Plenary Lecture

Impact of dietary fat on the development of non-alcoholic fatty liver disease in Ldlr\textsuperscript{−/−} mice

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The prevalence of non-alcoholic fatty liver disease (NAFLD) has increased in parallel with central obesity and is now the most common chronic liver disease in developed countries. NAFLD is defined as excessive accumulation of lipid in the liver, i.e. hepatosteatosis. The severity of NAFLD ranges from simple fatty liver (steatosis) to non-alcoholic steatohepatitis (NASH). Simple steatosis is relatively benign until it progresses to NASH, which is characterised by hepatic injury, inflammation, oxidative stress and fibrosis. Hepatic fibrosis is a risk factor for cirrhosis and primary hepatocellular carcinoma. Our studies have focused on the impact of diet on the onset and progression of NASH. We developed a mouse model of NASH by feeding Ldlr\textsuperscript{−/−} mice a western diet (WD), a diet moderately high in saturated and trans-fat, sucrose and cholesterol. The WD induced a NASH phenotype in Ldlr\textsuperscript{−/−} mice that recapitulates many of the clinical features of human NASH. We also assessed the capacity of the dietary \textit{n}-3 PUFA, i.e. EPA (20:5,\textit{n}-3) and DHA (22:6,\textit{n}-3), to prevent WD-induced NASH in Ldlr\textsuperscript{−/−} mice. Histologic, transcriptomic, lipidomic and metabolomic analyses established that DHA was equal or superior to EPA at attenuating WD-induced dyslipidemia and hepatic injury, inflammation, oxidative stress and fibrosis. Dietary \textit{n}-3 PUFA, however, had no significant effect on WD-induced changes in body weight, body fat or blood glucose. These studies provide a molecular and metabolic basis for understanding the strengths and weaknesses of using dietary \textit{n}-3 PUFA to prevent NASH in human subjects.

**Non-alcoholic steatohepatitis: Inflammation: Oxidative stress: Fibrosis: n-3 PUFA**

The Centres for Disease Control estimates that nearly 80 million adults\textsuperscript{(1)} and 13 million children\textsuperscript{(2)} in the USA are obese. Obesity is a risk factor for chronic metabolic diseases, such as CVD, metabolic syndrome (MetS), type 2 diabetes and non-alcoholic fatty liver disease (NAFLD). Our studies have focused on NAFLD. The prevalence of NAFLD has increased in parallel with incidence of central obesity\textsuperscript{(3,4)} and is now the most common fatty liver disease in developed countries\textsuperscript{(5)}. NAFLD is defined as excessive lipid accumulation in the liver, i.e. hepatosteatosis\textsuperscript{(6,7)}. NAFLD is the hepatic manifestation of MetS\textsuperscript{(8)}; MetS risk factors include obesity, elevated plasma TAG and LDL-cholesterol, reduced HDL-cholesterol, high blood pressure and fasting hyperglycemia\textsuperscript{(9)}. The prevalence of NAFLD in the general population is estimated to range from 6 to 30 % depending on the method of analysis and population studied\textsuperscript{(10)}. NAFLD ranges from benign hepatosteatosis to non-alcoholic steatohepatitis (NASH)\textsuperscript{(11)}, where NASH is defined as hepatosteatosis with inflammation and hepatic injury\textsuperscript{(12)}. Simple hepatosteatosis progresses to NASH in 30–40 % of patients\textsuperscript{(13)}, representing about 3–5 % of the general population\textsuperscript{(10)}. The type 2 diabetes population has a higher prevalence (≥60 %) of NAFLD and NASH.

**Abbreviations:** MetS, metabolic syndrome; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NOX, NADPH oxidase; TLR, Toll-like receptor; WD, western diet.

