Early biochemical observations point to nutritional strategies to manage non-alcoholic fatty liver disease

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Abbreviations used: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PUFA, polyunsaturated fatty acid; RCT, randomised controlled trial; TAG, triacylglycerol.

Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent liver disease globally. The first stage of NAFLD is steatosis, the accumulation of triacylglycerols within hepatocytes. Inflammation and oxidative stress both contribute to progression to more severe disease. In 2004 *Clinical Science* published two papers reporting on fatty acids and oxidative stress markers in the livers of patients with NAFLD; both these papers are highly cited. One paper reported an altered pattern of fatty acids within the livers of patients with NAFLD; there was a lower contribution of polyunsaturated fatty acids (PUFAs) including both n-6 and n-3 PUFAs and an altered balance between n-6 and n-3 PUFAs in favour of the former. Ratios of precursor PUFAs to their long chain more unsaturated derivatives were altered in NAFLD and were interpreted to indicate a reduced activity of the pathway of synthesis of long chain highly unsaturated PUFAs. The authors interpreted their findings to indicate that a low hepatic content of n-3 PUFAs has a causal role in NAFLD. The second paper reported lower hepatic anti-oxidant defences and increased markers of oxidative stress in NAFLD, consistent with a role for oxidative stress in the disease. Many studies have now explored the effect of supplemental n-3 PUFAs or antioxidants, including vitamin E, in patients with NAFLD with some benefits being reported. There remains much interest in n-3 PUFAs and antioxidants as preventive and therapeutic strategies in NAFLD and therefore it seems likely that citation of the two papers from 2004 will be sustained.

In 2004 *Clinical Science* published two papers from a Chilean group reporting on fatty acids and oxidative stress markers in the livers of patients with non-alcoholic fatty liver disease (NAFLD) [1,2]. According to a Web of Science search on 2 June 2022 these papers have been cited 500 and 358 times, respectively, making them the first and third most cited papers published in the journal in 2004. They are the fourth and twelfth most cited papers published in *Clinical Science* in the first decade of the 21st century.

NAFLD, considered to be the hepatic manifestation of the metabolic syndrome, covers the spectrum of liver disease from simple steatosis to hepatocellular carcinoma. NAFLD is now the most prevalent form of liver disease globally; it is estimated to affect about 25% of the world's adult population, although it is much more common in those living with obesity, where it may affect as many as 80% of individuals. NAFLD increases the risk of type-2 diabetes, cardiovascular disease and chronic kidney disease, amongst other impacts [3,4,5]. Recently there has been discussion that the term NAFLD does not reflect current knowledge and understandings, and "metabolic (dysfunction) associated fatty liver disease" has been suggested as a more appropriate overarching term [6,7]. Nevertheless, in the current article, the term NAFLD will be used since that is the term used in all human studies that are discussed. The first stage of the NAFLD spectrum is hepatic steatosis ("fatty liver"), the accumulation of triacylglycerols (TAGs) within hepatocytes. Inflammation and oxidative stress both contribute to progression of the disease to more severe forms, the first of these being non-alcoholic steatohepatitis (NASH), referred to simply as steatohepatitis.

Araya et al. [1] and Videla et al. [2] compared the biochemical characteristics of liver biopsies from control patients and patients with NAFLD, some of whom had steatosis and some of whom had progressed to steatohepatitis. The patients with NAFLD all underwent bariatric surgery involving the stomach while the control patients all underwent anti-reflux surgery. The focus of Araya et al. [1] was on the fatty acid composition of the liver; within tissues fatty acids are primarily esterified into phospholipids, mainly in cell membranes, or TAGs, which may be stored within cells as droplets. Patients with hepatic steatosis or steatohepatitis had over 5-times more hepatic TAG than the control patents [1]. There were very large differences in the fatty acid composition ("make up") of liver lipids: the data for each fatty acid were expressed as a percentage of all fatty acids present. Hence this does not take into account the 5-fold difference in hepatic TAG content between controls and patients with NAFLD. Patients with NAFLD had a much higher percentage of palmitic acid (16:0), and higher percentages of palmitolec acid (16:1n-7) and oleic acid (18:1n-9) than controls while they had lower percentages of linoleic acid (18:2n-6) and eicosapentaenoic acid (20:5n-3; EPA) and much

