Non-alcoholic fatty liver disease (NAFLD) is now the most common liver disease in both adults and children worldwide. As a disease spectrum, NAFLD may progress from simple steatosis to steatohepatitis, advanced fibrosis and cirrhosis. An estimated 20–35% of the general population has steatosis, 10% of whom will develop the more progressive non-alcoholic steatohepatitis associated with markedly increased risk of cardiovascular- and liver-related mortality. Development of NAFLD is strongly linked to components of the metabolic syndrome including obesity, insulin resistance, dyslipidaemia and type 2 diabetes. The recognition that NAFLD is an independent risk factor for CVD is a major public health concern. There is a great need for a sensitive non-invasive test for the early detection and assessment of the stage of NAFLD that could also be used to monitor response to treatment. The cellular and molecular aetiology of NAFLD is multi-factorial; genetic polymorphisms influencing NAFLD have been identified and nutrition is a modifiable environmental factor influencing NAFLD progression. Weight loss through diet and exercise is the primary recommendation in the clinical management of NAFLD. The application of systems biology to the identification of NAFLD biomarkers and factors involved in NAFLD progression is an area of promising research.

Non-alcoholic fatty liver disease: Metabolic syndrome: Systems biology

Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is the pathological accumulation of fat in the liver in the absence of alcohol intake. Described initially only 30 years ago(1,2), it is now the leading cause of liver disease in developed countries, with an estimated prevalence of 20–35% in the general population. The occurrence of NAFLD is strongly linked to obesity, insulin resistance and other components of the metabolic syndrome. From a nutrition and public health perspective the two major concerns are the rising incidence of NAFLD in children and the convincing evidence that NAFLD is an independent risk factor for CVD.

The term NAFLD encompasses a spectrum of histologically-defined liver disorders. Disease can progress from macrovesicular lipid accumulation in the hepatocytes (termed steatosis) to non-alcoholic steatohepatitis (NASH; steatosis in the presence of inflammatory infiltrate possibly with some fibrosis) to outright fibrosis, cirrhosis and even hepatocellular carcinoma. A combination of environmental and genetic factors determines individual risk of NAFLD development and progression, with a clear role for nutrition as a modifiable environmental risk factor. Pathogenesis of NAFLD was initially envisaged as a ‘two-hit process’(3) with fat accumulation in hepatocytes viewed as the primary insult and increased oxidative stress leading to inflammation being the second ‘hit’ in the progression to NASH and fibrosis. However, at the cellular level mechanisms influencing disease progression are clearly multi-factorial and dependent on numerous genetic and environmental interactions. Future analysis and modelling of NAFLD progression must take account of this complexity in a systems biology fashion.
Diagnosis

The majority of cases of NAFLD are identified after an incidental finding of either elevated liver enzymes on routine blood tests or suspected fatty liver on abdominal imaging in patients consuming little or no alcohol(4). After the exclusion of substantial alcohol consumption, which is now defined as >20 g/d in men and >10 g/d in women, alternative causes of fatty liver (nutritional, viral, metabolic, drug) must also be excluded before diagnosis of NAFLD. While liver enzymes such as alanine aminotransferase (ALT) and γ-glutamyl transpeptidase levels may be found to be elevated, they are not specific to NAFLD and the full histological spectrum of NAFLD has been observed in patients with normal ALT levels(5). Non-invasive imaging techniques used for diagnosis of NAFLD include ultrasound, computed tomography, MRI and proton magnetic resonance spectroscopy, each of which has its own set of advantages and disadvantages(6). While all these techniques yield information about fat distribution in the liver, MRI and proton magnetic resonance spectroscopy can quantify the total amount of fat in the liver. A population study using proton magnetic resonance spectroscopy to assess liver fat has found approximately one-third of subjects with hepatic steatosis, of whom 79% have normal ALT levels(7). These data highlight the concern that NAFLD prevalence as assessed by liver enzyme alterations is grossly underestimated.

