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Title Page

Title

From Sugar to Liver Fat and Public Health: Systems Biology Driven Studies in Understanding Non-Alcoholic Fatty Liver Disease Pathogenesis

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Running title

Systems Biology Driven Studies of NAFLD

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Abbreviations

DNL, de novo lipogenesis; EASL, European Association for the Study of the Liver; GCKR, glucokinase regulator; GSMN, genome-scale metabolic networks; HbA1c hemoglobin A1c; HCC, hepatocellular carcinoma; HSC, hepatic stellate cells; HSD17B13, hydroxysteroid 17-beta dehydrogenase 13; KC, Kupffer cells; MBOAT7, membrane bound O-acyltransferase domain-containing 7; NAFL non-alcoholic fatty liver; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBPK, physiologically based pharmacokinetic; PNPLA3, patatin-like phospholipase domain containing 3 protein; PPARα, peroxisome proliferator activated receptor alpha; QSSPN, quasi-steady state Petri nets; ROS, reactive oxygen species; TM6SF2, transmembrane 6 superfamily member 2 protein.

Abstract

Non-alcoholic fatty liver (NAFLD) disease is now a major public health concern with an estimated prevalence of 25-30% of adults in many countries. Strongly associated with obesity and the metabolic syndrome, the pathogenesis of NAFLD is dependent on complex interactions between genetic and environmental factors that are not completely understood. Weight loss through diet and lifestyle modification underpins clinical management; however, the roles of individual dietary nutrients (e.g. saturated and omega-3 fatty acids; fructose, vitamin D, vitamin E) in the pathogenesis or treatment of NAFLD are only partially understood. Systems biology offers valuable interdisciplinary methods that are arguably ideal for application to the studying of chronic diseases such as NAFLD, and the roles of nutrition and diet in their molecular pathogenesis. Although current *in silico* models are incomplete, computational tools are rapidly evolving and human metabolism can now be simulated at the genome scale. This article will review NAFLD and its pathogenesis, including the roles of genetics and nutrition in the development and progression of disease. In addition, the article introduces the concept of systems biology and reviews recent work utilising genome-scale metabolic networks (GSMNs) and developing multi-scale models of liver metabolism relevant to NAFLD. A future is envisioned where individual genetic, proteomic and metabolomic information can be integrated computationally with clinical data, yielding mechanistic insight into the pathogenesis of chronic diseases such as NAFLD, and informing personalized nutrition and stratified medicine approaches for improving prognosis.

Non-alcoholic fatty liver disease

Introduction

Non-alcoholic fatty liver (NAFLD) is defined by fat accumulation in the liver in the absence of excess alcohol consumption. Described histologically, NAFLD may range from simple steatosis (non-alcoholic fatty liver; NAFL), where there is fatty infiltration but no evidence of hepatocellular injury, to non-alcoholic steatohepatitis (NASH), where there is evidence of inflammation and ballooning, with or without fibrosis[1]. Although the early stage of NAFL is often considered benign, 25% of patients will progress to more serious disease[2,3]. NAFLD is now the second most common cause of chronic liver disease among individuals listed for liver transplantation in the United States[4], and in the United Kingdom (UK) and Europe, the number of NAFLD-related liver transplantation has increased dramatically within the last ten years[5]. Significantly, there are currently no licensed pharmaceutical agents specific for the treatment of NAFLD; although several agents, including dietary supplements, are in Phase 2 and Phase 3 clinical trials[6]. Given the close association between NAFLD and obesity, weight loss through dietary and lifestyle intervention is the mainstay of current clinical management[1,7,8].

Diagnosis

Currently available diagnostic tools (liver enzymes, imaging and biopsy) are either non-specific, expensive, or invasive. The lack of an acceptable, inexpensive diagnostic tool makes large-scale population studies difficult[9]. Elevated liver enzymes (aspartate and alanine transaminases) are often used to define 'suspected NAFLD' at a population level. However, the majority (79%) of individuals diagnosed with NAFLD by MRI in a large population study had normal transaminase levels[10], so relying on this measure significantly underestimates the burden of disease. Imaging is non-invasive but, in the case of MRI or magnetic resonance elastography, it can be expensive and not accessible to all. Alternatively, in the case of ultrasound and transient elastography (fibroscan), it can be somewhat insensitive for the staging of NASH and fibrosis. While liver biopsies are the gold standard for staging of NASH and fibrosis, required for licencing purposes in pharmacological trials[11], biopsies have their limitations, including issues with inter-rater reliability, sampling error, cost, and acceptability for monitoring the condition in the long term.

NAFLD is closely associated with obesity and metabolic disorders. In a large meta-analysis of 86 studies, with a sample size of more than 8.5 million persons from 22 countries, more than 80% of individuals with NASH and 51% of individuals with NAFL were obese. Type 2 diabetes co-occurred in 47% of NASH cases and 23% of NAFL cases; metabolic syndrome was found in 71% NASH patients and 41% of NAFL patients[12]. For these reasons, clinical guidelines for NAFLD diagnosis[1,7,8] do not advocate general population screening, but stress that NAFLD is to be suspected

in individuals with type 2 diabetes or the metabolic syndrome; defined as 3 or more of 5 risk factors for cardiovascular disease and type 2 diabetes: hypertension, hypertriacylglycerolaemia, lowered high-density lipoprotein cholesterol, raised fasting glucose, and central obesity defined by increased waist circumference[13].

Prevalence

Given the challenges of NAFLD diagnosis, the prevalence of NAFLD can only be estimated and estimates vary depending on the diagnostic tool used. Nonetheless, it is clear that the prevalence of NAFLD varies by region and ethnicity, and the global prevalence of NAFLD is estimated to be 24%[14]. The highest reported rates are in the Middle East (32%) and South America (31%), followed by Asia (27%), the US and the UK (24 and 23%)[14]. Recent reviews of the epidemiology of NAFLD have highlighted surprising high prevalence in Asia (27% pooled estimate[12]), with country-specific estimates ranging from 15-40% for China, 25-30% for Japan, and 27-30% for Korea and India[15]. Prevalence estimates in North America have ranged from 11-46% dependent on diagnostic modality and population studied; a recent meta-analysis with random effects model concluded a pooled average of 24% [19, 29%] by ultrasound but only 13% by blood testing[12]. Prevalence in the US also depends on ethnicity with Hispanic Americans at highest risk (53%) relative to Caucasians (44%) and African Americans (35%)[10]; while American Indians have a prevalence as low as 13%[16]. Genetic variability, discussed in detail later, likely explains some, but not all of the differences in risk. The heritability of liver fat and fibrosis has estimated to be 39-52% and 50% respectively[17,18], underscoring that the environment also plays a large role in NAFLD development. Estimates of global NASH prevalence range from 1.5-6.5%[14], with estimates of 6% and 2% prevalence for NASH and NASH-related cirrhosis in the United States[2]. In sum, NAFLD is a common chronic liver disease worldwide.

