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Dietary protein insufficiency: an important consideration in fatty liver disease?

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Abstract

Dietary protein insufficiency has been linked to excessive TAG storage and non-alcoholic fatty liver disease (NAFLD) in developing countries. Hepatic TAG accumulation following a low-protein diet may be due to altered peroxisomal, mitochondrial and gut microbiota function. Hepatic peroxisomes and mitochondria normally mediate metabolism of nutrients to provide energy and substrates for lipogenesis. Peroxisome biogenesis and activities can be modulated by odd-chain fatty acids (OCFA) and SCFA that are derived from gut bacteria, for example, propionate and butyrate. Also produced during amino acid metabolism by peroxisomes and mitochondria, propionate and butyrate concentrations correlate inversely with risk of obesity, insulin resistance and NAFLD. In this horizon-scanning review, we have compiled available evidence on the effects of protein malnutrition on OCFA production, arising from loss in mitochondrial, peroxisomal and gut microbiota function, and its association with lipid accumulation in the liver. The methyl donor amino acid composition of dietary protein is an important contributor to liver function and lipid storage; the presence and abundance of dietary branched-chain amino acids can modulate the composition and metabolic activity of the gut microbiome and, on the other hand, can affect protective OCFA and SCFA production in the liver. In preclinical animal models fed with low-protein diets, specific amino acid supplementation can ameliorate fatty liver disease. The association between low dietary protein intake and fatty liver disease is underexplored and merits further investigation, particularly in vulnerable groups with dietary protein restriction in developing countries.

Key words: **Protein restriction: Protein malnutrition: *De novo* lipogenesis: Odd-chain fatty acids: Metabolism**

Non-alcoholic fatty liver disease (NAFLD) remains the most frequently occurring chronic liver disease with global prevalence of 25·25 %[1]. Whilst NAFLD has become a global health concern, the incidence of this metabolic disorder is growing in developing countries particularly in the Middle East where the prevalence rate is reported to be highest followed by South America and Africa[1]. NAFLD is considered as a broad spectrum of liver conditions and is histologically subdivided into simple steatosis (with mild or no inflammation), non-alcoholic steatohepatitis with or without fibrosis and more advanced liver disease, cirrhosis. NAFLD is an asymptomatic, silent metabolic disease which is diagnosed by elevated levels of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase with alanine aminotransferase:aspartate aminotransferase ratio >1. Ultrasound or MRI and magnetic resonance elastography of the liver or an invasive liver biopsy may also be used for diagnostic purposes[2].

NAFLD, which is associated with diabetes, obesity and hyperlipidaemia, is considered as the hepatic manifestation of the metabolic syndrome. Consequently, diet is an important modifiable risk factor for NAFLD.

Several studies have reported that consumption of high carbohydrate (especially refined sugars) contributes to NAFLD[3]. In contrast, the effect of low dietary protein is less well understood, for example, during pig development, a low-maternal protein diet was found to be associated with increased liver lipid accumulation of the offspring in later life[4]. Low-protein diets result in amino acid deficiency intracellularly and trigger amino acid response gene expression to enable cells to either adapt to the nutrient stress or undergo apoptosis[5]. Whether adaptive responses mediated by amino acid response activation play a significant role in the development of fatty liver disease is unknown. Nevertheless, it has been shown that sensing of low amino acid levels via the

Abbreviations: ACOX1, peroxisomal acyl-CoA oxidase 1; BCAA, branched-chain amino acid; BCKDH, branch chain keto-acid dehydrogenase; CD36, cluster of differentiation 36; CPT, carnitine palmitoyltransferase; FABP, fatty acid binding protein; FATP, fatty acid transporter protein; GCN2, general control nonderepressible 2; NAFLD, non-alcoholic fatty liver disease; OCFA, odd-chain fatty acid.

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general control nonderepressible 2 (GCN2)/ATF4 pathway affects both lipogenesis, decreasing lipogenic gene expression and the activity of fatty acid synthase and increasing lipid metabolism[6]. When essential amino acid levels drop below the cell threshold, deacetylation of the corresponding tRNA occurs and these uncharged tRNA bind and activate the GCN2 kinase to initiate the amino acid response signal transduction cascade. Lipid synthesis was up-regulated in the livers of *Gcn2*$^{-/-}$ mice during prolonged leucine deprivation resulting in severe liver steatosis due to failure to up-regulate PPARα expression[6]. PPARα is abundant in hepatocytes where it regulates lipid metabolism including fatty acid degradation, synthesis, transport, storage, and lipoprotein metabolism[7]. Protein restriction prevents the expression of PPARα, a mediator of numerous hepatic responses to nutrient restriction. However, pre-clinical studies confirm that restoration of dietary protein in mice by supplementation with fish-derived protein increases hepatic expression of two PPARα target genes, peroxisomal acyl-CoA oxidase 1 (ACOX1) and mitochondrial carnitine palmitoyltransferase (CPT)2[8]. This is hepatoprotective, most likely due to stimulation of mitochondrial and peroxisomal β-oxidation of fatty acids[8]. However, it is noteworthy that the fish-derived protein study was undertaken in a transgenic TNFα over-expressing mouse model where altered mitochondrial fatty acid oxidation in animals with 20 % casein in the diet was due to inflammation. The higher activity of CPT2 and ACOX1 and observed increase in plasma concentrations of short and medium carnitine esters in mice fed the fish protein hydrolysate suggest that compared with a casein diet, this fish protein diet improved hepatic fatty acid oxidation that had been compromised by inflammatory TNFα.

It is also recognised that a low-protein diet has consequences in altering the composition and functions of the gut microbiota, which may affect the delivery of hepatoprotective SCFA to the liver (Fig. 1)[9,10]. This article explores the current knowledge of mechanisms underpinning diet-induced fatty liver disease, with a focus on low dietary protein intake and contributions of mitochondria, peroxisomes and gut microbiota to hepatic lipid accumulation. We propose that there is an untapped opportunity for improved protein nutrition to restore the metabolic homeostasis, gut microbiota and reduce the risk for fatty liver disease at risk groups, for example, in developing countries.