Ultrasound-based transient elastography and magnetic resonance elastography are currently being investigated for their ability to assess the stage of liver fibrosis(8,9). However, transient elastography has been shown to fail at a BMI >28 kg/m²(10) and magnetic resonance elastography remains experimental(11). Although algorithms combining biochemical markers and patient variables have been developed for predicting steatosis(12), steatohepatitis(13) and fibrosis(14) in NAFLD, they require independent population validation and still lack sensitivity and specificity for widespread use. Histological assessment of liver biopsies remains the gold standard for diagnosing and assessing the stage of NASH. However, liver biopsy is invasive and associated with morbidities (15) and rare cases of mortality(16), precluding its routine use in screening for NAFLD or for repeated assessment of either disease progression or response to therapy. Furthermore, as histological lesions are not evenly distributed in the liver, considerable sampling error and misdiagnosis exists(17). Consequently, the identification and validation of non-invasive biomarkers capable of diagnosing and assessing the stage of NAFLD is a research priority(18).

Non-alcoholic fatty liver: disease development

The pathogenesis of NAFLD begins with the accumulation of lipid in the liver. As only a minority of patients with hepatic steatosis progress to the necroinflammatory steatohepatitis and develop fibrosis, it was originally conceptualised that a second ‘hit’ is required to induce cellular events (e.g. oxidative stress) leading to inflammation, cell death and fibrosis(3). As with all complex diseases, it is now recognised that the phenotypic expression of NAFLD depends on a myriad of genetic, behavioural and environmental interactions(41).

While hepatic steatosis can be viewed simplistically as an imbalance between the processes of lipid accumulation and lipid disposal, the cellular and molecular regulation of hepatic metabolism is intricate and has been the subject of a recent extensive review(42). Fat in the liver is acquired from the diet via lipoproteins, by de novo lipogenesis or circulating NEFA, and is removed through VLDL secretion or by β-oxidation. Derangements in each of these pathways have been associated with NAFLD. Traditionally, it was considered that lipid accumulation in the liver occurs in the context of obesity and peripheral insulin resistance, with lipid coming from an elevated plasma NEFA pool caused by increased activity of hormone-sensitive lipase and increased lipolysis from engorged adipocytes. Multiple lines of evidence suggest that this explanation is likely to be too naïve; indeed, it has been postulated that systemic insulin resistance might be secondary to NAFLD(43).
Stable-isotope research in patients with NAFLD has shown that although 60% of the TAG in the liver comes from the peripheral NEFA pool, elevations in fasting de novo lipogenesis also contribute substantially\(^{44}\). Additional work has observed both an increase in adipose lipolysis and increased VLDL-TAG secretion from the liver in patients with NAFLD\(^{45}\). However, not only is the increase in VLDL-TAG secretion insufficient to account for the increase in hepatic TAG content in these patients, but the VLDL-TAG is derived predominantly from ‘non-systemic’ NEFA, either from de novo lipogenesis or lipolysis of visceral fat. This finding is interesting in the light of very recent proton magnetic resonance spectroscopy and MRI data showing that hepatic lipid content is directly correlated with visceral fat levels\(^{46}\). The study highlights ethnic differences in the manifestations of insulin resistance; although Hispanics and African-Americans have similar frequencies of insulin resistance, African-Americans are less likely to have NAFLD, hypertriacylglycerolaemia and elevated visceral fat depots. These data, as well as data showing NAFLD in non-obese individuals with normal glucose and lipid levels\(^{47}\), suggest that NAFLD can and does occur without the backdrop of insulin resistance.

Once steatosis is established multiple mechanisms contribute to lipid-induced cellular injury\(^{48}\). Increased mitochondrial β-oxidation of NEFA leads to an increase in reactive oxygen species. Mitochondrial defects have been observed in patients with NAFLD and impairment of the respiratory chain also produces reactive oxygen species. Mitochondrial dysfunction may also result in a reduction in mitochondrial ATP production, leading to cell death. In addition, the aldehyde by-products of lipid peroxidation increase production of pro-inflammatory cytokines and recruit inflammatory cells into the liver. Inflammation and the activation of hepatic stellate cells lead to collagen production and the initiation of fibrosis\(^{48}\).

