FISH AND LONG-CHAIN OMEGA-3 FATTY ACIDS
IN PEDIATRIC LIVER DISEASE

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ABSTRACT

Pediatric nonalcoholic fatty liver disease (NAFLD) and parenteral nutrition-associated liver disease (PNALD) are difficult to manage conditions that have been rising with increasing rates of childhood obesity and survival of infants with intestinal failure, respectively. This dissertation is comprised of five research studies, which examine: a) the burden of pediatric NAFLD in Hawai’i, b) the effect of dietary fish and long-chain omega-3 fatty acid intake (LCω3) on pediatric NAFLD, and c) the use of fish oil-based lipid emulsions (FOLEs) in infants with PNALD. In a cross-sectional analysis of 195 patients referred to a pediatric endocrinologist at Kapi’olani Medical Center for Women and Children (KMCWC) with metabolic syndrome, approximately two-thirds had elevated serum alanine aminotransferase (ALT) concentrations indicative of NAFLD, which makes it one of the most common obesity-related comorbidities in this population. Examination of 81 pediatric NAFLD patients referred to two pediatric gastroenterologists at KMCWC showed that these children were typically overweight (98%) and suffered from obesity-related comorbidities, particularly dyslipidemia, and often exhibited histological features of steatohepatitis. There was a significant reduction in serum ALT concentration in these patients over time; however, body weight tended to increase and was not associated with changes in serum ALT concentrations. In a prospective cohort of 200 Asian and White female adolescents recruited among members of Kaiser Permanente Oahu, greater fish consumption appeared to reduce two-year changes in waist circumference (n=103), a risk factor for NAFLD, although relatively few girls, mostly of Asian ethnicity, consumed the recommended eight ounces of fish per week (12.4%). Children who were attending one of the eight clinical centers of the
Nonalcoholic Steatohepatitis Clinical Research Network (n=223) reported similarly low rates of eating eight or more ounces of fish per week (9.9%). In this study, greater fish and long-chain omega-3 fatty acid intake were associated with less severe of portal and lobular inflammation on liver biopsy. Among ten infants diagnosed with PNALD at KMCWC who were treated with FOLE, earlier initiation of FOLE treatment was associated with reduced length of hospital stay. In summary, the findings of this dissertation demonstrated that a) pediatric NAFLD is a common consequence of obesity in Hawai‘i that is associated with serious disease sequelae, b) fish and LCω3s consumption is low in adolescents, which may increase risk for and severity of hepatic inflammation in NAFLD, and c) early initiation of FOLE treatment in infants with PNALD may reduce length of hospital stay. Strategies to improve fish and LCω3 intake in children and infants at risk of NAFLD and PNALD, respectively, are needed to reduce the burden of pediatric liver disease.
DEDICATION

To my family,
for their sympathetic ears,
sound advice, and
constant encouragement.
ACKNOWLEDGEMENTS

There are many individuals without whom this dissertation would not be possible. First and foremost, I would like to extend my deepest gratitude to my supervisor, Dr. Corilee Watters, for her wonderful guidance, support and patience. It has been a pleasure and a privilege to work with and learn from her. I would also like to thank the members of my dissertation committee, Dr. Michael Dunn, Dr. Rachel Novotny, Dr. Jeremy King and Dr. Lynne Wilkens, who have given their time generously, and contributed greatly to my professional development. Finally, I would like to express my appreciation and gratitude to the many individuals from the University of Hawai‘i, John A. Burns School of Medicine, Kapi‘olani Medical Center for Women and Children and Nonalcoholic Steatohepatitis Clinical Research Network who dedicated their time and efforts to my research. I have been truly fortunate to have such a wonderful group of mentors, and to benefit from their wisdom and expertise.
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<tr>
<td>AA</td>
<td>Arachidonic acid</td>
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<tr>
<td>ALA</td>
<td>α-linolenic acid</td>
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<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>AMDR</td>
<td>Acceptable macronutrient distribution range</td>
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<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<td>ApoB100</td>
<td>Apolipoprotein B100</td>
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<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
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<tr>
<td>ChREBP</td>
<td>Carbohydrate responsive element binding protein</td>
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<td>CoA</td>
<td>Coenzyme A</td>
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<td>CPT-1</td>
<td>Carnitine palmitoyl-transferase 1</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DNL</td>
<td>De novo lipogenesis</td>
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<td>DRI</td>
<td>Dietary reference intake</td>
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<td>EFAD</td>
<td>Essential fatty acid deficiency</td>
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<td>EPA</td>
<td>Eicosapentaenoic acid</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>FBG</td>
<td>Fasting blood glucose</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>FFA</td>
<td>Free fatty acid</td>
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<td>FFQ</td>
<td>Food frequency questionnaire</td>
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<td>FOLE</td>
<td>Fish oil-based lipid emulsion</td>
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<td>GPRs</td>
<td>G protein-coupled receptors</td>
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<td>HDL</td>
<td>High-density lipoprotein</td>
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<td>HOMA-IR</td>
<td>Homeostatic model of insulin resistance</td>
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<td>IHTG</td>
<td>Intrahepatic triglyceride</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>KMCWC</td>
<td>Kapi‘olani Medical Center for Women and Children</td>
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<tr>
<td>LCo3</td>
<td>Long-chain omega-3 fatty acid</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>LDL-C</td>
<td>Low-density lipoprotein cholesterol</td>
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<tr>
<td>LMS</td>
<td>Lambda, mu and sigma</td>
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<tr>
<td>LS means</td>
<td>Least squares means</td>
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<tr>
<td>MANOVA</td>
<td>Multivariate analysis of variance</td>
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<tr>
<td>MEC</td>
<td>Multiethnic Cohort Study</td>
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<tr>
<td>METs</td>
<td>Metabolic equivalents</td>
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<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>NAFLD</td>
<td>Nonalcoholic fatty liver disease</td>
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<td>NAS</td>
<td>Nonalcoholic fatty liver disease activity score</td>
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<td>NASH</td>
<td>Nonalcoholic steatohepatitis</td>
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<td>NASH CRN</td>
<td>Nonalcoholic Steatohepatitis Clinical Research Network</td>
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<tr>
<td>NEFA</td>
<td>Non-esterfied fatty acid</td>
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<td>NF-κB</td>
<td>Nuclear factor-κB</td>
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<td>NHANES III</td>
<td>National Health and Nutrition Examination Survey III</td>
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<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>PN</td>
<td>Parenteral nutrition</td>
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<tr>
<td>PNAC</td>
<td>Parenteral nutrition-associated cholestasis</td>
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<td>PNALD</td>
<td>Parenteral nutrition-associated liver disease</td>
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<td>PNHS</td>
<td>Pediatric nonalcoholic fatty liver disease histological score</td>
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<td>PNPLA3</td>
<td>Patatin-like phospholipase 3</td>
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<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SAFETY</td>
<td>Screening ALT for Elevation in Today’s Youth</td>
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<tr>
<td>SBS</td>
<td>Short bowel syndrome</td>
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<td>SCD1</td>
<td>Steaoryl-CoA desaturase-1</td>
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<td>SDS</td>
<td>Standard deviation score</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>SREBP-1c</td>
<td>Sterol regulatory element binding protein 1-c</td>
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<td>STEP</td>
<td>Serial transverse enteroplasty</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerides</td>
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<tr>
<td>TONIC</td>
<td>Treatment of NAFLD in Children</td>
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<tr>
<td>tTRL</td>
<td>Total triglyceride-rich lipoprotein</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>ULN</td>
<td>Upper limit of normal</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
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<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
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<tr>
<td>VLDL</td>
<td>Very low-density lipoprotein</td>
</tr>
<tr>
<td>VOLE</td>
<td>Vegetable oil-based lipid emulsion</td>
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</table>
CHAPTER 1: OVERVIEW OF RESEARCH

Introduction

The liver is arguably the most functionally diverse organ in the human body, making liver disorders particularly devastating for the individuals affected, and challenging for the healthcare team attempting to manage their care (1). Nonalcoholic fatty liver disease (NAFLD) and parenteral nutrition associated liver disease (PNALD) are both liver pathologies that may benefit from intake of the long-chain omega-3 fatty acids (LCω3s) found in fish, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (2-5). The purpose of this dissertation is to further explore the role of LCω3s in children with NAFLD and PNALD.

Rationale for the studies

*Pediatric nonalcoholic fatty liver disease*

Adolescent obesity is a major health concern in Hawai’i. According to the 2009 Hawai’i Youth Risk Behavior Survey, over one-quarter of adolescents are considered to have an at-risk weight (6). In overweight and obese adolescents, hepatic lipid regulation can become altered such that the production and uptake of fatty acids into the liver exceeds the liver’s capacity to oxidize and release them as very-low density lipoproteins, resulting in accumulation of fat known as NAFLD (7). Many children with NAFLD have concomitant hepatocellular injury, inflammation and fibrosis of the liver termed nonalcoholic steatohepatitis (NASH), which has a worse prognosis (8-10)).

Roughly 10% of adolescents in the United States (US) have NAFLD, although certain populations are disproportionately affected (11). The most prominent risk factors for NAFLD are excess body fat and the associated metabolic derangements (11-12).
Consequently, the prevalence of this condition in children has increased in relation to the recent rise in childhood obesity (13). Moreover, this growing cohort of obese adolescents with liver disease is difficult to treat given their other comorbidities (12,14).

Cross-sectional analyses have revealed additional disparities in the distribution of pediatric NAFLD. Race and ethnicity appear to be important determinants of the disease with Hispanic and Black children being vulnerable and protected from NAFLD, respectively (11,15). Male gender and parental NAFLD have also been identified as significant risk factors (11,15-16). Moreover, the severity and histopathological characteristics of NAFLD vary considerably between populations, and may reflect differences in disease presentation between populations (9-10,17).

The natural history of this disease in adolescents is poorly defined, but case reports and case series indicate that it can progress in a few years from steatosis to decompensated cirrhosis requiring liver transplantation (18-20). Sadly, liver transplantation is not a cure for the condition, and patients may redevelop the liver disease shortly afterwards (21). Moreover, children with NAFLD have reduced quality of life, and may be at greater risk of developing other medical complications including hepatocellular carcinoma, cholecystitis and type II diabetes mellitus (19,22-23).

Gradual weight loss through diet and exercise is the standard treatment for NAFLD (24). However, obesity is complex condition that results from individual, social and environmental interactions, making it very difficult to treat, let alone cure (25). This is particularly true for adolescents because they need adequate nutrition for growth and are at risk of developing disordered eating habits (26). While many children with NAFLD are able to lose weight in the short-term and reverse their liver disease, most will
not be able to sustain the weight loss, causing a return of NAFLD (19). This “weight-cycling” has been linked with additional complications among obese individuals in later life (27). A recent Cochrane systematic review of weight loss for NAFLD concluded that weight reduction is recommended for patients with NAFLD, but is not supported by strong clinical evidence (28).

The LCω3s from fish may be important mediators in the development and progression of NAFLD, and have been extensively investigated in animal studies (4). However, there is a paucity of research looking at the fish and LCω3 intake of children with NAFLD. Assessment of dietary intake can provide important insights into the role of LCω3s in the pathogenesis of pediatric NAFLD beyond what can be determined in experimental studies. Additional support for LCω3s in pediatric NAFLD comes from a clinical trial that reported a five-fold decrease in liver steatosis at doses of omega-3 fatty acids within the range of dietary intake (29-30).

Despite the health risk that NAFLD presents to the roughly one-quarter of adolescents in Hawai’i that are overweight, it has not been subject to formal investigation (6). Given the multi-ethnic population in Hawai’i, and the apparent role that race and ethnicity have in disease risk, research into NAFLD in Hawai’i youth may have important implications both locally and globally for the management of the condition. As with many chronic diseases, nutrients such as LCω3s may be important mediators in NAFLD pathogenesis, and need to be explored.

**Parenteral nutrition-associated liver disease**

Parenteral nutrition (PN), or intravenous nutrition support, is indicated in patients that are unable to meet their nutrition needs enterally, often related to inadequate length
of functional bowel, a condition known as intestinal failure (31). For individuals that are PN-dependent, it is a life saving therapy. However, prolonged use of PN is associated with metabolic and clinical complications (33). One of the most common and serious concerns of prolonged PN is PNALD, which occurs in nearly 30% of children (34), and 65% of infants with intestinal failure receiving PN (32). It is estimated that approximately 40% of infants with PNALD will go on to develop end-stage liver disease, which has a one-year case fatality rate of nearly 90% if unable to wean off of PN or receive a liver transplant (32).

The pathogenesis of PNALD is incompletely understood, although several risk factors have been identified. One of the leading hypotheses attributes PNALD to the lipid component of PN, which is a strong predictor of liver disease in children and adults on long-term PN (35-37). Restricting lipids is associated with the reversal of PNALD; however, this practice is problematic because they are an importance source of non-protein calories, and essential fatty acids (36,38). The high omega-6: omega-3 fatty acid ratio and phytosterol content in conventional vegetable oil-based lipid emulsions are generally thought to be contributing factors (39). Alternative lipid emulsions have been developed, but are not currently approved for use by the Food and Drug Administration (FDA) (40).

Early research comparing intravenous lipid emulsions found that the use of fish oil, which is rich in omega-3 fatty acids and free of phytosterols, prevented PN-induced cholestasis in piglets (41). However, it wasn’t until several years later the therapeutic potential of fish oil-based lipid emulsions (FOLEs) for PNALD was investigated in humans. In a landmark case series by Gura et al. (2006), two infants with severe
PNALD were provided Omegaven®, a 10% FOLE, and had a reversal of their liver disease (42). The beneficial effects of FOLE treatment prompted the FDA to permit the compassionate use of Omegaven® in patients with PNALD who had not responded to conventional therapies (40).

Since this time, several observational studies have been published reporting resolution of PNALD in patients receiving FOLE, although only three of these studies, all conducted at the same medical center, attempted to compare outcomes with a historical cohort of patients (43-45). The results of these case-control studies are promising, but should be interpreted with caution as there are many limitations including dissimilar baselines and lipid doses between cases and controls, and lack of long-term and clinically relevant outcomes (43-46). Additional studies are necessary to both confirm these findings, and to determine the optimal use of FOLE in PN-dependent patients.

In December 2009, the Kapi’olani Medical Center for Women and Children (KMCWC) established a protocol for the compassionate use of Omegaven® in infants with short bowel syndrome (SBS) who require long-term PN support. As there are no published reports of FOLE use in a population with Hawai’i’s multi-ethnic population, evaluation of the safety and efficacy of this treatment is required.

**Research questions**

There are three major research questions that this dissertation will attempt to address:

1. Is nonalcoholic fatty liver disease a health concern in Hawai’i adolescents?

2. Does intake of fish and long-chain omega-3 fatty acids affect pediatric nonalcoholic fatty liver disease and its risk factors (e.g., obesity)?
3. Are fish oil-based lipid emulsions safe and effective in the treatment of parenteral nutrition-associated liver disease in infants?

To better understand the approach to these questions taken for this dissertation, each will be discussed in more detail in the following sections.

*Is nonalcoholic fatty liver disease a health concern in Hawai‘i adolescents?*

Many factors need to be considered when evaluating the public health burden posed by a disease including prevalence, incidence, diagnosis, presentation, sequelae, complications, natural history, and treatment. Awareness of the health concern posed by pediatric NAFLD in the U.S. has grown dramatically over the last decade, which can largely be attributed to the research efforts of the Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN) (47). The NASH CRN is a network of 10 liver centers across the mainland U.S. that was established in 2001 by the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) to fulfill the need for further research into NAFLD (47).

Hawai‘i is often recognized as an excellent location for the study of diseases owing to its unique ethnic and cultural diversity. Consequently, this research will provide preliminary data on pediatric NAFLD in Hawai‘i as a means of evaluating the health risk of the disease in Hawai‘i, and identifying at risk ethnic groups not represented in the NASH CRN. Chapters three and four contain two studies that address this research question.

*Does intake of fish and long-chain omega-3 fatty acids affect pediatric nonalcoholic fatty liver disease and its risk factors?*

Along the continuum of basic to applied nutrition research, several approaches have been developed to evaluate the relationship between exposures (ie. nutrients) and
health outcomes (ie. diseases). Cross-sectional studies that include dietary measurements of children with NAFLD can identify foods and nutrients that may be capable of attenuating disease progression within the normal consumption range (29). Along with this, it is possible to explore biological mechanisms, additional benefits and potential harms of dietary exposures in free-living subjects.

Under normal physiological conditions, intake of LC\(\omega\)3s can be assessed using biomarkers such as erythrocyte fatty acid profile (48). However, NAFLD is a disease of lipid dysregulation, and the validity of these proxy indicators is unknown under these circumstances. Consequently, direct measurement of dietary intake is required to evaluate the impact of LC\(\omega\)3s in this population. Fortunately, the majority of LC\(\omega\)3s in the diet come from a single source, fish, which has a large inter-individual variability in intake, making it easier to detect differences (49-50). Supporting this, a Canadian study reported a standard deviation nearly two times greater than the mean intake of EPA in adolescents with NAFLD (29). Fish is a culturally significant food among certain populations in Hawai’i with roughly one-third of high-school students reporting fish consumption in the previous 24-hours, making it a suitable population to evaluate the impact of LC\(\omega\)3s in NAFLD (51-52). When assessing fish intake, it is important to consider the LC\(\omega\)3 content of the fish consumed because they have been found to vary dramatically in LC\(\omega\)3 content (53). However, total omega-3 fatty acid intake should also be assessed because \(\alpha\)-linolenic acid (ALA) found in vegetable oils (50), which is consumed in much larger amounts than LC\(\omega\)3s (49-50.), can be converted into EPA and DHA in the body (54). Chapters five and six contain two studies that address this research question.
Are fish oil-based lipid emulsions safe and effective in the treatment of parenteral nutrition-associated liver disease in infants?

The gold standard study design for evaluation of a medical treatment is the double blind, randomized placebo-controlled clinical trial. Unfortunately, for various reasons, it is not always feasible or ethical to conduct research using this model. In the case of FOLE for the treatment of infantile PNALD, the evidence has been rated Grade 2C, meaning that it is a weak recommendation based upon lower-quality evidence (55). Despite the lack of strong evidence for its efficacy, it is difficult to justify withholding of a potentially beneficial therapy for such a serious condition in this vulnerable population. Consequently, attempts should be made to utilize observational study designs to characterize associations and refine hypotheses until more is known.

The recent adoption of Omegaven use at Kapi‘olani Medical Center for Women and Children (KMCWC) provides a unique opportunity to evaluate the safety and efficacy of fish-oil based lipid emulsions in a multi-ethnic cohort of children with PNALD. Chapter seven contains one study that addresses this research question.
References


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Pediatric Nonalcoholic Fatty Liver Disease

Pathogenesis

Nonalcoholic Fatty Liver Disease

Nonalcoholic fatty liver disease (NAFLD) is a condition characterized by accumulation of triacylglycerides (TAGs) in hepatocytes that occurs in individuals that are not consuming significant amounts of alcohol, and are free from other known causes of hepatic TAG accretion, including viral, congenital and autoimmune liver diseases. Hepatocytes contain a pool of free fatty acids (FFAs), which originate from dietary TAGs, hepatic de novo lipogenesis (DNL), and adipocyte non-esterfied fatty acids (NEFAs) release (1). Within hepatocytes, the FFAs can be broken down for production of adenosine triphosphate (ATP) and ketone bodies, exported in very low-density lipoproteins (VLDLs) for extra-hepatic tissues, or stored as TAGs in lipid droplets (1). In NAFLD, the uptake and synthesis of fatty acids in hepatocytes exceeds their use for energy and ketone production, and release in VLDLs, resulting in increased TAG storage in lipid droplets, termed steatosis (1). Each of these processes are summarized below and depicted in Figure 2.1.
Figure 2.1 Summary of lipid metabolism in the liver

Sources of hepatic free fatty acids include dietary carbohydrates and triglycerides, as well as non-esterfied fatty acids released from adipose tissue. Dietary carbohydrates are absorbed as sugars, which are converted into free fatty acids within hepatocytes by de novo lipogenesis. Dietary triglycerides are absorbed in chylomicrons, which are released into extrahepatic tissues (e.g., muscle) by the action of lipoprotein lipase, resulting in some spillover of non-esterfied fatty acids. Remaining triglycerides are taken up by hepatocytes in chylomicron remnants, and can be hydrolyzed to generate hepatic free fatty acids. Within hepatocytes, free fatty acids can be broken down by β-oxidation, or esterfied to form triglycerides. Hepatic triglycerides may be packaged and excreted in very low-density lipoproteins, or stored as a lipid droplet. In nonalcoholic fatty liver disease, the input of free fatty acids and triglycerides into hepatocytes exceeds the capacity of hepatocytes to use them in β-oxidation and secrete them in very low-density lipoproteins, resulting in excess storage of triglycerides as lipid droplets (steatosis).
Sources of Hepatic Triglycerides - Input

Dietary TAGs, hepatic DNL, and adipocyte NEFAs are all potential sources of fatty acids that may be contributing to hepatic steatosis in pediatric NAFLD. These pathways are dynamic, interconnected and overlapping, making it difficult to determine to what extent each is necessary and/or sufficient to cause steatosis. In the fed state, a combination of dietary TAGs and hepatic DNL contribute the majority of fatty acids to hepatocytes, while NEFAs derived from adipocytes are the major source of fatty acids in the fasted state (2). Different approaches have been developed to evaluate these sources, although many involve indirect, static measurements. Recent kinetic studies using isotope methodologies have provided considerable insight into ectopic fat disposition in hepatocytes; however, most of these studies are small, making them difficult to interpret.

Dietary Triglycerides

Dietary TAGs are delivered to the liver in chylomicron remnants and as NEFAs. When consuming 30% of energy as fat, dietary TAGs are estimated to supply roughly 15% of liver TAGs in patients with NAFLD, but the relative contribution varies considerably from less than 5% to almost 30% (2). The NEFAs from dietary TAGs include the incidental release of fatty acids during chylomicron lipolysis in extrahepatic tissues, termed spillover, and the shorter chain fatty acids that are transported to the liver through the portal circulation (2). The amount of dietary TAGs reaching hepatocytes will depend on the amount and type of fat in the diet, and the uptake of dietary TAGs by extrahepatic tissues.

The contribution of dietary TAGs to the hepatocyte lipid stores is generally expected to be greatest on a low-carbohydrate, high-fat diet (3-4). However, fatty acid
composition and form of dietary fat (5-6), and the distribution of fat intake throughout the day have been reported to influence dietary TAG oxidation and deposit in extrahepatic tissues (4). Changes in lipid metabolism associated with obesity and NAFLD may also increase the proportion of dietary TAGs supplied to hepatocytes (7). In NAFLD, there is a reduction in endothelial lipases and fatty acid importers in adipose tissue, and an increase in endothelial lipases and fatty acid importers in hepatic tissue, which is theorized to shunt a greater proportion of fatty acids towards hepatocytes (8-12). Supporting this hypothesis, postprandial hepatic uptake of dietary TAGs was greater in patients with type 2 diabetes mellitus, a condition closely related to NAFLD (13-14).