Natural history

As with prevalence, defining the natural history of disease progression in NAFLD has been hampered by the reliance on liver biopsies. While only recently the disease was perceived as progressing somewhat linearly from NAFL to NASH, then to NASH plus fibrosis, and then to cirrhosis, and end stage liver disease requiring transplantation, including occasionally hepatocellular carcinoma (HCC)[19]; this perspective continues to evolve as outlined (Fig. 1). While simple steatosis in the absence of fibrosis is generally thought to have a more benign course of disease in terms of liver-specific outcomes and mortality[20,21], some patients with NAFL, so-called 'rapid progressors' can progress towards well-defined NASH with bridging fibrosis within a very few years[22]. In addition, as diagrammed (Fig. 1), based on current data it can-not be excluded that in some cases, perhaps dependent on genetic susceptibilities, a NASH liver may arise from a normal liver[23]. Moreover, an

increasing number of studies suggests that HCC can develop in a non-cirrhotic liver, further altering the early linear model of NAFLD natural history (Fig. 1)[24-26]. Increased risk for HCC in NASH likely relates to body weight, as 80% of patients with NASH are also obese[12]. A recent population-based cohort study of 5.24 million UK adults has demonstrated large increases in risk (HR >1·1 per 5 kg/m²) for liver cancer occurring in a linear fashion with increasing BMI[27].

Progression to severe liver disease in adults is in the order of decades[2,12,28]. Multiple large retrospective cohort studies (>600 patients, mean follow-up 20 years) have now demonstrated that it is fibrosis, rather than NASH, on index biopsy that is associated most strongly with increased risk of mortality and liver-related outcomes such as decompensation or transplant[21,29]. This work suggests NAFLD activity score is not clearly prognostic[29], and time to development of severe liver disease is dependent on fibrosis stage at presentation. Approximately 22–26 years for F0-1, 9.3 years for F2, 2.3 years for F3, and 0.9 years to liver decompensation in F4 fibrosis[21]. However, the risk of selection bias for follow-up liver biopsy in single-centre studies is substantial, and rates of progression may thus be overestimated in the general population. Some have expressed concern about the risk of over diagnosis in screening and monitoring individuals for NAFLD, when the majority will not develop advanced liver disease[30].

Conversely, a recent population study (n=3,041 adults >45) assessed fibrosis by transient elastography and demonstrated clinically relevant fibrosis in the community was a concerning 5.6%[31]. Furthermore, modelling indicates the burden of NASH, end-stage liver disease (decompensated cirrhosis, HCC), and liver-related deaths will continue to grow[32]. Importantly, while severe liver outcomes may be the third rather than the primary cause of death in NASH patients, worryingly the primary and secondary causes of death are cardiovascular disease and extra-hepatic cancers[29]. A growing body of evidence suggests the effects of NAFLD extend beyond the liver, and NAFLD precedes and/or exacerbates the development of type 2 diabetes, hypertension and cardiovascular disease[33]. From a public health perspective, NAFLD, in particular NASH, cannot be ignored.

Pathogenesis

NAFLD is a complex phenotype that arises from dynamic interactions between diet, lifestyle and genetic factors, and involving crosstalk between multiple organs and the intestinal microbiome. Mechanistically, NAFLD pathogenesis can be viewed as an imbalance between lipid accumulation and removal (Fig. 2). Fatty acids arise in the liver from either the diet (dietary fats delivered via chylomicrons or dietary sugars converted via *de novo* lipogenesis; DNL), or from the circulating non-esterified fatty acid (NEFA) pool. Under normal circumstances fatty acids (FA) are either oxidized

for energy or packaged into triacylglycerol (TAG) for export and circulation in very low-density lipoproteins (VLDL).

The seminal view of NASH pathogenesis was one of "two hits"[34], where steatosis was followed by oxidative stress leading to lipid peroxidation and inflammation. Layers of complexity, and 'multiple hits' are now recognized around these pathways; including genetic susceptibility, biological environment, behavioural factors, metabolism and the intestinal microbiome[35,36]. In particular over the last decade, the roles of lipotoxic intermediates[37,38] and hepatic fatty acid trafficking[39] in NAFLD pathogenesis has come to be appreciated (Fig. 2). Intermediates in the synthesis of TAG (lysophosphatidic acid, phosphatidic acid, lysophosphatidyl choline, ceramides and diacylglycerols) are now recognized to contribute to altered insulin signalling[37]. In addition, lipotoxic intermediates are released via extracellular vesicles also activating hepatic stellate cells (HSCs) and other parenchymal cells driving inflammation and fibrosis[38].

The dynamics of lipid droplet formation[40], and the role of autophagy in fat mobilization[41] are also very active areas of research. Identification of the genetic risk variants described below, has underscored that lipid droplets are not merely inert bundles of triacylglycerol; they contain other lipid species, most notably cholesterol esters, and are associated with a diverse array of proteins. Notably, lipolysis of TAG from both adipocyte and hepatocyte lipid droplets is more dynamic and complex than previously envisioned, and lipid droplet associated proteins play a role in NAFLD pathogenesis[42].

The progression of NAFLD involves an interplay of multiple cell types residing in the liver (Fig. 2). Lipotoxic intermediates, reactive oxygen species (ROS), endotoxins and adipokines, all drive recruitment and signalling of immune cells, including Kupffer cells (KC); along with the activation of HSCs (Fig. 2). Activated HSCs become fibroblasts, producing fibrogenic factors and collagen, and through apoptosis drive cirrhosis development[43]. The chronic oxidative metabolism observed in NAFLD enhances ROS production creating a pro-oxidative state[44]. This overall increase in pro-oxidative/pro-inflammatory state leads to intracellular damage, activating repair mechanisms that can become hyperactive, further driving fibrosis[43].

Genetic risk factors

Initially identified through genome-wide association scanning as contributing to individual and ethnic differences in hepatic fat content and susceptibility to NAFLD[45]; a missense mutation, leading to an isoleucine to methionine substitution at position 148, in the patatin-like phospholipase domain containing 3 protein (PNPLA3; I148M variant, rs738409), has now been independently verified as associated with NAFLD severity in multiple populations. Individuals who are homozygous for this allele have markedly increased steatosis levels compared with non-carriers[45] and the minor allele

frequency correlates positively with steatosis across populations[14]. This genetic variant is estimated to account for 30-50% of high-risk progression of NAFLD towards fibrosis, cirrhosis and HCC[46]. In addition, it has also been linked to alcoholic[47] and viral[48] liver disease severity as well as HCC[49]. This suggests the PNPLA3 variant is not specific to NAFLD, but more generally influences susceptibility to liver disease with environmental factors (viral or toxin exposure, nutrition/diet, microbiome) playing an integral, and perhaps deterministic role. Subsequent biochemical work has demonstrated that the PNPLA3 protein is associated with lipid droplets and has hydrolase (lipase) activity against TAG in hepatocytes and against retinyl esters in HSCs[50-52]. Disruption of PNPLA3 function leads to accumulation of TAG in hepatocytes; and the rs738409 risk allele is associated with severity of a variety of liver diseases[53].