Last, the pathogenesis of NAFLD is also mediated by transcription factors such as the sterol regulatory element-binding protein-1, the carbohydrate responsive element-binding protein and the PPAR, which are reviewed elsewhere\(^{49}\), and influenced by circulating cytokines and adipokines. The levels of all these molecules will depend on both genetic polymorphisms and the presence or absence of disease (e.g. obesity or immune), adding a further level of complexity to the pathogenesis of NAFLD. A review of clinical studies has shown low levels of the anti-inflammatory adiponectin and high levels of the pro-inflammatory resistin, TNFα and IL-6 cytokines in patients with NAFLD\(^{50}\). Genetic polymorphisms in these cytokines have also been investigated in patients with NAFLD, as described later.

Non-alcoholic fatty liver disease: progression and associated mortality

The prognosis of patients with NAFLD depends on the histological stage of the disease. Simple steatosis may have a relatively benign course with no increased risk of mortality, whereas NASH may progress quite rapidly and is associated with markedly increased risk of mortality\(^{51–54}\). Progression in NAFLD is difficult to assess, requiring years of follow-up and repeat biopsies that are prone to sampling errors\(^{17,55}\). The longest prospective study of the natural history of NAFLD using repeat biopsies had a mean follow-up of 13.7 years and involved 129 patients\(^{54}\). It was reported that 47% of patients presenting with steatosis progressed to NASH and 25–50% of patients presenting with NASH developed advanced fibrosis or cirrhosis in 8–13 years (Fig. 1).
Although the results underscore the fact that patients with steatosis can and do develop steatohepatitis, mortality was not found to be changed in the group presenting with steatosis, whereas survival was reported to be significantly lower in patients that had presented with NASH (70% v. 80% in the reference population, \( P = 0.01 \)). Patients with NAFLD-associated cirrhosis have been shown to have a very poor 10-year prognosis, with 50% needing a liver transplant, 20% dying from a liver-related cause and 7% developing hepatocellular carcinoma (Fig. 1)\(^{56} \). It is of great concern that a very recent study on the natural history of NAFLD in children has demonstrated that paediatric NAFLD is also progressive and can result in end-stage liver disease and death\(^{57} \). In the cohort of sixty-six children followed over a mean of 6.4 years, two children underwent liver transplantation and two children died, while observed serial biopsies showed progression in 80% of patients.

Multiple risk factors have associated with NAFLD progression including older age\(^{23,24,58} \), BMI\(^{24,53,58} \), insulin resistance\(^{54} \), diabetes\(^{59,60} \), metabolic syndrome\(^{61} \) and histological NASH on diagnosis\(^{52,54,62} \). A current systematic review of ten studies has examined risk factors for progression from NASH to advanced fibrosis\(^{63} \). Using multivariate analysis the review has found that only age and the presence of inflammation on initial biopsy are independent predictors of progression in 221 patients over a 5-3-year follow-up. The well-documented limitations associated with systematic reviews\(^{64} \), particularly the choice and heterogeneity of included studies in this case, may explain why obesity, BMI and diabetes were not found to be significant predictors in this analysis.

Data relating to the mortality risks associated with NAFLD are somewhat conflicting. The first population-based cohort study that examined NAFLD and mortality has determined survival among 420 patients with NAFLD, identified through imaging or biopsy, after a mean follow-up of 7.6 years\(^{65} \). In agreement with case series from specialist clinics\(^{52,54,60} \), NAFLD was found to be associated with lower survival and increased risk of mortality from liver disease. After 10 years of follow-up \((n = 161) \) survival was reported to be 77% v. 87% in the general population. Subsequently, three independent groups have analysed data from the US Third National Health and Nutrition Examination Survey for mortality risk among suspected cases of NAFLD based on elevated ALT levels\(^{66–68} \). While two groups have found NAFLD to be associated with an increase in overall mortality\(^{66,67} \), one of these two groups has found NAFLD to be associated with a very large increase in liver-associated mortality\(^{66} \), whereas the other group has reported a striking link between NAFLD among 45–54 year olds and CVD mortality\(^{67} \). Conversely, the third group has reported no association between elevated ALT levels and all-cause or CVD mortality but an association with liver-related mortality\(^{68} \). The differences may be attributable to variations in study design, patient selection strategy and statistical analysis. While these studies have the advantage of very large sample sizes from a representative sample of the general population, diagnosis of NAFLD is based on elevated ALT levels, which do not differentiate between steatosis and steatohepatitis and, as previously mentioned, are likely to severely underestimate NAFLD incidence and therefore the magnitude of mortality risk. However, the data overall show NAFLD is far from a benign diagnosis.