**Hepatic De Novo Lipogenesis**

Hepatocytes are capable of synthesizing fatty acids through polymerization of 2-carbon acetyl groups, termed hepatic DNL. Acetyl-coenzyme A (CoA) is a shared intermediate in the breakdown of carbohydrates, proteins and alcohol, and can be used for DNL or for the production of adenosine triphosphate (ATP), depending on the energy needs of the cell. However, the roles of protein and alcohol in acetyl Co-A production are generally ignored in NAFLD because their relative contribution is minimal compared to that of carbohydrates. The quantity and type of carbohydrate in the diet, as well as the uptake of glucose by extrahepatic tissues influence the amount of hepatic DNL. Children with NAFLD have greater skeletal muscle insulin resistance, which may contribute to the development of liver steatosis by directing a greater proportion of dietary carbohydrates towards the liver for hepatic DNL (15-16). When NAFLD patients consume a diet containing 55% of energy as carbohydrates, approximately one-quarter of hepatic TAGs are derived from DNL, although this value was found to range from almost 10% to 40%
(2). The expression of genes coding for rate-limiting enzymes in the hepatic DNL pathway are up regulated in NAFLD patients (12).

The transcription factors, carbohydrate responsive element binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c) coordinate the activation of glycolytic and lipogenic pathways for hepatic DNL in response to glucose and insulin. As a result, hepatic DNL exhibits diurnal variations, which correspond to the shifts in serum glucose and insulin concentrations between the postprandial and fasting states. However, patients with NAFLD appear to have a constant rate of hepatic DNL (2). Fasting hyperinsulinemia and hyperglycemia secondary to insulin resistance may contribute to this finding, as it is common in children with NAFLD (17-18). Although the effect of hyperinsulinemia on glucose metabolism is generally matched by a parallel increase in insulin resistance, hepatocytes may remain sensitive to the effects of insulin on SREBP-1c and lipogenesis, a phenomenon known as selective hepatic insulin resistance (19-21). Although the mechanism of selective hepatic insulin resistance is unknown, one factor that may contribute is adipocyte-derived adiponectin, which is reduced in pediatric NAFLD, limiting its inhibitory effect on SREBP-1c (22-23). In the fed state, hepatic DNL does not increase in NAFLD patients despite substantial increases in serum glucose and insulin concentrations, suggesting that there may be a threshold in glucose and insulin stimulated hepatic DNL (2).

Consumption of dietary carbohydrates as fructose instead of glucose may result in greater hepatic DNL (24). Unlike dietary fructose, most dietary glucose is removed from the blood for use by extrahepatic tissues, which limits the amount of carbohydrate available for hepatic DNL. Within hepatocytes, fructose and glucose differ significantly
in their regulation (24). The uptake and breakdown of glucose into acetyl-CoA in hepatocytes is carried out in relation to serum glucose and insulin concentrations, insulin sensitivity, and the energy state of the cell. Dietary fructose is able to bypass these regulatory controls because it enters glycolysis downstream of the rate-limiting enzymes glucokinase and phosphofructokinase, thereby producing greater amounts of acetyl-CoA (24). Additionally, fructose is able to activate transcription factors ChREBP and SREBP-1c, which promote hepatic DNL through a yet unidentified mechanism (24).

**Adipocyte Non-esterfied Fatty Acids**

The largest sole contributor to the hepatic fatty acid pool (approximately 60%, range 45-75%) among patients with NAFLD is from NEFAs derived from adipocytes (2). Adipose tissue is the primary site for the long-term storage of excess energy as TAGs in the body. Positive energy balance results in adipocyte hypertrophy and proliferation to accommodate greater amounts of TAGs, and adipocyte TAGs are hydrolyzed and released as NEFAs to provide energy to the body during periods of negative energy balance. Similarly, the net balance of fatty acids in adipocytes fluctuates between storage and release in response to shifts in serum substrate (e.g., glucose, chylomicrons/VLDL) and insulin concentrations in the fed and fasting states, respectively. Adipocyte lipolysis is mediated through the action of hormone-sensitive lipase, and is influenced by perilipin, a protein that coats lipid droplets thereby restricting access to enzymatic breakdown (25).

Excess fat storage can trigger a phenotypic change in adipose tissue characterized by infiltration with pro-inflammatory macrophages and lymphocytes cells and shifts in the secretion of adipokines (e.g., ↑ resistin and leptin; ↓ adiponectin) and cytokines (e.g., ↑ interleukin-6, tumor necrosis factor-α (TNF-α), and interferon-γ; ↓ interleukin-10).
These alterations cause adipocyte insulin resistance, which suppresses the action of insulin on hormone-sensitive lipase and perilipin, resulting in net lipolysis and release of NEFAs (25). This pathophysiology is most often associated with visceral fat, and may be partly related to lipidome remodeling associated with adipocyte hypertrophy (27-28). The contribution of visceral fat to the plasma NEFA pool is proportional to visceral fat stores, and was found to range from <10% to almost 50% (29). In relation to NAFLD, liver steatosis is independently and positively correlated with visceral fatness and adipocyte size (30-31). Pediatric NAFLD patients generally have excess body fat around the viscera, and have increased NEFA release from adipocytes in both the postprandial and postabsorptive states (32). Adipose tissue insulin resistance and corresponding increased adipocytes lipolysis is thought to be one of the primary causes of excess NEFAs release from adipocytes in NAFLD patients (19,21).

**Fates of Hepatic Triglycerides - Output**

Hepatic TAGs are derived from multiple sources including FFAs from NEFA uptake and hepatic DNL, and TAGs delivered in chylomicron and VLDL remnants. A limited capacity of the liver to oxidize FFAs or secrete TAGs in VLDLs, or a propensity to store TAGs could contribute to the development of NAFLD (1). Within hepatocytes, FFAs can be generated by TAG hydrolysis, and TAGs can be synthesized by FFA esterification (33). Alternatively, FFAs can be oxidized to produce acetyl CoA, and TAGs can be exported in VLDLs or stored in lipid droplets (33). The partitioning of intracellular lipids along these different pathways is highly variable, and under the influence of multiple regulatory factors (1,33). The study of lipid metabolism in the liver is hindered by the need for liver biopsy to measure it directly in humans; however,
laboratory and animal studies have provided considerable insight into these processes. In
the fed state, greater proportions of hepatic lipids are directed towards VLDL production
and TAG storage, while lipid oxidation is more prominent when fasting (33).

**Oxidation of Hepatic Free Fatty Acids**

The oxidation of hepatic FFAs occurs primarily within the mitochondria in a
process known as β-oxidation. The translocation of FFAs from the cytosol into the
mitochondria by carnitine palmitoyl-transferase 1 (CPT-1), known as the carnitine
shuttle, represents a major branching point in the metabolic fate of FFAs (1). The
activity of carnitine shuttle is tightly linked to hepatic DNL through allosteric inhibition
by the DNL intermediate malonyl-CoA on CPT-1 (1). Consequently, hepatic β-oxidation
is negatively regulated by factors that up regulate DNL, namely carbohydrates and
insulin. Additionally, CPT-1 and enzymes involved in hepatic β-oxidation are directly up
regulated by the transcription factor, peroxisome proliferator-activated receptor-α
(PPAR-α), which is activated by dietary unsaturated fatty acids, and their derivative
compounds (33). As such, the ingestion of a meal, particularly one that contains large
amounts of carbohydrate, especially fructose, will suppress hepatic β-oxidation, whereas
this process will be activated in the fasting state, or following a high-fat meal that
contains little or no carbohydrates (24,34). The adipokine, adiponectin, may help
promote FFA oxidation in hepatocytes by increasing intracellular AMP-activated protein
kinase (AMPK), a compound that raises the activity of PPAR-α (35). The end product of
β-oxidation is acetyl-CoA, which can enter the tricarboxylic acid cycle to produce ATP,
or be used for ketogenesis.
Mitochondrial dysfunction is a common complication observed in NAFLD patients that is thought to precede the development of steatosis (36-37). However, it is unclear whether alterations in hepatic β-oxidation contribute to liver steatosis in NAFLD, as this process cannot be directly measured, and indirect methods have generated mixed results. Studies estimating hepatic lipid oxidation using plasma concentrations of the ketone body, 3-hydroxybutyrate, under fasting conditions and following insulin infusion have suggested reduced (38), normal (39), or greater rates in patients with NAFLD (40). Although the overall rates of hepatic lipid oxidation do not appear to be reduced in NAFLD, β-oxidation may be suppressed relative to the greater input of hepatic FFAs in NAFLD. As previously discussed, hepatic DNL is increased and does not decrease in the fasting state in NAFLD, resulting in a constant excessive production of malonyl-CoA, which may inhibit the action of CPT-1 (1-2). Moreover, patients with NAFLD have been found to have a decreased expression of PPAR-α and CPT-1, a finding that may be related to the reduced levels of adiponectin seen in these patients (22,35,41-42).

Removal of Hepatic Triglycerides via Very Low-Density Lipoproteins

Hepatic FFAs that are not oxidized are esterfied, usually forming TAGs (33). Consequently, factors that suppress hepatic lipid oxidation tend to increase the amount of hepatic TAGs, and visa versa (33). Within hepatocytes, TAGs derived from FFA esterification and chylomicron and VLDL remnants can be packaged into a VLDL for delivery to extrahepatic tissues, or stored as lipid droplets (1). The former pathway represents an important compensatory mechanism for disposal of excess hepatic TAGs to prevent liver steatosis. In this process, hepatic TAGs are bound to apolipoprotein B100 (ApoB100) through the action of microsomal triglyceride transfer protein, followed by
the addition of the core TAGs (1). Both ApoB100 and microsomal triglyceride transfer protein are negatively regulated by insulin (43). Additionally, synthesis of ApoB100 is stimulated by NEFA delivery to the liver, and is degraded in the absence of hepatic TAGs or in the presence of oxidative stress (43).

The secretion of TAGs in VLDLs increases linearly with intrahepatic TAG content, but appears to plateau at levels indicative of NAFLD (44). Consequently, the export of TAGs in VLDLs is greater among patients with NAFLD; however, this is presumably insufficient to have prevented liver steatosis (44). The increase in TAG export appears to be due to the production of large, TAG-rich VLDLs as opposed to greater ApoB100 (44). Although many of the factors that contribute to ApoB100 synthesis including insulin resistance and elevated NEFA concentrations are present in NAFLD, this may be counteracted by ApoB100 degradation related to oxidative stress (43). A limited ability of the liver to secrete large, TAG-rich VLDLs due to the physical constraints of the liver sinusoids has been proposed as a possible mechanism underlying the apparent plateau in TAG export (45). Further supporting an increase in TAG export in VLDLs is the hypertriglyceridemia that is often observed in pediatric NAFLD patients (46-47).

Storage of Hepatic Triglycerides in Lipid Droplets

Hepatic TAGs that are not exported in VLDLs can be stored in lipid droplets. This provides a reserve of TAGs for metabolic processes while protecting the liver from the accumulation of cytotoxic FFAs during periods of lipid excess (48). The storage of FFAs in the liver is facilitated by the action of steaoryl-CoA desaturase-1 (SCD1), the enzyme that converts saturated fatty acids into monounsaturated fatty acids, which are
more easily incorporated into lipid droplets (48). Disruption of SCD1 activity prevents hepatic steatosis in mouse models of NAFLD at the expense of greater hepatitis and liver fibrosis (48). Once formed, TAGs can be released from lipid droplets in a manner that appears to be analogous to adipocytes (43). In hepatocytes, lipid droplet breakdown appears to involve multiple factors including lipases/esterases (e.g., adipose triglyceride lipase) and lipid droplet protein coats (e.g., perilipin); however, this process is not yet well understood (43). Adiponutrin is a protein that is associated with the endoplasmic reticulum (ER) and lipid droplets that exhibits TAG hydrolase and transacylation activity (49). Although the exact function of adiponutrin has yet to be elucidated, it has received considerable attention due to the presence of a common gain-of-function polymorphism that increases the risk of NAFLD (49). Preliminary findings suggest that adiponutrin may have important roles in both the release of TAG from lipid droplets, and the secretion of TAGs in VLDLs (49-50). The transcription factors, SREBP-1c and ChREBP, discussed previously have been found to up regulate SCD1 (SREBP-1c only) and adiponutrin in the liver, and may function to regulate lipid droplet formation and breakdown through these enzymes (43,51-52).

Nonalcoholic Steatohepatitis

While steatosis is the defining feature of NAFLD, disease severity is largely determined by hepatocellular injury, and liver inflammation and fibrosis (53). The term nonalcoholic steatohepatitis (NASH) was established to differentiate steatosis from steatosis with histological features of advanced liver disease (54). The ‘two-hit hypothesis’ developed by Day and James (1998) was the initial model developed to describe the pathogenesis of NAFLD, and progression to NASH (55). According to this
disease model, ectopic fat disposition in the liver is the first hit, which primes the liver to secondary insults that result in NASH (55). A few years later, this model was modified to include the fact that the two processes are likely occurring simultaneously, and that steatosis may not necessarily contribute directly to NASH pathogenesis (56-57).

The study of NASH pathogenesis is hindered by the need to liver biopsy for diagnosis and evaluation, the varying presentation of the condition, and the multitude of changes that are occurring. As a result, factors leading to NASH are incompletely understood; however, the interplay of cytokines, FFAs and oxidative stress, and resultant organelle dysfunction are thought to contribute greatly (56). Many of these factors appear to influence one another, resulting in feed forward signaling that propagates the disease (56). The role of cytokines, FFAs, oxidative stress and organelle dysfunction in the pathogenesis of NASH is described below, and depicted in Figure 2.2.
The pathogenesis of nonalcoholic steatohepatitis is thought to involve the actions of pro-inflammatory cytokines, free fatty acids and reactive oxygen species. In liver tissue, increased pro-inflammatory cytokines trigger inflammatory and hepatic stellate cells activation, resulting in inflammatory cell infiltration/activation with corresponding pro-inflammatory cytokine production and fibrogenesis, respectively. Within hepatocytes, inflammatory signaling pathways (e.g., c-Jun N-terminal kinase) are upregulated by pro-inflammatory cytokines, which promote additional pro-inflammatory cytokine secretion, oxidative stress, mitochondrial dysregulation and apoptosis. Increased free fatty acids related to alterations in lipid metabolism in nonalcoholic fatty liver disease also stimulates inflammatory signaling pathways, and independently contributes to oxidative stress and mitochondrial dysregulation. Finally, reactive oxygen species propagate these changes by increasing hepatic free fatty acids, activating inflammatory signaling pathways, and adding to organelle dysfunction.
The Role of Cytokines in Nonalcoholic Steatohepatitis

In NAFLD, the alteration in visceral adipose tissue phenotype results in a persistent secretion of pro-inflammatory cytokines directly into the hepatic portal circulation (26). Although numerous cytokines are thought to contribute to NASH pathogenesis, the effects of TNF-α have been most extensively studied. Binding of TNF-α to surface receptors on Kupffer cells, hepatic stellate cells and hepatocytes activate intracellular inflammatory signaling molecules including nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK) (36,57-58). Activation of these pathways in Kupffer cells stimulates the secretion of chemokines and other pro-inflammatory molecules, which recruit immune cells to the liver (e.g., monocytes, neutrophils, eosinophils, dendritic cells and leukocytes), and cause chronic low-level inflammation (59-61). Hepatic stellate cells are responsible for producing the extracellular matrix in the liver, and transform into collagen-producing myofibroblastic cells in response to pro-inflammatory cytokines, promoting fibrogenesis (56,62). In hepatocytes, TNF-α binding leads to the production of reactive oxygen species (ROS) through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and to an increase in hepatic FFAs by promoting hepatic insulin resistance (56,58). Moreover, cytokine-activated inflammatory signaling pathways impair the function of mitochondria and endoplasmic reticulum (ER), and promote cell apoptosis (56,58,62). Finally, activation of inflammatory signaling molecules in hepatocytes causes the production of additional pro-inflammatory cytokines, perpetuating the cycle of inflammation (58). Patients with NAFLD have been found to have a greater expression of TNF-α in adipocytes and hepatocytes, which is also associated with histological features of NASH (63).
The Role of Free Fatty Acids in Nalcoholic Steatohepatitis

As previously mentioned, liver steatosis is thought to protect the liver from the cytotoxic effects of FFAs, collectively referred to as lipotoxicity (48). Excess FFA input in NAFLD may overwhelm the ability of hepatocytes to safely export or store them in lipid droplets, resulting in accumulation of FFAs and their metabolites (e.g., diacylglycerol, ceramide) (64). One possible culprit in this process is an imbalance between the production of saturated FFAs in DNL, and the activity of SCD1, which facilitates TAG synthesis (48,64). Several possible mechanisms may contribute the to effects of FFAs on NASH pathogenesis. Similar to cytokines, saturated FFAs are able to activate the intracellular inflammatory signaling pathways (58). In addition to the effects of JNK on mitochondrial function, FFAs are able to trigger the mitochondrial uncoupled protein response, a mechanism that is thought to aid in the removal of excess FFAs (65). Finally, buildup of FFAs in hepatocytes increases the production of ROS by promoting lipid oxidation, and by leading to the activation of NADPH oxidase (65). The role of FFAs in NASH pathogenesis is supported by experiments demonstrating that exposure of hepatocytes to saturated fatty acids, and blockage of hepatic TAG synthesis in mice causes hepatocellular injury, inflammation and fibrosis characteristic of NASH (48).

The Role of Oxidative Stress in Nalcoholic Steatohepatitis

There are numerous sources of reactive oxygen species (ROS) in hepatocytes, which are known to have important roles in cell signaling (61). When production of ROSs exceeds the capacity of antioxidant systems to safely remove them, free radicals accumulate causing damage to deoxyribonucleic acid (DNA), lipids and proteins thereby disrupting normal cell function, a condition known as oxidative stress (61). Functional abnormalities in mitochondria, and increased extra-mitochondrial lipid oxidation and
NADPH oxidase activity are thought to be important contributors to ROS production in NASH (41,61). Oxidative stress activates JNK in hepatocytes, adding to the stimulatory effects of cytokines and FFAs on intracellular inflammatory signaling (58,61). Furthermore, ROS cause structural and functional abnormalities in mitochondria and ER, and impair the synthesis of proteins involved in lipid metabolism (e.g., ApoB100), resulting in FFA accumulation (61). Increased markers of hepatic oxidative stress have been reported in pediatric NAFLD patients (66).

The Role of Organelle Dysfunction in Nonalcoholic Steatohepatitis

Mitochondria are the primary source of lipid oxidation in hepatocytes, and contribute importantly to the removal of FFAs. Liver biopsies of patients with NAFLD often exhibit morphological changes in mitochondria underlying the functional abnormalities that are observed in these patients (67). Mitochondrial uncoupled protein response is activated by cytokines, FFAs and oxidative stress, and decouples ATP synthesis from the electron transport chain, and therefore β-oxidation (58,61-62,65). This aids in the removal of FFAs at the expense of greater release of ROS from the electron transport chain, and impaired ATP production (62,68). Additionally, the uncoupled protein response causes the release of pro-apoptotic factors from the mitochondrial membrane (e.g., cytochrome C) (62). Excess ROS in mitochondria also result in lipid peroxidation and deoxyribonucleic acid (DNA) damage, which further impairs mitochondrial function (62).

The ER is the primary site of protein and lipid synthesis in hepatocytes, and is therefore directly linked to most cellular functions. Patients with NASH have activation of the unfolding protein response in ER, referred to as ER stress, a reaction to cellular
stressor (e.g., cytokines, oxidative stress) that alters ER synthetic pathways (58,61). The ER stress is thought to contribute to hepatic FFAs by increasing hepatic DNL through the proteolytic activation of SREBP-1c, and reducing VLDL production and lipid droplet formation by decreasing the synthesis of ApoB100 and perilipin, respectively (61). Additionally, ER stress directly activates JNK signaling pathway described previously, linking the cellular stress to inflammatory response and apoptosis (58). Consequently, mitochondria and ER dysfunction appear to be central to NASH pathogenesis, and are important in mediating the effects of cytokines, FFAs and oxidative stress.

**Diagnosis and Assessment**

Pediatric NAFLD can be difficult to diagnose in practice. It may be suspected in at-risk children that are found to have elevated liver enzymes and radiological evidence of steatosis, although no biochemical test is specific for NAFLD (69). Instead, NAFLD is identified by exclusion, meaning that other common causes of liver disease including alcohol, medications, viruses, and metabolic and autoimmune disorders are ruled out leaving NAFLD as the likely condition (69). Definitive diagnosis and assessment of pediatric NAFLD requires liver biopsy; however, this is not performed routinely in clinical practice due to the perception that the cost, burden and risk of liver biopsy exceeds the clinical utility of biopsy results (70). Clinical guidelines for the diagnosis of pediatric NAFLD suggest that liver biopsy may be performed at the discretion of the medical team for differential diagnosis, when advanced liver disease is suspected, prior to pharmacological/surgical treatment, and for research purposes (69).

Alanine aminotransferase (ALT) is the most commonly used liver disease biomarker for NAFLD screening (69,71). This enzyme is found within hepatocytes, but
is released into the blood as a result of liver damage. The threshold of ALT that is used to screen for NAFLD varies considerably between facilities, a finding that has been attributed to differences in serum ALT values of the local reference populations (72). A survey of 43 children’s hospitals in the U.S. found that the median upper limit of normal for ALT was 53 U/L, and that values ranged considerably from 30 to 90 U/L (71). When hospital-based cutoffs for ALT were compared to the 95th percentile of healthy adolescents in the U.S., there was a dramatic increase in sensitivity for NAFLD in boys (32 to 80%) and girls (36 to 92%) with only minor loss of specificity (92 to 79% and 96 to 85%, respectively) (71). Low sensitivity of conventional upper limits of normal for ALT may result in many cases of NAFLD being missed, a problem that is compounded by the low rates of ALT screening for NAFLD in at-risk patients visiting pediatricians (2%), pediatric endocrinologists (10%) and pediatric gastroenterologists (23%) (73). Although ALT is recommended as an indicator of NAFLD (69), and has been found to be associated with histological features of NASH (17,74), the sensitivity of ALT for NASH is modest and severe liver disease is occasionally observed in pediatric NAFLD patients with normal ALT values (75). Additional biochemical and clinical indicators of liver steatosis, injury, inflammation and fibrosis have been investigated for use in screening for NAFLD or for differentiating NAFLD from NASH, but are generally limited to research applications currently (76-80).

One clinical measurement that is recommended in screening for NAFLD is liver ultrasound (69). Ultrasonography provides a relatively low-cost, safe means of measuring liver fat, and has been found to have moderate sensitivity (80%) and specificity (86%) for diagnosing moderate to severe steatosis in pediatric NAFLD (81). Additional
radiological methods including computed tomography and magnetic resonance imaging and spectroscopy are able provide more precise measurements of liver fat, but are generally limited to use in research as they are more expensive (82).

