonetheless, qualitative and quantitative analyses are required to understand the mechanisms underlying a disease. Metabolite profiling focuses on the analysis of a group of metabolites related to a specific metabolic pathway. In this approach, target metabolites are selected beforehand and are assessed using specific analytical methods.

The analytic techniques used to study the metabolome are mass spectroscopy (MS), nuclear magnetic resonance (NMR), or a combination of both[88,89]. Each technique has its own strengths and weaknesses[88,90]. An advantage of NMR technique, is that it can be used to study tissues, including liver, without destroying the sample with the proton high-resolution magic-angle spinning probe (HR-MAS)[90,91].

Metabolomics is a very powerful tool for the study of metabolic diseases[90,92], yet applications of metabolomics to NAFLD is an understudied area. Nonetheless, some studies demonstrate the importance of measuring metabolites for better characterization of the disease. NAFLD is a metabolic illness, hence metabolomics as a technique offers the opportunity to better understand the metabolic alterations in NAFLD progression[87,92,93] and patient stratification[89]. MS and NMR have been used to study NAFLD progression in rodent models. Articles yielded from the keyword search using the term "metabolomics" and "rodent models" are shown in (Table 2). Metabolomics studies have been carried out in dietary, chemical, genetic and combined models of NAFLD. Including metabolic alterations could broaden the search for specific metabolomics biomarkers which would help in disease diagnosis.

Despite the diversity of models used in previous metabolomics studies on NAFLD rodent models (Table 2), some common findings can be extracted. Fatty acids are stored as triacylglycerols in the liver when not catabolized by β -oxidation. Consequently, fatty liver seems to be a rearrangement of lipids in the liver and not just fat storage. Most studies in liver tissue of rodent models have revealed massive accumulation of triacylglycerols (see liver extract studies in Table 2). The well-known adipocyte origin of some of these triacylglycerols suggests AT as a potential source of triacylglycerols deposited in the liver in NAFLD. Furthermore, almost all studies in NAFLD rodent models report alterations in other metabolites like glucose, lactate, pyruvate, and alanine, suggesting that NAFLD is involved in cytosolic glycolysis and oxidative stress[97,112,119]. Metabolism of branched-chain amino acids also seems to be altered in NAFLD. A previous study including human subjects and animal models in the context of hepatic insulin resistance demonstrated a link between BCAA and the tri-carboxylic acid cycle[106,108]. The integration of findings in human and rodent model studies seems very complex. In a translational human-animal study, Han *et al*[123] studied the progression of fatty liver and liver steatosis, finding changes in metabolic networks related to amino acids and bile acids. However, these results were significantly different between animals and humans. Among others, taurine, a well-known amino acid with protective and antioxidant properties, was increased in humans but not in rat models. Finally, consistent finding in different rodent and human studies on NAFLD is an increased level in serum of bile acids, important molecules which signal many processes in the liver and are involved in lipid and glucose homeostasis.

CONCLUSION

NAFLD is the most prevalent liver disease worldwide. Approaches from different perspectives have led to increased insight into many aspects of the disease. Knowledge of the disease has increased with the use of animal models, especially those in rodents. Although, the perfect animal model does not exist, some models perfectly mimic several aspects of NAFLD development and have become very useful tools to address the disease in the search for biomarkers of the early reversible stages. Studying metabolism in these models provides a direct reflection of what happens inside the cell. Metabolomics seems an important tool for studying metabolic pathways and crosstalk between organs affected in animal models of NAFLD, and for identifying and validating relevant biomarkers with biological understanding.

Table 2 Studies related to “non-alcoholic steatohepatitis”, “rodent models” and “metabolomics”