**Genetic and environmental factors associated with non-alcoholic fatty liver disease development and progression**

The development of NAFLD is strongly linked to obesity and insulin resistance. However, as there are obese individuals and individuals with diabetes who do not have NAFLD, and since NAFLD can occur in normal-weight individuals with normal glucose and lipid levels\(^{47} \), there are obviously multiple genetic and environmental factors determining NAFLD development and progression (Fig. 2).

Initial evidence for a genetic component to NAFLD comes from familial clustering studies\(^{69,70} \) and the ethnic variation in NAFLD prevalence\(^{7} \). Various genetic...
single-nucleotide polymorphisms have been investigated in NAFLD including single-nucleotide polymorphisms in the adiponectin\textsuperscript{71,72} IL-6\textsuperscript{73} TNF\textsubscript{a}\textsuperscript{74} and apoE\textsuperscript{75} genes. ‘Candidate gene’ studies in small cohorts have yielded somewhat conflicting results in different populations. The first genome-wide association scan conducted in 2008 in a large multiethnic population targeting NAFLD has identified the patatin-like phospholipase domain-containing protein 3 (also known as adiponutrin) gene as being strongly associated with hepatic TAG content\textsuperscript{76}. Allelic variants of the patatin-like phospholipase domain-containing protein 3 gene have been found to be associated with high and low amounts of hepatic fat in Hispanics and African-Americans respectively, and to be correlated with the high and low prevalence of NAFLD in these populations. Remarkably, patatin-like phospholipase domain-containing protein 3 has also been independently identified in a separate population-based genome-wide scan as one of two genetic loci influencing plasma levels of ALT\textsuperscript{77}. Undoubtedly, as genome sequencing continues to get cheaper and faster, studies of this type will reveal more genes that are causally related to the pathogenesis of NAFLD.

Nutrition and physical activity are important environmental factors that determine risk in NAFLD. Excess food intake and lack of exercise contribute to weight gain, which has been shown to contribute to the progression of liver fibrosis in patients with NAFLD\textsuperscript{54}. Specific dietary factors may also play either protective or antagonistic roles in the development and progression of NAFLD. Nutrient intakes in individuals with NAFLD have been assessed in Italian\textsuperscript{78}, Japanese\textsuperscript{79}, Israeli\textsuperscript{80} and US\textsuperscript{81} populations; no UK cohort has been assessed to date. An increased consumption of meat and soft drinks and low consumption of fish were found to be associated with NAFLD cases compared with controls. Unsurprisingly, low intakes of PUFA and high intakes of saturated fat and cholesterol were also shown to be associated with NAFLD. Data from two randomised controlled trials in patients with NAFLD have shown beneficial effects from 6 months of dietary supplementation with n-3 PUFA compared with dietary advice alone\textsuperscript{82,83}. Reductions in fatty liver observed by ultrasonography as well as improvements in serum ALT and TAG levels were found to be greater in the patients supplemented with n-3 PUFA. In both trials the patients receiving n-3 PUFA were found to be more likely to have complete fatty liver regression, suggesting a beneficial therapeutic effect for n-3 PUFA supplementation in NAFLD. Other studies have shown higher-carbohydrate and lower-fat diets to be associated with more progressive disease\textsuperscript{84,85}. Conversely, very recent animal data have shown that in both mice\textsuperscript{86} and non-human primates\textsuperscript{87} exposure to a maternal high-fat diet leads to a disturbing development and progression of NAFLD in the offspring.