Three other common genetic variants also robustly associate with the development and progression of NAFLD and other liver diseases[36]. Intriguingly, these genes all encode proteins involved in the regulation of hepatocyte lipid metabolism and are linked to the severity of multiple liver diseases. In particular the rs58542926 variant of the transmembrane 6 superfamily member 2 protein (TM6SF2) results in a loss-of-function, inducing higher liver TAG content and lower circulating lipoproteins[54] through disrupted hepatocyte secretion of TAG and VLDL. Somewhat paradoxically, carriers of this mutation are at greater risk of liver disease but lower risk of cardiovascular events[55]. In addition, a common polymorphism (rs641738, C>T) variant in the membrane bound O-acyltransferase domain-containing 7 (MBOAT7) gene has also been recently associated with alcoholic liver disease[56], NAFLD severity[57,58] and HCC[59]. The variant reduces protein expression and alters phosphatidylinositol concentrations in the liver[57]. Variation in the glucokinase regulator (GCKR) gene, which regulates de novo lipogenesis by controlling the influx of glucose in hepatocytes, has also been associated with NAFLD in multiple studies[60-62]. The associated variant (rs780094) appears to be in linkage disequilibrium with a common missense loss-of-function GCKR mutation (rs1260326) that effects it ability to negatively regulate glucokinase, resulting in an increase in hepatocyte glucose uptake and glycolytic flux, promoting lipogenesis and hepatic steatosis[63].

Possessing multiple risk alleles increases risk severity for NASH, fibrosis[64] and HCC[59]. While it is hoped that in the near future polygenic risk scores may improve clinical stratification and management, there is undoubtedly genetic complexity yet to be elucidated. For example, only in March of 2018, Regeneron scientists reported their identification of splice variant rs72613567 (T>A) in the hydroxysteroid 17-beta dehydrogenase 13 (*HSD17B13*) gene and its association with reduced levels of alanine transaminase and protection against chronic liver disease[65]. The association was identified by exome sequencing of 46,544 participants with corresponding electronic health records, and then replicated in four independent cohorts. The rs72613567 variant results in a truncated protein

with loss of enzymatic function that is associated with reduced risk of NASH and fibrosis, but not steatosis, suggesting the variant allele protects against progression to more clinically advanced stages of chronic liver disease. Interestingly, previous work had identified 17-beta-hydroxysteroid dehydrogenase 13 as overexpressed from hepatic lipid droplets from fatty liver patients and shown that adenovirus driven overexpression in mice induced a fatty liver phenotype[66]. The physiological substrate(s) for the enzyme remain unknown, but *in vitro* it has activity against numerous steroid and bioactive lipids (e.g., leukotriene B_4) [65]. These data highlight again the role of lipid intermediates and lipid droplet dynamics in the pathogenesis of NAFLD, and open the possibility of targeting HSD17B13 therapeutically.

Nutrition and non-alcoholic fatty liver disease

While genetic mechanisms continue to be described, it is important to acknowledge the interplay between genetic background and environmental factors. Although, genetic risk for NAFLD influences pathogenesis, the phenotypic threshold is strongly influenced by environmental factors such as adiposity, insulin resistance and diet[36]. For example, recent work has demonstrated that for three of the aforementioned risk variants (PNPLA3, TM6SF2, GCKR), adiposity as measured by BMI greatly amplified the genetic risk[67]. With NAFLD disease progression linked closely to obesity and type 2 diabetes, it is clear that diet and lifestyle are key modifiable risk factors.

Weight loss for the treatment of NAFLD

Hyper-energetic diets, containing high levels of saturated fat, refined carbohydrates and sugar sweetened beverages, are strongly implicated in NAFLD pathogenesis. Weight gain and obesity are closely associated with NAFLD progression, therefore dietary and lifestyle changes aimed at weight loss are fundamental to all clinical management guidelines for NAFLD[1,7,8]. This includes eating a healthy diet and increasing physical activity to prevent and resolve NAFLD, regardless of BMI, as advised by both the UK's National Institute for Health and Care Excellence[8] and the European Association for the Study of the Liver (EASL)[7]. Significant reduction in steatosis and hepatic markers of NAFLD have generally been observed with 5-10% weight loss [68,69]; although weight reductions of >10% may be required for resolution of NASH and reducing fibrosis and portal inflammation[70]. In general, combining dietary and physical activity interventions appears most effective, as are interventions of longer duration and greater intensity (multicomponent; more contact time, ≥14 times in 6 months); although trial heterogeneity can confound systematic review[68,69,71]. Because achieving and maintaining 5-10% weight loss is a significant challenge for many[69,72], a pertinent question is whether or not improving the nutritional quality of the diet and/or increasing physical activity may improve NAFLD in the absence of weight loss[73].

While the focus of this review is the role of nutrition and dietary modification, increasing physical activity is an important component of lifestyle change aimed at weight loss and clinical improvement of NAFLD. Randomized clinical trials assessing the effects of resistance training, aerobic exercise or a combination of both have reported improvements in liver enzyme levels and reduced intrahepatic TAG measured by magnetic resonance spectroscopy[68,74]. Positive effects have been reported in patients engaging in physical activity only once a week[75], and meta-analysis shows this to be independent of significant weight change[74]. Mechanistically this is plausible, as exercise has potent anti-inflammatory effects and protects against many chronic inflammatory diseases[76,77]. Nonetheless, meta-analysis also suggests benefits are substantially greater with weight loss, particularly where weight loss exceeds 7%; with meta-regression demonstrating reductions in liver fat proportionally related to the magnitude of weight loss induced[74].

Macronutrient composition and the Mediterranean diet

The benefits of altering macronutrient composition and dietary patterns in NAFLD has been explored. In particular the Mediterranean diet is attractive given the body of evidence suggesting this dietary pattern reduces metabolic risk factors and cardiovascular disease risk[78-82]. On this theoretical basis and only one randomised trial[83] in 12 NAFLD subjects at the time, the EASL Clinical Practice Guidelines made a strong recommendation that, in addition to aiming for a 7-10% weight reduction, 'macronutrient composition should be adjusted according to the Mediterranean diet'[7].

Primarily a plant-based diet characterized by high intakes of vegetables, legumes, fruits, nuts and whole grains, along with olive oil as the main source of added fat; the Mediterranean diet is typified by low intakes of dairy and meat products, higher intakes of fish and seafood, and moderate (red) wine consumption. In terms of macronutrients it tends to be much higher in fiber (>33g/d), lower in carbohydrates, higher in total and monounsaturated fat (~37% and ~18%), but lower in saturated fat (9%) than typical Western diets[81]. As reviewed in detail by Zelber-Sagi[81] the evidence base for the Mediterranean diet and NAFLD remains limited and largely observational. Nonetheless, the data to date are consistently in favor of a beneficial effect from the Mediterranean diet for treating NAFLD, even without accompanying weight reduction.