Nutrient metabolism in the liver

Following digestion of dietary fat, proteins or carbohydrates in the gut, the end product nutrients released such as glucose, amino acids, NEFA, SCFA and odd-chain fatty acids (OCFA) such as propionic acid from fermentation by gut microbiota are taken up into the periportal blood supply that drains to the liver. Within the liver, nutrient homeostasis is maintained by metabolism to acetyl CoA and then to ATP through mitochondrial oxidative phosphorylation; acetyl CoA anabolism leading to fat accumulation or distribution of lipids in the systemic circulation through VLDL. The liver is the first major organ after the periportal vein to receive gut-derived nutrients. Therefore,

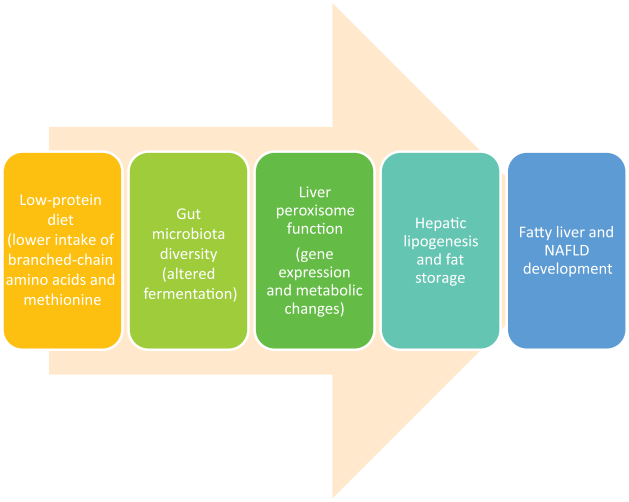

Fig. 1. Low-protein diet-induced changes to liver mitochondrial and peroxisomal activities and gut microbiota may contribute to hepatic liver fat accumulation. NAFLD, non-alcoholic fatty liver disease.

controlled biogenesis of hepatic organelles and expression of uptake/transport proteins and metabolic enzymes are essential to minimise TAG accumulation, formation of reactive oxygen species, inflammation and development of NAFLD[11]; and PPARα, an indirect sensor of amino acid levels, plays a significant role in regulating lipid metabolism in the liver.

Hepatic fatty acid uptake and impairment during non-alcoholic fatty liver disease

Dietary fat contributes to NAFLD and fatty acid uptake mechanisms that are reviewed here to appreciate how low-protein diets may contribute to fatty acid uptake during fatty liver disease development.

The main plasma membrane transporters of NEFA into the liver postprandially and during obesity are fatty acid transporter proteins (FATP), caveolins, fatty acid translocase/cluster of differentiation 36(CD36) and fatty acid binding protein (FABP), whose expression is regulated by PPARα. Of the six member FATP family, only FATP2 and FATP5 are highly expressed in the liver[12]. Knockdown of FATP2 in mice decreases the uptake of NEFA and ameliorates hepatic steatosis induced by a high-fat diet[12]. Similarly, FATP5 knockout mice are resistant to diet-induced obesity and hepatic TAG storage accumulation[13], highlighting the significant role played by FATP5 in liver lipid accumulation. The second family of lipid transporters, caveolins, have three members, namely caveolins 1, 2 and 3, and play an important role in the formation of lipid droplets[11]. Caveolin 1 is increased in the liver of mice with NAFLD, mainly in the centrilobular region, where the steatosis tends to be severe[14]. There are contradictory reports on the significance of caveolin 1 for NAFLD based on knockout studies[15,16]; this may reflect the different dietary induction protocols with caveolin 1 being important for NAFLD development in high-fat but not in fasting diets.

Fatty acid translocase/CD36 accelerates NEFA uptake via facilitated diffusion. Elevated hepatic expression of CD36 has

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been observed in NAFLD and is associated with enhanced uptake of NEFA[17]. High-fat-diet-fed mice develop hepatic steatosis in parallel with increased CD36 mRNA and protein expression[18], promoting a positive feed-forward loop for lipid accumulation. After uptake, the cytosolic FABP facilitate intracellular transport of NEFA for metabolism and to lipid-sensitive transcription factors. Targeted deletion of the liver isoform, liver fatty acid binding protein (LFABP) in mice results in hepatic lipid accumulation in female mice[19] further illustrating the importance of transport protein expression to prevent fatty liver disease. Liver expression of FATP, caveolin, LFABP or CD36 is regulated by the common transcription factors FOXA1, CEBPα and PPARα, and there is evidence that CEBPα and PPARα are in turn regulated by the amino acid response to low dietary protein[5]. Together, this evidence suggests that dietary protein malnutrition will influence fatty acid uptake and storage in the liver, at least in part due to altered PPARα activity.

Mitochondrial fatty acid metabolism and dysfunction in non-alcoholic fatty liver disease

Mitochondrial dysfunction has been associated with the pathogenesis of NAFLD[20,21] and hepatic steatosis[22–24]. Mitochondrial-derived reactive oxygen species contribute to the progression of NAFLD[25]. In conditions of dietary excess or obesity, oxidative phosphorylation promotes further reactive oxygen species production by mitochondria[25]. This is likely to be exacerbated by increased conversion of accumulating palmitate to ceramide and inhibition of PPARα[26], in turn inhibiting metabolic organelle biogenesis and activity.

Mitochondria play a central role in aerobic ATP production from fatty acid β-oxidation (acyl-chain length of ≤C20), ketogenesis and gluconeogenesis from pyruvate and tricarboxylic acid cycle intermediates.

After fatty acid uptake by the hepatocyte, CoA is added by fatty acyl-CoA synthase, forming long-chain acyl-CoA. Then, CPT1 catalyses conversion of the long-chain acyl-CoA to long-chain acylcarnitine to facilitate transport across the inner mitochondrial membrane. An inner mitochondrial membrane CPT2 then converts the long-chain acylcarnitine to acyl-CoA for β-oxidation. It has been reported that a low-protein diet (3 %) in weanling rodents impaired hepatic expression of CPT1 and CPT2 genes and promoted fatty liver development[27]. Fatty liver induced by this 3 % protein diet was attenuated when the rat diets were supplemented with dietary medium-chain TAG[27]. While the evidence from low protein dietary studies so far is limited, existing data suggest a role of PPARα regulation and mitochondria in mediating the effect of paternal protein malnutrition on offspring hepatic metabolism[28].

Mitochondrial amino acid metabolism

The branched-chain amino acids (BCAA) valine, leucine and isoleucine constitute between 15 and 25 % of total amino acids from dietary protein intake[29]. During BCAA catabolism, each BCAA is first converted into its respective α-keto acid through mitochondrial branched-chain aminotransferase in extrahepatic tissue. The α-keto acid is further metabolised by a rate-limiting step of BCAA catabolism via branched-chain keto-acid dehydrogenase (BCKDH) in liver, skeletal muscle, kidney and adipose tissue. Notably, a decrease in BCKDH activity is seen in several diseases such as maple syrup urine disease, type 2 diabetes and obesity, measured as increases in circulating BCAA levels[30]. A recent study has identified higher valine and isoleucine concentrations in adolescents with NAFLD independently of the presence of diabetes. In addition, the BCAA metabolic signature was negatively correlated with insulin sensitivity[31]. There are at least two explanations for the presence of higher concentrations of circulating BCAA in blood during obesity: either down-regulation of BCAA catabolic enzymes[32] or more likely the inhibition of rate-limiting BCKDH in liver. The activity of BCKDH is regulated by inhibitory phosphorylation by BCKDH kinase, and preliminary studies have successfully targeted BCKDH kinase using a selective inhibitor (BT2) to increase BCKDH activity, reduce BCAA, inhibit lipogenesis and improve glycaemic control[33].