The defining feature of NAFLD is the presence of greater than 5% of hepatocytes that contain macrovesicular steatosis (53). Attempts to establish standardized criteria for diagnosing NASH are hindered by differences in disease presentation, inter-observer variability, and lack of knowledge on the natural history of the disease (53,83). To better study NASH, grades were developed to rate the presence/severity of the different histological features of NAFLD (53). Based on these grading criteria, several histological features have been identified that independently predict diagnosis of NASH including portal and lobular inflammation, ballooning degeneration and fibrosis (53,84). Along with this, scoring systems were created that quantify disease severity in NAFLD by combining the grades of multiple histological features of NAFLD (53,85). Two such scoring systems are the NAFLD activity score (NAS) that uses the unweighted sum of grades for steatosis (0-3), lobular inflammation (0-2) and hepatocyte ballooning (0-2) (53); and the pediatric NAFLD histological score (PNHS), which adds portal inflammation to the NAS criteria (85). The PNHS was found to be superior to NAS in identifying pediatric patients with NASH, highlighting the importance of portal inflammation in measuring disease severity in this population (84-85). Although the NAS and PNHS provide a useful means ranking NAFLD patients for research purposes, they are not recommended for evaluation or monitoring of NAFLD in clinical practice (83).
In the assessment of NASH, two patterns of steatohepatitis have been identified, which are classified based on their location in the liver as zone 3 (panacinar distribution) and zone 1 (portal distribution) (83). Zone 3 steatohepatitis generally presents with lobular inflammation and perisinusoidal fibrosis, and may be considered borderline steatohepatitis, or definite NASH depending on the severity of these features, and the presence of lesions indicative of hepatocellular injury, particularly ballooning degeneration (83). Zone 1 borderline steatohepatitis is the form of NASH that is most commonly observed in children, and is characterized by portal inflammation and fibrosis that is often mild, and little or no ballooning degeneration and Mallory-Denk bodies (83,86). It is important to note that these patterns are highly varied, and many children present with features of zone 3 steatohepatitis (87-88).

Disease Sequelae and Natural History

The symptoms and health-related consequences of pediatric NAFLD are important considerations in evaluating the burden this disease. Many of these factors are poorly characterized in pediatric NAFLD, in part due its relatively recent discovery and recognition as an important complication of obesity (89-90). Although only a minority of children with NAFLD will develop life-threatening complications related to advanced liver disease, many of the concerns of pediatric NAFLD occur without progression to these late stages of liver disease.

Pediatric NAFLD patients often present with non-specific symptoms, which may make it difficult for patients to appreciate the consequences of having the disease (91). Commonly reported symptoms include irritability (73%), fatigue (68%), headache (60%), difficulty concentrating (55%), and muscle aches and cramps (53%) (91). Compared to
their healthy controls, children with NAFLD suffer from lower health-related quality of life, particularly psychosocial health, a finding that was related to symptom severity (91). Another study found that health-related quality of life was also reduced compared to healthy obese children, although this difference did not reach statistical significant (p = 0.10) (92). In the latter study, NAFLD patient reported worse depression on the Children’s Depression Inventory test that seemed to stem from negative mood, reduced self-esteem, and feelings of ineffectiveness (92). Additionally, children with NAFLD had lower scores on all three subsets of the Body-Esteem Scale for Adolescents and Adults (appearance, attribution and weight), which assesses the effect of body weight on self-esteem (92).

The studies on symptoms in these patients, and effects on psychosocial health and quality of life are difficult to interpret due to the numerous obesity-related co-morbidities that present alongside NAFLD, including insulin resistance, dyslipidemia and hypertension (17-18, 46,74,93). Metabolic syndrome is a disease that occurs when several of these disease risk factors develop in the same individual. Pediatric NAFLD patients are estimated to have five times greater odds of having metabolic syndrome compared to age, sex and body mass index (BMI)-matched controls (46). Additionally, amongst children with NAFLD, metabolic syndrome and its constituents are associated with greater hepatocellular injury, inflammation and fibrosis (17-18,74,93). Due to the overlap between NAFLD and metabolic syndrome, it has been proposed that NAFLD be used as a defining feature of this condition (94). Furthermore, there is some indication that ectopic fat deposition in the liver, and resultant changes in liver function may be an important mediator of the metabolic complications of obesity (95-96). Supporting this
hypothesis, studies have found that the apparent relationship between visceral adipose tissue and features of metabolic syndrome can be explained by differences in intrahepatic fat, and that intrahepatic fat is associated with these factors, independently of visceral adipose tissue (95-96). However, prospective studies are necessary to confirm the effect of NAFLD as a risk factor for obesity-related morbidity.

Little is known about the natural history of NAFLD, but case reports suggest that NAFLD can develop in children as young as two years old, and can progress rapidly into liver cirrhosis (97-99). One retrospective follow up study has been conducted in children (100). Of the 66 pediatric NAFLD patients included in this study, two died and two received liver transplantation for cirrhosis, far lower than the expected liver transplantation-free survival rate in general U.S. population (100). Repeat liver biopsies were performed in five of these patients, most of which indicated worsening fibrosis (4/5), including one patient without fibrosis that developed liver cirrhosis in less than five years (100). Although liver transplantation can be life saving, it does not address the underlying factors contributing to NAFLD, and disease recrudescence commonly occurs in these patients (100-102). In addition to liver cirrhosis, NAFLD is a risk factor for hepatocellular carcinoma, the most common form of liver cancer (103-104).

**Epidemiology**

Epidemiology is the study of the distribution and determinants of disease in a population. In recent years, several epidemiological studies of pediatric NAFLD have appeared in the literature, raising awareness of its present burden, and providing important insights into its etiology and pathogenesis. Additionally, the information gleaned from the epidemiological research on NAFLD in children can help to better
direct healthcare resources and future investigations into the prevention and treatment of this disease.

Incidence and Prevalence

The burden of disease in a population is directly related to the number of individuals in that population that are afflicted by it. In the past 30 years, NAFLD went from discovery to recognition as one of the most common complications of obesity in children (89,94). Along with the rise in awareness, there has been a dramatic increase in the proportion of children being diagnosed with NAFLD (105). At least some of the growing prevalence can be attributed to an increased incidence of NAFLD stemming from the rise in childhood obesity, which is estimated to have tripled in 6-19 year olds during this time (106). The current prevalence of pediatric NAFLD is estimated to be approximately one in ten, although certain groups have higher rates than others (107-108). In particular, children that are overweight and obese have been found to have a increased prevalence of NAFLD (16% and 38%, respectively) compared to their normal weight counterparts (5%) (107). This finding is unsurprising given then role of obesity in the pathogenesis of NAFLD. Pediatric NAFLD is also unevenly distributed by sex and ethnicity (107-111), observations that will be discussed in more detail in the following sections.

An important consideration when evaluating the burden of NAFLD is the distribution of disease severity among those afflicted. The spectrum of pediatric NAFLD ranges from isolated steatosis to borderline NASH containing mild liver inflammation and fibrosis to definitive NASH, which includes moderate to severe inflammation and fibrosis with overt signs of hepatocellular injury (67). Several epidemiological studies
have obtained liver biopsies in a sufficient number of NAFLD patients to obtain a picture of its distribution (see Table 2.1 below). Although the majority of these studies do not classify NAFLD according to the current classification scheme, they do report on histological features of NAFLD. As seen in Table 2.1, the majority of patients with NAFLD have some degree of hepatocellular injury, inflammation and fibrosis with a minority of subjects (5-20%) having advanced liver fibrosis. It appears that the hospital-based studies may have been biased towards more severe ballooning degeneration, but the rates of inflammation and fibrosis were similar to that reported in the general population. There did not appear to be a clear difference in disease presentation with regards to location; however, the group in Italy reported slightly lower rates of fibrosis, particularly bridging fibrosis or cirrhosis (87). A separate study of 149 patients with NAFLD in the U.S. found a relatively even distribution of NAFLD between isolated steatosis (26%), borderline zone 3 NASH (18%), borderline zone 1 NASH (24%), and definitive NASH (32%) (112).

Table 2.1 Distribution of disease severity in pediatric nonalcoholic fatty liver disease

<table>
<thead>
<tr>
<th>Population (ref)</th>
<th>Inflammation</th>
<th>Fibrosis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Port</td>
<td>Lob</td>
<td>Port</td>
</tr>
<tr>
<td>General Population</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[107] U.S. (n=78M/19F), 17.2 y</td>
<td>68%</td>
<td>-</td>
<td>59%</td>
</tr>
<tr>
<td>Hospital</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[86] U.S. (n=65M/35F), 12 y</td>
<td>70%</td>
<td>36%</td>
<td>60%</td>
</tr>
<tr>
<td>[87] Italy (n=59M/25F), 11.7 y</td>
<td>85%*</td>
<td>42%</td>
<td>14%</td>
</tr>
<tr>
<td>[88] U.S. / Canada (n=68M/50F), 12 y</td>
<td>84%</td>
<td>92%</td>
<td>56%</td>
</tr>
<tr>
<td>[113] Korea (n=67M/13F), 12 y</td>
<td>43%</td>
<td>86%</td>
<td>77%</td>
</tr>
<tr>
<td>[114] U.S. (n=114), 12 y</td>
<td>-</td>
<td>49%</td>
<td>53%</td>
</tr>
</tbody>
</table>

Port=Portal, Lob=Lobular, Peri=Perisinusoidal, Br/Cir=Bridging fibrosis or cirrhosis, B-Degen=Ballooning degeneration
*85% necroinflammation noted, but inflammation not separated regionally
Sex is an important determinant of NAFLD risk among children. Specifically, boys have consistently been shown to have higher prevalence of NAFLD than girls (107-111). The reported magnitude of this disparity ranged considerably from 1.4 to 7.7 times greater odds of NAFLD in males (107-111). The actual prevalence is probably on the lower end of this range because many of these studies, particularly those reporting higher odds ratios, used ALT to define NAFLD without taking into consideration the fact that normal range of ALT is higher in boys compared to girls (108-109,111,115). Few studies have attempted to ascertain whether the presentation of NAFLD varies by sex. A greater propensity towards zone 3 NASH was reported among girls in one study (107), while another study noted no zonal patterns in NASH by sex (113). All three studies that examined the effect of sex on disease severity failed to detect any differences (87-88,113). Given the limited number of girls in these studies (n = 13-40 subjects), it is unlikely that they were adequately powered to detect these outcomes, which may have contributed to the null findings (87-88,113,115).

Sex hormones are thought to be largely responsible for the observed sex-dimorphic prevalence of pediatric NAFLD. The increase in disease prevalence seen in low estrogen states in females (e.g., post menopause, Turner Syndrome, Tamoxifen therapy, polycytic ovarian syndrome), and the protective effect of hormone replacement therapy, provides a strong basis for this hypothesis (116-119). Some of the proposed protective effect of estrogen on NAFLD is related to its influence on adipose tissue (27). During puberty in girls, estrogen stimulates a rapid proliferation of adipocytes, particularly in the lower body, resulting in a large number of immature adipocytes in this region (27). This mass of adipose tissue is thought to provide a buffer against the
development of hypertrophied adipocytes in the visceral cavity that contribute to NAFLD pathogenesis, a concept that is supported by the greater amount of visceral adipose tissue seen in males (27). Along with this, the prevalence of NAFLD has been seen to increase with puberty in boys, but was found to be lowest among postpubertal girls (110). While the effect of estrogen on adipogenesis may have a role in reducing NAFLD risk in females, other factors appear to be contributing because males have been found to have greater intrahepatic fat independent of intra-abdominal fat content (120). One possible reason for this discrepancy could be the direct effect of estrogen on hepatocytes. In a series of experiments using orchidectomized rats and human liver cells, binding of estradiol to estrogen receptor-α resulted in a decrease in hepatic DNL by phosphorylating and inactivating acetyl CoA carboxylase, the rate-limiting enzyme in this process (121).

Differences in diet between boys and girls may also contribute to the dissimilar NAFLD rates; however, this idea has yet to receive much attention in the literature. A cross-sectional analysis of Hispanic boys and girls with and without NAFLD, defined as an ALT >97.5%ile for age-and-sex, reported similar macronutrient intakes in all groups based on two, 24-hr dietary recalls (122). More robust analyses including liver biopsy and additional dietary exposures are needed to determine the extent to which diet is a factor influencing the observed differences in NAFLD prevalence by sex.

Pediatric Nonalcoholic Fatty Liver Disease by Ethnicity

Pediatric NAFLD is also unevenly distributed with regards to ethnicity, with Hispanics being at greater risk and Blacks being at lower risk of NAFLD compared to Whites and Asians (107-109,123). At the extremes, Hispanic children are estimated to have a five times greater prevalence of NAFLD than Black children (107). In addition to
differences in pediatric NAFLD risk, ethnic groups may vary in disease presentation. This concept was explored in one study, which found predominantly isolated steatosis in Blacks, zone 3 NASH in Whites, and zone 1 NASH in Hispanics and Asians (86). Another study reported a trend towards a significant difference (p=0.10) in the prevalence of moderate to severe fibrosis in children from Asian (66%), White (45%) and Hispanic ethnic groups (32%) (88). Although these results suggest that ethnicity influences NAFLD risk and presentation, they should be interpreted cautiously because the numbers of children in these studies, particularly from Asian and Black ethnic backgrounds, were generally too small to provide representative samples or control for potential confounders (86,88). Moreover, there appears to be considerable variability in the prevalence of NAFLD within each ethnic group (107). For example, one study found that the proportion of Asians with NAFLD ranged from 4% in Cambodian or Vietnamese children up to 20% in Filipino children (107).

Differences in NAFLD risk by ethnicity do not appear to be related to disparities in metabolic risk factors (e.g., visceral adiposity, insulin resistance), suggesting that genetics and environmental exposures may be important in mediating these effects (124). Among the genetic factors that have been linked to NAFLD, a common variant polymorphism in the patatin-like phospholipase 3 (PNPLA3) gene, which codes for the protein, adiponutrin, has been identified as a likely candidate (125). As previously mentioned, adiponutrin appears to be involved in both lipid droplet breakdown and VLDL secretion in hepatocytes, making it an important determinant of TAG handling in the liver (49-50). The PNPLA3 gene contains a single-nucleotide polymorphism that takes the form of a C-to-G (isoleucine-to-methionine) substitution, which gives rise to
gain-of-function variant that increases NAFLD risk (125). In a study of 85 obese children, the PNPLA3 minor allele (G) increased in frequency in parallel to the trends in NAFLD risk by ethnicity from Black (0.183) to White (0.324) to Hispanic children (0.483) (125). In this study, the proportion of Black, White and Hispanic subjects with the CC genotype that had liver steatosis, defined as a hepatic fat fraction ≥5.5% on magnetic resonance imaging (MRI), was 7%, 12% and 50%, respectively, indicating that other, yet unexplored factors are contributing to the association between ethnicity and pediatric NAFLD (125). Among environmental exposures, dietary choices and meal patterns are known to vary dramatically by ethnicity in children, although this has yet to be really explored with regards to pediatric NAFLD risk (126).

**Dietary Intake in Pediatric Nonalcoholic Fatty Liver Disease**

Nutritional epidemiology is the study of the distribution of usual intake and nutritional determinants of disease in specified populations, and can be a useful means of identifying potential modifiable risk factors for nutritional intervention. Numerous cross-sectional studies have been conducted examining the relationships between food and nutrient intakes and NAFLD risk and severity in children (112,122,127-131). The findings from these studies are summarized in Table I of the Appendix, and provide an indication of dietary modifications that might be beneficial in the prevention and management of pediatric NAFLD.

Based on these studies, it appears that children with NAFLD consume diets that contain the recommended amounts of protein (15-20% of kcal), fat (30-35% of kcal) and carbohydrates (50-55% of kcal) (112,122,129-132). A considerable proportion of fats and carbohydrates came from saturated fat (30-45% of fat) and sugars (10-25% of
carbohydrates), whereas and dietary fiber (10-15 g/d) intakes were generally reported to be low (112,128-132). Saturated fat and sugars, especially fructose, have been found to be very lipogenic, and are thought to promote NAFLD (133-138). Conversely, dietary fibers, particularly those that are viscous, may attenuate the lipogenic effect of sugars by delaying gastric emptying thereby slowing the rate of sugar absorption, and reducing postprandial hyperglycemia and hyperinsulinemia (139). Additional dietary factors that are thought to be important in NAFLD pathogenesis include antioxidants such as vitamins C and E due to their function in removing the reactive oxygen species that are involved in the progression of NASH (140). Pediatric NAFLD patients generally report adequate intakes of vitamin C (90-125 mg/d), but vitamin E intakes (5-10 mg/d) are well below the estimated average requirements for children (112,128-129,132).

Despite the observed dietary intakes and biological plausibility, studies examining the associations between saturated fat, sugars, dietary fiber and antioxidant intakes and pediatric NAFLD risk and severity are somewhat inconclusive. For saturated fat, one study reported a significant positive dose response with liver steatosis grade (128), while others found no such trend (112,130). The former study also noted higher sugar and lower fiber intakes in subjects with NAFLD compared to those without NAFLD, but these associations were no longer significant after adjusting for age, sex and other dietary variables (128). Among pediatric NAFLD patients, saturated fat, sugar and dietary fiber intake were generally not associated with disease severity (112,129), although a negative correlation between consumption of dietary fiber and lobular inflammation grade was seen in one study (112). Similarly, dietary antioxidants were not related to NAFLD risk (128), or most indicators of disease severity (112,128-129), but one study did find a
protective effect of vitamin C on ballooning degeneration, and vitamin E on steatosis (112).

*Treatment of Pediatric Nonalcoholic Fatty Liver Disease*

Previous sections of this review have highlighted the serious burden of pediatric NAFLD, and consequent need for effective treatment options. Similar to other chronic diseases, the pathogenesis of NAFLD is highly complex, and likely involves the interaction of numerous known and unknown component causes (140). While this offers a multitude of potential targets for disease intervention, it also means that no single treatment is likely to be effective in all cases of NAFLD.

*Weight Loss in Pediatric Nonalcoholic Fatty Liver Disease*

The current primary treatment option for pediatric NAFLD is weight reduction through lifestyle modification. Not only is obesity one of the strongest predictors of NAFLD risk in children (107-108), there is a growing body of evidence suggesting that the phenotypic changes in adipose tissue related to obesity directly contribute to liver steatosis and the development of NASH (26,141-142). Moreover, childhood obesity is thought to lead to several other chronic conditions and adult obesity, providing additional benefits of weight reduction (143-144). However, obesity is not itself a modifiable behavior, but is another chronic condition with its own set of component causes and treatments. Unfortunately, there is no cure for obesity, and many attempts at weight reduction are unsuccessful in the long-term (145-146). Treatment of obesity is particularly challenging in children, as it requires the establishment of an energy deficit in a population that is still be growing. Adding to this, children with NAFLD have greater
weight-related negative self-esteem, which appears to influence their willingness to carry on with weight loss treatment attempts (92).

There have been many clinical trials investigating the effectiveness of weight reduction on pediatric NAFLD (see Appendix, Table II) (87,127,147-154). These clinical trials ranged in duration from 1-24 months, and varied in their measurements of body weight and NAFLD, but all used a combination of diet and physical activity, and reported substantial reductions in weight status and resolution of NAFLD. When examined, the greatest improvements in NAFLD were seen among subjects that achieved the greatest weight loss, indicating a graded exposure-response relationship (87,147-148,151,153). The majority of these studies were one-group pretest-posttest trials (87,127,147-149,152-154), which were often investigations of weight loss in obese children that contained NAFLD measurements as an outcome (127,149,152-154). The two trials that had controls for comparison reported a significant treatment effect on BMI z-scores, ALT or liver ultrasound grade (150-151).

Despite the indication of a benefit from weight reduction on NAFLD in children, this practice is still not considered to be evidence-based due to a lack of high quality randomized controlled trials and liver histology measurements (155). Additionally, the effectiveness of weight loss attempts in clinical practice may not reflect the results observed in clinical trials. This was investigated in a retrospective chart review of 66 pediatric NAFLD patients with a mean follow up of 6.4 years (100). Approximately half of the patients were able to lose at least 10% of their initial body weight after one year of treatment, which included individualized diet and exercise recommendations (100). Most of these patients (86%) had a significant reduction or normalization of ALT at this time,
suggesting a benefit of weight loss (100). However, by the final follow up, the majority had regained weight (76%) and nearly half had ALT levels return to baseline values (100). Another study found that among pediatric NAFLD patients receiving diet and physical activity advice, those who were able to reduce their BMI actually reported greater depression scores than those whose BMI increased (92). Although the reason for this finding is unknown, it does warrant consideration when implementing lifestyle interventions geared towards weight loss in this population. Finally, it is possible that changes in diet and physical activity rather than decreases in fat mass are responsible for most of the apparent beneficial treatment effects (156). Supporting this hypothesis, subjects in the placebo arm of the Treatment of NAFLD in Children (TONIC) trial who received standard care including recommendations for diet and physical activity had significant reductions in ALT and generally saw improvements in histological features of NAFLD despite steadily gaining weight over the 120-week treatment period (157).

Alternative Treatments for Pediatric Nonalcoholic Fatty Liver Disease

Adjunctive therapies to lifestyle intervention for pediatric NAFLD have also been explored, and may be useful in these patients. Antioxidant and insulin sensitizers are some of the better-investigated NAFLD treatments. Antioxidants reduce oxidative stress, which is thought to have an important role in NASH pathogenesis (61). Several clinical trials have investigated the use of antioxidants, particularly vitamins C and E, in pediatric NAFLD patients, and generated mixed results (157-161). The study by Nobili et al. (2006, 2008) failed to find a beneficial effect of one-year vitamin C and E supplementation on ALT and histological features of NAFLD, whereas the study by Lavine et al. (2011) reported significant improvements in ballooning degeneration, NAS
and diagnosis of NASH following 96-weeks of vitamin E supplementation. Both clinical trials were randomized and placebo-controlled with blinded treatment allocation to the participants, investigators and examiners, and included lifestyle recommendations as part of standard care. The limited number of participants attending the two-year follow up examinations (59%, n=53) may have contributed to the negative findings in the trial by Nobili et al. (2006, 2008). Alternatively, the difference in these two studies may be related to the intensity of the lifestyle interventions as Nobili et al. (2006, 2008) reported 4.5 and 5.5 kg weight losses in the placebo and treatment groups, respectively, whereas there was a 12.6 kg increase in body weight in the study by Lavine et al. (2011). The latter hypothesis is supported by the findings of an earlier study, which found improvements in ALT following vitamin E supplementation only among those patients that were unable to lose the recommended amount of weight (159).