Recent work suggests that switching to either an isocaloric low-fat or Mediterranean diet for 12 weeks, even *ab libitum,* can reduce liver fat (25% in low-fat and 32% in the Mediterranean diet; p=0.32) and alanine transaminase levels with minimal weight loss (1.6-2.1 kg). The Mediterranean diet did have better adherence and additional cardiometabolic benefits with improvements seen in total cholesterol, serum triglyceride, hemoglobin A1c (HbA1c) and the Framingham risk score[84]. While the intervention was not designed for weight loss, and there was no difference in the caloric intakes measured at baseline and 12 weeks, both groups lost a small (2%) amount of weight, lower

than that typically associated with NAFLD improvement. Although no differences were observed in the reductions of liver fat and body weight between the dietary groups, improvements in total cholesterol, plasma triglycerides, and HbA1c levels were observed in the Mediterranean diet group.

Saturated fat

What both 'low-fat' (<35%) and the Mediterranean diet often have in common, is reduced (<10%) saturated fat relative to the Western diet. Although dietary sugars, in particular fructose discussed in the next section, have been scrutinised for their role in driving *de novo* lipogenesis and NAFLD pathogenesis[85,86], overfeeding saturated fat is more metabolically harmful to the liver[87]. Specifically, using stable isotopes in combination with MRI, Luukkonen and colleagues showed that 3 weeks of overfeeding (1000kcal/day) with saturated fat, simple sugars or unsaturated fats increased liver fat by 55%, 33% and 15% respectively. Furthermore, overfeeding saturated fat induced insulin resistance and endotoxemia, and increased multiple plasma ceramides[87]. Recent focus on the negative metabolic effects of a high sugar diet has led to debate over historical dietary guidelines, which recommend low-fat and low saturated fat diets for the prevention of cardiovascular disease[88,89]. It bears noting that 'low-fat' is considered <35% of daily energy from fat with an 'acceptable distribution' of 20-35% and low-saturated fat is considered 7-10% of total energy. In the US[90] and the UK[91] adults consume an average of 34-35% of daily energy intake from fat. As highlighted by Maldonado and colleagues[92], neglected in the often polarized debates around sugar or fat[93,94], is the fact that at a population level, identifying individual culpable nutrients is problematic. The vast majority of adults in developed countries consume excess energy from foods high in both sugar and fat, fundamentally contributing to increasing obesity and NAFLD. Where low-fat versus low-carbohydrate has been examined in a NAFLD context, the results are similar to that seen in the meta-analysis of weight loss trials in diabetes[72]; whereas low carbohydrate may induce a greater weight loss in short term (12 weeks), in long term the net weight loss tends to be similar to that from low-fat[68,71].

Fructose and dietary sugars

Nonetheless, given the excessive consumption of sugar in general[86], messages of reducing sugar sweetened beverages and added sugars, consuming 'healthy' (e.g. complex) carbohydrates alongside lowering saturated fat intake and consuming more 'healthy fats' (e.g. monounsaturated and omega-3 fatty acids) seem highly prudent. It is noted that beyond the obvious culprits of sugar sweetened beverages, biscuits and sweeties or candies, even foods with healthful components such as yogurts can have surprisingly high amounts of added sugars[95]. 0Lowering intakes of fructose and high glycemic index foods in the diet have been shown to have beneficial effects in NAFLD patients[96,97].

Whereas the the EASL Clinical Practice Guidelines specifically suggest 'exclusion of NAFLD-promoting components (processed food, and food and beverages high in added fructose)'[7]; the UK guidelines cited a lack of scientific studies meeting their inclusion and exclusion criteria, in not yet making specific recommendations[8]. Fructose has been scrutinised because fructose consumption has risen in parallel with obesity, it is metabolized differently by liver and, at high experimental doses, exacerbates obesity and NAFLD[85]. Furthermore, genetic predisposition may make some populations more susceptible to fructose consumption and liver disease than others[98].

However, it remains challenging to separate out the effects of specific monosaccharides from the effects of excess energy. The experimental doses typically shown to be lipogenic (20% total energy) far exceed the population median amounts consumed and individuals rarely consume single sugars in isolation[86]. When excess energy has been carefully controlled for in randomized controlled human feeding trials, no differential effects are seen between the lipogenic effects of fructose and glucose[99]. A systematic review of controlled fructose feeding trials with NAFLD related endpoints examined 13 trials in total, including 7 isocaloric trials where fructose exchanged for other carbohydrates and 6 hypercaloric trials; diet supplemented with excess energy (21–35% energy) from high-dose fructose (104–220 g/day)[100]. It concluded that in healthy participants isocaloric exchange of fructose for other carbohydrates does not induce NAFLD changes, however, extreme doses providing excess energy increase steatosis and liver enzymes; in agreement with computational modelling of hepatocyte lipogenesis in response to excess glucose and fructose, described in more detail below[92].

There is worldwide agreement on the need to reduce the consumption of dietary sugars to prevent obesity and in particular reduce the consumption of sugar sweetened beverages to reduce the incidence of type 2 diabetes[86]. Whereas strict restriction of free sugars (to <3% of total energy) for 8 weeks has recently been shown to decrease hepatic steatosis in adolescents[101], it is not clear in the context of the prevention or treatment of NAFLD, whether public health messages focusing on fructose monosaccharides rather than free sugars and total energy is useful. An overall message should be that given the majority of populations worldwide are consuming too much total sugar, and given the dramatic increase in NAFLD and type 2 diabetes, reducing free sugar intake and choosing a more healthful diet in terms of macro- and micronutrients will be beneficial. Sugar sweetened beverages in particular, convey an additional risk for type 2 diabetes, most especially in young people, and should be restricted for the prevention of obesity and eliminated altogether in the treatment of existing NAFLD.

Supplemental nutrients: omega-3 PUFAs, vitamin E & vitamin D

A variety of vitamins and micronutrients have been implicated in NAFLD pathogenesis. This is either because of epidemiological data associating a deficiency with disease or because of plausible anti-steatotic, anti-inflammatory or anti-fibrotic mechanisms that (a supplemental dose of) dietary nutrients or other components may confer in a disease state.

NAFLD patients have been shown to have lower intakes of fish[102] and omega-3 PUFAs[103] in comparison to controls and therefore PUFA supplementation has been explored. Two independent groups have systematically reviewed control intervention trials that examined omega-3 fatty acid supplementation for the treatment of NAFLD[104,105]. Both meta-analyses included 18 independent trials with >1400 participants and concluded that supplementation of omega 3 PUFAs reduced steatosis as measured by ultrasound or MRI, and liver enzymes[104,105]. Disappointingly, in the four trials that examined histological markers, omega-3 PUFA supplementation did not improve inflammation, ballooning or fibrosis[104]. Strikingly, responders and non-responders to supplementation that correspond to improvements in liver markers were clearly evident in the well-designed trial by Scorletti and colleagues[106]. As discussed below, personalized nutrition for the prevention of chronic disease in the near future might account for inherent (genetic, epigenetic or microbiome mechanisms) inter-individual variation.