Metabolism of BCAA adds to the lipogenic acetyl-CoA and propionyl CoA pools. Using isotopomer spectral analysis[34], BCAA were shown to be involved in the synthesis of OCFA and even-chain fatty acids in adipocytes. Propionyl CoA derived solely from isoleucine and valine catabolism is a primer for fatty acid synthase; thus, C5 : 0 may then be extended in the synthesis of longer chain OCFA (Fig. 2). This pathway is also used in the metabolism of methionine, although its contribution to the total OCFA pool is unknown. The OCFA C15 : 0 and C17 : 0 are both associated with good metabolic health, possibly mediated through PPARα activation[35]. It has been shown that plasma circulating OCFA correlate inversely with a range of metabolic conditions including fatty liver disease and insulin resistance[35]. It remains to be determined whether, in conditions where BCAA metabolism is inhibited or when dietary protein intake is low, OCFA synthesis is also lower. These findings suggest a novel pathway for investigating the protective effect of dietary protein against hepatic lipid accumulation, through enhanced BCAA metabolism and the production of OCFA.

Mitochondrial metabolism of fatty acids and BCAA is impaired in obesity and fatty liver disease but whether these changes are causal or a consequence of disease is unknown. Nevertheless, metabolic inhibition in liver mitochondria correlates with lower circulating OCFA and elevated BCAA. As OCFA are protective, the potential for targeting BCAA metabolism to increase OCFA in fatty liver disease merits consideration.

Peroxisomes in fatty acid α- and β-oxidation

The peroxisome, a single membrane-bound organelle, plays important roles in plasmalogen and bile acid synthesis, β-oxidation of very long-chain fatty acids, α-oxidation of branched-chain fatty acids and BCAA, and in maintaining cellular redox homeostasis[36]. Peroxisomes work in concert with mitochondria to provide substrates for oxidative metabolism by regulating early metabolism of long-chain fatty acids, branched-chain fatty acids and possibly also BCAA[37]. They are also enriched in catalase which supports the detoxification of the

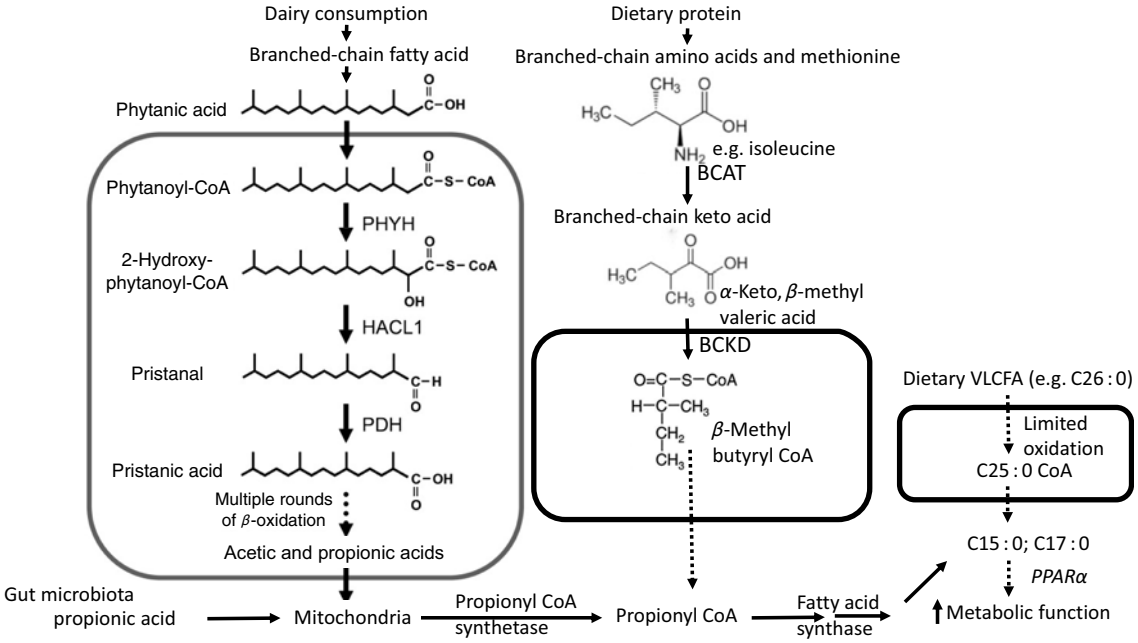

Fig. 2. Pathways for synthesis of C15 : 0 and C17 : 0 in the liver. PHYH, phytanoyl-CoA α-hydroxylase; HACL1, 2-hydroxyphytanoyl-CoA lyase; PDH, pristanal dehydrogenase; BCAT, branched-chain amino acid aminotransferase; BCKD, branched-chain ketoacid dehydrogenase; VLCFA, very long-chain fatty acids.

reactive oxygen species hydrogen peroxide produced during metabolism thereby mitigating the risk for oxidative damage[38].

Very long-chain fatty acids, including C22 : 0, C24 : 0 and C26 : 0, undergo *β*-oxidation in peroxisomes because very long-chain fatty acids are not substrates for CPT1, and it is the only acylcarnitine fatty acid product of CPT1 that can be transported into mitochondria. Peroxisomes also lack a citric acid cycle and respiratory chain; hence, the end products of *β*-oxidation in peroxisomes including NADH, acetyl-CoA, propionyl-CoA and other longer chain acyl-CoA are shuttled from peroxisomes to mitochondria for complete oxidation[36]. The rate-limiting enzyme in peroxisomal fatty acid *β*-oxidation, acyl-coA oxidase 1 which produces hydrogen peroxide as a by-product, regulates metabolism, spontaneous hepatic steatosis and hepatocellular damage over time[36].