Rodent model	Produced by	Animals used	Biological sample	Platform used	Ref.
Dietary	HFD	C57BL/6 mice, 6-wk-old	Liver extract and serum	UPLC-QTOF-MS and GC-MS	Kim <i>et al</i> [94]
	HFD and Paigen diet	BALB/c mice, 6-wk-old	Liver extract and urine	¹ H-NMR	Klein <i>et al</i> [95]
	HFD and HCD	C57BL/6N mice, 6-wk-old	Urine	¹ H-NMR	Jung <i>et al</i> [96]
	HFD and HCD	Wistar rats, 6-wk-old	Liver extract	¹ H-NMR	Bertram <i>et al</i> [97]
	HFD	C57BL/6S1ac mice, 4-wk-old	Urine	¹ H-NMR and UPLC-QTOF-MS	Li <i>et al</i> [98]
	HFD	C57BL/6J mice, 6-wk-old	Serum	UHPLC-QTOF-MS and GC-MS	Lai <i>et al</i> [99]
	High-fructose and saturated fatty acid diet	Sprague-Dawley rats	Liver extract	HR-MAS and ¹ H-NMR	Tranchida <i>et al</i> [100]
	HFHCC diet	C57BL/6J mice, 8-wk-old	Liver extract and plasma	GC-TOF MS and CSH-QTOF MS	Tu <i>et al</i> [101]
	HFD	Sprague-Dawley rats, 4-6-wk-old	Liver extract	LC-MS	Wan <i>et al</i> [102]
	HFD	Sprague-Dawley, 6-wk-old	Urine and feces	¹ H-NMR	Chen <i>et al</i> [103]
	MCD	C57BL/6J mice, 8-wk-old	Feces	GC-MS	Ye <i>et al</i> [104]
	HFD	Swiss albino mice	Serum and feces	¹ H-NMR	Carvalho <i>et al</i> [105]
	High fat-sucrose diet	Sprague-Dawley rats, 6-wk-old	Serum	HPLC-QTOF-MS	Xu <i>et al</i> [106]
	MCD and atherogenic diet	C57BL/6J mice, 10-wk-old	Liver extract	MS	Montandon <i>et al</i> [107]
	HFD	Sprague-Dawley rats, 6-8-wk-old	Serum	LC-MS	Cui <i>et al</i> [108]
	HFD	Sprague-Dawley, Fisher 344 and Brown-Norway rats, 5-wk-old	Liver extract	LC-MS	Boyce <i>et al</i> [109]
Genetic	Db/db mice	C57BL/6J mice, 10-wk-old	Liver extract	¹ H-NMR and UPLC-QTOF-MS	Kim <i>et al</i> [110]
	Ob/ob mice	B6.Cg- <i>Lepob</i> /J mice, 8-wk-old	Liver extract	HR-MAS and ¹ H-NMR	Gogiasvili <i>et al</i> [111]
Chemical	DEN	Sprague-Dawley rats, 4-wk-old	Liver extract	¹ H-NMR	Wang <i>et al</i> [112]
	CCl ₄	Wistar rats	Plasma	UPLC-QTOF-MS	Li <i>et al</i> [113]
	CCl ₄	Sprague-Dawley rats, 4-wk-old	Urine	GC-TOF MS	Jiang <i>et al</i> [114]
	CCl ₄	Wistar rats	Liver extract	GC-MS	Song <i>et al</i> [115]
	CCl ₄	Sprague-Dawley rats	Urine	¹ H-NMR	Wu <i>et al</i> [116]
	CCl ₄	Wistar rats	Urine	GC-MS	Fang <i>et al</i> [117]
	CCl ₄	Sprague-Dawley rats, 1-yr-old	Serum and urine	UPLC-QTOF-MS	Chang <i>et al</i> [118]
	CCl ₄	Sprague-Dawley rats, 7-wk-old	Serum	¹ H-NMR	Li <i>et al</i> [119]
	CCl ₄	Sprague-Dawley rats	Serum	¹ H-NMR	Liu <i>et al</i> [120]
	DEN	Sprague-Dawley rats, 6-wk-old	Serum	¹ H-NMR	Yang <i>et al</i> [121]
Combined model	Combined (genetic + dietary) with HCD	Acyl1 knockouts mice on a C57BL6/J background, 4-wk-old	Serum	LC-MS	Zhao <i>et al</i> [122]

CCl₄: Carbon tetrachloride; CSH-QTOF MS: Reverse-phase lipid chromatography-quadrupole/time-of-flight mass spectrometry; DEN: Diethylnitrosamine; GC-MS: Gas chromatography-mass spectrometry; GC-TOF MS: Gas chromatography-time-of-flight mass spectrometry; HCD: High-carbohydrate diet; HFD: High-fat diet; HFHCC: High-fat, high cholesterol, cholate diet; HPLC-QTOF-MS: High-performance liquid chromatography quadrupole time of flight mass spectrometry; ¹H-NMR: Proton nuclear magnetic resonance; LC-MS: Liquid chromatography-mass spectrometry; MCD: Methionine, and choline-deficient diet; MS: Mass spectroscopy; UPLC-QTOF-MS: Ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry.

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Country/Territory of origin: Spain

ORCID number: Maria Martin-Grau 0000-0002-8781-5952; Vannina G Marrachelli 0000-0002-4097-3312; Daniel Monleon 0000-0002-4803-1573.

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