Insulin resistance is thought to promote liver steatosis by increasing adipose tissue lipolysis and hepatic DNL, and decreasing the breakdown of FFAs via $\beta$–oxidation (19). Research on the use of insulin sensitizers for NAFLD in children has focused on the biguanides (157,162-164), although thiazolidinediones have been investigated for NAFLD treatment in adults (165). Similar to antioxidant supplementation, the use of biguanides has generated inconsistent results with reductions in liver steatosis reported in some (163), but not other studies (157,162). Importantly, the clinical trial that found a beneficial effect recruited children with insulin resistance rather than NAFLD. It is quite reasonable to presume that the benefits of insulin sensitizers would be limited to those patients that have insulin resistance, something that was not examined in the studies that reported no treatment effects of biguanides (157,162).
**Long-Chain Omega-3 Fatty Acids and Nonalcoholic Fatty Liver Disease**

The long-chain omega-3 fatty acids (LCω3), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), may help to prevent, delay and/or reverse the development of NAFLD in children. A protective effect of LCω3s on pediatric NAFLD is supported by epidemiological and clinical studies, and may be related to numerous biological mechanisms (26,166-168). Fish is one of the primary sources and main contributors on LCω3s in the diet, making it a potentially viable target for medical nutrition therapy in children with NAFLD (169). Despite the promising evidence for LCω3s, current dietary guidelines for pediatric NAFLD do not contain recommendations for fish intake (170).

**Biological Mechanisms of Action of Long-Chain Omega-3 Fatty Acids**

There is an abundance of biological effects of LCω3s that could protect against pediatric NAFLD relating to their integration into phospholipid membranes, and ability to alter cellular activities both directly and through receptor-mediated pathways (26). The extent to which each of these mechanisms influences NAFLD pathogenesis is difficult to determine because they occur simultaneously and have overlapping downstream effects in biological systems, which include reductions in inflammation and insulin resistance, as well as changes in carbohydrate and lipid metabolism. The ability of LCω3s to operate though multiple mechanisms and tissues may be particularly beneficial in NAFLD, which appears to have multiple contributing factors (26).

**Integration of Long-Chain Omega-3 Fatty Acids into Phospholipid Membranes**

One of the possible metabolic fates of dietary fatty acids is to become incorporated into phospholipid membranes (171-172). As a result, the composition of cell phospholipid membranes is influenced by the distribution of fatty acids in the diet.
This is especially true for omega-3 and omega-6 fatty acid because the body lacks the enzymes necessary to synthesize them (171). Although $\alpha$-linolenic acid, an 18-carbon fatty acid that constitutes approximately 95% of omega-3 fatty acid intake, can be modified through a series of connected desaturation and elongation steps to produce EPA and DHA in the body, this process is relatively limited in humans (169,171). Moreover, this metabolic pathway is under competitive inhibition by linoleic acid, an 18-carbon omega-6 fatty acid that is contained in high amounts in the average Western diet (169,173-174).

The incorporation of LC$\omega$3s into phospholipid membranes is thought to affect numerous body processes by directly modifying the physical structural and chemical properties of the membranes, and by acting as a substrate in the formation of cellular signaling molecules (174-175). Due to their relatively large size and number of double bonds, phospholipids containing LC$\omega$3s tend to pack together poorly and dissociate themselves from less polar molecules resulting in phospholipid membranes that are relatively thin that have high fluidity and increased permeability (175). The importance of these properties in biological systems is currently poorly understood, but is thought to influence the transmembrane flux of molecules (175). Clearer is the effect of LC$\omega$3s on lipid rafts, cholesterol and saturated fatty acid-rich clusters that house cell membrane proteins and are thought to assist in the coordination of cell signaling (172,175). The incorporation of LC$\omega$3s into cell phospholipid membranes alters lipid raft functioning by displacing cholesterol and causing declustering of the raft (176). There are numerous mechanisms by which lipid raft activity could link LC$\omega$3 to NAFLD, although cellular responses to lipid raft changes are largely unknown.
Historically, many of the biological actions of LCω3 were attributed to their function in phospholipid membranes as precursors in the production of secondary messengers (172). Specifically, the 20-carbon omega-3 and omega-6 fatty acids, EPA and arachidonic acid (AA), respectively, were known to compete for enzymatic activity of cyclooxygenases and lipoxygenases to derive distinct classes of signaling molecules including prostaglandins, thromboxanes and leukotrienes, collectively termed eicosanoids (174). Once produced, eicosanoids are released into the surrounding tissue where they bind to a variety of G protein-coupled receptors (GPRs) on nearby cells, and trigger downstream effects. The EPA-derived eicosanoids are less potent promoters of neutrophil chemotaxis and inflammation than those generated from AA, which led to the concept that the anti-inflammatory effects of LCω3s are principally due to the displacement of AA from phospholipid membranes, and greater production of EPA-derived eicosanoids (172). In recent years, new classes of secondary messengers, including some that are derived from DHA, have been discovered that have potent anti-inflammatory properties and are able to actively resolve inflammation (Bannenberg et al., 2010; 172,). These LCω3s derivatives, classified as resolvins, protectins and maresins, are produced similarly, but do not exhibit the classic wedge shape of other eicosanoids, and have their own set of receptor binding activities (177). In a series of animal studies by Gonzalez-Periz et al. (2009), resolvins and protectins were found to mimic the actions of DHA in reversing NAFLD, suggesting that these metabolites may have an important role in mediating treatment effects of LCω3s (178).

_Intracellular Activities of Long-Chain Omega-3 Fatty Acids_
LCω3s and their derivatives regulate numerous transcription factors, particularly those involved in carbohydrate and lipid metabolism. Within hepatocytes, LCω3s suppress ChREBP and SREBP-1c activity resulting in reduced hepatic DNL (179-183). These transcription factors also stimulate adiponutrin synthesis, and therefore LCω3s may increase lipid droplet breakdown and decrease VLDL secretion in hepatocytes (49-51,181). Supporting this concept, the ratio of omega-6: omega-3 fatty acids in the diet of children with NAFLD have been found to influence relationship between PNPLA3 genotype and liver steatosis (184). Additionally, LCω3s are known to reduce triglyceride concentrations by decreasing VLDL secretion, although the post-ER, pre-secretory degradation of ApoB100 by LCω3 peroxidation is thought to have an essential role in this process (185).

The PPAR nuclear receptor superfamily is another group of LCω3-sensing transcription factors, which are expressed in several cell types where they influence a variety of cellular processes including glucose and lipid metabolism, and inflammatory response (186-188). Stimulation of PPAR-α increases fatty acid oxidation in hepatocytes and skeletal muscle fibers (34,187-188), and suppresses inflammation in hepatocytes (188,190-191). Similarly, PPAR-γ has been found to reduce inflammatory signaling pathways in macrophages and adipocytes (186,192). Additionally, PPAR-γ activation results in increased insulin sensitivity in adipocytes and skeletal muscle fibers (186,188,192), and greater secretion of adiponectin (186,192-193). Several PPAR-agonists have been investigated in animal and human studies, and found to have a protective effect on NAFLD (188).
Binding of Long-Chain Omega-3 Fatty Acids to Cell Surface Receptors

The understanding of how LCω3s can elicit changes in cell processes was expanded considerably by the recent identification and characterization of a cell surface receptor with a LCω3 binding domain, GPR120 (194). In a series of in vitro and animal experiments, Oh et al. (2010) convincingly demonstrated that the GPR120 found on adipocytes and macrophages are involved in mediating several beneficial effects of DHA, including prevention of NAFLD (194). In this study, binding to GPR120 was found to have an insulin sensitizing effect and inhibit inflammatory signaling pathways (194). Using bone marrow transplantation from GPR120 knockout mice, the anti-inflammatory effect of GPR120 were shown to be sufficient to prevent skeletal muscle and hepatic insulin resistance caused by a high fat diet (194). Although EPA was not directly evaluated, it was found to have similar GPR120 binding affinity, and therefore would be expected to produce a similar response (194).

Systemic Effects of Long-Chain Omega-3 Fatty Acids on Nonalcoholic Fatty Liver Disease

The various biological mechanisms of LCω3 noted above may protect against NAFLD by bringing about changes in several tissue including adipose, skeletal muscle and liver tissue. A theoretical model of the systemic physiological changes related to LCω3s, and their subsequent effects on NAFLD pathogenesis is depicted in Figure 2.3 and described in detail below.
Figure 2.3 Effects of long-chain omega-3 fatty acids in nonalcoholic fatty liver disease pathogenesis

ChREBP=Carbohydrate-responsive element-binding protein, FFA=Free fatty acid, GPR=G protein-coupled receptor, NEFA=Nonesterfied fatty acid, Nonalcoholic fatty liver disease=NAFLD, PPAR=Peroxisome proliferator-activated receptor, SREBP=Sterol regulatory element-binding protein
Long-chain omega-3 fatty acids (LCω3s) are thought to protect against NAFLD by altering transcription factor activity (e.g., PPAR-α and -γ, ChREBP and SREBP), binding to GPR 120, and modifying the production/secretion of fatty acid derivatives from cell membranes (e.g., eicosanoids, resolvins, protectins and maresins). In adipose tissue, LCω3s reduce pro-inflammatory cytokines and NEFA release, and increase the secretion of adiponectin. These changes as well as the direct action of LCω3s are thought to alter skeletal muscle tissue causing greater uptake/storage of glucose and uptake/oxidation of NEFAs, respectively. Within hepatocytes, the indirect and direct effects of LCω3s promote a shift from FFA formation towards oxidation. This shift, combined with a reduction in NEFAs, decreases hepatic FFAs, the main driver of steatosis in NAFLD. The effect of LCω3s on transcription factors may also directly contribute to their anti-steatotic action by altering the expression of adiponutrin. In addition to reducing liver steatosis, LCω3s may reduce hepatocellular injury, and liver inflammation and corresponding fibrosis characteristic of steatohepatitis by the direct and indirect actions of LCω3s on inflammatory and parenchymal cells in the liver.
Obesity and the corresponding phenotypic changes in adipose tissue are thought to be central to obesity-related pathologies including NAFLD (21,26,196). Several animal studies have observed decreases in diet-induced obesity with greater LC\(\omega\)3 intake (196-199). The exact mechanism underlying these findings have not been elucidated; however, reduced de novo lipogenesis and increased lipid oxidation by LC\(\omega\)3s could be contributing factors (197). In addition to reducing adiposity, LC\(\omega\)3s may directly oppose the pathological phenotypic changes in adipose tissue by reducing inflammation and insulin resistance (26,194). The net effect of LC\(\omega\)3s in adipose tissue include a decrease in inflammatory cytokines and NEFAs release, and an increase in adiponectin production, which has been observed in animals (178,194,196,200-205), and humans (203,207-208).

Skeletal muscle is a metabolically important tissue due to its large energy demand, and ability to take up and store glucose as glycogen (209-210). Skeletal muscle insulin resistance associated with pediatric NAFLD reduces the uptake of glucose into skeletal muscle fibers, contributing to hyperglycemia (16,19,21). An increase in intracellular FFAs is thought to be the primary cause of skeletal muscle insulin resistance in obesity (209), although pro-inflammatory cytokines have also been postulated to also contribute (209). LC\(\omega\)3s may improve skeletal muscle insulin sensitivity by decreasing systemic NEFAs and pro-inflammatory cytokines, and promoting FFA oxidation (206-207,209-213). Additionally, LC\(\omega\)3s increase adiponectin production, which has an insulin sensitizing effect on skeletal muscle (206,214). These findings are supported by observations that the DHA content of muscle cells is associated with greater insulin sensitivity (209,215), and intake of LC\(\omega\)3s are associated with decreased insulin
resistance, and increased glucose disposal and glycogen synthesis in skeletal muscle (178,194,211,216-218).

The changes adipose and skeletal muscle tissue functioning described above, as well as the direct actions of LCω3s in the liver oppose hepatic steatosis characteristic of NAFLD, and the progression of inflammation, fibrosis, and hepatocellular injury seen in NASH (26). The anti-steatotic effects of LCω3s can be primarily attributed to a decrease in NEFA release from adipocytes, and shift from DNL toward FFA oxidation in hepatocytes (26,166-167). The reduction in hepatic DNL occurs secondarily to decreases in serum concentrations of glucose and insulin resulting from improvements in skeletal muscle carbohydrate metabolism, and by the direct inhibition of ChREBP and SREBP-1c by LCω3s and adiponectin (26,166-167). Suppression of hepatic DNL promotes FFA oxidation by reducing the inhibitory effects of malonyl-CoA on this pathway (1). Oxidation of FFAs is also triggered directly through PPAR-α activation by LCω3s (26,42,166-167). The effect of LCω3s on ectopic fat deposition in the liver is supported by findings that mice fed an omega-3 fatty acid depleted diet develop hepatic steatosis, which was associated with insulin resistance, increased expression of SREBP-1c and DNL, and reduced expression of PPAR-α and FFA oxidation (182).

In addition to decreasing liver steatosis, the changes in carbohydrate and lipid metabolism induced by LCω3s may reduce hepatic FFAs, and therefore slow the progression of NASH (48). At the same time, the anti-inflammatory properties of LCω3s reduce Kupffer cell activation by pro-inflammatory cytokines, thereby blocking the cycle of inflammation and immune cell infiltration/activation, and consequent pro-fibrotic transformation of hepatic stellate cells (57,60). This also diminishes hepatocellular injury.
caused by inflammatory signaling pathways in hepatocytes. The proposed beneficial effects of LCω3s on NAFLD and NASH are supported by findings from epidemiological and clinical studies in humans, which are discussed in more detail in the following sections.

Epidemiological Evidence of Long-Chain Omega-3 Fatty Acids

A direct link between LCω3s and NAFLD in humans was first reported by Singer et al. (1974) who observed a reduction in EPA content of liver biopsy samples obtained from diabetic patients with liver steatosis compared to those without (220). Since this time, several additional studies have examined the fatty acid profile of liver biopsy samples from individuals with and without NAFLD, and have confirmed these initial findings (42,221-225). In NAFLD patients, the amount of LCω3s in the liver has been found to be lower among those with steatohepatitis compared to those with steatosis (221,223). Collectively, these findings suggest that LCω3 depletion may increase NAFLD risk, and promote the progression towards NASH, although the nature of these relationships cannot be inferred based on these associations alone.

The cause of LCω3 depletion in patients with NAFLD may be a consequence of NAFLD pathogenesis, and/or inadequate dietary intake of omega-3 fatty acids. It appears that at least some of the observed difference in LCω3 content can be attributed to decreased LCω3 synthesis and increased LCω3 peroxidation related to NAFLD. The delta-6 and delta-5 desaturases, key enzymes in the production of long-chain polyunsaturated fatty acids from their precursors including the synthesis of EPA and DHA from ALA, have markedly reduced activity in the livers of NAFLD patients, and are thought to contribute to LCω3 depletion (225). This is supported by findings of
reduced total hepatic long-chain polyunsaturated fatty acids in NAFLD (221-22,224) and NASH (221). The delta-6 and delta-5 desaturase enzymes are up regulated by PPAR-α and insulin signaling, factors that are suppressed in NAFLD and activated by LCω3 (225). Consequently, a reduction in hepatic desaturase activity in NAFLD is proposed to mediate a constant cycle of LCω3 depletion and NAFLD progression in these patients (225). In addition to a reduction in LCω3 synthesis, LCω3s may be broken down in the liver by lipid peroxidation resulting from oxidative stress, which are reportedly increased in NAFLD (221-225).

The effects of NAFLD on LCω3 status may be compounded by an inadequate dietary intake. Currently, there is only one study that has examined LCω3 intake in pediatric NAFLD (129). Using three-day food records, Mager et al. (2010) found that children with NAFLD consumed less than the recommended amount of omega-3 fatty acids, and that higher intakes of LCω3s were associated with lower serum ALT values (129). Based on these results alone, it is not possible to determine whether or not lower dietary intake of LCω3s contribute to the differences in liver LCω3 content seen in NAFLD. However, regardless of the cause of diminished hepatic LCω3s, these findings suggest that increasing LCω3s intakes may be beneficial in pediatric NAFLD patients.

Clinical Evidence of Long-Chain Omega-3 Fatty Acids

Clinical trials provide the strongest form of evidence in determining if an exposure is causally related to an outcome. To date, at least nine clinical trials, including one in children, have investigated the effect of LCω3s in NAFLD (226-234). These studies are summarized in Table III of the Appendix, and are described in detail below.
The first clinical trial examining the efficacy of LCω3s in the treatment for NAFLD was an open-label trial published in 2004 (226). In this study, patients with biopsy-confirmed NAFLD and hypertriglyceridemia (>200 mg/dL) were given 5 mL of MaxEPA® (Twin Laboratories, NY, USA) thrice daily, which provides 2.3 g of EPA and 1.6 g of DHA per day. Subjects that were obese (BMI >25.0kg/m2) also received advice for weight loss. A total of 23 patients (9M/14F, 38-66 years, BMI 25 ± 2.6 kg/m²) were recruited, and 22 completed the 24-week trial (one subject moved away). At the end of the study, there was no change in BMI, but significant (p<0.01) reductions were reported in biomarkers of liver disease and dyslipidemia. Moreover, 35% of participants had normalization of ultrasound liver echogenicity, an indicator of liver steatosis.

Two years later, another open-label trial was conducted in a group of 42 patients (23M/19F; 32-77 years; BMI 28.6 ± 4.7 kg/m²) with a positive ultrasound for liver steatosis (227). In this study, subjects were provided 1 g of LCω3 containing 37.5% EPA and 62.5% DHA for 12 months. A separate group of 14 NAFLD patients that did not want to participate in the trial (9M/5F, 31-75 years, BMI 28.2 ± 4.0 kg/m²) were used as controls. At 12 months, neither group had a significant change in BMI. However, compared to controls, the group that received LCω3 supplementation had a significant decrease in biomarkers of liver disease, dyslipidemia and insulin resistance, and greater improvement in liver perfusion measured by echo-Doppler. Furthermore, while there were no changes in liver ultrasound steatosis in the control group, the treatment group liver steatosis decreased in 64% of subjects including one-quarter of subjects who no longer had liver steatosis detected on ultrasound. Compliance with treatment in this study was confirmed by pill count and decrease in ω-6: ω-3 of plasma lipids.
Four more trials assessing LCω3 treatment for NAFLD were published in 2008 including one open-label trial (229), one placebo-controlled trial (230), and two randomized, double blind, placebo controlled trials (228,231). The open-label trial provided 23 patients (14M/9F, 27-74 years, BMI 28.1 ± 3.2 kg/m²) who had persistently elevated ALT and NASH with 2.7 g of highly purified EPA-ethyl ester daily for 12 months. All subjects completed the trial without side effects, and were compliant with treatment as indicated by an increase in plasma EPA concentration. There was no change in body weight, but significant decreases were reported for biochemical indicators of liver disease, dyslipidemia and inflammation, and more than half of these subjects had reduced liver steatosis with 22% no longer having any steatosis. Seven participants agreed to have a follow up liver biopsy. In this sub-group, the majority of subjects had improvements in histological features of NAFLD including steatosis (5/7), lobular inflammation (4/7), hepatocyte degeneration (5/7) and fibrosis (6/7), corresponding to a decrease in NAS from 6.1 ± 1.3 to 3.7 ± 1.4.

The placebo-controlled trial recruited 22 participants from the Dallas Heart Study that had magnetic resonance spectroscopy performed (230). This study included a four-week run-in period in which subjects received a placebo consisting of mostly 18-carbon, trans monounsaturated fatty acid (72%) to identify subjects unlikely to comply with the treatment protocol. Of the initial cohort, 16 subjects (73%, 4M/12F, 50 years, BMI 36.2 ± 8.5 kg/m²) were deemed eligible, and completed the treatment protocol, which included 9 g of fish oil containing 51% EPA and 24% DHA per day for eight weeks. At the end of the treatment period, subjects had significant reductions in hypertriglyceridemia, but not changes in low-density lipoprotein (LDL-C)- or high-density lipoprotein (HDL-C)-
cholesterol, or liver steatosis. Importantly, the median liver fat concentration at baseline was estimated to be 7.9% with many subjects falling below levels indicative of NAFLD, reducing the potential effect size for this outcome.

Spadaro et al (2008) randomized 40 patients (n=20 per group) with persistently elevated ALT and ultrasound liver steatosis to receive dietary intervention alone, or dietary intervention plus 2 g of LCω3 supplements daily for six months (228). The dietary intervention consisted of 50% carbohydrate, 30% fat and 20% protein, and calorie restriction for overweight subjects (≥ 25kg/m^2). Thirty-six participants successfully completed the trial (18 per group, 19M/17F, BMI 30.0 ± 4.1 kg/m2). The treatment and control groups were comparable at baseline, had similar compliance with the dietary intervention, and both had decreases in BMI. However, only the LCω3 group had significant resolution of liver disease, dyslipidemia, insulin resistance and inflammation. Unfortunately, the authors did not compare the two groups to one another, limiting the interpretation of these findings.

In the other double blind, placebo-controlled trial conducted in 2008, 144 patients were randomized to receive dietary intervention with either 6 g of seal oil supplements or placebo daily for 24 weeks (231). Similar to Spadaro et al. (2008), a hypocaloric diet providing 25-30 kcal/kg was recommended for overweight subjects. Inclusion criteria for this study included persistently elevated ALT or aspartate aminotransferase (AST), liver steatosis on ultrasound, and dyslipidemia. Subjects in the treatment group (47M/19F, 45 years; BMI 26.4 ± 3.1 kg/m^2) were not different than subjects in the placebo group for any of the study parameters at baseline (50M/18F, 44 years, 26.0 ± 2.7 kg/m^2). BMI did not change during the study for either group; however, both groups had significant
improvements in serological and radiological indicators of liver disease, dyslipidemia and symptoms. Disease symptoms were evaluated as the unweighted sum of liver discomfort or pain, weakness, abdominal distention and nausea, which were rated on a Likert scale from asymptomatic (zero) to severe (three). Compared to the control group, subjects in the treatment group had greater reductions in liver ultrasound steatosis, triglycerides and disease symptoms scores, but smaller decreases in ALT. The differences in ALT may have been confounded by the non-significantly higher baseline ALT values in the control group (80 ± 51 U/L) compared to the treatment group (62 ± 36 U/L).

In 2009, Cussons et al (2009) conducted a randomized double blind, placebo-controlled crossover trial of women with polycystic ovary syndrome (PCOS) that were recruited from current patient lists, as well as a newspaper advertisement (232). Subjects received 4 g of LCω3s (56% DHA, 27% EPA) or 4 g of olive oil for eight weeks at a time with an eight-week washout period. Unlike previous trials, no attempt was made to rule out other common causes of liver disease. Moreover, only a subset of 12 patients (35 years, BMI 38.2 ± 7.2 kg/m²) had liver steatosis detected on magnetic resonance spectroscopy (>5% liver fat). The LCω3 treatment resulted in a significantly greater reductions in liver fat percentage, triglycerides, and insulin resistance compared to the olive oil treatment, but no differences were noted in ALT, systemic inflammation or cholesterol parameters.