Vitamin E is a powerful antioxidant that helps protect cells against free radical damage, one of the pathogenic insults that drives NAFLD progression. There have now been several well-designed multi-centre trials in both adults and children examining vitamin E supplementation at pharmacological doses that could not be obtained through diet[107]. Several meta-analyses show benefit from supplemental vitamin E on steatosis, inflammation, and ballooning in NASH, although the extent to which vitamin E benefits fibrosis remains unclear[108-110]. Consequently, UK, EU and US clinical guidelines indicate vitamin E as a therapeutic option once a patient is in second or tertiary care for NASH[1,7,8], but with the awareness of potential risks for long-term vitamin E supplementation[107]. Recommended doses are typically 800 IU/d as opposed to recommended nutrient intakes of ≤15mg/d (22.4 IU/d) in the UK. While the American guidelines specify vitamin E only for NASH patients without diabetes[1], the UK guidelines consider vitamin E an option for patients with and without diabetes[8].

A growing body of research suggests a relationship between vitamin D deficiency and chronic liver disease, in particular NAFLD, with low levels of serum 25-hydroxyvitamin D (25OHD) strongly associated with hepatic inflammation[111-113]. Low levels of dietary vitamin D[114] and serum 25OHD are widespread, and vitamin D deficiency and insufficiency have been observed in pediatric NAFLD[115]. In addition, polymorphisms within vitamin D metabolic pathway genes associate with the histological severity of pediatric NAFLD[115]. However, the results of oral vitamin D

supplementation trials on adult NAFLD patients are conflicting[116,117]. Some studies have demonstrated a correlation between NAFLD and NASH severity and lower levels of vitamin D[118]. However, others, including a meta-analysis with 974 adult patients find no such relationships[119,120].

The determinants of 25OHD bioavailability are complex; genetic variation determines serum levels of vitamin D binding protein thus influencing bound and 'free' 25OHD[121]. Inter-individual vitamin D concentrations are highly variable and the degree to which they change over the decades through which NAFLD may progress, is unknown. The mechanisms behind the role of vitamin D in NAFLD pathogenesis are not yet fully understood and there are likely to be both hepatic and extra-hepatic mechanisms involved. Interestingly, vitamin D has been shown to have antifibrotic effects of vitamin D in both rodent[122,123] and human[124] HSCs. While there are clearly likely to be multiple pathways to fibrogenesis in NAFLD[125], together these studies show a role for vitamin D in liver disease pathogenesis and suggest common polymorphisms influencing vitamin D homeostasis may be relevant to NAFLD. Although supplementation with vitamin D has not been demonstrated an effective intervention in the limited studies done in adult patients with NAFLD to date; further research is warranted into whether targeted supplementation, either in genetically susceptible or pediatric populations may be indicated.

While, data from large well controlled trials are limited, it may be that classical intervention trials for single nutrients are doomed to fail in light of the high inter-individual genetic variation in the metabolism of many of these nutrients; in combination with individual epigenetic, microbiome and environmental, namely dietary, effects. As illustrated for omega-3 supplementation[106], population studies will include non-responders that may mask the positive (or negative) effects of dietary supplements in others. As will be discussed, the goal of personalised nutrition is to stratify dietary intervention according to such genetic, 'omic' and clinical information in the first instance to maximise therapeutic benefit.

Systems biology

Systems biology is the application of mathematical or computational modelling to biological systems, and has evolved as a complementary method of understanding a biological organism. Reflecting its roots in mathematical graph theory, cybernetics and general systems theory; within systems biology, biological systems, whether a signalling network, a cell, an organ, or an organism, are visualized and modelled as integrated and interacting networks of elements from which coherent function emerges[126]. As illustrated in Fig. 3A, from a systems point of view, a human may be deconstructed into a series of networks at organ, cellular and the molecular or genetic levels; equally, humans are parts within larger social networks. Underpinning systems biology are advanced mathematical theory and computational approaches that aim to model organism function and predict behaviour. Early in

its evolution, computational systems biology was envisioned as working best if integrated in an iterative cycle of model development and prediction, with experimental ('wet lab') investigation and model refinement (Fig. 3B)[127]. This iterative cycle moves from hypothesis-led experiments generating data that can both yield biological insights, and can be further utilized in the reconstruction of mathematical network models (such as the extended Petri net model of insulin signalling illustrated in Fig. 3C) for predictive simulation, model refinement and more biological insight that informs further experimental hypotheses.

Systems medicine and personalized nutrition

Systems pharmacology and systems medicine are subtypes of systems biology underpinning current efforts in what has been alternately termed stratified, personalized or precision medicine[128]. Emerging out of the genomics revolution, came the recognition that whereas currently used pharmaceuticals are based on clinical trials involving large cohorts, these neglect the underlying genetic and environmental heterogeneity represented within the population. This heterogeneity explains the existence of responders and non-responders to drug intervention, as well as drug off-target effects. Precision medicine aims for the stratification of patients into tightly molecularly defined groups (based on multiple types of 'omics' data), with effective interventions or treatments defined for each[129]. While currently used stratified medicines are largely within the cancer field and rely on genetic testing of a relatively limited number of genes, ultimately it is envisioned that the integrative analyses of different types of data: clinical, genomic, proteomic, metabolomic; will yield system insights (Fig. 3D). Beyond the genomic vision of stratified medicine, systems medicine in its grandest vision, has been described as personalized, predictive, preventive and participatory medicine ('4P' medicine) and intriguingly perhaps for Nutritional Scientists, has an aim of quantifying wellness in addition to understanding disease[130,131].

Arguably, the Nutritional Sciences are ideal for the use of systems approaches given the complex, dynamic nature of diet where small effects may be magnified on a chronic time scale; and furthermore, occur against a backdrop of tremendous genetic diversity both of humans and their intestinal microbiomes[132]. The vision and aim of personalised nutrition mimics that of personalised medicine, e.g. tailoring diets in a way that optimises health outcomes for the individual based on their 'omics' data[133]. Currently it is estimated only 40% of a cohort may respond to a dietary intervention; analogous to observed nonresponse or off-target effects to pharmaceutical compounds. This is attributed to inter-individual variation in a host of variables (sex, habitual dietary habits, genetics, epigenetics, and gut microbiota) effecting individual absorption, distribution, metabolism, and excretion of compounds and metabolites[134]. Personalised nutrition therefore, presents both grand opportunities and challenges; e.g. how to capture small, accumulative factors that only manifest into

disease over a matter of years, while distinguishing differential effects of one nutritional component from hundreds of others[135].