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than the general population\(^{14}\). NASH patients have higher mortality rates than NAFLD patients; and both are higher than the general population\(^{15–17}\). NASH can progress to cirrhosis and hepatocellular carcinoma\(^4,13\). Over a 10 year period, cirrhosis and liver related death occurs in 20 and 12 % of NASH patients, respectively\(^18\). Cirrhosis resulting from NASH is projected to be the leading cause of liver transplantation in the USA by 2020\(^19\). Given the increasing prevalence of NASH and its negative clinical outcomes, NASH is rapidly becoming a significant public health burden\(^20\).

**Multi-hit hypothesis for non-alcoholic steatohepatitis development**

The development of NASH has been proposed to follow a multi-hit model\(^{21–23}\). The 1st Hit involves excessive neutral lipid accumulation which sensitises the liver to the 2nd Hit\(^{22}\) (Fig. 1). The 2nd Hit is characterised by hepatic insulin resistance, inflammation, oxidative stress leading to in hepatic damage that is associated with increased blood levels of hepatic enzymes/proteins, e.g. alanine aminotransferase\(^3,4,24\). The resulting hepatocellular death and necrosis promotes the 3rd Hit which involves activation of resident stellate cells and subsequent deposition of extracellular (fibrotic) matrix. Fibrosis is a tissue repair mechanisms that results in scarring; it is mediated by hepatic stellate cell activation and myofibillar cell infiltration of the liver. These cells produce extracellular matrix proteins, including collagen (collagen 1A1), elastin and smooth muscle α2 actin\(^{25}\). Dietary (excess fat, cholesterol, glucose and fructose), metabolic (plasma and hepatic fatty acid profiles, hepatic ceramide, oxidised LDL, bile acid metabolites), endocrine (insulin, leptin and adiponectin), gut (endotoxin, microbial metabolites) and genetic (e.g. patatin-like phospholipase domain containing 3 polymorphisms) factors have been implicated as triggers for NASH progression\(^{26–34}\).

Hepatosteatosis develops because of an imbalance of hepatic lipid metabolism leading to the accumulation of hepatic neutral lipids as TAG and diacylglycerols and cholesterol esters. In human subjects with NAFLD, about 60 % of the fat appearing in the liver is derived from circulating NEFA mobilised from adipose tissue; 26 % are from de novo lipogenesis and 15 % are from the diet\(^{35}\). Hepatic fatty acid oxidation and VLDL assembly and secretion represent pathways for removal of liver fat. Hepatosteatosis develops when lipid storage exceeds lipid export or fatty acid oxidation. Both hepatic and peripheral insulin resistance also contribute to the disruption of these metabolic pathways\(^36\).

NASH patients consume a lower ratio of PUFA to SFA when compared with the general population\(^{37,38}\). Furthermore, consumption of a low ratio of dietary n-3 PUFA to n-6 PUFA is also associated with NAFLD development, while increased consumption of dietary long-chain n-3 PUFA decreases hepatic steatosis\(^{39–41}\). Pachikian et al\(^{42}\) recently reported that removal of all n-3 PUFA from a mouse diet promoted insulin resistance and hepatosteatosis in C57Bl/6J mice. While this diet lowered hepatic n-3 PUFA, including α-linolenic acid (18 : 3, n-3), EPA (20 : 5, n-3) and DHA (22 : 6, n-3), it did not affect hepatic n-6 PUFA content, i.e. linoleic acid (18 : 2, n-6) or arachidonic acid (20 : 4, n-6). Several hepatic transcription factors are regulated by C20–22 n-3 PUFA, including PPAR-α, sterol regulatory element binding protein-1, carbohydrate regulatory element binding protein and Max-like factor \(X^{43}\). PPAR-α is a fatty acid-regulated nuclear receptor.
Activation of PPAR-α increases expression of enzymes involved in fatty acid oxidation. Sterol regulatory element binding protein-1 and the carbohydrate regulatory element binding protein /Max-like factor X heterodimer regulate the expression of genes involved in de novo lipogenesis and TAG synthesis. Dietary n-3 PUFA suppress the nuclear abundance of sterol regulatory element binding protein-1 and carbohydrate regulatory element binding protein /Max-like factor X leading to the attenuation of expression of genes involved in fatty acid and TAG synthesis. Lowering hepatic n-3 PUFA, as reported by Pachikian et al. (42), promotes hepatosteatosis by suppressing hepatic fatty acid oxidation and stimulating fatty acid and TAG synthesis and storage. While trans-fatty acid consumption is associated with insulin resistance and CVD, the impact of trans-fatty acid consumption on NAFLD in human subjects is less clear (44). In mice, however, trans-fatty acid consumption is associated with hepatic steatosis and injury (45,46).