The most recent investigation of LCω3 treatment in adults NAFLD was published in 2010 (233). This was another double blind, placebo-controlled trial of patients with persistently elevated ALT and liver ultrasound steatosis. In this study, subjects were randomized to receive 6.5 mL of olive oil (5M, 54 years, BMI 29.3 ± 3.9 kg/m²), or olive
oil enriched with 0.83 g of omega-3 fatty acids consisting of 0.47 g of EPA and 0.24 g of DHA (4M/2F, 55 years, 29.3 ± 4.1 kg/m²) for 12 months. At follow up, subjects receiving olive oil had no significant changes in any of the outcomes, whereas those receiving olive oil enriched with omega-3 fatty acids had a reduction in liver steatosis and transaminases, greater liver perfusion on echo-Doppler, and increased HDL-cholesterol and adiponectin concentrations. The improvements in liver disease biomarkers, HDL-cholesterol and adiponectin in the group that received omega-3 fatty acids were significant compared to the group that received olive oil only. Although other disease biomarkers including triglycerides, total and LDL-C, insulin resistance and ROS did not change in either group, the triglyceride concentrations were significantly reduced in the treatment group when compared to controls.

In 2012, Nobili et al. published the first and only trial investigating the efficacy of LCω3s in pediatric NAFLD (234). This was a doubly blinded study that randomized 60 children with biopsy-proven NAFLD to receive either 0.5 g (8M/12F, 11 years, BMI z-score 1.81) or 1 g of DHA (8M/12F, 11 years, BMI z-score 1.63), or germ oil daily for six months (8M/12F, 13 years, BMI z-score 1.76). During this time, subjects were asked to follow a balance, low-calorie diet and be physically active. Compared to controls, subjects receiving 0.5 g and 1 g of DHA had greater reductions in liver ultrasound steatosis and insulin sensitivity, although there was no difference in the changes in BMI z-score or ALT. The two DHA treatments did no differ from one another except for a higher blood DHA content in the group receiving 1 g of DHA.

While these clinical trials certainly shed light as to the efficacy of LCω3s on pediatric NAFLD, there is still a need for more research. Most of these studies were
conducted in adults (226-233), and had significant methodological limitations including small sample size (226,229,232), and a lack of control comparison (226,228-230), lack of randomization (227), potential for placebo effect (227-228), and lack of measurements of compliance (226,228,231-232). A variety of different sources of LCω3s were used in these studies with doses and duration ranging from 0.25 g per day (227) up to 6.75 g per day (230), and eight weeks (230,232) up to one year (227,229), respectively. Significant improvements in NAFLD were reported in most studies based on change in histological (229), radiological (226-229, 231-234) and serological (226-229,233) indicators of liver disease. Moreover, many of these clinical trials observed additional benefits of LCω3s for other obesity-related conditions including dyslipidemia (226-227,229-234), insulin resistance (227-228,232,234) and inflammation (228-229). Only one study reported any negative findings, which was a smaller reduction in ALT compared to controls that occurred despite greater improvements in liver steatosis, triglycerides and disease symptoms (231). Based on these findings, a large clinical trial with histological end points should be conducted to evaluate the potential use of LCω3s on pediatric NAFLD.

Conclusion

In summary, NAFLD is a common complication of obesity in children that may be difficult to recognize and evaluate despite having potentially serious health consequences. Although conventional management of weight loss through diet and exercise may be an efficacious, the long-term effectiveness of this approach in clinical setting is largely unproven. The LCω3s found in fish are theoretically able to address many of the known factors contributing to NAFLD, and may be superior to other adjunct therapies such as antioxidants and insulin sensitizers, which target only one feature of the
The beneficial effects of LCω3s on pediatric NAFLD are supported by epidemiological and clinical evidence in humans, although additional research, particularly from robust clinical trials, is needed before LCω3s can be recommended as a treatment for this disease.

**Parenteral Nutrition-Associated Liver Disease**

*Background*

The ability to support nutrition needs intravenously, or “parenterally”, is a relatively recent advancement in medicine that has yielded considerable improvements in morbidity and mortality of patients with intestinal failure who are unable to meet their nutrition needs enterally due to inadequate length of functional bowel relative to their nutrient requirements (235). One of the major challenges facing early parenteral nutrition (PN) manufacturers was the design of lipid emulsions that could be safely administered intravenously to prevent essential fatty acid deficiency (EFAD) (236-237). This obstacle was overcome in the 1970s with the development of vegetable oil-based lipid emulsions (VOLEs) derived from soybean oil and safflower oil (236), which are still the conventional lipid emulsions in use in the US today (238). Although PN is a life-sustaining therapy for infants with intestinal failure (239), its use is associated with numerous metabolic and infectious complications (240).

Parenteral nutrition-associated liver disease (PNALD) is a group of hepatobiliary disorders that occur in patients receiving PN support (240). Infantile PNALD is usually cholestatic, sometimes referred to as parenteral nutrition-associated cholestasis (PNAC), and is commonly associated with periportal inflammatory infiltrate and fibrosis (241-242). Consequently, infants with PNALD often present with jaundice secondary to
conjugated hyperbilirubinemia, and have elevated concentrations of alkaline phosphatase and γ-glutamyl transpeptidase (240). The diagnosis of PNALD in infants is generally defined as a conjugated bilirubin concentration of greater than 2.0 mg/dL (240). Additional liver disease biomarkers such as alanine aminotransferase, aspartate aminotransferase and coagulation indices are also monitored in these patients to assess hepatocellular injury and liver decompensation (243).

The present burden of PNALD in pediatric populations is directly related to the prevalence and incidence of intestinal failure, and ability to prevent and treat PNALD in PN-dependent patients. Approximately 80% of pediatric intestinal failure occurs in neonates with short bowel syndrome (SBS) who have congenital and perinatal gastrointestinal conditions including intestinal atresia, meconium ileus, gastroschisis, malrotation/volvulus, Hirschsprung’s disease, and necrotizing enterocolitis (244). Improvements in the initial prognosis of these conditions have resulted in an increased incidence of PN-dependency, and therefore risk of PNALD in infants (245). Overall, SBS is relatively rare in newborns, occurring in an estimated 24.5 per 100,000 live births; however, certain groups such as infants born before 37-weeks gestation are at much higher risk (353.7 vs 3.5 per 100,000 live births) (246). PNALD develops in about 65% of infants with SBS after approximately two months of receiving PN support, and progresses to liver failure in 40% of cases, which has a case fatality rate of nearly 90% of infants that remain PN-dependent for one year (247). Along with this, the case fatality rate of infants with SBS is estimated to be 20-40% with almost two-thirds of deaths (60%) being attributed to hepatic failure (244,246-247).
The low incidence, and considerable morbidity and mortality of intestinal failure in infants are significant hindrances to the study of the etiology and pathogenesis of PNALD (244). Nonetheless, several factors have been identified that are thought to contribute to PNALD including prematurity, inadequate length of functional gastrointestinal tract and/or lack of enteral feeding, PN component deficit (carnitine, taurine, choline) and excess (calories, dextrose, lipid/phytosterols, copper, manganese, aluminum), continuous PN feeding, and infection/sepsis (248-249). The extent to which each of these factors is a necessary and sufficient causal component of PNALD risk is unknown, and it is probable that they contribute to different hepatobiliary pathologies rather than a singular condition. For example, PNALD related to excess calories, particularly in the form of carbohydrates, would be expected to manifest with liver steatosis and hypertriglyceridemia due to the stimulatory effects on hepatic de novo lipogenesis and simultaneous inhibition of hepatic lipid oxidation, whereas aluminum toxicity is thought to promote liver cholestasis secondary to canalicular microvilli damage (240,250).

Role of Lipid Emulsions in Parenteral Nutrition-Associated Liver Disease

An excess of lipid in PN has been identified as a potential risk factor for PNALD (251-253). The mechanism underlining this observation is not well understood; however, the high omega-6: omega-3 fatty acid ratio and phytosterol content of conventional VOLEs have been proposed as contributing factors (254).

Currently available VOLEs in the US are derived from soybean oil (Intralipid®, Baxter Healthcare/Fresenium Kabi, Deerfield Illinois, US), or a 1:1 combination of soybean and safflower oil (Liposyn II®, Hospira Inc., Lake Forest, Illinois, US), which
have an omega-6: omega-3 fatty acid ratio of approximately 5.5-16: 1 (255). The omega-6 fatty acids of these VOLEs are thought to propagate the effects of inflammation and oxidative stress in PNALD pathogenesis through the production of pro-inflammatory eicosanoids (256-258). The role of inflammation and oxidative stress in hepatocellular injury and fibrosis in PNALD are similar to that of pediatric NAFLD described earlier and depicted in Figure 2.2. In additional to their effects on hepatic inflammation, the 2-series prostaglandins derived from omega-6 fatty acids have been found to reduce secretion of bile at the canalicular membrane of hepatocytes, thereby promoting cholestasis (259-260).

Phytosterols are a family of sterols found in plants that are structurally and functionally analogous to cholesterol in animals (261). Under normal physiological conditions, only a very small amount of phytosterols is consumed and absorbed (262), and are removed from the body with bile (261). The phytosterols in PN lipids are able to bypass the gastrointestinal tract, and exceed the capacity of the liver to secrete them in bile, causing them to accumulate in the body (261). Excess phytosterols displace cholesterol in the phospholipid membranes of erythrocytes, which reduce their half-life and increase the amount of bilirubin in bile (261). Within hepatocytes, phytosterols inhibit cholesterol 7α-hydroxylase, an enzyme necessary for the production of bile acids, resulting in impaired bile acid-dependent bile flow (261). The combination of reduced biliary flow and poor solubility of phytosterols and bilirubin is thought to contribute to biliary sludge and cholestasis in PNALD (261). Supporting this, a positive correlation between serum phystosterol and direct bilirubin concentrations in PN-dependent infants
has been observed, although this relationship could be confounded as serum phytosterol content is a biomarker for total lipid dose in these patients (263).

Due to the observed association between VOILEs and PNALD, restriction of PN lipid has been suggested for the prevention and treatment PNALD (264). While this approach was found to be associated with significantly reduced total bilirubin concentrations, it also may resulted in EFAD (265). Furthermore, this approach increases the amount of dextrose that needs to be provided in PN in order to remain eucaloric, which may promote liver steatosis and dyslipidemia (140). Consequently, ensuring adequate intake for growth, development and recovery is a major challenge in PN-dependent infants and children with PNALD, and may necessitate aggressive approaches to bowel adaptation (e.g., surgery) and advancement of enteral feeding, which are not always possible and may be present additional risks to these patients (266). The development of alternative lipid emulsions such as those derived from fish oil may offer new ways of mitigating and managing PNALD (254).

**Biological Mechanisms of Action of Fish Oil-Based Lipid Emulsions**

Fish oil-based lipid emulsions (FOLEs) are thought to reduce hepatobiliary disease in PN-dependent patients through a variety of mechanisms (254). Indirectly, fish oils are low in omega-6 fatty acids and contain none of the phytosterols found in vegetable oils, precluding their negative effects on the liver (238,254). It is probable that this indirect effect of using FOLE is responsible for the relative increase in biliary flow seen with its use (267). In addition to removing potentially harmful substances in PN, FOLEs may reduce hepatic inflammation and oxidative stress contributing to PNALD directly (256-257). The anti-inflammatory properties of FOLEs can be attributed to the
presence of LCω3s, which decrease hepatic inflammation by altering cyclooxygenase- and lipoxygenase-derived signaling molecules (e.g., eicosanoids, resolvins, protectins, maresins), binding GPR120 and activating PPAR-α (187,254). Additionally, despite the large number of double bonds in LCω3, use of FOLE does not appear to increase lipid peroxidation, and is associated with reduced oxidative stress (268). This finding may be related to both the anti-inflammatory properties of LCω3 as well as the high vitamin E content of FOLE compared to VOLE (238).

Clinical Evidence of Fish Oil-Based Lipid Emulsion Use in Infants

Given the potential advantages of FOLEs over conventional VOLEs, there has been considerable interest in evaluating its use in PN-dependent patients. As of 2004, the US Food and Drug Administration has permitted the compassionate use of the FOLE, Omegaven® (Fresenius Kabi, Bad Homburg, Germany), in PNALD patients that are unresponsive to conventional treatments (258). Since this time, Omegaven® has been widely adopted as rescue therapy for infants with PNALD (258), and preliminary case reports (269-276), case series (277-280) and case-control studies (281-283) have been published reporting on its safety and efficacy.

The results of published case series and case-control studies investigating FOLE use are reported in Table IV of the Appendix. Clinical experience with FOLE has thus far been generally positive with most studies reporting normalization of direct bilirubin concentrations indicative of cholestasis resolution (277-283). FOLE treatment in infants with PNALD has also been associated with improvements in other liver disease biomarkers (277,280-282) and dyslipidemia (279,282-283). Moreover, there was a reduction in liver transplantations and liver-related mortality compared to historical
controls (281-283). When initially introduced, there was concern that the use of FOLE would result in EFAD and impaired growth in infants (258). However, preliminary reports indicate that triene: tetrane ratio and mead acid concentrations indicative of EFAD do not increase in infants receiving FOLE treatment (278-279,282), and that growth is not negatively affected (278). Importantly, the vast majority of clinical evidence of FOLE use in infants comes from Boston Children’s Hospital including two of the four case series (278-279) and all three of the case-control studies (281-283). Despite these promising findings, a recent systematic review of FOLE use in PNALD concluded that additional studies, which include long-term, clinically relevant outcomes, are still needed (284).

**Conclusion**

In summary, PNALD is a relatively common and serious complication in PN-dependent infants, which may be related to the high omega-6 fatty acid and phytosterol content of conventional VOLEs. Substitution of conventional lipids for FOLEs is theorized to reduce PNALD by decreasing exposure to omega-6 fatty acids and phytosterols, and through the anti-inflammatory and antioxidant effects of LCω3s and vitamin E in FOLE, respectively. Several observational studies suggest that FOLE use is safe and efficacious in infants with PNALD; however, additional studies with clinically relevant outcomes are necessary.
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CHAPTER 3: LIVER DISEASE AMONG CHILDREN IN HAWAII DIAGNOSED WITH METABOLIC SYNDROME

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Abstract
The purpose of this study was to evaluate the prevalence of and factors related to liver
disease among children in Hawai‘i with metabolic syndrome. The medical charts of
children diagnosed with metabolic syndrome by an outpatient endocrinologist between
January 2000 and December 2010 were reviewed. Liver disease prevalence was
estimated based on serum alanine aminotransferase (ALT) levels, which were then
assessed for associations with demographic (age, gender, ethnicity), anthropometric
(body mass index), biochemical (fasting blood glucose, hemoglobin A1c, triglycerides,
and total, LDL- and HDL-cholesterol), and clinical (blood pressure) characteristics of
subjects. Serum ALT was available for 167 of the 195 subjects. The proportion of
subjects with liver disease (105/167 (63%)) was greater than many traditional features of
metabolic syndrome including hypertriglyceridemia (73/177 (41%)), hypertension
(37/194 (19%)) and hyperglycemia (37/170 (22%)). Serum ALT values were positively
associated with age (p=0.030), and liver disease was more common among boys than
girls (62/91 (68%) vs 43/76 (57%)), although this difference was not statistically
significant (p=0.123). There was a significant difference in liver disease across ethnicities
(p=0.029), and appeared to be more common in children with Pacific Islander surnames
(14/16 (88%)), and less common in children with Hispanic surnames (7/20 (35%)).
Diastolic blood pressure was the only obesity-related disease parameter associated with
serum ALT after adjusting for age and gender (p=0.018). In conclusion, liver disease
was common among children diagnosed with metabolic syndrome in Hawai‘i. Age,
gender and ethnicity may be important determinants of liver disease risk, and should be
investigated further.
Introduction

Childhood obesity is a major health concern in Hawai‘i. According to the Hawai‘i Youth Risk Behavior Survey, over one-quarter of adolescents are overweight or obese.\(^1\) The term metabolic syndrome has been adopted to describe the clinical and biochemical derangements related to excess body fat. The definition of metabolic syndrome in children has yet to be clearly determined, but usually includes measures of central adiposity, insulin resistance, dyslipidemia and hypertension.\(^2\) Nonalcoholic fatty liver disease (NAFLD) is another consequence of obesity that is closely linked to metabolic syndrome, and has been proposed as a defining feature of the condition.\(^3\)\(^-\)\(^5\)

Most children with NAFLD suffer from non-specific symptoms such as fatigue, which contribute to a lower physical and psychosocial health, and reduced quality of life.\(^6\) The prevalence and clinical spectrum of pediatric NAFLD varies considerably between populations, but affects roughly 40% of obese adolescents in the US, and presents with hepatic inflammation and/or fibrosis in the majority cases.\(^7\)\(^-\)\(^10\) Despite rapidly becoming the leading cause of liver disease in adolescents, screening for elevated hepatic transaminases in serum (transaminasemia) indicative of NAFLD, namely alanine aminotransferase (ALT), is still rarely carried out during general pediatric visits of at-risk subjects.\(^11\)

Presently, no studies have been published looking at the epidemiology of NAFLD among children in Hawai‘i. In view of the varied risk of pediatric NAFLD and the unique ethnic makeup of Hawai‘i, it is important to evaluate its present burden.\(^12\)\(^-\)\(^14\) The purpose of this study is to evaluate the prevalence of and factors related to elevated serum ALT
values indicative of liver disease in children diagnosed with metabolic syndrome in Hawai’i.

Methods
The study sample included consecutive patients referred to the outpatient pediatric endocrinologist at Kapi’olani Medical Center for Women and Children (KMCWC) between January 2000 and December 2010 who were described as having “metabolic syndrome” during the initial consultation at age 1-19 years old. Demographic, anthropometric, biochemical and clinical data were collected from the patient’s medical charts. The University of Hawai’i and Hawai’i Pacific Health Institution Review Boards approved this study.

Height and weight were converted into body mass index (BMI) standard deviation score (SDS) for age and gender according to the 2000 CDC growth charts using the lambda, mu and sigma (LMS) technique.\(^{15}\) Systolic and diastolic blood pressures were converted into SDSs for age, gender and height as described previously.\(^{16}\) The prevalence of metabolic syndrome features was evaluated based on the criteria from Graham et al (2009), except for central obesity, which was approximated using the body mass index (BMI), a measure of excess body weight, because waist circumference was not available (Table 3.3).\(^{12}\) Subject ethnicity was determined using the surname list method developed for the Multiethnic Cohort Study (MEC), and grouped according to the National Institutes of Health (NIH) standards for race and ethnicity as Asian (Chinese, Filipino, Japanese), Hispanic, Pacific Islander (Hawaiian, Samoan), or White (surname not found).\(^{17-18}\) Although both White and African American ethnicities were not available in the MEC surname list, it is expected that the majority of these subjects would have been
White based on the ethnic makeup of Hawaii. Surnames that corresponded to more than one ethnicity were assigned to the dominant ethnicity in Hawai ’i.

The Upper Limit of Normal (ULN) for serum alanine aminotransferase (ALT) varies considerably between facilities, largely due to differences in reference populations used. As per a telephone communication with the laboratory manager Teresa Walsh (December 18, 2012), Clinical Laboratories of Hawai’i, which provides laboratory services to KMCWC, use an ULN for serum ALT of 51 U/L for boys and 31 U/L for girls. In this study, subjects were classified as liver disease cases or controls based on the 95th percentile for healthy boys (<25.8 U/L) and girls (<22.1 U/L) in the US. Compared to the ULN for ALT used in many children’s hospitals, this cutoff was found to provide higher sensitivity for chronic liver disease with only a slight loss of specificity.

Subject characteristics were summarized according to liver disease status as frequencies (percentage) for categorical variables, and means ± standard deviation or medians (interquartile range) for parametric and nonparametric continuous variables, respectively (Table 3.1). Continuous study variables were classified as parametric (systolic and diastolic blood pressure SDS) or nonparametric (age, BMI SDS, fasting blood glucose, hemoglobin A1c, total-, LDL- and HDL-cholesterol, serum triglycerides, alanine aminotransferase) based on Shapiro-Wilk test and visual assessment of frequency distribution graphs. Liver disease cases were compared to controls using chi-square analysis for categorical variables, independent two-sample t-test for parametric continuous variables, Wilcoxon rank sum test for nonparametric continuous variables, and logistic regression analysis for all variables, adjusting for age and gender (Table 3.1). The relationship between serum ALT and continuous variables was also analyzed using
Spearman’s rank correlation coefficient and linear regression analysis, adjusting for age and gender (Table 3.2). Due to the differences in the ULN for serum ALT, direct comparison of serum ALT between boys and girls was not conducted.\textsuperscript{21} Prevalence of liver disease in relation to other features of metabolic syndrome was determined using the ULN of serum ALT from both Clinical Laboratories of Hawai‘i (boys 51 U/L, girls 31 U/L), and the 95\textsuperscript{th} percentiles for healthy children in the US (boys <25.8 U/L, girls <22.1 U/L) (Table 3.3). Finally, subjects with and without serum ALT measurements available were compared across study parameters for sensitivity analysis. Statistical tests were carried out using SAS version 9.2, and graphs were created using using Microsoft Excel for Mac 2011 v. 14.2.5.

Results

A total of 195 children (12.1±3.6 years old, 103/195 (53\%) boys, Table 3.1) were referred to the pediatric endocrinologist, and described as having metabolic syndrome during the initial consultation during this period. Data was available at the first appointment for >80\% of subjects for all study variables, including ALT (167/195 (86\%), Table 3.1). The group that was missing ALT measurements was not significantly different (p <0.05) in any of the variables measured (data not shown).

Despite slightly higher thresholds, boys were more likely to have elevated ALT values indicative of liver disease (62/91 (68\%)) than were girls (43/76 (57\%)), although this difference was not statistically significant (p = 0.123, Table 3.1, Figure 3.1). Similarly, the presence of liver disease was not associated with age (p = 0.369, Table 3.1), although there was a slight positive correlation between serum ALT and age (p = 0.030, Table 3.2). Liver disease was associated with ethnicity (p = 0.012, Table 3.1).
Compared to White children (33/52 (63%)), Pacific Islander children tended to be more likely (14/16 (88%), \(p = 0.069\)), and Hispanic children were less likely (7/20 (35%), \(p = 0.029\)) to have elevated ALTs suggestive of liver disease (Table 3.1). Multivariable analyses of study parameters adjusting for age and gender produced similar results (Tables 3.1 and 3.2).