Modelling liver metabolism

In more recent years, systems biology approaches have been applied to human metabolism at the genome scale. Genome-scale metabolic networks (GMSNs) may be thought of as essentially an organized list of metabolic reactions derived from all available data of an organism's metabolism into a mathematically structured network. Constraint-based flux balance analysis is used to predict metabolic fluxes *in silico*, while the GSMN is constrained mathematically based on experimental data sets. The liver as an organ is central to both human metabolism and overall homeostasis; and the first liver-specific genome-scale metabolic networks (GMSNs) were published in 2010[136,137]. While one of these was derived from a generic GSMN by automated methods integrating tissue-specific datasets[136]; the model presented by Gille and colleagues[137] was based on exhaustive manual curation of transcript, protein, biochemical and physiological data and contains 2539 reactions and 777 individual metabolites.

More recently, the liver-specific iHepatocytes2322[138] was reconstructed, comprising 7,930 reactions, 2,895 unique metabolites in 8 different compartments mapped to 2,322 genes. This was done in semi-automated fashion but, significantly, utilized proteomics expression data from hepatocytes from the Human Protein Atlas[139] to establish tissue specificity. This incredibly comprehensive reconstruction paid particular attention to manual curation of reactions involving lipids. Both of these GSMNs have been utilized in the context of NAFLD related research. While GSMNs continue to evolve as powerful tools, it is important to note that metabolism is only one of the many networks considered in systems biology (Fig. 3A) and flux balance analysis is limited in being static and not reflecting the dynamic metabolic response to altered cell signaling. A very active area of systems research is focused on developing novel tools and algorithms for integrating and simulating models at multiple scales and linking GSMNs to gene regulatory networks and/or PBPK models in systems pharmacology/toxicology and kinetic signaling networks[140-142].

Application of systems approaches to NAFLD

It has only been in very recent years that GSMNs have been used along with relevant omics data in the context of NAFLD. The aforementioned iHepatocytes2322[138] was reconstructed specifically to interrogate liver transcriptomic data from 19 healthy subjects and 26 patients with varying degrees of NAFLD. Using a metabolite reporting algorithm, a pair-wise comparison was used to identify reporter metabolites. Network subgroup analyses predicted disruptions in the non-essential amino acids: serine, glutamate and glycine (along with others), along with metabolites in the folate pathway related to the interconversion of serine, glycine and glutamate. Phosphatidylserine, an essential

494 component of lipid droplets was also identified as disrupted, with the mRNA for enzymes involved
495 in its synthesis found downregulated in the NASH patients. Similarly, several enzymes that either use
496 serine as substrate or produce it as a product were transcriptionally downregulated. Collectively, the
497 authors inferred an endogenous serine deficiency and suggested serine supplementation as a possible
498 intervention in NASH. Chondroitin and heparin sulphate levels were also identified as potential
499 NAFLD biomarkers, although these have not yet been independently validated.
500 Impressive follow up work from the same group has now shown in an untargeted
501 metabolomics analysis of individuals with either low (mean 2.8%, n=43) or high (mean 13.4%, n=43)
502 liver fat as measured by MRI, decreased levels of glycine and serine, along with betaine and N-
503 acetylglycine associated with higher levels of steatosis[143]. In addition to the metabolomic
504 measurements, *in vivo* VLDL kinetics were measured via stable isotope infusion in 73 of the
505 individuals. These experimentally measured VLDL secretion rates along with individually defined
506 NEFA uptake rates (based on body composition and secretion rates of NEFA from adipose and
507 muscle) were used to constrain the iHepatocytes2322 GSMN. The resulting personalized GSMNs
508 were then simulated using the secretion rate of VLDL as an objective function in order to identify
509 hepatic metabolic alterations between individuals with high and low steatosis. Liver fluxes were
510 predicted for each subject and several reactions, consistent with an increased demand for
511 nicotinamide adenine dinucleotide (NAD+) and glutathione, correlated to steatosis and net fat influx.
512 Relating this back to amino acid precursors and the lower levels of serum serine and glycine,
513 in a proof-of-concept study in six subjects with obesity, Mardinoglu and colleagues observed both a
514 decrease in liver fat (mean 26.8 to 20.4%) and aspartate and alanine transaminase levels after 14 days
515 of serine supplementation (~20 g of L-serine, 200 mg/kg/day)[143]. The authors suggest serine could
516 be combined with N-acetylcysteine, nicotinamide riboside and L-carnitine as a supplement to aid in
517 mitochondrial fatty acid uptake and oxidation and increased generation of glutathione may have
518 benefit for either the prevention or treatment of NASH. While pilot trials have examined N-
519 acetylcysteine[144,145] and L-carnitine[146,147] supplementation in NAFLD separately with mixed
520 results, they have not been examined in combination. Amino acid disturbances, particularly to
521 glutamate, serine and glycine continue to be explored in relation to NAFLD liver disease severity in
522 different populations[148,149]. Returning to the ideas of systems medicine and personalized nutrition,
523 and the example of responders and non-responders to omega-3 supplementation, an open question is
524 whether or not a subgroup of NAFLD patients are likely to benefit (respond) to such an intervention
525 more than others. It is hoped with advances in systems biology the identification of such patient
526 subgroups will be feasible in the near future.
527 Other work has also integrated transcriptomic data with experimentally measured *in vivo* flux
528 measurements from NAFLD patients[150] utilising GSMNs. Hyötyläinen and workers used Recon1

and measured flux ratios of metabolites and bile acids across the hepatic venous splanchnic bed in nine subjects with NAFLD that were fasted and then underwent euglycemic hyperinsulinemia. The work developed a metabolic adaptability score and found steatosis is associated with overall reduced adaptability. Steatosis induced mitochondrial metabolism, lipolysis and glyceroneogenesis; plus, a switch from lactate to glycerol as a substrate for gluconeogenesis. In this, and the work of Mardinoglu and colleagues, GSMNs were utilized for the mechanistic interpretation of clinical (transcriptomic and metabolomic) NAFLD data. However, these models are static, reflecting liver adaptation at an endpoint, and do not give insight into the dynamic reprogramming of global metabolism and metabolic adaptation to maintain homoeostasis in response to stimulation as recently addressed by Maldonado and colleagues[92].