Branched-chain fatty acids, which cannot undergo *β*-oxidation directly due to the location of the methyl group at position 3, undergo *α*-oxidation in peroxisomes by removing the terminal carbon to generate a 2-methyl fatty acid before entering into the *β*-oxidation pathway[36]. For instance, phytanic acid which is a 3-methyl branched-chain fatty acid undergoes *α*-oxidation beginning with the formation of phytanoyl-CoA, followed by hydroxylation to produce 2-hydroxyphytanoyl-CoA, a reaction catalysed by the enzyme phytanoyl-CoA 2-hydroxylase. Subsequently, 2-hydroxyphytanoyl-CoA is lysed by the enzyme 2-hydroxyacyl-CoA lyase to pristanal and formyl-CoA and then hydrolysed into formic acid and CoASH. Pristanal is oxidised to pristanic acid (2, 6, 10, 14-tetramethylpentadecanoic acid) by a yet undefined peroxisomal aldehyde dehydrogenase. Finally after activation to its CoA-ester, pristanoyl-CoA undergoes three cycles of *β*-oxidation in peroxisomes prior to transport of end products to mitochondria for complete oxidation[36]. Both propionyl CoA and pristanal produced in peroxisomes by phytanoyl-CoA 2-hydroxy lyase activity are important precursors for biosynthesis of OCFA[39] (Fig. 2). It remains to be determined whether impaired peroxisomal function reduces levels of circulating protective OCFA. Nevertheless, deficits in peroxisomal biogenesis have been associated with liver disease development[40]. In later sections, we explore the relationship between protein malnutrition and deficits in peroxisomal function and number.

Odd-chain fatty acids and non-alcoholic fatty liver disease

Two key odd-chain SFA that have become increasingly important biomarkers for predicting metabolic diseases including fatty liver disease are pentadecanoic acid (C15 : 0) and heptadecanoic acid (C17 : 0)[35]. Lower levels of OCFA are associated with reduced insulin sensitivity and increased fatty liver. Similarly, serum levels of C15 : 0 and C17 : 0 are inversely correlated with NAFLD activity scores and hepatocyte ballooning scores in humans[41]. OCFA originating from microbial fermentation have been used as biomarkers for dairy fat intake in humans[42]. As described previously, OCFA biosynthesis is catalysed by fatty acid synthase from propionic acid[43] and in humans, the SCFA propionic acid can be generated via BCAA degradation, peroxisomal oxidation of cholesterol side-chain during bile acid formation and peroxisomal *α*-decarboxylation, followed by successive *β*-oxidative degradation of the branched-chain fatty acid, phytanic acid (Fig. 2)[35]. Propionic acid produced via gut microbiotic fermentation of dietary fibre may then absorbed and metabolised by the liver to produce further OCFA[42]. The relative contribution of propionic acid produced during metabolism of BCAA to the serum levels of C15 : 0 and C17 : 0 remains unknown. Nevertheless, it

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has been suggested that a high-protein diet can minimise diet-induced development of fatty liver and even reverse pre-existing steatosis state[42]. In order to investigate whether the beneficial effect of high-protein diet is mediated via production of OCFA, the anaplerotic lipid C7 : 0 was investigated in a high-fat diet-induced fatty liver disease mouse model. Despite beneficial effects of dietary C7 : 0 on expression of hepatic PPARα that regulates peroxisomal and mitochondrial biogenesis, increased acyl-CoA oxidase expression (rate-limiting step in peroxisomal metabolism of fatty acids), CD36 expression (fatty acid uptake) and a decline in plasma acetyl-carnitines, there was no change in liver fat accumulation[44]. A complex picture is emerging where adequate dietary protein contributes to the synthesis of protective OCFA, but in models of liver disease that are induced by high-fat diet, no protective effect of the OCFA C7 : 0 was observed. The interpretation of C7 : 0 supplementation study is further complicated by the observation that a high-fat diet, which was used to induce liver fat accumulation, reduces C15 : 0[45]. C7 : 0 supplementation to prevent NAFLD during a high-fat diet is not likely to be effective; however, where low protein is a causative factor for fatty liver disease, further investigation of the effect of C7 : 0 supplementation is merited.

Dietary protein effects on liver peroxisome and mitochondrial function

Peroxisomal biogenesis is regulated by PPARα and coordinated with mitochondrial biogenesis through PGC1α activity[46,47].

van Zutphen *et al.* showed diminished hepatic peroxisome content and impaired peroxisomal function in rats following a 4 weeks low-protein diet (5 % energy content as protein) and attributed the cause of hepatic steatosis to loss or dysfunction of the peroxisomes. Using electron microscopy, peroxisomes in the periportal area of the liver were rarely observed in low-protein diet animals compared with controls as indicated by decreased immunofluorescence staining of the peroxisomal membrane protein PEX14. Also, protein levels of peroxisomal membrane protein 70 and the antioxidant enzyme catalase were decreased after 4 weeks of a low-protein diet[40], increasing the risk for oxidative damage and lipid accumulation. In the same study, in the mitochondria, there was a reduction in both complex I and complex IV activity. Lower expression of medium-chain specific acyl-CoA dehydrogenase, medium and short-chain L-3-hydroxyacyl-coA dehydrogenase and enoyl-CoA hydratase were observed, suggesting that peroxisomes and mitochondria are affected by conditions of low dietary protein availability[40]. More studies are required for further understanding of the impact of protein restriction on the biogenesis of peroxisomes and on mitochondrial function.

Dietary protein restriction and hepatic lipid accumulation

Both human and animal studies have shown that a low-protein diet (below 9 % of total energy) causes an increase in hepatic lipid accumulation. In human studies, a diet generally low in protein and high in carbohydrates, which may been seen in the elderly with significant co-morbidity and is present at all ages in developing countries, has been shown to cause liver lipid accumulation[48]. Moreover, children in India that presented with severe malnutrition were found to show several metabolic disturbances including increased oxidative stress and hepatic steatosis[48].

In animal models, feeding a 5 % of low-protein diet to Wistar rats for 4 weeks resulted in increased hepatic TAG accumulation[27]. Similarly, feeding Sprague–Dawley male rats with a low-protein diet for 4 weeks also led to hepatic TAG accumulation which was associated with down-regulation of hepatic microsomal TAG transfer protein and increased expression of acetyl CoA carboxylase[49]. Similarly, Kwon *et al.* reported that an 8 % low-protein diet resulted in steatohepatitis with severe steatosis in lactating female Sprague–Dawley rats[50]. Despite the emerging evidence from animal models, there is a potential of confounding in the interpretation of the data; for example, a low-protein diet in mice for 16 weeks led to increased body weight, adiposity and fatty liver[51] but the lack of dietary protein was substituted with a high carbohydrate content in the diet. It has been suggested that decreasing protein and replacing energy loss with a higher proportion of sucrose may be the driving force of the emerging high incidence of fatty liver disease in developing countries. In the studies we have explored here, protein was substituted by sucrose in two studies[27,50] and by a variety of starches in four other studies[9,40,49,55] demonstrating that replacing protein with a specific source of simple sugars was not solely responsible for the effects of low-protein diets observed. Is the development of fatty liver in this model due to low protein or high carbohydrate or a combination of both? To address this, some studies have explored the effect of supplementation with protein extract or amino acids on low-protein-induced fatty liver disease.

Effect of amino acid supplementation on low-protein-induced fatty liver disease

A previous study in an animal model of paternal inheritance has shown that supplementing a low-protein diet with specific amino acids, for example, methionine, moderated fat accumulation in major metabolic tissues such as the liver, muscle and subcutaneous adipose tissue of the adult offspring[52]. This may be due to changes in gene expression through altered promotor methylation.