The majority of patients was obese (BMI ≥95th percentile for age and gender; 191/194 (98.5%)), and had multiple co-morbidities consistent with the diagnosis of metabolic syndrome (Table 3.3). Apart from obesity, the most common features of metabolic syndrome were low HDL-cholesterol (≤40 mg/dL boys, ≤50 mg/dL girls; 122/177 (69%)) and hypertriglyceridemia (≥150 mg/dL; 73/177 (41%)) (Table 3.3). Hypertension (systolic or diastolic blood pressure ≥95th percentile for age, gender and height) and fasting hyperglycemia (≥110 mg/dL) were less common, presenting in 37/194 (19%) and 37/170 (22%) of patients, respectively (Table 3.3). The majority of subjects had elevated serum ALT values suggestive of liver disease (boys ≥25.8 U/L, girls ≥22.1 U/L) was 105/167 (63%) (Table 3.3). Even at the more conservative ULN for serum ALT used locally by Clinical Laboratories of Hawai’i (boys 51 U/L, girls 31 U/L), an estimated 49/167 (29%) of subjects had liver disease, making it a relatively common complication in this population (Table 3.3).

As expected, subjects with raised serum ALT values had higher BMIs (2.54 (2.27-2.75) SDS vs 2.35 (2.13-2.61) SDS, \(p = 0.036\)), and were worse for all disease factors measured, although this was only statistically significant for fasting blood glucose (96 (87-111) mg/dL vs 89 (83-97) mg/dL, \(p=0.007\)), and diastolic blood pressure (0.47 ± 0.91 SDS vs 0.13 ± 0.93 SDS, \(p = 0.025\)) (Table 3.1). After adjusting for age and gender,
fasting blood glucose was no longer associated with the ALT group \( (p = 0.216) \) (Table 3.1). When examined as a continuous variable, serum ALT was correlated with fasting blood glucose \( (p = 0.005) \), total cholesterol \( (p = 0.045) \), serum triglycerides \( (p = 0.044) \), and systolic and diastolic blood pressure \( (p = 0.047 \text{ and } p = 0.001) \), but not BMI \( (p = 0.265) \) (Table 3.2). Only the association between serum ALT and diastolic blood pressure remained statistically significant when analyzed using linear regression controlling for age and gender \( (p = 0.018) \) (Table 3.2).

**Discussion**

The findings of this retrospective chart review confirm that elevated ALT is common in pediatric patients diagnosed with metabolic syndrome in Hawai‘i. The thresholds for transaminasemia in this study were based on the 95\(^{th}\) percentile of ALT in healthy adolescents from the National Health and Nutrition Examination Survey 1999-2006, which is lower than the ULN of ALT that are generally used in US children’s hospitals.\(^{21}\) The recommendation to adopt these lower cutoffs comes from the Screening ALT for Elevation in Today’s Youth (SAFETY) study, which reported vast improvements in sensitivity for pediatric NAFLD in boys (32% to 80%) and girls (36% to 92%), while still maintaining moderate specificity of 79% and 85%, respectively.\(^{21}\) The ULN used by Clinical Laboratories of Hawaii is 51 U/L for boys and 31 U/L for girls, which still yields a prevalence of 29% for transaminasemia, making it a more common co-morbidity than either hypertension or fasting hyperglycemia. The relatively normal fasting glucose and hemoglobin A1c values are surprising given the high BMIs of children in this sample, although insulin resistance may have been disguised by insulin hypersecretion.\(^{22}\) Unfortunately, other glycemic measurements such as serum insulin
concentrations and oral glucose tolerance tests were not available to test this hypothesis.

There was a weak positive correlation between serum ALT and diastolic blood pressure, but not other obesity-related disease parameters. This study did not have the statistical power to assess these relationships, which may be partly related to the homogeneity of the sample. Supporting this, nearly every subject was obese. Additionally, fasting blood glucose may not be an appropriate indicator for assessing insulin resistance as it has been demonstrated previously to fail to detect significant relationships with ALT when present. The association between ALT and blood pressure may be related to elevated angiotensin II, which has been proposed to promote oxidative stress, inflammation and fibrosis in the liver.

The observation that serum ALT levels were correlated with age is consistent with other studies, which report increasing onset of NAFLD through the second decade of life. Pediatric NAFLD has been found to be more common among boys and Hispanic adolescents, and least likely to present in Black adolescents. In this sample, boys were not more likely to have liver disease, and children with Pacific Islander surnames tended to have higher ALT values. Hispanic ethnicity appeared to be protective in this sample, which was an unexpected finding. Importantly, ethnicity based on subject surname is likely to result in some misclassification, particularly in Hawai’i where almost one-quarter of the population is ethnically mixed. The distinction between Hispanic and Filipino ethnicity based on surnames is particularly tenuous given the early occupation of the Philippines by the Spanish. The differences in the distribution of NAFLD among adolescents by age, gender and ethnicity may be related to developmental changes with respect to sex hormones, visceral fat deposition, insulin sensitivity, and/or hepatic
antioxidant defenses, although this has not been clearly elucidated.\textsuperscript{28-30}

There are several limitations of this study that are noteworthy. Most patients did not have the testing necessary to rule out other causes of liver disease. However, given the high BMIs and age of the patients, NAFLD likely contributed to the majority of the observed prevalence of transaminasemia. Additionally, patients who were missing data may have been healthier, and therefore were not tested for co-morbidities, contributing to overestimation of the prevalence of these conditions in our sample. However, liver disease screening was not associated with any of the variables measured in sensitivity analysis. Finally, this is a pilot study based on a limited sampling frame and size. While it is able to provide preliminary data on liver disease on patients with metabolic syndrome, it did not have adequate power to evaluate relationships between most variables, and may not be representative of pediatric centers in other parts of Hawai‘i.

Screening for NAFLD in this population occurred much more frequently than what has been reported in other pediatric hospitals, indicating a relatively good awareness of the condition locally in this clinical setting.\textsuperscript{3} The prevalence of liver disease in this sample lend support to this practice. Future studies are needed to further evaluate the risk of NAFLD by ethnicity, and to evaluate the follow up patients that are found to have elevated ALT levels.
References


evaluation, and treatment of high blood pressure in children and adolescents.


### Table 3.1 Characteristics of subjects by serum alanine aminotransferase status

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal ALT</th>
<th>Elevated ALT</th>
<th>Normal v Elevated ALT (P-value)</th>
<th>Univariate Analysis</th>
<th>Logistic Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>62</td>
<td>105</td>
<td></td>
<td>0.369</td>
<td>0.362</td>
</tr>
<tr>
<td><strong>Gender (n (% Male)</strong></td>
<td>62</td>
<td>105</td>
<td></td>
<td>0.123</td>
<td>0.129</td>
</tr>
<tr>
<td><strong>Ethnicity (n (%))</strong></td>
<td>62</td>
<td>105</td>
<td></td>
<td>0.012</td>
<td>0.029</td>
</tr>
<tr>
<td>Asian</td>
<td>28 (45%)</td>
<td>51 (49%)</td>
<td></td>
<td>0.898</td>
<td>0.800</td>
</tr>
<tr>
<td>Hispanic</td>
<td>13 (21%)</td>
<td>7 (7%)</td>
<td></td>
<td>0.029</td>
<td>0.049</td>
</tr>
<tr>
<td>Pacific Islander</td>
<td>2 (3%)</td>
<td>14 (13%)</td>
<td></td>
<td>0.069</td>
<td>0.093</td>
</tr>
<tr>
<td>White (reference)</td>
<td>19 (31%)</td>
<td>33 (31%)</td>
<td></td>
<td>0.036</td>
<td>0.048</td>
</tr>
<tr>
<td><strong>Body Mass Index (SDS)</strong></td>
<td>62</td>
<td>104</td>
<td></td>
<td>0.007</td>
<td>0.216</td>
</tr>
<tr>
<td><strong>Fasting Blood Glucose (mg/dL)</strong></td>
<td>54</td>
<td>94</td>
<td></td>
<td>0.119</td>
<td>0.882</td>
</tr>
<tr>
<td><strong>Hemoglobin A1c (%)</strong></td>
<td>48</td>
<td>92</td>
<td></td>
<td>0.519</td>
<td>0.483</td>
</tr>
<tr>
<td><strong>Total Cholesterol (mg/dL)</strong></td>
<td>56</td>
<td>100</td>
<td></td>
<td>0.663</td>
<td>0.592</td>
</tr>
<tr>
<td><strong>LDL-Cholesterol (mg/dL)</strong></td>
<td>52</td>
<td>97</td>
<td></td>
<td>0.114</td>
<td>0.117</td>
</tr>
<tr>
<td><strong>HDL-Cholesterol (mg/dL)</strong></td>
<td>53</td>
<td>99</td>
<td></td>
<td>0.144</td>
<td>0.228</td>
</tr>
<tr>
<td><strong>Triglycerides (mg/dL)</strong></td>
<td>53</td>
<td>99</td>
<td></td>
<td>0.152</td>
<td>0.226</td>
</tr>
<tr>
<td><strong>Systolic Blood Pressure (SDS)</strong></td>
<td>62</td>
<td>104</td>
<td></td>
<td>0.025</td>
<td>0.039</td>
</tr>
<tr>
<td><strong>Diastolic Blood Pressure (SDS)</strong></td>
<td>62</td>
<td>104</td>
<td></td>
<td>0.025</td>
<td>0.039</td>
</tr>
</tbody>
</table>

**SDS** = standard deviation score (body mass index adjusted for age and gender); systolic and diastolic blood pressures adjusted for age, gender and height,

**ALT** = alanine aminotransferase

Cutoffs for elevated alanine aminotransferase based on 95th percentile of healthy U.S. adolescents (ALT ≥ 25.8 U/L for boys, ≥ 22.1 U/L for girls).

Values are presented as the number of subjects (percentage) for categorical variables, and means ± standard deviation or medians (interquartile ranges) for parametric and nonparametric continuous variables based on Shapiro-Wilk test and visual assessment of frequency distribution graphs, respectively. Univariate analysis was carried out using chi-square analysis, independent two-sample t-test and Wilcoxon rank sum tests for categorical, and parametric and nonparametric continuous variables, respectively. Logistic regression analysis included age and gender as covariates. Asian, Hispanic and Pacific Islander ethnic groups were assessed in relation to Whites for univariate and logistic regression analyses.
Table 3.2 Associations between alanine aminotransferase and study parameters

<table>
<thead>
<tr>
<th>Study Parameter</th>
<th>n</th>
<th>Correlation Coefficient (p-value)</th>
<th>Regression Coefficient (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>167</td>
<td>0.168 (0.030)</td>
<td>1.528 (0.022)</td>
</tr>
<tr>
<td>Body Mass Index (SDS)</td>
<td>166</td>
<td>0.070 (0.265)</td>
<td>1.288 (0.802)</td>
</tr>
<tr>
<td>Fasting Blood Glucose (mg/dL)</td>
<td>148</td>
<td>0.231 (0.005)</td>
<td>0.048 (0.336)</td>
</tr>
<tr>
<td>Hemoglobin A1c (%)</td>
<td>140</td>
<td>0.102 (0.231)</td>
<td>0.646 (0.687)</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dL)</td>
<td>156</td>
<td>0.161 (0.045)</td>
<td>0.085 (0.218)</td>
</tr>
<tr>
<td>LDL-Cholesterol (mg/dL)</td>
<td>149</td>
<td>0.103 (0.209)</td>
<td>0.055 (0.527)</td>
</tr>
<tr>
<td>HDL-Cholesterol (mg/dL)</td>
<td>152</td>
<td>-0.077 (0.347)</td>
<td>-0.073 (0.801)</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>152</td>
<td>0.163 (0.044)</td>
<td>0.025 (0.314)</td>
</tr>
<tr>
<td>Systolic Blood Pressure (SDS)</td>
<td>166</td>
<td>0.155 (0.047)</td>
<td>1.931 (0.382)</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (SDS)</td>
<td>166</td>
<td>0.249 (0.001)</td>
<td>6.200 (0.018)</td>
</tr>
</tbody>
</table>

SDS = standard deviation score (body mass index adjusted for age and gender; systolic and diastolic blood pressures adjusted for age, gender and height)  
1Spearman’s rank correlation coefficient; 2Linear regression analysis adjusted for age and gender.
Table 3.3 Proportion of subjects with features of metabolic syndrome and elevated alanine aminotransferase

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Prevalence</th>
<th>Cutoff for Metabolic Syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin Resistance</td>
<td>37 / 170 (22%)</td>
<td>Fasting blood glucose ≥110 mg/dL.</td>
</tr>
<tr>
<td>Central Obesity</td>
<td>191 / 194 (98%)</td>
<td>BMI ≥95th percentile for age / gender</td>
</tr>
<tr>
<td>Low HDL-cholesterol</td>
<td>122 / 177 (69%)</td>
<td>HDL-cholesterol ≤40 mg/dL for boys, ≤50 mg/dL for girls</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>73 / 177 (41%)</td>
<td>Triglycerides ≥150 mg/dL.</td>
</tr>
<tr>
<td>Hypertension</td>
<td>37 / 194 (19%)</td>
<td>SBP or DBP ≥95th percentile for age / gender / height</td>
</tr>
<tr>
<td>Elevated ALT&lt;sup&gt;1&lt;/sup&gt;</td>
<td>105 / 167 (63%)</td>
<td>ALT ≥25.8 U/L for boys, ≥22.1 U/L for girls</td>
</tr>
<tr>
<td>Elevated ALT&lt;sup&gt;2&lt;/sup&gt;</td>
<td>49 / 167 (29%)</td>
<td>ALT &gt;51 U/L for boys, &gt;31 U/L for girls</td>
</tr>
</tbody>
</table>

SBP = Systolic Blood Pressure, DBP = Diastolic Blood Pressure, ALT = Alanine Aminotransferase
<sup>1</sup>95<sup>th</sup> percentile of healthy U.S. adolescents<sup>19</sup>, <sup>2</sup>Clinical Laboratories of Hawai‘i Upper Limit of Normal<sup>21</sup>
The median (interquartile range) for serum ALT in boys was 35 U/L (22-56 U/L), and in girls was 24 U/L (17-38 U/L).
CHAPTER 4: THE EFFECT OF WEIGHT LOSS ON PEDIATRIC NONALCOHOLIC FATTY LIVER DISEASE

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¹University of Hawaii at Manoa; ²Kapi‘olani Medical Center for Women and Children
Abstract
This study evaluated the effect of weight loss on pediatric nonalcoholic fatty liver disease (NAFLD). Subjects included 81 overweight NAFLD patients referred to two pediatric gastroenterologists from 2000-2010. Data on subjects were obtained from review of medical charts. The effect of weight loss was assessed at 1-4 months, 5-8 months, 9-12 months and beyond one year as the change in weight, BMI z-score (for age-and-sex), and alanine aminotransferase, and the relationship between the change in body weight and BMI z-score, and the change in alanine aminotransferase. Subjects were mostly obese (99%), male (86%) and Asian (63%), and had median age of 14.1 (11.2-16.2) years and alanine aminotransferase of 105 (78-153) U/L at referral. Alanine aminotransferase decreased $32\pm66 \ (p=0.016)$, $30\pm65 \ (p=0.134)$, $37\pm75 \ (p=0.0157)$ and $45\pm69 \ (p=0.014)$ for subjects with follow up data at 1-4 months (n=47), 5-8 months (n=26), 9-12 months (n=19) and beyond one year (n=19), respectively. During these time periods, neither body weight (-0.2 to +7.1 kg) or BMI z-score (-0.12 to -0.05) was significantly reduced, nor were changes in these variables associated with the change in alanine aminotransferase. These findings suggest that weight and BMI z-score may not be sufficient indicators of treatment response in pediatric NAFLD patients.
Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common complication of pediatric obesity characterized by the inappropriate accumulation of fat in hepatocytes in the absence of other known causes of steatosis [1-3]. Hepatic steatosis is closely related to metabolic syndrome, and may contribute to the pathogenesis of other obesity-related conditions [4-7]. Prior investigation of children diagnosed with metabolic syndrome in Hawai‘i found that approximately two-thirds had elevated serum alanine aminotransferase (ALT) values suggestive of NAFLD [8].

The majority of children with NAFLD suffer from psychological, physical and pain-related symptoms, which contribute to a lower physical and psychosocial health, and reduced quality of life [9]. When compared to obese controls, pediatric NAFLD patients were found to have greater depression and influence of body weight on self-esteem [10]. Moreover, hepatic steatosis may be accompanied by inflammation and/or fibrosis, which can progress to liver cirrhosis requiring transplantation [1, 11-13]. Along with this, children with NAFLD were found to have reduced age- and sex-standardized survival free of liver transplantation [13].

Similar to other obesity-related conditions, weight loss is the primary treatment strategy for overweight children with NAFLD, and has been found to have good efficacy in clinical trials [14-16]. However, the effectiveness of weight reduction efforts on pediatric NAFLD in free-living individuals is not well established. One retrospective chart audit noted that nearly half (49%) of the pediatric NAFLD patients were able to lose 10% of their initial body weight, and that the vast majority of them (86%) saw a reduction in liver aminotransferases [13]. However, by the time of the final follow up, most of these patients (76%) re-gained the lost weight with many patients (46%) having recrudescence of liver aminotransferases [13]. Weight loss treatment is particularly challenging to implement and evaluate in pediatrics because children are
still developing, and changes in body weight may be confounded by increases in height. For this reason, age and sex standardized BMI should be used to monitor lifestyle interventions, especially in the long-term [15-16].

The purpose of this study is to evaluate the effectiveness of weight loss on reducing serum alanine aminotransferase (ALT) in pediatric NAFLD. In addition to providing preliminary data on pediatric NAFLD in Hawai’i, this study will examine the relationship between changes in body weight and BMI z-score for age and gender, and serum ALT in the outpatient setting.

Methods
The source population for this study was patients referred to two pediatric gastroenterologists at Kapi’olani Medical Center for Women and Children (KMCWC) with elevated serum ALT or fatty liver between January 2000 and December 2010 (n = 124). Subjects with positive testing for hepatic viral infections, autoimmune hepatitis or metabolic liver diseases, or taking hepatotoxic medications (33/124 (27%)) were excluded. Additionally, as the primary purpose of this study was to evaluate the effectiveness of weight reduction on serum ALTs, subjects were excluded if they were not overweight (BMI <85th percentile for age-and-sex; 2/124 (2%)), or had normal serum ALTs (<25.8 U/L for boys, <22.1 U/L for girls; 4/124 (3%)). The upper limit of normal for serum ALT was based on the 95th percentile for healthy boys and girls in the United States, which has been demonstrated to have superior sensitivity with only minor losses in specificity for diagnosis of NAFLD compared to the higher cutoffs used in most children’s hospitals [17]. Finally, four subjects (3%) were missing data, leaving 81/124 (65%) of the source population available for analysis. The University of Hawai’i Committee on Human Studies and Hawaii Pacific Health Research Institute provided ethical approval for this study.
Demographic, anthropometric, biochemical and clinical data on subjects were collected from medical charts. Body weight and height were used to calculate BMI z-scores according to the 2000 CDC growth charts for age and sex using the lambda, mu and sigma (LMS) technique [18]. Systolic and diastolic blood pressures were converted into z-scores for age, sex and height as per the National High Blood Pressure Education Working Group on High Blood Pressure in Children and Adolescents recommendations [19]. Self-reported ethnicity was available in the charts of 30/81 (37%) subjects. The remaining subject ethnicities were determined using the surname list method designed for the Multiethnic Cohort Study [20]. When a surname corresponded to more than one ethnic group, the subject was assigned to the dominant ethnicity in Hawaiʻi [21]. There were 11/40 (28%) subjects whose surnames were not found on the surname list. These subjects were considered White because both White and African American ethnicities are not represented in the surname list, and Whites are more common in Hawaiʻi [20-21]. Subjects were then classified as American Indian, Asian (Chinese, Japanese, Korean, Filipino), Hispanic, Pacific Islander (Native Hawaiian, Samoan), or White as per the the National Institutes of Health (NIH) guidelines for race and ethnicity [22].

Characteristics of subjects at the time of referral to the gastroenterologist were summarized as frequency and percentages for categorical variables, mean and standard deviations for parametric continuous variables, and median and interquartile ranges for nonparametric continuous variables (Table 4.1). The designation of continuous variables as parametric (body weight, height, BMI z-score, HDL-cholesterol and diastolic blood pressure) or nonparametric (age, fasting blood glucose, total- and LDL-cholesterol, triglycerides, systolic blood pressure, and alanine aminotransferase) was based on Shapiro-Wilk test, and confirmed by analysis of frequency distribution graphs. The prevalence of co-morbidities including obesity,
fasting hyperglycemia, dyslipidemias and hypertension was determined using previously published criteria in pediatric NAFLD patients (Table 1) [23-24]. Serum alanine aminotransferase values of the sample at the time of referral to the gastroenterologist were presented in a histogram for visualization of frequency distribution (Figure 1). Liver biopsy was performed on a subset of subjects (n=13), and the presence of inflammation and fibrosis was assessed based on liver histology reports.

The effectiveness of weight loss intervention was assessed at time intervals of 1–4 months, 5–8 months, 9–12 months, and beyond one year as body weight, BMI z-score and serum ALT using the independent two-sample t-test and Wilcoxon rank sum test for parametric and nonparametric continuous variables, respectively (Table 4.2). Only subjects with both anthropometric and serum ALT measurements obtained as part of the same clinic visit were included in this analysis. When a subject had more than one complete set of data for a given time interval, the visit furthest from the time of the initial consultation was used. The average change in subject height was also included to provide perspective on vertical growth. Sensitivity analysis was performed by comparison of subjects with follow-up data to those without follow-up data at each time interval using the independent two-sample t-test and Wilcoxon rank sum test for parametric and nonparametric variables, respectively (Table 4.3).

The relationships between the changes in body weight and BMI z-score, and the changes in serum ALT were examined at each time point using Pearson correlation and linear regression adjusting for initial serum ALT, and baseline values for body weight and BMI z-score for the corresponding parameter (e.g., analysis of change in body weight and change in serum ALT included initial body weight and initial serum ALT as covariates) (Table 4.2). The changes in body weight and BMI z-score, and changes in serum ALT at each time interval were graphed as
a scatterplot for visual presentation of data (Figures 1 and 2). Statistical tests were carried out using SAS v. 9.2, and graphs were created using Microsoft Excel for Mac 2011 v. 14.2.5.

Results

The characteristics of the subjects at the time of referral are presented in Table 4.1. The majority of subjects were male (70/81 (86%)) and Asian (51/81 (63%)), and the median age was 14.1 years (interquartile range 11.2–16.2 years). Almost everyone had a BMI at or above the 95th percentile for age and sex (80/81 (99%)), and many had obesity-related comorbidities, although this could only be assessed in 27-34/81 (33-42%) and 59/81 (73%) of the subjects for laboratory and clinical parameters, respectively. Among the comorbidities assessed, hypertriglyceridemia (20/32 (63%)), hypercholesterolemia (21/34 (62%)) and low HDL-cholesterol (14/27 (52%)) were present in more than half of the subject tested, whereas hypertension (22/59 (37%)), high LDL-cholesterol (8/30 (27%)) and fasting hyperglycemia (6/33 (18%)) were less common. The serum ALT value of most patients was well above the upper limits of normal (Figure 4.1), and the median serum ALT was 105 U/L (interquartile range 78-153 U/L). Of the 13 subjects with liver biopsies, 13/13 (100%) had features of inflammation and 10/13 (77%) had fibrosis, which was noted to be at the stage of bridging fibrosis in three subjects (data not shown).