lower percentages of arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3; DHA). Consequently, the total polyunsaturated fatty acid (PUFA) percentage was different between patients with NAFLD and controls, as were the percentages of n-6 and n-3 PUFAs. Patients with NAFLD had much lower percentages of all of these and they had a much elevated ratio of n-6 to n-3 PUFAs. There was no difference reported in the fatty acid composition of liver lipids between patients with steatosis and steatohepatitis, except that the ratio of n-6 to n-3 PUFAs was higher in the latter group. The ratios of PUFA pathway products (arachidonic acid and EPA+DHA) to substrates (linoleic acid and α-linolenic acid, respectively) were much higher in the control patients than in patients with NAFLD, which might suggest an impairment of long chain PUFA synthesis in NAFLD. However, another possibility is that the differences in PUFA composition simply reflect differences in the types of lipid present in the livers of these different patient groups. Phospholipids are typically quite rich in PUFAs, whereas TAGs are rich in saturated and monounsaturated fatty acids which are abundant in the diet and can be produced endogenously through de novo synthesis from simple sugars. It is obvious that patients with NAFLD accumulate hepatic TAGs, as already mentioned for the patients studied by Araya et al. [1]. Thus, the relative contribution of TAG fatty acids to the total fatty acids reported is much greater in the patients with NAFLD than in the controls, and so it would be expected that in NAFLD total hepatic lipid would contain a higher percentage (and a higher absolute amount) of palmitic, palmitoleic and oleic acids and lower percentages of linoleic, arachidonic, eicosapentaenoic and docosahexaenoic acids. This is precisely what is seen. Araya et al. [1] also report the fatty acid composition for hepatic TAGs and a summary of the fatty acid composition of hepatic phospholipids. This additional detail goes some way towards clarifying the reason for the remarkable differences in fatty acids observed in total lipid fatty acids. In hepatic TAGs, compared with controls, patients with NAFLD had a similar percentage of myristic, palmitic, palmitoleic and oleic acids. They had a much higher percentage of linoleic acid and very much lower percentages of α-linolenic, arachidonic, eicosapentaenoic and docosahexaenoic acids. This is interpreted to indicate restricted conversion of essential PUFA precursors to their long chain, more unsaturated derivatives. However different findings for the two precursors (linoleic and α-linolenic acids) do not fully support that interpretation. Unfortunately, the full composition of the hepatic phospholipids is not presented. However, the summary data show no differences in percentages of total saturated, monounsaturated or polyunsaturated fatty acids between controls and patients with NAFLD. Nevertheless, significant differences in the percentages of n-6 and n-3 PUFAs are reported (n-6 PUFAs were

much higher in NAFLD and n-3 PUFAs lower) with a consequently higher ratio of n-6 to n-3 PUFAs in patients with NAFLD. There were no differences reported in the fatty acid composition of liver TAGs or phospholipids between patients with steatosis and steatohepatitis. It is worth noting that the summary data for n-6 and n-3 PUFAs reported for hepatic phospholipids are somewhat unusual compared with phospholipids reported in other human cells and tissues. The marked differences in hepatic fatty acids in patients with NAFLD reported by Araya et al. [1] may be indicative of alterations in hepatic fatty acid and complex lipid metabolism. As already mentioned, the authors suggest that there is altered synthesis of bioactive long chain PUFAs in NAFLD; this is a possibility because that pathway is insulin sensitive [8] and NAFLD involves hepatic insulin resistance [9]. However, the influence of the different types of lipid present in the liver in NAFLD needs to be considered and also whether NAFLD impacts the synthesis and turnover of complex lipids such as phospholipids. Other possibilities are that the oxidative stress and inflammation that occur as part of NAFLD (see later) deplete the liver of long chain PUFAs either by destroying them through peroxidation or by using them as substrates for synthesis of inflammatory mediators. Whether these processes would differentially affect n-6 or n-3 PUFAs is uncertain as both are prone to peroxidation (EPA and DHA more so that arachidonic acid) and both are substrates to produce inflammatory lipid mediators. Whatever the reason, having insufficient long chain n-3 PUFAs (EPA and DHA) in the liver could contribute to development and progression of NAFLD: EPA and DHA favour partitioning of fatty acids towards β-oxidation and away from TAG synthesis [10,11] and both EPA and DHA are anti-inflammatory [12] and give rise to pro-resolution mediators [13].