High dietary cholesterol promotes hepatic inflammation (58,47-49) and contributes to NASH development (50). In the Ldlr−/− mouse model, high fat–high cholesterol feeding results in a robust NASH phenotype (51). Kupffer cells, i.e. resident hepatic macrophage, become engorged with oxidised-LDL, which induces inflammatory cytokine secretion. These locally secreted cytokines act on other hepatic cells and cause cellular injury. Kupffer cells also secrete chemokines (e.g. monocyte chemoattractant protein-1) that recruit monocytes to the liver, further promoting an inflammatory environment in the liver. As such, reducing hepatic inflammation is an obvious target for NASH therapy.

Over the past 30 years there has been a dramatic increase in obesity and NAFLD in the USA (3,32-36). These changes in health status are associated with increased carbohydrate and total energy consumption, but not total fat consumption. Elevated carbohydrate, and specifically fructose, consumption has been linked to the development of NAFLD and NASH progression (57-59). The liver expresses the fructose-specific transporter (Glu5) and is responsible for metabolising up to 70% of dietary fructose (58,59). Fructose metabolism is independent of insulin. When compared with glucose, fructose more readily enters the pathway for de novo lipogenesis and TAG synthesis. Fructose promotes all aspects of MetS including hepatosteatosis, insulin resistance, dyslipidaemia, hyperglycaemia, obesity and hypertension (60). In contrast to fructose, hepatic glucose metabolism is well-regulated by insulin; glucose is also converted to glycogen for storage. Excess glucose consumption does not promote hepatosteatosis as aggressively as excess fructose consumption. Fructose also affects several biochemical events that exacerbate NASH development, including formation of reactive oxygen species and advanced glycation end-products (61-64).

**Treatment strategies for non-alcoholic fatty liver disease**

General therapeutic strategies for NAFLD/NASH start with lifestyle management (diet and exercise) and treating the co-morbidities associated with NAFLD/NASH, e.g. obesity, type 2 diabetes, dyslipidemia. The best strategy for managing NASH, however, has not been established (65). Clinical approaches to manage NAFLD/NASH focus on: (1) a reduction in overall body weight by using dietary and exercise therapy; (2) control blood glucose and dyslipidemia (cholesterol and TAG) by using pharmaceutical and/or dietary supplements, such as metformin, fibrates, thiazolidinediones, statins, and/or n-3 PUFA; (3) suppression of inflammation by using Toll-like receptor (TLR) modulators or n-3 PUFA; and (4) suppression of oxidative stress by using vitamin E and other antioxidants (66-70). Therapeutic regulators of fibrosis, however, are less well-defined (71,72).