Building on their previous work establishing the use of quasi-steady state Petri nets (QSSPN) to integrate and simulate gene regulatory networks and/or PBPK models with constraint-based GSMNs[140-142]; the group have developed novel multi-scale models to predict the hepatocyte's response to fat and sugar[92]. In one case, from experimental -omics data and the literature, they manually curate a comprehensive network reconstruction of the peroxisome proliferator activated receptor alpha (PPARα) regulome. Integrated to the HepatoNet1[137] GSMN, the resulting multi-scale model reproduced metabolic responses to increased fatty acid levels and mimicked lipid loading *in vitro*. Adding to the conflicting literature on the role of PPARα in NAFLD, the model predicted that activation of PPARα by lipids produces a bi-phasic response, which initially exacerbates steatosis[92]. The data highlight potential challenges for the use of PPARα agonists to treat NAFLD and illustrate how dynamic simulation and systems approaches can yield mechanistic explanations for drug off-target effects. While the PPARα regulome module was sufficiently large to preclude complete deterministic parameters for every reaction; illustrating the flexibility of QSSPN, the authors also simulate a kinetic multi-scale model of monosaccharide transport and insulin signalling integrated to the HepatoNet1 GSMN. Interestingly, while the model predicted differential kinetics for the utilisation of glucose and fructose, TAG production was predicted to be similar from both monosaccharides. This finding is supported both by the author's experimental data presented alongside the simulations[92], as well as other clinical and intervention data[99,100]. These data imply that it is the quantity, not type of sugar that drives fat accumulation in liver cells and NAFLD per se.

The focus here has been on reviewing recent work applying the simulation of GSMNs in NAFLD-related research. Computational approaches and network reconstructions are rapidly evolving and current models have strengths and weaknesses that will resolve in future iterations. More work is needed comparing results from different reconstructions and establishing best choice of objective functions for human applications. Integrating constraint-based analyses of GSMNs with

whole body PBPK models or gene regulatory and signalling network models in multi-scale fashion for dynamic simulations and insights into pathogenesis over time is a current research goal.

Conclusions

The interdisciplinary methods of systems biology are rapidly evolving and have recently been applied to the study of NAFLD. Technology is rapidly evolving and a not too distance future is envisioned where individual genetic, proteomic and metabolomic information can be integrated computationally with clinical data. Ideally this will inform personalized nutrition and precision medicine approaches for improving prognosis to chronic diseases such as NAFLD, obesity and type 2 diabetes. Several genetic variants mediating susceptibility to liver diseases have been identified and validated, opening up possibilities for the use of polygenic risk scores to stratify patients once disease is identified. Progression of NAFLD is dependent on environmental factors and it should be stressed that NAFLD is reversible through lifestyle change. As has recently been argued for type 2 diabetes, a systems disease requires a 'systems solution'[151]. While intervention studies demonstrate that high-intensity combination interventions, including behaviour change alongside dietary and lifestyle change, are most efficacious for treating NAFLD; undoubtedly, broader societal systems level changes are urgently required to reduce the current burden and prevent obesity and related morbidities such as NAFLD and type 2 diabetes going forward.

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Conflict of Interest

None.

Authorship

595 JBM had sole responsibility for all aspects of the preparation of this manuscript.

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597
598 **Figure Legends**

599 **Fig. 1** The dynamic spectrum of non-alcoholic fatty liver disease (NAFLD). The liver can accumulate
600 fat (non-alcoholic fatty liver; NAFL) in the absence or presence of inflammation (non-alcoholic
601 steatohepatitis; NASH) and fibrosis. These processes are reversible as indicated by the dashed arrows.
602 Poor and over-nutrition can influence the development and progression of NAFLD as indicated by
603 the red arrows; whereas weight loss and a healthy diet is the mainstay of successful NAFLD treatment
604 as indicated by the green arrows. Evidence from clinical trials in NAFLD suggest even fibrosis can
605 regress. Questions remain about whether the development of steatohepatitis is an independent
606 maladaptive process from the development of steatosis; and whether hepatocellular carcinoma (HCC)
607 can develop directly from NAFL and NASH without the development of fibrosis.

608 **Fig. 2** Diet and non-alcoholic fatty liver disease pathogenesis. Fatty acids (FAs) arise in the liver from
609 [1] de novo lipogenesis (DNL) of dietary sugars, [2] dietary fat via chylomicrons and [3] the non-
610 esterified fatty acid (NEFA) pool derived primarily from adipose tissue. In the context of normal
611 physiology, FAs are either [4] oxidized for energy or [5] esterified into triacylglycerol (TAG) and
612 exported in very low-density lipoprotein (VLDL) particles into circulation. In the context of excess
613 energy, [6] TAG is stored in lipid droplets. Lipid intermediates, reactive oxygen species (ROS),
614 endotoxins and adipokines all contribute to [7] inflammation, hepatic stellate cell (HSC) and Kupffer
615 cell (KC) activation leading to liver fibrosis. Pathogenesis is influenced by genetic and epigenetic
616 mechanisms and is influenced by the microbiome.

617 **Fig. 3** Systems biology and systems medicine. **A** Humans may be deconstructed into a series of
618 networks at genetic, molecular, cellular and organ levels; equally, humans are places within larger
619 social networks. **B** Systems biology ideally is an iterative cycle from hypothesis-led experiments
620 generating data that can both yield biological insights, and can be further utilized in the reconstruction
621 of mathematical models for predictive simulation, model refinement and more biological insight that
622 informs further experimental hypotheses. **C** A kinetic network model of insulin signalling
623 reconstructed in a Petri net formalism, reprinted with permission[92]. Coloured ovals used to highlight
624 modules used by Kubota and colleagues[152]. **D** Systems medicine and systems pharmacology
625 integrate genetic, clinical and 'omic' data into network models, representing an *in silico* human, that
626 can yield emergent insights. For example, simulations may predict responders/non-responders to a
627 drug or identify mechanisms of action underpinning drug off target effects.
628

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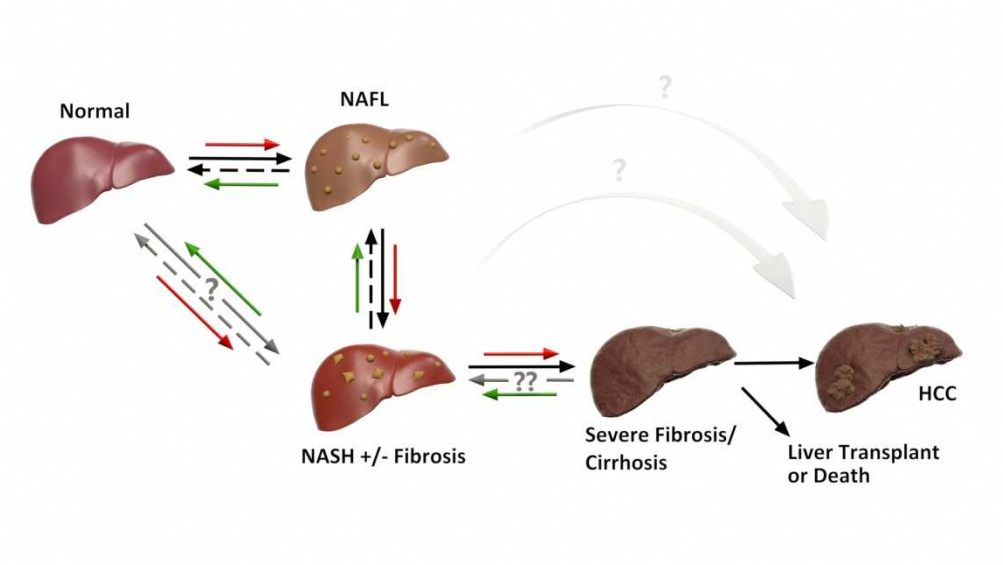