The amino acid derivative, betaine (trimethylglycine), occurs naturally in most living organisms. It is synthesised during the oxidation of choline[53]. Betaine and choline are methylating agents and are important for regulating DNA methylation and downstream gene expression. Choline is directed to maintain the S-adenosyl methionine cycle; both choline-deficient diets and methionine- and choline-deficient dietary models may be useful for understanding the role of dietary amino acids for human NAFLD partly due to their histological similarity with these diseases[55,54].

A study by Madeira *et al.* has demonstrated that betaine and arginine supplementation, either individually or combined, into reduced protein diets (160 *v.* 130 g/kg of crude protein) decreased plasma total lipids and total cholesterol in lean pigs[56]. In the presence of either betaine or arginine supplementation,

Table 1. Dietary protein alters gut microbiota; resulting inflammatory effect associated with non-alcoholic fatty liver disease

Dietary protein	Effect on gut microbiota	Metabolic or inflammatory effect
High protein (humans) (>20 % of total energy from protein)	Increased levels of *Clostridium* spp. and *Bacteroides* spp.[69] Decreased level of *Bifidobacterium* spp., *Roseburia* spp. and *Eubacterium* spp.[69]	Low butyrate production
Normal protein (mice) (20 % total energy from protein)	Bacteroidetes and Firmicutes decreased, whilst Verrucomicrobia, Tenericutes, and Proteobacteria increased after 14 d (post-weaning mice). Verrucomicrobia and Tenericutes decreased after further 10 d, whilst Firmicutes and Proteobacteria increased this time[9]	
Protein deficient (animals)	Post-weaning increase in Verrucomicrobia.	Increased lipocalin-2 and myeloperoxidase in the stool (inflammatory markers)[9]
• (2 % of total energy from protein)	No decrease in Bacteroidetes but post weaning loss of Firmicutes observed[9]	Decrease in isobutyrate, isovalerate and branched chain proportion
• (13 % of energy as protein)	Increased in genera of *Prevotella* and *Coprococcus* (in the caecum) as well as Sarcina, Subdoligranulum, *Coprococcus*, and Mogibacterium (in the colon) but decreased genera abundance of *Lactobacillus* (in the caecum) and *Streptococcus* in the colon of pigs[71]	

except for fatty acid synthase which was down-regulated by protein reduction, no effects on hepatic fatty acid composition and gene expression levels of lipid-sensitive factors were induced by the protein-restricted diet[56]. Another study showed that supplementation with fish-derived protein was hepatoprotective during a low-protein diet[8]. Together these studies indicate that in animals receiving low-protein diets, the associated development of fatty liver may be prevented by simple amino acid supplementation. The beneficial effects of supplementing with methyl donors that are absent during protein malnutrition may be explained, at least in part, by epigenetic regulation of hepatic metabolic gene expression. Conversely, it has been demonstrated in rat pups from dams previously fed low-protein diets during lactation; hypomethylation of PGC1-α was observed in the offspring, decreased m etabolic gene expression and subsequently impaired mitochondrial fatty acid oxidation[57]. It is not known whether a similar pattern of gene promotor hypomethylation may be induced by protein malnutrition in human liver.

Effect of protein variation in the diet on gut microbiota

In humans, dietary patterns shape the gut microbiota[58–61]. Previous studies have demonstrated that malnutrition in children can change the structure and function of the bacterial populations that are found in the gut with subsequent changes in the nutrient flow, for example, SCFA to the host[48,62]. Notably, fat absorption is impacted by the microbiota and has been linked to the development of steatosis[63].

Many gut microbiota also digest complex carbohydrates, such as dietary fibre, to release the OCFA and SCFA such as acetic, propionic, butyric and valeric acids[64]. These SCFA may act on receptors in the gut or be taken up directly through monocarboxylate transporters into the periportal vein for delivery to the liver and entry into metabolic pathways. Whether gut microbiota-derived OCFA are a source of circulating C15 : 0 or C17 : 0 is unclear[65]. Nevertheless, it has been shown in mice fed a high-fat diet that butyrate generated by probiotics can promote peroxisomal biogenesis in a PPARα-dependent manner, thereby facilitating further lipid metabolism[66].

The protective effect of gut-derived SCFA against development of fatty liver disease is, at least in part, due to their activation of the G-protein coupled receptor, GPR43, present in liver and adipose tissue[67]. In germ-free mice without gut microbiota or associated production of SCFA and in a GPR43 knockout mouse model, increases in pro-inflammatory cytokine production and immune cell recruitment to the liver were observed[67]. As inflammation plays a major role in the progression of early stage fatty liver to fibrotic liver disease, dietary patterns that influence gut microbiota may exert distant effects on hepatic metabolism via their metabolites, for example, SCFA[68].

To date, few studies in humans and animals have investigated any association between dietary proteins, liver metabolism and gut microbiota in relation to metabolic diseases including fatty liver disease; several studies have focused on a high-protein/low-carbohydrate diet rather than low-protein/high-carbohydrate diet. The limited numbers of protein-varied diet studies available have shown that duration and extent of protein malnutrition influence the gut microbiota and their function including secretion of SCFA and proinflammatory mediators (Table 1)[9,70,71]. On the one hand, a higher-protein diet (>20 % of energy as protein) that led to weight loss in obese subjects was suggested to pose a risk for metabolic health through altering the gut microbiota and reducing the levels of protective SCFA production in the gut[69]. On the other hand, specific populations at risk for protein malnutrition, including children in developing countries (Malawi and India) and older adults merit further study[48,62,72]. They may benefit from increased dietary protein through effects on gut microbiota producing OCFA/SCFA and modulation of lipid accumulation in the liver.

Associative evidence between low dietary protein and fatty liver disease from population studies

Epidemiological studies have reported an association between poor outcomes and high intake of animal but not plant protein and commonly describe these effects in individuals with existing metabolic disease[73,74]. These studies highlight the

need for assessing individual protein requirement, since an increase in dietary protein may have adverse outcomes for some populations, for example, younger people and those with chronic kidney disease, but potentially beneficial effects in those over 65 years of age or who have dietary protein insufficiency. In contrast, a low-protein diet is used in the management of organic acidaemias; however, to the best of our knowledge, no study has reported the outcome of fatty liver associated with protein restriction after the long-term management. Any analysis of fatty liver in these patients is in part confounded by the use of liver transplantation to address the metabolic deficit in patients and because conservative management is achieved not only by dietary protein restriction and metronidazole but also by L-carnitine supplements. A systematic review of the general population concluded that higher protein diets probably improve adiposity, blood pressure and TAG levels, but that these effects are small and need to be weighed against potential for adverse effects[75]. Nevertheless, our focus here has been to understand the effects of improving dietary protein intake on fatty liver disease risk in people who are malnourished and who have low protein intake. In Europe, the most recent analyses of National Dietary Surveys suggest that protein intake is adequate over all age ranges examined[76], although there is some evidence that in older adults with frailty syndrome, protein intake may be reduced. Dietary surveys are limited in developing countries; however, a review of dietary surveys conducted on the adult South African population from 2000 to 2015 revealed that out of the total energy intake of men and women, the % energy from protein ranged from 10·9 to 18·3 %; fat from 17 to 37·1 %; and carbohydrate from 47·0 to 69 %, highlighting the variation that exists in country[77].