**Development of a mouse model of non-alcoholic steatohepatitis**

We have used wild type C57BL/6J mice and mice with global ablation of the LDL receptor (Ldlr−/−, on the C57BL/6J background) to study dietary factors and molecular mechanisms involved in the onset and progression of diet-induced chronic fatty liver diseases (41,73-80). We have assessed three diets for their capacity to promote a NASH phenotype that recapitulates human NASH: (1) the high fat diet (60 % energy as fat (Research Diets; D12492)) typically used to promote diet-induced obesity and type 2 diabetes (79); (2) a high fat–high cholesterol diet (Research Diets) used to induce fatty liver with elevated oxidative stress (49,81); and (3) the western diet (WD; Research Diets; D12079B) to induce NASH. The WD is moderately high in saturated and trans-fat (41 % total energy), sucrose (30 % total energy) and cholesterol (0.15 g%, w/w). Our studies established that the wild type mice developed hepatosteatosis and relatively mild hepatic inflammation and fibrosis when compared with WD-fed Ldlr−/− mice (Table 1). The combination of the WD and the Ldlr−/− mice yields a NASH- and MetS-like phenotype; a phenotype characterised by obesity, hyperglycaemia, dyslipidaemia, hepatosteatosis, hepatic inflammation, damage and fibrosis (77). Since human subjects (3,4,14) and Ldlr−/− mice (49,75-80,82) develop NAFLD and NASH in a context of obesity and insulin resistance, Ldlr−/− mice may be a useful preclinical model to investigate the development, progression and remission of NASH under defined laboratory conditions.

The WD is similar to a fast-food based diet (83) and human diets linked to obesity in the USA (84,85). Both the WD and fast-food mouse models induced a NASH phenotype that recapitulates many of the phenotypic features of human NASH, including hepatic micro- and macro-steatosis, hepatocyte ballooning, hepatic injury including infiltration of leucocytes (inflammation), oxidative stress and branching fibrosis (77,82). Moreover, NASH is associated with a major enrichment of both plasma and liver with SFA and MUFA and hepatic depletion of n-3 and n-6 PUFA (49,77,78), a phenomenon that has been described in human NASH (86,87).
isoenergetic. The concentration of C20,22 n-3 PUFA in the WD is comparable with the dose consumed by patients taking Lovaza™ (GSK) for treating dyslipidemia(93). Supplementing human diets with a DHA-enriched fish oil (6 g/d for 8 weeks) increased plasma DHA from 4 to 8 mol% (96,97). Human subjects consuming EPA + DHA ethyl esters (4 g/d for 12 weeks) increased plasma EPA + DHA from 5-5 to 16-2 + 2-1 mol% (98). In our studies, mice consuming DHA at 2% total calories for 16 weeks increased plasma EPA, docosapentaenoic acid (DPA; 22 : 5, n-3) + DHA from 6-2 to 15-2 mol%. As such, our protocol for C20,22 n-3 PUFA supplementation yields a change in blood C20,22 n-3 PUFA comparable with that seen in human subjects consuming C20,22 n-3 PUFA at 4-6 g/d.

WD induces a robust NASH phenotype that recapitulates human NASH (Fig. 2)(77). Addition of EPA or DHA to the WD did not affect body weight, body fat or blood glucose, but the n-3 PUFA supplemented diets reduced WD-induced plasma lipids, hepatic lipids, inflammation, oxidative stress and fibrosis(77,78). Moreover, these studies also established that DHA was equal or superior to EPA at attenuating all WD-induced NASH markers.

Feeding mice n-3 PUFA does not prevent western diet-induced endotoxinemia

Systemic inflammation is a major driver of NASH. Inflammatory signals contributing to NASH progression include: gut-derived microbial products (endotoxin, other bacterial toxins (Fig. 1) (30,99); oxidised-LDL (51,74); adipokines (leptin/adiponectin) and cytokines (TNFa)(100) and products from hepatocellular death(53,101). Feeding Ldlr−/− mice the WD leads to a 14-fold increase in plasma endotoxin. Including EPA or DHA in the WD did not prevent diet-induced endotoxinemia(78). The appearance of bacterial lipids (endotoxin, a TLR-4 agonist)(102) in the plasma may represent a disturbance in gut physiology such as a change in microbial population, increased gut permeability (leaky gut), or simply co-transport of microbial lipids with chylomicron(30,103,104). A link between the gut microbiome and NAFLD has been established(30,105,106).

n-3 PUFA attenuate hepatic inflammation

Analysis of the liver showed that including EPA or DHA in the WD attenuated WD-induced expression of multiple genes linked to inflammation including TLR (TLR-2, -4, -9) and TLR components (cluster of differentiation-14 (CD14); binds endotoxin), downstream targets of TLR; like NF-κB (p50 and P65 subunits) nuclear abundance, downstream targets of NF-κB (chemokines (monocyte chemoattractant protein-1)), inflammasome NACHT, LRR and PYD domains-containing protein (NLRP3) and hepatic expression of cytokines, e.g.