Araya et al. [1] report that the livers of patients with NAFLD have a higher lipid peroxidation index and higher protein carbonylation than those from controls, suggesting higher hepatic oxidative stress in NAFLD. These markers did not differ between patients with steatosis and steatohepatitis, which is somewhat surprising since development of the latter is linked with oxidative stress. These observations link with the findings of Videla et al. [2], although it is not clear if these two studies used samples from the same patients. Patients with hepatic steatosis had higher hepatic protein carbonylation and lower glutathione levels and superoxide dismutase activity than controls. Patients with hepatic steatohepatitis had higher hepatic protein carbonylation and cytochrome P450 2E1 content and activity and lower glutathione levels and superoxide dismutase and catalase activities. These observations suggest that anti-oxidant defences are lower in the livers of those with NAFLD and are consistent with

oxidative stress playing a role in NAFLD and being linked with increasing severity of disease (i.e. progression from steatosis to steatohepatitis). Oxidative stress is itself damaging but it is intimately linked with inflammation: oxidative stress induces inflammation and inflammation induces oxidative stress. Both oxidative stress and inflammation could deplete long chain PUFAs. Hence the observations of these two papers [1,2] could be linked.

Araya et al. [1] and Videla et al. [2] are observational studies reporting on hepatic biochemical profiles. Neither paper provides strong mechanistic insights to explain the observations. Furthermore, although both papers are suggestive that the differences from control livers that they report (depletion of long chain PUFAs, PUFA imbalances, lower anti-oxidant protection, increased markers of oxidative stress) are a cause of NAFLD, some of them, particularly the PUFA differences could be a consequence rather than a cause. Araya et al. [1] is highly cited because it provides striking evidence of fatty acid alterations in the liver of patients with NAFLD, with a derangement that results in lower amounts of n-3 PUFAs and a higher ratio of n-6 to n-3 PUFAs. If this is causal in the development and progression of NAFLD, then it would invite interventions with n-3 PUFAs to lower risk, slow progression and maybe even reverse the disease. Vidella et al. [2] is highly cited firstly because it provides evidence of lower hepatic anti-oxidant capacity and increased hepatic oxidative stress in patients with NAFLD, especially NASH, and secondly because it invites interventions with antioxidants to lower risk, slow progression and maybe even reverse the disease.

Interventions with both n-3 PUFAs and the antioxidant vitamin E have been performed in patients with NAFLD with the aim of improving the condition if the liver and reversing the disease. Seventeen controlled trials of EPA and DHA in adults or children with NAFLD published between 2004 and 2016 have been extensively reviewed elsewhere [14]. In 2012 Parker et al. [15] published the first meta-analysis on randomised controlled trials (RCTs) of n-3 fatty acids (EPA and DHA) and fatty liver. They included 9 trials, 6 in patients with NAFLD and 3 in patients with related conditions. These trials used a wide range of EPA+DHA doses (0.83 to 13.7 g/day) and were of variable duration (2 to 12 months). Seven trials were included in a meta-analysis exploring the effect of n-3 fatty acids on liver fat, measured using different approaches. Six of these trials individually reported that n-3 fatty acids reduced liver fat compared with control, so it is no surprise that aggregating the findings of these trials identified a significant effect of n-3 fatty acids. Since then, more trials have been performed and at least another eight meta-analyses of n-3 fatty acids in NAFLD have been published. Most of these report that the combination of EPA and DHA (or DHA alone in some studies mainly in children) decreases liver fat in patients with NAFLD (e.g. [16,17]). The success of

these trials with supplemental n-3 fatty acids supports the view of Araya et al. [1] that low hepatic status of EPA and DHA is causally linked with accumulation of liver fat.

Many controlled trials of antioxidants, many including vitamin E either alone or in combination with other antioxidants, in adults or children with NAFLD have been published. In 2012 Musso et al. [18] published a meta-analysis on RCTs of many lifestyle, nutritional and pharmacological interventions and NAFLD. They included 5 trials of vitamin E and identified a modest reduction in liver fat and lobular inflammation. There are at least another ten meta-analyses of vitamin E and NAFLD. Many of these report some benefit from vitamin E in adults with NAFLD (e.g. [19-21]). The success of these trials with supplemental vitamin E supports the view of Videla et al. [2] that oxidative stress is causally linked with progression of fatty liver disease.

NAFLD is an important heath condition without a licenced medical treatment. The key initial feature is hepatic accumulation of TAGs and both inflammation and oxidative stress are involved in the progression from simple steatosis [22]. In 2004 Araya et al. [1] and Videla et al. [2] published important observations of altered hepatic PUFA and antioxidant defence profiles in patients with NAFLD along with increased markers of oxidative stress. The altered fatty acid profiles were generally similar in patients with steatosis and steatohepatitis whereas oxidative stress appeared to be greater in patients with steatohepatitis. These observations provide support to test interventions to correct the PUFA and antioxidant defence derangements that exist in NAFLD in order to find preventive and therapeutic options for this common disease. There remains much interest in n-3 PUFAs [23] and antioxidants [24] in this regard and therefore it seems likely that citation of these two papers will be sustained for some time.

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