Two population-based studies have explored the effects of increasing dietary protein after weight loss in people with diabetes[79,78]. In the PREVIEW study, the prospective association between increased plant protein intake and improvement in diabetes indicators and fatty liver was reported to be independent of BMI[78,80]. In a secondary analysis of data from the randomised controlled DIOGenes trial, which investigated dietary protein composition and glycaemic index on weight loss maintenance and cardiometabolic risk factors in obesity, plant protein offered the greatest health benefits, while a higher plant protein intake from non-cereal products instead of cereal products (matched by a decrease in other sources of protein intake) benefited by body weight maintenance and blood pressure[79]. In a 5-year follow-up study, it was reported that substitution with 10 g carbohydrate with plant (but not animal) protein did not lead to any weight change and all-cause mortality was reduced[81].

Another study explored liver fat deposition and showed that a high-protein diet compared with an isoenergetic high-carbohydrate diet, without any change in weight, reduced intrahepatic lipid storage, consistent with our suggestion that improving protein intake in low-income countries may reduce risk for NAFLD[82]. To date, there is a lack of detailed biochemistry to describe the underpinning association between low dietary protein, lipid deposition and whether this relates impaired OCFA production.

Conclusion

The importance of dietary composition on lipid storage in metabolic health and diseases cannot be over-emphasised. Altered lipid metabolism is strongly associated with NAFLD, obesity, diabetes, the metabolic syndrome and other cardiometabolic diseases, and diet plays a critical role in the development of these diseases. Several human and animal studies have shown that high-fat and high-carbohydrate diets are associated with the development of NAFLD. These diets affect lipid metabolism resulting in accumulation of TAG in the liver. Here, we suggest that the effects of insufficient dietary protein for fatty liver disease risk are underexplored in specific populations that have a protein-restricted diet, either for medical reasons or because of limited availability of food resources in developing countries.

We have gathered evidence that suggests mitochondria, peroxisomes and altered gut microbiota may each play a role in the development of NAFLD in response to protein restriction and possibly mediated through epigenetic effects and changes to circulating SCFA and OCFA.

The limited existing data suggest that targeting specific sources of protein and amino acid intake provides an opportunity for reducing risk of fatty liver disease, particularly in vulnerable groups[75] and those with restricted protein diets in developing countries. Such studies would also need to address the type, source and quality of amino acid/protein used. For example, plant protein but not animal protein has been reported to reduce mortality risk in people with diabetes[81]. The feasibility of improving dietary protein as an approach to NAFLD is further supported by the recent report that an increase in daily protein intake to 25 % by weight was sufficient to reduce intrahepatic lipid accumulation in people with diabetes following a weight-reduction diet[80]. This beneficial effect on fat storage in the liver was observed during a 2-year follow-up period of weight maintenance and was independent of BMI.

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References

1. Younossi ZM, Koenig AB, Abdelatif D, *et al.* (2016) Global epidemiology of nonalcoholic fatty liver disease—meta-analytic assessment of prevalence, incidence, and outcomes. *Hepatology* **64**, 73–84.
2. Sattar N, Forrest E & Preiss D (2014) Non-alcoholic fatty liver disease. *BMJ* **349**, g4596.
3. Chiu S, Sievenpiper JL, de Souza RJ, *et al.* (2014) Effect of fructose on markers of non-alcoholic fatty liver disease