In view of the role of oxidative stress in NAFLD pathogenesis several studies have examined antioxidant supplementation as a therapeutic intervention. A recent Cochrane review of six very small and extremely diverse clinical trials has found no evidence either to recommend or advise against the use of antioxidant supplements in patients with NAFLD\textsuperscript{88}. However, data from pilot studies\textsuperscript{89,90} and one double-blind randomised placebo-controlled trial\textsuperscript{91} have shown sufficient positive effects of vitamin E supplementation in patients with NAFLD for further investigation in two ongoing multicentre trials. The Pioglitazone versus Vitamin E versus Placebo for the Treatment of Non-diabetic Patients with Non-alcoholic Steatohepatitis Trial\textsuperscript{92} in adults and the Treatment of Non-alcoholic Fatty Liver Disease in Children Trial\textsuperscript{93} both involve 96 weeks of treatment and will measure improvements in histology as well as ALT levels. While the former trial has three arms and includes a placebo group, the latter trial in children will compare metformin treatment with vitamin E supplementation. Data from these trials, whether negative or positive, should end the debate on the potential efficacy of vitamin E supplementation in NAFLD.

Small intestinal bacterial overgrowth may be an additional environmental factor involved in NAFLD pathogenesis, and dietary supplements such as probiotics could have a beneficial effect. Evidence from animal studies shows that small intestinal bacterial overgrowth increases gut permeability leading to portal endotoxaemia and increased circulating inflammatory cytokines, both of which have been implicated in the progression of NAFLD\textsuperscript{94}. Several studies have reported an association between small intestinal bacterial overgrowth and the progression of NAFLD\textsuperscript{95–97} and in one small human study treatment with antibiotics has been shown to result in an increase in fasting insulin levels\textsuperscript{97}. Limited evidence from animal models and one preliminary human study suggest that supplementation with the probiotic VSL\#3\textsuperscript{8} (a combination of nine strains of various bifidobacteria, lactobacillus and Streptococcus thermophilus; VSL Pharmaceuticals, Inc., Gaithersburg, MD, USA) may possibly be beneficial in NAFLD, but data are mixed\textsuperscript{98–100}. More research in this area is warranted.

Non-alcoholic fatty liver disease, the metabolic syndrome and CVD risk

The metabolic syndrome was originally defined as a cluster of CVD risk factors with insulin resistance as the underlying pathogenic factor\textsuperscript{101}. The third report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) defines metabolic syndrome as being any three or more of five variables: increased waist circumference; hypertriglyceridaemia; hypertension; low HDL-cholesterol; hyperglycaemia\textsuperscript{102}. Metabolic syndrome is highly prevalent among patients with NAFLD in multiple populations and is strongly associated with progressive disease\textsuperscript{61,103–105}. Furthermore, a Japanese prospective study (n 3147) has demonstrated that patients with metabolic syndrome, as defined by Adult Treatment Panel III\textsuperscript{102}, are four to eleven times (men and women respectively) more likely to have developed NAFLD on follow-up\textsuperscript{106}. Likewise, children with metabolic syndrome, as defined by Adult Treatment Panel III\textsuperscript{102}, have been shown to be five times more likely to have NAFLD\textsuperscript{38}. This strong relationship between NAFLD and the metabolic syndrome has led to the...
description of NAFLD as the hepatic component of the metabolic syndrome.

Indeed, in the last 5 years NAFLD has emerged as an independent risk factor for CVD. Several studies have observed increased carotid intima-media thickness and carotid plaques in patients with NAFLD, including children\(^{39,107–111}\). Although there has been some conflicting work\(^{112}\), a systematic review has found that carotid plaques are two to three times more likely to be observed in patients with NAFLD and intima-media thickness is strongly associated with NAFLD\(^{113}\). Furthermore, multiple population studies have observed increased CVD events in patients with NAFLD diagnosed by ultrasonography and have demonstrated that the increased risk for CVD in NAFLD is independent of conventional risk factors and components of the metabolic syndrome\(^{114–116}\). These data add to population data that have shown that elevated liver enzymes are also associated with increased risk for CVD\(^{97,117}\).

As the treatment of NAFLD involves correcting the same metabolic factors as those involved in CVD, it is prudent that all patients with NAFLD be evaluated for early atherosclerosis. Likewise, patients presenting with the metabolic syndrome or a high Framingham risk score\(^{118}\) should be evaluated for the presence of NAFLD. Children identified with NAFLD or the metabolic syndrome should be strongly counselled to avoid smoking, increase their physical activity and improve their nutrition in order to prevent the development of CVD.