Table 1. Comparison of mouse models of non-alcoholic steatohepatitis*

<table>
<thead>
<tr>
<th>Gene Expression (Fold change)</th>
<th>Wild type</th>
<th>Ldlr−/−</th>
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<tbody>
<tr>
<td>Diet</td>
<td>RD</td>
<td>HF</td>
</tr>
<tr>
<td>Scd1 mRNA</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mcp1 mRNA</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>Col1A1 mRNA</td>
<td>1</td>
<td>7</td>
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Rationale for using n-3 PUFA to prevent non-alcoholic steatohepatitis

Our studies have assessed the capacity of C20,22 n-3 PUFA to prevent diet-induced NASH. C20,22 n-3 PUFA are pleiotropic regulators of cell function affecting membrane structure and multiple cellular regulatory mechanisms(43). The impact of C20,22 n-3 PUFA on lipid metabolism and inflammation is well documented making these dietary fats an attractive nutritional approach to combat NASH(43). Meta-analyses and other clinical studies suggest n-3 PUFA may have promise in reducing hepatic fat content in the NAFLD patient. These clinical studies, however, lack the capacity to assess the cellular, molecular and metabolic changes associated with NASH. As such, studies in mice may provide insight into the molecular and metabolic processes associated with the onset, progression and remission of NASH and thus fill critical gaps in the field of chronic fatty liver disease.

n-3 PUFA attenuate western diet-induced non-alcoholic steatohepatitis in Ldlr−/− mice

We assessed the capacity of EPA and DHA to prevent NASH in Ldlr−/− mice(77). The dietary level of EPA or DHA was at approximately 2% of total energy; olive oil was added to control diets to ensure all diets were isoeenergetic. The concentration of C20,22 n-3 PUFA in the WD is comparable with the dose consumed by patients taking Lovaza™ (GSK) for treating dyslipidemia(93).
TNFα and IL1β\(^{77,78}\). As such, EPA and DHA attenuated WD-induced hepatic inflammation by down-regulating key cellular mediators of inflammation, including TLR, CD14 (CD14 mRNA and protein), NF-κB-p50 nuclear abundance.

\( n^{-3} \) PUFA have selective effects on hepatic oxidative stress

Hepatic oxidative stress is associated with NASH progression\(^{107}\). Feeding mice the WD increased hepatic...
expression of transcripts linked to oxidative stress, e.g. NADPH oxidase (NOX) subunits (Nox2, P22phox, P40phox and P67phox). The WD also induced the expression of nuclear factor-erythroid derived 2 (Nrf2), a key transcription factor involved in the anti-oxidant response pathway (49,77). Induction of Nrf2 was associated with increased expression of downstream targets of Nrf2 action, including hemeoxygenase-1 (Hmox1), glutathione-S transferase-1 (Gst1a) (78). Dietary n-3 PUFA had no effect on WD-mediated induction of hepatic Nrf2, Hmox1 or Gst1a. However, both EPA and DHA significantly attenuated WD-mediated induction of all NOX subunits (77). Thus, EPA and DHA do not attenuate the Nrf2-regulated anti-oxidant pathway, but target the NOX pathway to lower hepatic oxidative stress.