(NAFLD): a systematic review and meta-analysis of controlled feeding trials. *Eur J Clin Nutr* **68**, 416.
4. Wang J, Cao M, Zhuo Y, *et al.* (2016) Catch-up growth following food restriction exacerbates adulthood glucose intolerance in pigs exposed to intrauterine undernutrition. *Nutrition* **32**, 1275–1284.
5. Haro D, Marrero PF, Relat J, *et al.* (2019) Nutritional regulation of gene expression: carbohydrate-, fat- and amino acid-dependent modulation of transcriptional activity. *Int J Mol Sci* **20**, E1386.
6. Guo F & Cavener DR (2007) The GCN2 eIF2alpha kinase regulates fatty-acid homeostasis in the liver during deprivation of an essential amino acid. *Cell Metab* **5**, 103–114.
7. Montagner A, Polizzi A, Fouché E, *et al.* (2016) Liver PPARα is crucial for whole-body fatty acid homeostasis and is protective against NAFLD. *Gut* **65**, 1202–1214.
8. Bjørndal B, Berge C, Ramsvik MS, *et al.* (2013) A fish protein hydrolysate alters fatty acid composition in liver and adipose tissue and increases plasma carnitine levels in a mouse model of chronic inflammation. *Lipids Health Dis* **12**, 143.
9. Mayneris-Perxachs J, Bolick DT, Leng J, *et al.* (2016) Protein- and zinc-deficient diets modulate the murine microbiome and metabolic phenotype. *Am J Clin Nutr* **104**, 1253–1262.
10. Houghton D, Stewart C, Day C, *et al.* (2016) Gut microbiota and lifestyle interventions in NAFLD. *Int J Mol Sci* **17**, 447.
11. Ipsen DH, Lykkesfeldt J & Tveden-Nyborg P (2018) Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease. *Cell Mol Life Sci* **75**, 3313–3327.
12. Falcon A, Doege H, Fluitt A, *et al.* (2010) FATP2 is a hepatic fatty acid transporter and peroxisomal very long-chain acyl-CoA synthetase. *Am J Physiol-Endocrinol Metab* **299**, E384–E393.
13. Doege H, Baillie RA, Ortegon AM, *et al.* (2006) Targeted deletion of FATP5 reveals multiple functions in liver metabolism: alterations in hepatic lipid homeostasis. *Gastroenterology* **130**, 1245–1258.
14. Qiu Y, Liu S, Chen HT, *et al.* (2013). Upregulation of caveolin-1 and SR-B1 in mice with non-alcoholic fatty liver disease. *Hepatobiliary Pancreat Dis Int* **12**, 630–636.
15. Asterholm I, Mundy WDI, Weng J, *et al.* (2012). Altered mitochondrial function and metabolic inflexibility associated with loss of caveolin-1. *Cell Metab* **15**, 171–185.
16. Li M, Chen D, Huang H, *et al.* (2017). Caveolin1 protects against diet induced hepatic lipid accumulation in mice. *PLOS ONE* **12**, e0178748.
17. Miquilena-Colina ME, Lima-Cabello E, Sánchez-Campos S, *et al.* (2011) Hepatic fatty acid translocase CD36 upregulation is associated with insulin resistance, hyperinsulinaemia and increased steatosis in non-alcoholic steatohepatitis and chronic hepatitis C. *Gut* **60**, 1394–402.
18. Wilson CG, Tran JL, Erion DM, *et al.* (2015) Hepatocyte-specific disruption of CD36 attenuates fatty liver and improves insulin sensitivity in HFD-fed mice. *Endocrinology* **157**, 570–585.
19. Martin GG, Atshaves BP, Landrock KK, *et al.* (2015) Loss of l-FABP, SCP-2/SCP-x, or both induces hepatic lipid accumulation in female mice. *Arch Biochem Biophys* **580**, 41–49.
20. Nassir F & Ibdah J (2014) Role of mitochondria in nonalcoholic fatty liver disease. *Int J Mol Sci* **15**, 8713–8742.
21. Begriche K, Igoudjil A, Pessayre D, *et al.* (2016) Mitochondrial dysfunction in NASH: causes, consequences and possible means to prevent it. *Mitochondrion* **6**, 1–28.
22. Koliaki C, Szendroedi J, Kaul K, *et al.* (2015) Adaptation of hepatic mitochondrial function in humans with non-alcoholic fatty liver is lost in steatohepatitis. *Cell Metab* **21**, 739–746.
23. Pérez-Carreras M, Del Hoyo P, Martín MA, *et al.* (2003) Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* **38**, 999–1007.
24. Simões IC, Fontes A, Pinton P, *et al.* (2018) Mitochondria in non-alcoholic fatty liver disease. *Int J Biochem Cell Biol* **95**, 93–99.
25. Koliaki C, Szendroedi J, Kaul K, *et al.* (2015) Adaptation of hepatic mitochondrial function in humans with nonalcoholic fatty liver is lost in steatohepatitis. *Cell Metab* **21**, 739–746.
26. Williams B, Correnti J, Oranu A, *et al.* (2017) A novel role for ceramide synthase 6 in mouse and human alcoholic steatosis. *FASEB J* **32**, 130–142.
27. Kuwahata M, Kubota H, Amano S, *et al.* (2011). Dietary medium-chain triglycerides attenuate hepatic lipid deposition in growing rats with protein malnutrition. *J Nutr Sci Vitaminol* **57**, 138–143.
28. Contreras AV, Torres N & Tovar AR (2013) PPAR-α as a key nutritional and environmental sensor for metabolic adaptation. *Adv Nutr* **4**, 439–452.
29. Holeček M (2018) Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. *Nutr Metab* **15**, 33.
30. She P, Van Horn C, Reid T, *et al.* (2007) Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. *Am J Physiol-Endocrinol Metab* **293**, E1552–E1563.
31. Goffredo M, Santoro N, Trico D, *et al.* (2017) A branched-chain amino acid-related metabolic signature characterizes obese adolescents with non-alcoholic fatty liver disease. *Nutrients* **9**, E642.
32. Herman MA, She P, Peroni OD, *et al.* (2010) Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels. *J Biol Chem* **285**, 11348–11356.
33. White PJ, McGarrah RW, Grimsrud PA, *et al.* (2018) The BCKDH kinase and phosphatase integrate BCAA and lipid metabolism via regulation of ATP-citrate lyase. *Cell Metab* **27**, 1281–1293.
34. Crown SB, Marze N & Antoniewicz MR (2015) Catabolism of branched chain amino acids contributes significantly to synthesis of odd-chain and even-chain fatty acids in 3T3-L1 adipocytes. *PLOS ONE* **10**, e0145850–e0145850.
35. Jenkins B, West JA & Koulman A (2015) A review of odd-chain fatty acid metabolism and the role of pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) in health and disease. *Molecules* **20**, 2425–2444.
36. Wanders RJ (2014) Metabolic functions of peroxisomes in health and disease. *Biochimie* **98**, 36–44.
37. Smith JJ & Aitchison JD (2013) Peroxisomes take shape. *Nat Rev Mol Cell Biol* **14**, 803.
38. Walker CL, Pomatto LCD, Tripathi DN, *et al.* (2018) Redox regulation of homeostasis and proteostasis in peroxisomes. *Physiol Rev* **98**, 89–115.
39. Jenkins B, de Schryver E, Van Veldhoven PP, *et al.* (2017). Peroxisomal 2-hydroxyacyl-CoA lyase is involved in endogenous biosynthesis of heptadecanoic acid. *Molecules* **22**, E1718.
40. van Zutphen T, Ciapaite J, Bloks VW, *et al.* (2016) Malnutrition-associated liver steatosis and ATP depletion is caused by peroxisomal and mitochondrial dysfunction. *J Hepatol* **65**, 1198–1208.
41. Yoo W, Gjuka D, Stevenson HL, *et al.* (2017) Fatty acids in non-alcoholic steatohepatitis: focus on pentadecanoic acid. *PLOS ONE* **12**, e0189965.
42. Pfeuffer M & Jaudszus A (2016) Pentadecanoic and heptadecanoic acids: multifaceted odd-chain fatty acids. *Adv Nutr* **7**, 730–734.
43. Al-Lahham SH, Peppelenbosch MP, Roelofsen H, *et al.* (2010) Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. *Biochim Biophys Acta* **1801**, 1175–1183.