Fig. 1 The dynamic spectrum of non-alcoholic fatty liver disease (NAFLD). The liver can accumulate fat (non-alcoholic fatty liver; NAFL) in the absence or presence of inflammation (non-alcoholic steatohepatitis; NASH) and fibrosis. These processes are reversible as indicated by the dashed arrows. Poor and over-nutrition can influence the development and progression of NAFLD as indicated by the red arrows; whereas weight loss and a healthy diet is the mainstay of successful NAFLD treatment as indicated by the green arrows. Evidence from clinical trials in NAFLD suggest even fibrosis can regress. Questions remain about whether the development of steatohepatitis is an independent maladaptive process from the development of steatosis; and whether hepatocellular carcinoma (HCC) can develop directly from NAFL and NASH without the development of fibrosis.

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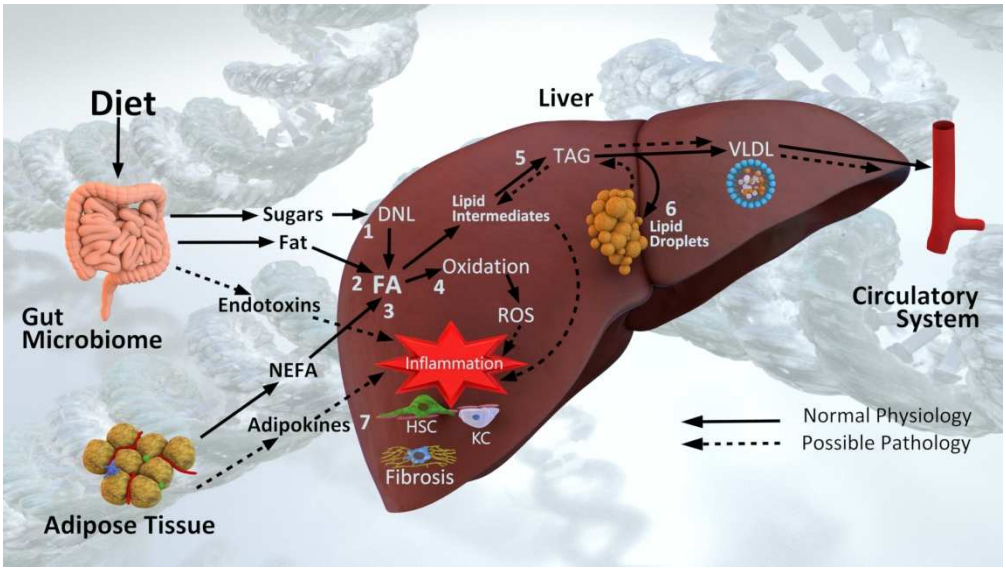

Fig. 2 Diet and non-alcoholic fatty liver disease pathogenesis. Fatty acids (FAs) arise in the liver from [1] de novo lipogenesis (DNL) of dietary sugars, [2] dietary fat via chylomicrons and [3] the non-esterified fatty acid (NEFA) pool derived primarily from adipose tissue. In the context of normal physiology, FAs are either [4] oxidized for energy or [5] esterified into triacylglycerol (TAG) and exported in very low-density lipoprotein (VLDL) particles into circulation. In the context of excess energy, [6] TAG is stored in lipid droplets. Lipid intermediates, reactive oxygen species (ROS), endotoxins and adipokines all contribute to [7] inflammation, hepatic stellate cell (HSC) and Kupffer cell (KC) activation leading to liver fibrosis. Pathogenesis is influenced by genetic and epigenetic mechanisms and is influenced by the microbiome.

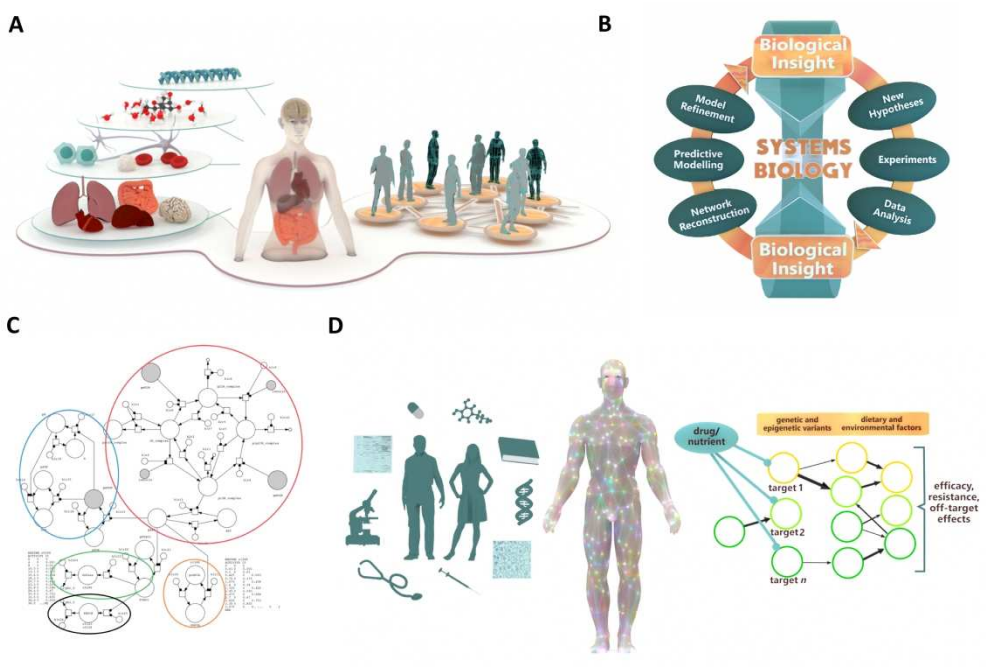

Fig. 3 Systems biology and systems medicine. A Humans may be deconstructed into a series of networks at genetic, molecular, cellular and organ levels; equally, humans are places within larger social networks. B Systems biology ideally is an iterative cycle from hypothesis-led experiments generating data that can both yield biological insights, and can be further utilized in the reconstruction of mathematical models for predictive simulation, model refinement and more biological insight that informs further experimental hypotheses. C A kinetic network model of insulin signalling reconstructed in a Petri net formalism, reprinted with permission(92). Coloured ovals used to highlight modules used by Kubota and colleagues(152). D Systems medicine and systems pharmacology integrate genetic, clinical and 'omic' data into network models, representing an in silico human, that can yield emergent insights. For example, simulations may predict responders/non-responders to a drug or identify mechanisms of action underpinning drug off target effects.