Follow up data was available for 47/81 (58%) subjects from 1-4 months, 26/81 (32%) subjects from 5-8 months, and 19/81 (23%) subjects from 9-12 months and beyond one year (Table 4.2). Baseline serum ALT was consistently higher in subjects that had follow up data compared to those that did not, and this difference was statistically significant for the 1-4 months (p=0.026) and 5-8 months (p=0.026) time intervals, and trended towards significance in subjects with follow up data beyond one year (p=0.056) (Table 3). In the 5-8 month time interval, baseline body weight (p=0.024) and BMI z-score (p=0.072) tended to be lower in the group that
had follow up data available (Table 4.3).

Subject’s gained weight consistently over the time intervals, but the BMI z-scores tended to be lower as this was at least matched by vertical growth (Table 4.2). None of these changes reached statistical significance. Average serum ALT values were reduced substantially in the follow up time periods (-45 to -30 U/L), although there was a considerable variability in subject response (standard deviations 65 to 75 U/L) (Table 4.2). Despite the large inter-individual variance, the change in serum ALT was statistically significant in the 1-4 months ($p=0.016$) and beyond one year ($p=0.014$) time periods (Table 4.2).

The associations between changes in body weight and BMI z-score, and serum ALT at each time interval are depicted in Figures 2 and 3. As expected, decreases in body weight and BMI z-score generally corresponded to decreases in serum ALT (Table 4.2). However, the correlations coefficients were small for both the change in body weight (0.076 to 0.371) and BMI z-score (-0.012 to 0.235), and did not reach statistical significance at any time interval (Table 4.2). Adjusting for baseline values in linear regression analysis did not change these findings (Table 4.2).

**Discussion**

The results of this study provide a preliminary description of pediatric NAFLD in Hawai’i. Asians made up the majority of subjects in this sample, but Whites, Hispanics and Pacific Islanders were also fairly prevalent, reflecting the diverse ethnic makeup of Hawai’i [21]. Similar to what has been observed elsewhere, subjects were mostly obese, male and in their second decade of life, and many suffered from other comorbidities, particularly dyslipidemia [2,4-5,13,25-26]. Liver biopsy performed in these pediatric patients often revealed inflammation and fibrosis characteristic of steatohepatitis including three subjects with bridging fibrosis [1].
The reason that pediatric NAFLD is more common in males and increases early in the second decade of life is not known, although several mechanisms have been proposed. This period of childhood development is associated with changes in body fat deposition, insulin sensitivity and hepatic antioxidant defenses, which may confer greater susceptibility to alterations in lipid metabolism and liver disease [27-30]. Boys and girls appear to differ in these physiological changes [27-30].

These results suggest that pediatric NAFLD patients in Hawai‘i generally present with hepatic inflammation and fibrosis, and often suffer from other conditions. The amount of hepatic inflammation and fibrosis exceeds that which has been reported in large histological studies conducted in Asian populations and on the mainland United States [1,31]. However, this was not a representative sample of patients, and the decision to obtain a liver biopsy was biased towards patients that were thought to have more advanced disease. The high prevalence of comorbidities is expected given elevated BMIs in the sample, and is consistent with other reports of pediatric NAFLD [4-5]. Additionally, it is not surprising that dyslipidemias were among the most common comorbidities because NAFLD is a disorder of lipid metabolism [32].

The serum ALT of subjects tended to decrease from the time of referral suggesting improvements in liver disease, although this was not related to changes in body weight or BMI z-score. These findings contrast prior clinical trials investigating the effects of weight loss on pediatric NAFLD, which have reported strong associations between reductions in body weight and BMI z-score, and indicators of liver disease [14-16]. Unlike the patients in this study, subjects in those clinical trials were volunteers that received comprehensive treatment and monitoring regiments, which are generally not reflective of standard care [14-16]. This distinction is supported by the findings of the trial by Reinehr et al. (2009), which included a
control group consisting of children unable to participate in the lifestyle intervention program [16]. While the treatment group had significant reductions in BMI z-score, serum ALT and liver ultrasound at one and two years follow up, the control group remain unchanged for all outcomes [16]. The Treatment of Nonalcoholic Fatty Liver Disease in Children (TONIC) study, a multicenter clinical trial conducted by the Nonalcoholic Steatohepatitis Clinical Research Network, further supports our findings [33-34]. Although the TONIC study was designed to investigate the effects of vitamin E and metformin in pediatric NAFLD, all participants received standard diet and exercise advice for weight loss and were monitored over a 96-week period [34]. The placebo group had improvements in serum ALT (-35.2 U/L (95% CI -56.9 to -13.5 U/L)) and histological features of NAFLD despite negligible changes in BMI z-score (-0.01 (95% CI -0.08 to 0.06)) and increased body weight (+12.7 kg (95% CI 9.7 to 15.6 kg)) [33].

There are several possible explanations for the lack of association between body weight and BMI z-score, and serum ALT that was observed in this study. Although adipose tissue, particularly around the viscera, is thought to contribute to the pathogenesis of NAFLD, it is unlikely that the reversal of liver disease associated with weight loss in pediatric NAFLD patients seen in clinical trials can be solely attributed to the shrinking of fat stores [14-16,35-36]. Lifestyle interventions targeted towards weight reduction often include recommendations to increase aerobic exercise and reduce dietary intake of added sugars, which may have independent effects on pediatric NAFLD [37-38]. Furthermore, it is possible that changes in body weight and BMI z-score were not sensitive to decreases in body fatness due to increases in muscle mass [39]. Finally, this was a pilot study based on limited sample of patients that had follow up data, which increases the risk of β-errors. This was more pronounced at the later time intervals (contained only 23% of initial sample), and was biased towards subjects that required
continued follow up due to greater disease severity or non-response to treatment (Table 3). However, the small correlated coefficients that were observed underscore the weak relationship between these variables.

These findings may reflect an important distinction between efficacy and effectiveness of weight loss in pediatric NAFLD. In the controlled setting of a weight loss intervention trial, many overweight and obese pediatric NAFLD patients were able to achieve clinically relevant reductions in body weight or BMI z-score, which were associated with improvements in serological and radiological indicators of liver disease [14-16]. However, in this sample of mostly obese outpatient pediatric NAFLD patients, body weight tended to increase and BMI z-score remained similar across the time intervals, a finding that was also observed in the TONIC trial [33]. It is possible that the associations between the changes in body weight and BMI z-score, and liver disease only exist in the range of weight loss that is generally found in intervention trials. Alternatively, the relationship between the changes in physical activity and diet that reduce liver disease, and changes in body weight and BMI z-score may be stronger in the controlled setting of a clinical trial.

The results of this study indicate that changes in body weight are not an appropriate primary outcome in obese pediatric NAFLD patients. Although body weight is easily measured, and may reflect lifestyle changes in experimental conditions, its role in free-living patients treated in the outpatient setting has not been established. Overemphasis on weight reduction, which is unlikely to be maintained in a growing child, may have lasting, negative effects on these patients that have already been identified as being more sensitive to weight-related poor self-esteem [10,13]. These findings need to be replicated in a larger, prospective study that directly measures diet and physical activity. Based on these findings, greater emphasis should be placed
on patient adherence with modifiable behaviors that are thought to contribute to NAFLD.
References


Table 4.1 Demographic, anthropometric, biochemical and clinical characteristics of subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>n</th>
<th>Value</th>
<th>Normal Range</th>
<th>Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>81</td>
<td>14.1 (11.2-16.2)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Sex (n (%) Male)</td>
<td>81</td>
<td>70 (86%)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Ethnicity (n (%))</td>
<td>81</td>
<td></td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>American Indian</td>
<td>1</td>
<td>1 (1%)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Asian</td>
<td>51</td>
<td>63%</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Hispanic</td>
<td>10</td>
<td>12%</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Pacific Islander</td>
<td>6</td>
<td>7%</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>13</td>
<td>16%</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>81</td>
<td>86.5 ± 26.3</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Height (m)</td>
<td>81</td>
<td>1.60 ± 0.14</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>BMI (z-score)</td>
<td>81</td>
<td>2.30 ± 0.40</td>
<td>&lt;1.64</td>
<td>80/81 (99%)</td>
</tr>
<tr>
<td>Glucose, Fasting (mmol/L)</td>
<td>33</td>
<td>5.4 (4.9-5.8)</td>
<td>&lt;6.1</td>
<td>6/33 (18%)</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>34</td>
<td>4.8 (4.1-5.7)</td>
<td>&lt;4.4</td>
<td>21/34 (62%)</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>30</td>
<td>2.7 (2.4-3.4)</td>
<td>&lt;3.4</td>
<td>8/30 (27%)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>27</td>
<td>1.1 ± 0.3</td>
<td>M &gt;1.0; F &gt;1.3</td>
<td>14/27 (52%)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>32</td>
<td>1.9 (1.5-2.5)</td>
<td>&lt;1.7</td>
<td>20/32 (63%)</td>
</tr>
<tr>
<td>Systolic Blood Pressure (z-score)</td>
<td>59</td>
<td>1.19 (0.72-1.84)</td>
<td>&lt;1.64</td>
<td>22/59 (37%)</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (z-score)</td>
<td>59</td>
<td>0.70 ± 1.07</td>
<td>&lt;1.64</td>
<td></td>
</tr>
<tr>
<td>Alanine Aminotransferase (U/L)</td>
<td>81</td>
<td>105 (78-153)</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

BMI adjusted for age and sex using the 2000 Centers for Disease Control and Prevention growth charts and lambda, mu and sigma method.\(^{18}\)
Systolic and diastolic blood pressures adjusted for age, sex and height as per the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents recommendations.\(^{19}\)
Values are presented as number (percent) for categorical variables, mean ± standard deviation for parametric continuous variables and median (interquartile range) for nonparametric continuous variables. Continuous variables were classified as nonparametric based on Shapiro-Wilk test, and confirmed by analysis of frequency distribution graphs.
Normal ranges as per Schwimmer et al. (2003) and Graham et al. (2009).\(^{23 \text{-} 24}\) Abnormal values are frequency (percent) of subjects outside the normal range. Hypertension was present if either systolic or diastolic values were above normal.\(^{24}\)
### Table 4.2 Effectiveness of weight loss treatment in children with nonalcoholic fatty liver disease

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Δ Weight (kg)</th>
<th>Δ Height (m)</th>
<th>Δ BMI (z-score)</th>
<th>Δ ALT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T&lt;sub&gt;1-4&lt;/sub&gt; (n = 47)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Value</td>
<td>-0.2±2.9 (0.976)</td>
<td>+0.01±0.01 (0.740)</td>
<td>-0.05±0.11 (0.532)</td>
<td>-32±66 (0.016)</td>
</tr>
<tr>
<td>Correlation</td>
<td>0.076 (0.611)</td>
<td>-0.012 (0.937)</td>
<td>-0.05 (0.740)</td>
<td></td>
</tr>
<tr>
<td>Regression</td>
<td>4.72 (0.107)</td>
<td>68.8 (0.377)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T&lt;sub&gt;5-8&lt;/sub&gt; (n = 26)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Value</td>
<td>+1.4±4.8 (0.825)</td>
<td>+0.02±0.02 (0.583)</td>
<td>-0.07±0.15 (0.493)</td>
<td>-30±65 (0.134)</td>
</tr>
<tr>
<td>Correlation</td>
<td>0.560 (0.071)</td>
<td>0.235 (0.247)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regression</td>
<td>3.28 (0.187)</td>
<td>101.1 (0.213)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T&lt;sub&gt;9-12&lt;/sub&gt; (n = 19)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Value</td>
<td>+3.3±4.7 (0.680)</td>
<td>+0.03±0.03 (0.425)</td>
<td>-0.05±0.15 (0.718)</td>
<td>-37±75 (0.157)</td>
</tr>
<tr>
<td>Correlation</td>
<td>0.077 (0.753)</td>
<td>0.107 (0.662)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regression</td>
<td>1.08 (0.617)</td>
<td>51.3 (0.424)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T&gt;1 (n = 19)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Value</td>
<td>+7.1±9.0 (0.471)</td>
<td>+0.07±0.06 (0.219)</td>
<td>-0.12±0.21 (0.453)</td>
<td>-45±69 (0.014)</td>
</tr>
<tr>
<td>Correlation</td>
<td>0.371 (0.118)</td>
<td>0.176 (0.471)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Regression</td>
<td>3.10 (0.073)</td>
<td>69.0 (0.320)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*T<sub>1-4</sub> = time interval 1-4 months, T<sub>5-8</sub> = time interval 5-8 months, T<sub>9-12</sub> = time interval 9-12 months, T>1 = time interval beyond one year, SD = standard deviation, ALT = alanine aminotransferase

BMI adjusted for age and sex using the 2000 Centers for Disease Control and Prevention growth charts and lambda, mu and sigma method. Values are the change in parameter presented as mean ± standard deviation with p-value in parentheses based on independent two-sample t-test and Wilcoxon rank sum test for parametric and nonparametric continuous variables, respectively. Association between changes in body weight and BMI z-score, and changes in serum alanine aminotransferase assessed by Pearson correlation and linear regression, and presented as correlation coefficients and regression coefficients with p-values in parentheses. Linear regression adjusted for initial serum alanine aminotransferase, and baseline values for body weight and BMI z-score of the corresponding analysis.
### Table 4.3 Comparison of subjects included and not included in the analysis according to time interval

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Included</th>
<th>Not Included</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1-4 Months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample Size</td>
<td>47</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>86.5 ± 25.4</td>
<td>86.4 ± 27.8</td>
<td>0.984</td>
</tr>
<tr>
<td>BMI (z-score)</td>
<td>2.29 ± 0.37</td>
<td>2.31 ± 0.45</td>
<td>0.817</td>
</tr>
<tr>
<td>Alanine Aminotransferase (U/L)</td>
<td>124 (79-189)</td>
<td>92 (73-122)</td>
<td>0.026</td>
</tr>
<tr>
<td><strong>5-8 Months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample Size</td>
<td>26</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>76.9 ± 23.2</td>
<td>91.0 ± 26.6</td>
<td>0.024</td>
</tr>
<tr>
<td>BMI (z-score)</td>
<td>2.18 ± 0.40</td>
<td>2.35 ± 0.40</td>
<td>0.072</td>
</tr>
<tr>
<td>Alanine Aminotransferase (U/L)</td>
<td>124 (96-196)</td>
<td>100 (73-148)</td>
<td>0.026</td>
</tr>
<tr>
<td><strong>9-12 Months</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample Size</td>
<td>19</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>82.0 ± 25.4</td>
<td>87.8 ± 26.6</td>
<td>0.379</td>
</tr>
<tr>
<td>BMI (z-score)</td>
<td>2.17 ± 0.45</td>
<td>2.33 ± 0.38</td>
<td>0.120</td>
</tr>
<tr>
<td>Alanine Aminotransferase (U/L)</td>
<td>109 (77-196)</td>
<td>104 (78-152)</td>
<td>0.359</td>
</tr>
<tr>
<td><strong>Beyond 1 Year</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample Size</td>
<td>19</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Body Weight (kg)</td>
<td>79.4 ± 31.3</td>
<td>88.6 ± 24.4</td>
<td>0.185</td>
</tr>
<tr>
<td>BMI (z-score)</td>
<td>2.37 ± 0.48</td>
<td>2.27 ± 0.38</td>
<td>0.240</td>
</tr>
<tr>
<td>Alanine Aminotransferase (U/L)</td>
<td>118 (88-198)</td>
<td>102 (77-151)</td>
<td>0.056</td>
</tr>
</tbody>
</table>

BMI adjusted for age and sex using the 2000 Centers for Disease Control and Prevention growth charts and lambda, mu and sigma method.\(^{18}\)

Values are presented as mean ± standard deviation for parametric continuous variables and median (interquartile range) for nonparametric continuous variables. Continuous variables were classified as nonparametric based on Shapiro-Wilk test, and confirmed by analysis of frequency distribution graphs. Comparison of groups was conducted using independent two-sample t-tests and Wilcoxon rank sum tests for parametric and non-parametric variables, respectively.
Figure 4.1 Frequency distribution of serum alanine aminotransferase (U/L) in subjects
**Figure 4.2** Scatterplot of subject’s change in body weight and change in serum alanine aminotransferase at time intervals of 1-4 month, 5-8 months, 9-12 months and greater than 1 year.

1 – 4 Months (n = 47)  
Correlation Coefficient (p-value) = 0.076 (0.611)

5 – 8 Months (n = 26)  
Correlation Coefficient (p-value) = 0.360 (0.071)

9 – 12 Months (n = 19)  
Correlation Coefficient (p-value) = 0.077 (0.753)

>1 Year (n = 19)  
Correlation Coefficient (p-value) = 0.371 (0.118)

ALT = alanine aminotransferase

The association between changes in body weight and changes in serum alanine aminotransferase was assessed by Pearson correlation, and is presented as correlation coefficient (p-value).
Figure 4.3 Scatterplot of subject’s change in BMI z-score and change in serum alanine aminotransferase at time intervals of 1-4 month, 5-8 months, 9-12 months and greater than 1 year

1 – 4 Months (n = 47)
Correlation Coefficient (p-value) = -0.012 (0.937)

5 – 8 Months (n = 26)
Correlation Coefficient (p-value) = 0.235 (0.247)

9 – 12 Months (n =19)
Correlation Coefficient (p-value) = 0.107 (0.662)

>1 Year (n =19)
Correlation Coefficient (p-value) = 0.176 (0.471)

ALT = alanine aminotransferase
BMI adjusted for age and sex using the 2000 Centers for Disease Control and Prevention growth charts and lambda, mu and sigma method. The association between changes in BMI z-score and changes in serum alanine aminotransferase was assessed by Pearson correlation, and is presented as correlation coefficient (p-value).
CHAPTER 5: ESTIMATION OF FISH INTAKE IN ASIAN AND WHITE FEMALE ADOLESCENTS, AND ASSOCIATION WITH TWO-YEAR CHANGES IN BODY FATNESS AND BODY FAT DISTRIBUTION: THE FEMALE ADOLESCENT MATURATION (FAM) STUDY

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\textsuperscript{1}University of Hawaii at Manoa
Abstract

Background: Fish is an important source of long-chain omega-3 fatty acids in the diets of female adolescents, which are thought to have an effect on adipose tissue deposition.

Objective: The purpose of this study was to evaluate fish intake in Asian and White female adolescents, and to determine if fish intake has an effect on changes in body fatness and distribution in this population.

Design: A cross-sectional analysis of fish intake using three-day food records (n=200), and prospective study of baseline fish intake on anthropometric measurements two years later was conducted (n=103).

Participants/Setting: Subjects obtained among female adolescents (9-14 years) that were recruited from Kaiser Permanente Oahu membership database in 2000-01 as part of the Female Adolescent Maturation (FAM) study (n=349).

Statistical Analysis: Fish intake and the proportion of subjects eating eight ounces of fish per week was compared between Asian, White and Mixed Asian/White ethnic groups using Kruskal Wallis test and Wilcoxon rank sum test, and Chi-square test, respectively. The effect of fish intake on anthropometric measurements was assessed using Spearman’s rank correlation coefficient, and linear regression analyses, adjusting for demographic, puberty, anthropometric, activity and dietary parameters.

Results: Asians consumed more fish (0.85 (0.00-4.74) oz/wk) than Whites (0.00 (0.00-0.40) oz/wk; p=0.0001), and were more likely to eat eight ounces of fish per week (13/68 vs 2/51, respectively; p=0.014). Greater fish intake corresponded to smaller changes in waist circumference when controlling for age, ethnicity, puberty, activity, energy intake and baseline waist circumference (p=0.026), but not after adjusting for additional dietary parameters (p>0.01).
**Conclusions:** Most female adolescents do not consume the recommended amount of fish, a problem that was more common in Whites than Asians. Greater fish intake may have a protective effect on abdominal obesity, but further study is needed.
Introduction

Obesity is a major concern among female adolescents in the United States where an estimated one-third have a body mass index (BMI) at or above the 85th percentile for age and sex.\(^1\) Although overweight and obesity are typically defined using the BMI, body fatness and distribution of fat in the body are the underlying factors that link excess body weight to disease risk.\(^2-4\) Dietary patterns are thought to be an important determinant of obesity risk through their effect on energy balance, but little is known about the impact of dietary components on the partitioning of excess energy toward adipose tissue, and on fat distribution in the body.

Fish is a nutritionally significant component in the diet because it is one of the few sources and main contributors to long-chain omega-3 fatty acid intake.\(^5\) In recognition of the importance of fish in nutrition and health, the United States Department of Agriculture,\(^6\) Academy of Nutrition and Dietetics and Dietitians of Canada,\(^7\) and American Heart Association and American Academy of Pediatrics\(^8\) recommend that adolescents consume approximately two servings or eight ounces of fish per week. However, a study of more than 1,000 ninth grade students found that only 36% reported that they eat fish at least once per week despite the fact that most (89%) believed that fish was healthy.\(^9\)

Long-chain omega-3 fatty acids are known to affect lipid metabolism in the body through their effects on transcription factors including peroxisome proliferator-activating receptors, and sterol-regulatory element binding proteins, which reduce lipogenesis and promote fat oxidation.\(^10-11\) Studies using animal models of obesity have observed a decrease in body fat\(^12-14\) and visceral fat measured by dissection\(^12-13\) with long-chain omega-3 fatty acid intake. These effects have not been investigated in humans. However, Asian-American females, particularly those that follow a more traditional Asian diet, have higher fish intakes than Whites\(^15-16\), but
partition a greater portion of body fat in the trunk.\textsuperscript{17} Fish vary dramatically in their long-chain omega-3 fatty acid content\textsuperscript{18}, which may explain this contradiction.

The purpose of this study was to compare the distribution and adequacy of fish intake in Asian, White and Mixed Asian/White female adolescents, and to examine the effect of fish intake on changes in body fatness and distribution of body fat in this population over a two-year period.

**Methods**

Data for this study was collected as part of the Female Adolescent Maturation (FAM) study, which has been described previously.\textsuperscript{17} Briefly, female adolescents (9-14 years) were recruited from the Kaiser Permanente Oahu membership database in 2000-2001 (Exam 1, n=349), and followed prospectively. Subjects that were known to have chronic diseases or asthma, or who were prescribed steroid or antiepileptic medications were not recruited. Only subjects that identified themselves as Asian, White, or a mixture of only Asian and White ethnicities (n=214, 61.5\%) were included. An additional 14 subjects that did not complete the dietary analysis were excluded, leaving 200 (57.5\%) Asian and/or White female adolescents for analysis of fish intake. Of this sample of 200 subjects, 104 (52.0\%) participated in the first follow-up examination (Exam 2), which was conducted two years plus or minus two months later. One of these subjects was missing anthropometric variables, so 103/200 (51.5\%) were used to evaluate the effect of fish intake on changes in body fatness and fat distribution. The Kaiser Permanente Hawaii and University of Hawaii Institutional Review Boards approved this study.