44. Comhair TM, Garcia Caraballo SC, Dejong CHC, *et al.* (2017) The odd-carbon medium-chain fatty triglyceride triheptanoin does not reduce hepatic steatosis. *Clin Nutr* **36**, 229–237.
45. Jenkins B, Aoun M, Feillet-Coudray C, *et al.* (2018) The dietary total-fat content affects the *in vivo* circulating C15:0 and C17:0 fatty acid levels independently. *Nutrients* **10**, E1646.
46. Wu Z, Puigserver P, Andersson U, *et al.* (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **98**, 115–124.
47. Mandard S, Müller M & Kersten S (2004) Peroxisome proliferator-activated receptor α target genes. *Cell Mol Life Sci* **61**, 393–416.
48. Ghosh TS, Gupta SS, Bhattacharya T, *et al.* (2014) Gut microbiomes of Indian children of varying nutritional status. *PLOS ONE* **9**, e95547.
49. Kang W, Lee MS & Baik M (2011) Dietary protein restriction alters lipid metabolism and insulin sensitivity in rats. *Asian-Australas J Anim Sci* **24**, 1274–1281.
50. Kwon DH, Kang W, Nam YS, *et al.* (2012) Dietary protein restriction induces steatohepatitis and alters leptin/signal transducers and activators of transcription 3 signaling in lactating rats. *J Nutr Biochem* **23**, 791–799.
51. Huang X, Hancock DP, Gosby AK, *et al.* (2013) Effects of dietary protein to carbohydrate balance on energy intake, fat storage, and heat production in mice. *Obesity* **21**, 85–92.
52. Watkins AJ & Sinclair KD (2014) Paternal low protein diet affects adult offspring cardiovascular and metabolic function in mice. *Am J Physiol Heart Circ Physiol* **15**, H1444–H1452.
53. Ueland PM (2011) Choline and betaine in health and disease. *J Inherit Metab Dis* **34**, 3–15.
54. Kulinski A, Vance DE & Vance JE (2004) A choline-deficient diet in mice inhibits neither the CDP-choline pathway for phosphatidylcholine synthesis in hepatocytes nor apolipoprotein B secretion. *J Biol Chem* **279**, 23916–23924.
55. Lyall MJ, Cartier J, Richards JA, *et al.* (2017) Methyl donor deficient diets cause distinct alterations in lipid metabolism but are poorly representative of human NAFLD. *Wellcome Open Res* **2**, 67.
56. Madeira MS, Rolo EA, Lopes PA, *et al.* (2018) Betaine and arginine supplementation of low protein diets improves plasma lipids but does not affect hepatic fatty acid composition and related gene expression profiling in pigs. *J Sci Food Agric* **98**, 598–608.
57. Pooya S, Blaise S, Moreno Garcia M, *et al.* (2012) Methyl donor deficiency impairs fatty acid oxidation through PGC-1alpha hypomethylation and decreased ER-alpha, ERR-alpha, and HNF-4alpha in the rat liver. *J Hepatol* **57**, 344–351.
58. David LA, Maurice CF, Carmody RN, *et al.* (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**, 559.
59. Schnorr SL, Candela M, Rampelli S, *et al.* (2014) Gut microbiome of the Hadza hunter-gatherers. *Nat Commun* **5**, 3654.
60. Carmody RN, Gerber GK, Luevano JM Jr, *et al.* (2015) Diet dominates host genotype in shaping the murine gut microbiota. *Cell Host Microbe* **17**, 72–84.
61. Wu GD, Chen J, Hoffmann C, *et al.* (2011) Linking long-term dietary patterns with gut microbial enterotypes. *Science* **334**, 105–108.
62. Smith MI, Yatsunenko T, Manary MJ, *et al.* (2013) Gut microbiomes of Malawian twin pairs discordant for kwashiorkor. *Science* **339**, 548–554.
63. Kolodziejczyk AA, Zheng D, Shibolet O, *et al.* (2019) The role of the microbiome in NAFLD and NASH. *EMBO Mol Med* **11**, e9302.
64. Hoverstad T & Midtvedt T (1986) Short-chain fatty acids in germfree mice and rats. *J Nutr* **116**, 1772–1776.
65. Kimura I, Ozawa K, Inoue D, *et al.* (2013) The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. *Nat Commun* **4**, 1829.
66. Weng H, Endo K, Li J, *et al.* (2015) Induction of peroxisomes by butyrate-producing probiotics. *PLOS ONE* **10**, e0117851.
67. Maslowski KM, Vieira AT, Ng A, *et al.* (2009) Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. *Nature* **461**, 1282–1286.
68. Jenkins BJ, Seyssel K, Chiu S, *et al.* (2017) Odd chain fatty acids; new insights of the relationship between the gut microbiota, dietary intake, biosynthesis and glucose intolerance. *Sci Rep* **7**, 44845.
69. Russell WR, Gratz SW, Duncan SH, *et al.* (2011) High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. *Am J Clin Nutr* **93**, 1062–1072.
70. Li Q, Lauber CL, Czarnecki-Maulden G, *et al.* (2017) Effects of the dietary protein and carbohydrate ratio on gut microbiomes in dogs of different body conditions. *mBio* **8**, e01703–e01716.
71. Zhou L, Fang L, Sun Y, *et al.* (2016) Effects of the dietary protein level on the microbial composition and metabolomic profile in the hindgut of the pig. *Anaerobe* **38**, 61–69.
72. Claesson MJ, Jeffery IB, Conde S, *et al.* (2012) Gut microbiota composition correlates with diet and health in the elderly. *Nature* **488**, 178.
73. Budhathoki S, Sawada N, Iwasaki M, *et al.* (2019) Association of animal and plant protein intake with all-cause and cause-specific mortality. *JAMA Intern Med* **179**, 1509–1518.
74. Alferink LJ, Kiefte-de Jong JC, Erler NS, *et al.* (2019) Association of dietary macronutrient composition and non-alcoholic fatty liver disease in an ageing population: the Rotterdam Study. *Gut* **68**, 1088–1098.
75. Santesso N, Akl EA, Bianchi M, *et al.* (2012) Effects of higher- versus lower-protein diets on health outcomes: a systematic review and meta-analysis. *Eur J Clin Nutr* **66**, 780–788.
76. Rippin HL, Hutchinson J, Jewell J, *et al.* (2017) Adult nutrient intakes from current national dietary surveys of European populations. *Nutrients* **9**, E1288.
77. Mchiza ZJ, Steyn NP, Hill J, *et al.* (2015) A review of dietary surveys in the adult South African population from 2000 to 2015. *Nutrients* **7**, 8227–8250.
78. Sluik D, Brouwer-Brolsma EM, Berendsen AAM, *et al.* (2019) Protein intake and the incidence of pre-diabetes and diabetes in 4 population-based studies: the PREVIEW project. *Am J Clin Nutr* **109**, 1310–1318.
79. van Baak MA, Larsen TM, Jebb SA, *et al.* (2017) Dietary intake of protein from different sources and weight regain, changes in body composition and cardiometabolic risk factors after weight loss: the DIOGenes study. *Nutrients* **9**, E1326.
80. Drummen M, Dorenbos E, Vreugdenhil ACE, *et al.* (2018) Long-term effects of increased protein intake after weight loss on intrahepatic lipid content and implications for insulin sensitivity: a PREVIEW study. *Am J Physiol Endocrinol Metab* **315**, E885–E891.
81. Campmans-Kuijpers MJ, Sluijs I, Nöthlings U, *et al.* (2015) Isocaloric substitution of carbohydrates with protein: the association with weight change and mortality among patients with type 2 diabetes. *Cardiovasc Diabetol* **14**, 39.
82. Martens EA, Gatta-Cherifi B, Gonnissen HK, *et al.* (2014) The potential of a high protein-low carbohydrate diet to preserve intrahepatic triglyceride content in healthy humans. *PLOS ONE* **9**, e109617.