**n-3 PUFA attenuate hepatic fibrosis**

Hepatic fibrosis develops as a result of hepatocellular death brought on by inflammation and oxidative stress. Key regulators of fibrosis include transforming growth factor β1, connective tissue growth factor, platelet-derived growth factor, oxidative stress (NOX), inflammatory mediators (endotoxin, TLR agonist), leptin and Notch signalling (34,74,108,109). While EPA and DHA supplementation attenuated WD-mediated induction of hepatic inflammation and oxidative stress, only DHA attenuated hepatic fibrosis. The anti-fibrotic effect of DHA was assessed by quantifying the expression of key markers of hepatic fibrosis, including the expression of collagen 1A1, tissue inhibitor of metalloprotease-1, plasminogen activator inhibitor-1 and transforming growth factor β1; as well as trichrome staining of liver for fibrosis (49,77). These studies reveal an important difference in the capacity of EPA and DHA to attenuate NASH-associated hepatosteatosis, inflammation, oxidative stress and fibrosis.

**The western diet and n-3 PUFA affect all major hepatic metabolic pathways**

To gain additional insight into NASH, we used a global non-targeted metabolomic approach to examine the impact of the WD and C20:22 n-3 PUFA on hepatic metabolism. The analysis identified 320 known biochemicals (78). Both the WD and C20:22 n-3 PUFA significantly affected the hepatic abundance of metabolites in all major metabolic pathways including amino acids and peptides, carbohydrate and energy, lipid, nucleotide and vitamins and cofactors. Fig. 3 illustrates the impact of diet on hepatic biochemistry associated with lipid, carbohydrate, amino acid and vitamin and cofactor metabolism. In each of the four pathways examined, at least 50 % of the biochemicals were affected by the WD. The WD either increased or decreased the hepatic abundance of these metabolites. A closer examination of lipid metabolites shows that WD feeding increased forty-three of 136 lipid metabolites, while inclusion of DHA in the WD attenuated the induction of 72 % of the forty-three metabolites. The WD also lowered hepatic levels of thirty-one lipids; DHA attenuated the WD effect on 87 % of the thirty-one lipid metabolites. Similar effects were seen with carbohydrates, amino acids, vitamins and cofactors.

Overall, the metabolomic analysis expanded our understanding of the impact of the WD and DHA on hepatic metabolism. The onset of NASH is associated with major changes in overall hepatic metabolism and dietary DHA supplementation was able to reverse many of these WD-induced effects on hepatic metabolism. In addition to the pathways listed earlier, our analysis identified several key metabolites (oxidised lipids, advanced glycation end products, sphingolipids) that were regulated by WD and n-3 PUFA. Future studies will focus on evaluating the role these metabolites play in NASH progression and remission.

**Summary**

NAFLD and its progression to NASH is a major public health concern. To help better understand the molecular and metabolic basis for the disease process, we developed a mouse model of NASH. The WD induces a robust NASH phenotype in Ldlr<sup>-/-</sup> mice that recapitulates human NASH. Addition of DHA to the WD attenuates NASH development without promoting weight loss or a reduction in body fat. While EPA and DHA did not attenuate WD-induced markers of systemic inflammation (endotoxin), dietary n-3 PUFA attenuated WD-induced hepatic inflammation by targeting key mediators of hepatic inflammation; specifically a key transcriptional mediator of inflammation (NF-κB-p50) and several downstream NF-κB targets, e.g. TLR receptors (TLR-2, -4, -9) and cofactors (CD14) and inflammasome components (NLRP3). The WD induced several oxidative stress pathways (Nrf2, Nrf2-regulated pathways and NOX-subtype). DHA attenuated the NOX-pathway while preserving the Nrf2-regulated anti-oxidant pathway. Finally, dietary DHA, but not EPA, attenuated WD-induced hepatic fibrosis. Together, these findings suggest that DHA may have potential for use as a therapeutic agent to treat human NASH.

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**Conflicts of Interest**

None.
Authorship

All authors contributed to the writing and editing of the manuscript.

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