The potential of systems biology

Systems biology is the study of a biological system (cell, tissue, organism) viewed as an integrated and interacting network of genes, proteins and biochemical reactions. This methodology combines computational modelling with experimental biology in order to discover emergent properties that arise from studying a system as a whole; it is these properties that ultimately determine how a system is controlled or regulated. A systems approach begins by constructing a predictive model from which a hypothesis is formed and then tested experimentally by perturbing the system. Experimental techniques are generally the global approaches of genomics, proteomics and metabolomics. Data obtained from these multiple approaches are integrated and compared with the original model, which is refined in an iterative process. In the context of human health it is believed that systems biology is essential to future drug discovery and, indeed, to a future of personalised, predictive and preventative medicine\(^{119}\). In this vision drug targets would be rational and identified through the analysis of normal and disease-perturbed networks, while an individual could have their genome sequenced and in combination with extensive blood analyses would be able to obtain a ‘probabilistic future health history’. Although systems biology is in its infancy and this vision still seems far off, global approaches have been applied to NAFLD and have produced data that will be relevant to the future creation of an integrated ‘systems’ model of NAFLD.

Proteomic technologies are still evolving and the analysis of a complete proteome, in the manner of DNA microarray analysis of complete genomes, has yet to be realised. However, as disease biomarkers and drug targets are most likely to be proteins, proteomics is viewed as central to systems biology and key to predictive medicine\(^{120}\). In the context of NAFLD proteomic techniques have been used to analyse adipose\(^{121}\), liver\(^{122}\) and most recently serum\(^{123}\) samples in patients with NAFLD. Ultimately, it is most likely that a serum diagnostic or prognostic tool for NAFLD would comprise a panel of proteins, and this research highlights the power of systems methodologies for identifying candidate protein biomarkers. Importantly, a ‘potential biomarker panel’ comprising six proteins has been demonstrated to have greater diagnostic utility than ALT to differentiate between patient groups\(^{123}\). It is also noteworthy that two independent studies\(^{122,123}\) have identified lumican, a protein that activates transforming growth factor \(\beta\) and smooth muscle actin, to be elevated in both liver and serum from patients with NASH. These studies have given insight into NAFLD pathogenesis and have identified candidate disease biomarkers worthy of prospective validation.

Conclusion

NAFLD is highly prevalent, with an estimated 20–35% of the UK population affected. Research on the natural history of NAFLD has demonstrated that even simple steatosis cannot be considered benign, as 47% of these patients will progress to NASH within 8–13 years. An estimated 25–50% of patients with NASH will progress to advanced fibrosis and cirrhosis. In real numbers these estimates represent 300 000–1 .8 · 10\(^6\) cases in the UK, 50% of whom will require a liver transplant to avoid death. It is clear that a diagnosis of NAFLD is associated with increased risk of mortality from liver- and cardiovascular-related events and the British Liver Trust has demonstrated that liver disease is the only major cause of death still increasing yearly in the UK\(^{124}\). An additional public health concern is the rising incidence of NAFLD in children who are not exempt from risk of CVD, the need for liver transplants or early death. Part of the challenge in assessing the burden of NAFLD in the population is the lack of a non-invasive screening test for NAFLD.

While NAFLD incidence is strongly linked to insulin resistance and the metabolic syndrome, the development and progression of NAFLD involves a myriad of molecular and cellular events that are influenced by both genetic and environmental factors. In the long term the determination of the critical factors involved in disease progression will identify possible therapeutic targets for treatment of NAFLD and may form the basis of prognostic tests. Proteomic studies are beginning to identify serum and liver proteins found altered in NAFLD. The modelling of NAFLD protein and gene regulatory networks in a systems biology manner has the potential to identify diagnostic, prognostic and therapeutic targets for NAFLD and more research of this type is urgently needed.
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References

36. Damaso AR, de Prado WL, de Piano A et al. (2008) Relationship between nonalcoholic fatty liver disease prevalence


88. Lirussi F, Azzalini L, Orando S et al. (2007) Antioxidant supplements for non-alcoholic fatty liver disease and/or steatohepatitis. *Cochrane Database of Systematic Reviews* 2007, issue 1, CD004996. Chichester, West Sussex: John Wiley and Sons, Ltd.