Information on subject’s age, ethnicity and physical activity was collected through a questionnaire in Exam 1. Ethnicity was reported by parents/guardians based on proportion of
Asian or White ethnicity in the biological mother and father, which was used to determine the percent Asian ethnicity of the subject. For example, a girl with a father that was 50% Asian and 50% White, and a mother that was 100% Asian would be considered to be 75% Asian and 25% White. The proportion of Asian ethnicity was used to classify subjects as Asian (100% Asian), White (0% Asian) or Mixed (1-99% Asian). Asian ethnicities included Japanese, Korean, Chinese, Filipino, Indian, Thai, and Vietnamese. Total physical activity was calculated as the sum of reported activities that were performed at least 10 times in the previous year by converting frequency and duration of activity into metabolic equivalents (METs) based on values published by Lee et al. (1992).

Subjects were asked to complete a three-day food record (Thursday, Friday and Saturday) with dietary supplement questionnaire one-week prior to Exam 1 and Exam 2 with the assistance of their parent or guardian(s). To improve accuracy, detailed examples of food records and dietary supplement questionnaires, and measuring tools including measuring cup and spoon, and a ruled edge of paper were provided. Food records were analyzed at the University of Hawaii Cancer Center using the Shared Nutrition Food Composition Data Base (v. 1999), which contains food group and nutrient data from the USDA databases that have been supplemented with foods commonly eaten by ethnic groups in Hawaii. Fish intake was sub-categorized into high and low omega-3 fatty acid content as per the MyPyramid Food Guidance System Education Framework. The use of dietary supplements made from fish oil, or containing omega-3 fatty acids was obtained from the dietary supplement questionnaire.

Mean daily fish consumption was multiplied by seven to provide more tangible amounts, and to allow for direct comparison to dietary recommendations, which are based on weekly intake. The frequency of fish consumption was determined based on the proportion of the
three, 24-hour dietary records that contained any fish. Among subjects that consumed fish, the mean amount of fish consumed on a fish consumption day was calculated by dividing three-day fish intake by the number of days in which fish was eaten. Additionally, the proportion of high and low omega-3 fatty acid fish consumption days with at least a three-ounce intake of high and low omega-3 fatty acid fish was determined. The decision to use a three-ounce was made post-hoc due to the limited number of four-ounce consumption days for high (n = 2) and low (n = 8) omega-3 fatty acid fish. Finally, subject high-omega-3 fatty acid fish intake was divided their total fish intake and multiplied by 100% to estimate the percent of fish consumed that were high in omega-3 fatty acids containing sources.

Physical examinations were performed on subjects to evaluate body composition and pubertal development. Body weight and height were measured using a digital scale (Seca) and stadiometer (Measurement Concepts) in kg and cm, respectively, by a standardized anthropometrist. Waist circumference was taken at the midpoint between the ribs and the iliac crest using inextensible measuring tape (Hoechst) in cm. Skinfold thickness was measured using Lange Skinfold Caliper (Beta Technology) in mm at the subscapular, triceps, biceps and iliac sites. If duplicate measurements of body weight, height, waist circumference or skinfold thickness were more than two units apart, a third measurement was taken, and the average of the two closest values was used. Total, trunk and peripheral fat mass was assessed using dual energy x-ray absorptiometry (DXA, Lunar Prodigy) during Exam 2 only, and the ratio of trunk-to-peripheral fat was calculated as per Novotny et al. (2006).\textsuperscript{17} Pubertal development was determined by a nurse practitioner according to Tanner staging criteria for breast and pubic hair.\textsuperscript{21}
Body weight and height were used to calculate BMI (weight in kg / height$^2$ in m$^2$), which was converted into z-scores for age and sex using the lambda, mu and sigma method.$^{22}$ Skinfold thickness measurements were combined to provide estimates of total (Σ subscapular, triceps, biceps and iliac crest), trunk (Σ subscapular and iliac crest), and trunk-to-peripheral ratio (Σ subscapular and iliac crest / Σ triceps and biceps) of body fat. Using the data from Exam 2, these estimates were compared to DXA measurements using Spearman’s rank correlation coefficient, and found to have strong correlations for total (Rho = 0.843; p<0.0001) and trunk (Rho = 0.839; p <0.0001) fat mass, and a moderate correlation for trunk-to-peripheral fat mass (Rho = 0.464; p <0.0001). DXA measurements for total and trunk fat mass were converted into percent body fat and trunk fat, respectively for analysis. Lastly, the scores for breast and pubic hair Tanner stage were added together to provide an overall Tanner score as a measure of pubertal development.

For the descriptive component of the study, a cross-sectional analysis was conducted on the 200 Asian and/or White female adolescents with dietary data from Exam 1. Subjects were categorized according to ethnicity as Asian, White or Mixed Asian/White, and demographic, anthropometric, physical activity, dietary and development characteristics were summarized and compared across groups. Continuous variables were classified as parametric (age, BMI z-score, percent of energy intake from protein, fat and carbohydrates) or nonparametric (waist circumference, physical activity, energy intake, frequency, consumption day quantity and weekly intake of fish, percent of high-omega-3 fatty acid fish, Tanner score) based on the Shapiro-Wilks test and analysis of frequency distribution graphs. Data were summarized as mean ± standard deviation and median (interquartile range), and compared across Asian, White and Mixed Asian/White ethnic groups using analysis of variance and Kruskal-Wallis test, and between Asian and White ethnic groups using independent two-sample t-test and Wilcoxon rank sum test.
for parametric and nonparametric variables, respectively. The frequency of fish intake and number of subjects consuming the recommended eight ounces of fish per week were presented as numbers and frequency (percent), and differences across ethnic groups were assessed using Chi-square test.\textsuperscript{6-7} Additionally, the consumption day quantity of fish intake in the Asian and White ethnic groups independent of frequency of fish consumption was compared using two-way analysis of variance of the log transformed quantity of fish intake. The least squares means (LS means) for the consumption day quantity of fish for the Asian and White ethnic groups were back-transformed, and presented in the original scale.

Overall patterns of fish consumption were analyzed using data from subjects that reported eating any fish in their three, 24-hour dietary records (n = 100). Fish intake can be regarded as the product of the probability of eating fish and the usual intake amount on days when fish is consumed, although these variables are not necessarily independent of one another.\textsuperscript{23} To determine if these factors were related in this population, the frequency of fish intake (1/3, 2/3 or 3/3 days), and the mean consumption day quantity were analyzed using Spearman’s rank correlation coefficient. Consumption day quantity of fish was further analyzed to determine the proportion of days in which at least three-ounces of fish were eaten for total, and high and low omega-3 fatty acid fish. These values were presented as a frequency (percent), and the proportion of three-ounce consumption day quantities were compared between high and low-omega-3 fatty acid fish using Chi-square test. Additionally, the relationship between the consumption day quantity of fish, and the percent of high omega-3 fatty acid fish was assessed using Spearman’s rank correlation coefficient. Finally, for subjects with dietary data for Exam 1 and 2 (n=103), estimates of dietary intake of fish collected at Exam 2 were compared to Exam 1
value using Spearman’s rank correlation coefficient to evaluate the two-year reliability of three, 24-hour dietary records for fish.

Subjects that participated in both Exam 1 and 2 (n = 103) were evaluated prospectively for the analytical component of this study, which examined the relationship between fish intake and changes in body fatness and body fat distribution. Anthropometric measurements were summarized for Exam 1 and 2 as mean ± standard deviation and median (interquartile range) for parametric (BMI z-score, trunk-to-peripheral fat ratio (skinfold thickness and DXA), and percent body fat and trunk fat (DXA)) and nonparametric continuous variables (waist circumference and total and trunk fat (skinfold thickness)), which was based on Shapiro-Wilks test and analysis of frequency distributions. The mean ± standard deviation change in BMI z-score, waist circumference and skinfold thickness parameters from Exam 1 to Exam 2 was determined, and assessed using the independent two-sample t-test and Wilcoxon rank sum test for parametric and nonparametric variables, respectively.

The relationship between fish intake collected at Exam 1, and BMI z-score, waist circumference, total, trunk and trunk-to-peripheral fat mass ratio determined by skinfold measurements, and percent body fat, percent trunk fat and trunk-to-peripheral fat mass ratio determined by DXA was assessed using Spearman’s rank correlation coefficient and two linear regression analyses. The change in anthropometric parameters was used as the dependent variable in these analyses, except for DXA measurements, which were based on values from Exam 2 because they were not collected in Exam 1. The first linear regression analysis (Model 1) adjusted for parameters collected during Exam 1 (age, percent Asian ethnicity, physical activity, energy intake, Tanner score and anthropometric value corresponding to the outcome of interest), and the change in Tanner score between Exam 1 and Exam 2. For DXA measurements,
skinfold thickness estimates of total, trunk and trunk-to-peripheral ratio of fat mass from Exam 1 were included in Model 1 as the baseline anthropometric values corresponding to evaluation of percent body fat, percent trunk fat and trunk-to-peripheral fat mass ratio, respectively, because DXA measurement were not collected during Exam 1. The second linear regression analysis (Model 2) included the all of the variables from Model 1 plus additional dietary variables thought to be related to energy balance and fat partitioning including percent of calories from protein, fat, carbohydrates, saturated fatty acids, polyunsaturated fatty acids and discretionary fat, added sugar, iron and fiber density of the diet, and soda consumption. Variables that were determined to be nonparametric based on Shapiro-Wilks test and analysis of frequency distribution graphs including fish consumption, waist circumference, total and trunk fat (skinfold thickness), physical activity, energy intake, Tanner score, percent of energy from polyunsaturated fatty acids, iron density of the diet, and soda consumption were log-transformed for the linear regression analyses. Due to some zero-values for fish and soda consumption, one unit was added prior to log-transformation.

Sensitivity analysis was performed comparing demographic, anthropometric, dietary and development parameters from the descriptive component of the study between subjects participating in Exam 1 and 2 (n = 103) to those that attended Exam 1 only (n = 97). Variables were compared using Chi-square test for categorical variables, and independent two-sample t-test and Spearman’s rank correlation coefficient for parametric and nonparametric continuous variables, respectively. All statistical tests were performed using Statistical Analysis Software version 9.2 (SAS v. 9.2).
**Results**

The characteristics of subjects that participated in FAM 1 (n = 200) are outlined in Table 5.1 according to ethnic group. There were no differences between Asian or White female adolescents with regards to age, BMI z-score, waist circumference, energy intake, percent of energy from fat or carbohydrates, or Tanner score. However, Asians had lower levels of physical activity (27.3 (13.8-44.2) METs) compared to Whites (40.0 (27.5-62.7) METs; p = 0.002), and a slightly greater proportion of energy intake coming from protein (15.6 ± 2.9 vs 14.5 ± 3.1, p = 0.047). Subjects with Mixed Asian/White ethnic background had a physical activity level that fell between the Asian and White groups (32.2 (16.1-53.0) METs).

Fish intake varied significantly between the ethnic groups (p <0.001) with Asians reporting greater fish consumption (0.85 (0.00-4.74) oz/wk) than Whites (0.00 (0.00-0.40) oz/wk; p = 0.0001) (Table 5.1). This appeared to be related to both a greater frequency of fish consumption, and quantity of fish intake among female adolescents in the Asian group (p = 0.006 and p = 0.021, respectively) (Table 5.1). Along with this, 13/68 (19.1%) Asians had fish intakes that met the recommended eight ounces of fish per week, compared to only 2/51 (3.9%) of Whites (p = 0.014) (Table 5.1). After controlling for differences in frequency of fish consumption, the quantity of fish intake still tended to be higher in Asians (LS mean = 1.035 oz) compared to Whites (LS mean = 0.535 oz), although the difference was no longer statistically significant (p = 0.092). The proportion of subjects that did not indicate any fish consumption during the three-day dietary data collection was 22/68 (32.4%) for Asians, 32/51 (62.7%) for Whites, and 46/81 (56.8%) for Mixed Asian/Whites (Table 1). The Mixed Asian/White group had a marginally higher consumption day quantity of fish intake (1.87 (0.50-2.02) oz/wk) compared to the Asian group (0.97 (0.35-1.85) oz/wk), although this difference was not statistically significant (p = 0.192) (Table 5.1). The percent of fish that were high in omega-3...
fatty acids was 40.2 ± 37.1% (data not shown), and did not vary with ethnicity (p = 0.971) (Table 5.1). Only two subjects reported consuming dietary supplements that contained omega-3 fatty acids. In both cases, these dietary supplements contained a relatively insignificant dose (≤50 mg) of the 18-carbon alpha-linolenic acid rather than the bioactive long-chain omega-3 fatty acids found in fish.

Subjects that reported any fish consumption (n = 100) were grouped together and analyzed collectively. There were 141 total fish consumption days, of which only 23 (16.3%) met or exceeding three ounces. On days when high and low omega-3 fatty acid fish were consumed, only 4/103 (3.9%) of high omega-3 fatty acid fish daily totals were three ounces or more compared to 16/105 (15.2%) of low omega-3 fatty acid fish (p = 0.005). Similarly, the amount of fish consumed on days when fish was eaten was negatively associated with the percent of high omega-3 fatty fish (Rho = -0.211, p = 0.035). When subjects were categorized according to the number of days in which they ate any fish (1/3, 2/3 or 3/3 days), more frequent fish intake was associated with greater mean consumption day quantity of fish (Rho = 0.239 (p = 0.016)).

Subjects that participated in both Exam 1 and 2 (n = 103) were not found to differ significantly from the Exam 1 only group (n = 97) with respect to demographic, anthropometric, dietary or development parameters that were compared (p > 0.05, Table 5.3). Among subjects with dietary data for both Exam 1 and 2 (n = 102), the two-year reliability of the three-day food records was found to be weak for fish intake (Rho = 0.205 (p = 0.039)), and non-significant for fish frequency (Rho = 0.153 (p = 0.124)) and consumption day quantity (n = 33, Rho = -0.137 (p = 0.439)). Waist circumference and skinfold thickness parameters increased substantially between from Exam 1 to Exam 2 (p < 0.0001), but BMI z-score remained similar (0.18 ± 1.03 vs
At the time of Exam 2, total and trunk fat were determined by DXA to be $28.9 \pm 8.1\%$ and $13.3 \pm 14.4\%$, respectively (Table 5.2).

Fish intake during Exam 1 was not associated the change in BMI z-score, waist circumference or skinfold thickness parameters between Exam 1 and Exam 2, or the DXA parameters collected at Exam 2 ($p > 0.05$), although there was a trend towards reduced percent body fat with increasing fish consumption ($Rho = -0.180$ ($p = 0.069$)) (Table 5.2). After controlling for subject age, percent Asian ethnicity, physical activity, energy intake, Tanner score and the anthropometric value corresponding to the outcome of interest at Exam 1, and change in Tanner score between Exam 2 and Exam 2, the apparent trend between fish intake and percent body fat was attenuated ($p = 0.280$), but a statistically significant inverse association with waist circumference was found ($p = 0.026$) (Table 5.2). None of the anthropometric parameters were related to fish intake when adjusting for additional dietary variables, which included percent of calories from protein, fat, carbohydrates, saturated fatty acids, polyunsaturated fatty acids and discretionary fat, added sugar, iron and fiber density of the diet, and soda consumption ($p > 0.10$, Table 5.2).

**Discussion**

The study provides a detailed summary of fish intake in Asian and White female adolescents that includes important contextual information regarding the frequency, quantity and omega-3 fatty acid content of fish consumed for future investigations in this area. Additionally, preliminary human data is presented, which examines the hypothesis that the long-chain omega-3 fatty acids found in fish reduce total and visceral adiposity.

As expected based on prior studies, Asians had greater fish intake than Whites.\textsuperscript{15-16} This appears to be related to both a greater frequency and quantity of fish intake in the Asian group.
The proportion of subjects that did not report any fish in their three-day food records accounted for much of the difference in the frequency in fish intake with nearly two-thirds of Whites not consuming fish compared to less than one-third of Asians. Consistent with others observations of episodically consumed foods, there was a positive correlation between frequency and quantity of fish intake in this study.\textsuperscript{23} Interestingly, after adjusting for frequency of fish intake, Asians still had consumption day quantities of fish that were almost two-fold greater than Whites. Although the difference in quantity of fish intake was not statistically significant, this study was underpowered to test this due to the limited number for subjects in the higher frequency groups. Overall, Asians were almost five-times more likely than Whites to report fish consumption that met the recommended eight ounces of fish per week.\textsuperscript{6-7} However, this still only represented a minority of subjects.

Dietary recommendations to consume fish twice per week are based on the assumption of four-ounce serving sizes.\textsuperscript{7} However, the reported quantities of fish intake in this study rarely exceeded three-ounces, suggesting that more regular fish consumption may be necessary to achieve the target of eight ounces of fish per week in this population.\textsuperscript{6-7} Moreover, although high and low omega-3 fatty acid fish were consumed on a similar number of days, the majority of the fish was from low omega-3 fatty acid species because they tended to be eaten in larger quantities. Differences in fish intake by ethnicity did not appear to account for this observation as the proportion of fish from high omega-3 fatty acid species were similar across ethnic groups. Long-chain omega-3 fatty acids content is one of the main reasons for the widespread recommendations for adolescents to consume fish.\textsuperscript{6-8} These results suggest that the source of fish may be an important target for dietary interventions attempting to increase long-chain omega-3 fatty acid intake.
The anthropometric parameters considered in this study include a diverse selection of body composition measurements, which provide a fairly comprehensive assessment of body fatness and distribution. The two-years between Exam 1 and Exam 2 appears to have been an appropriate period for the investigation of female adiposity as it corresponded to a significant amount of fat being deposited, and an increase in trunk-to-peripheral fat ratio. Moreover, subjects in this study varied considerably in the amount and distribution of fat deposited as evidenced by the large standard deviations, providing the opportunity to evaluate the effects of environmental factors such as fish intake. Importantly, the mean BMI z-scores remained relatively constant in this time suggesting that these changes in body composition were consistent with normal growth and development.

Despite these apparent advantages, fish intake was not correlated with changes in body fatness or distribution in this study. After adjustment for key variables including subject age, ethnicity, physical activity, energy intake, pubertal development and starting anthropometric measurements, greater fish intake was associated with lower waist circumference, but not other obesity parameters. These results suggests that nutrients in fish may reduce the amount of excess energy that is partitioned towards adipose tissue in abdominal region, although this does not appear to correspond to greater storage of adipose tissue in the thoracic or peripheral regions of the body. Given the effect of abdominal obesity on obesity-related comorbidities, this finding could have important public health implications, particularly given the relatively low intakes of fish that were reported.\textsuperscript{2-4} However, the association of fish intake with waist circumference was no longer evident when controlling for additional dietary factors related to energy balance and fat partitioning. Although this indicates that the relationship between fish intake and abdominal
obesity was the result of confounding, it is possible that the overfitting of the linear regression model with regressor variables contributed to the null findings.

One possible explanation for the limited support of the biological mechanism data in this study is the relatively low amount of long-chain omega-3 fatty acids consumed by these subjects compared to the experimental diets used in the animal studies.\textsuperscript{12-14} To put this in context, it is estimated that individuals eating eight ounces of fish per week will obtain approximately 500 mg of long-chain omega-3 fatty acids, which would constitute less than 1\% of mean daily fat intake reported by subjects in their three-day food records.\textsuperscript{7} While a small number of subjects may have achieved this level of intake, it is still far less than the approximately 5-35\% of long-chain omega-3 fatty acid that were provided in the experimental diets for these animal studies.\textsuperscript{12-14} If this dose is necessary for the observed beneficial effects of long-chain omega-3 fatty acids on adiposity, it is unlikely to be achieved through conventional diet alone.

There are limitations of this study that warrant consideration in the interpretation of the results. Most notably, fish intake was measured with error, which may have biased the results towards null findings. In a sample of this size, three-day food records can provide good estimates of many dietary exposures, and are often used as the reference for validation of food frequency questionnaires.\textsuperscript{24} However, for episodically consumed foods such as fish, dietary intake is the product of the probability of intake, and the amount of food consumed on days when it is eaten.\textsuperscript{23} Three days of food intake permit only a crude breakdown of the probability of fish intake as demonstrated by the weak, non-significant two-year reliability for estimation of fish frequency in this study. As further indication, the proportion of subjects that did not consume fish during the three days of dietary data collection greatly exceeds the 17\% and 18\% of Rhode Island adolescents that report consuming fish never or only a few times per year based on food
Despite this limitation, the reliability for fish intake was found to be statistically significant, suggesting that the three-day food records were still able to rank individuals by fish intake. Moreover, it is possible that some of the variance in reliability estimates reflect true dietary changes in fish intake over the two-year period. Additional dietary record days, or supplementation of the food records with frequency questionnaire data may have improved the precision of fish intake estimates. Another important consideration is the unique blend of Asian and White ethnic groups, and food environment of Hawaii. Several of the findings from this study may not be directly applied to Asian and White female adolescents on the mainland United States or elsewhere.

The results of this study showed that most Asian and White female adolescents did not consume the recommended eight ounces of fish per week. This was due to infrequent intake of fish, and serving sizes that were generally much smaller than the four-ounce reference. Additionally, although high and low omega-3 fatty acid fish were consumed on same number of days, the majority of fish came from low omega-3 fatty acid sources because low omega-3 fatty acid fish were eaten in larger amounts than high omega-3 fatty acid fish. As expected, ethnicity was an important determinant of fish intake with Asians reporting greater frequency, consumption day quantity and overall intake of fish compared to Whites. Despite promising research on long-chain omega-3 fatty acids, fish intake did not predict two-year changes in body and trunk fatness. Given the relatively high doses of long-chain omega-3 fatty acids provided to animals in these experiments, dietary supplements may be necessary to evaluate the potential anti-obesity effects of long-chain omega-3 fatty acids in humans. Greater fish intake corresponded to smaller two-year changes in waist circumference when controlling for
key confounding variables, although this association was no longer significant when adjusting for additional dietary factors.

In conclusion, interventions to improve the long-chain omega-3 fatty acid intake of Asian and White female adolescents should target the frequency, quantity and long-chain omega-3 fatty acid content of fish being consumed, particularly in Whites. At current levels of dietary intake, it does not appear that fish consumption has a beneficial effect on two-year changes in body or trunk fatness in Asian and White female adolescents, although a reduction in the storage of fat in the abdominal region may occur, which requires additional study to confirm.
References


15. Mahaffey KR, Clickner RP, Jeffries RA. Adult women’s blood mercury concentrations vary regionally in the United States: Association with patters of


Table 5.1 Summary of demographic, anthropometric, dietary and development characteristics of Asian, White and Mixed Asian/White female adolescents

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
<th>Asian</th>
<th>n</th>
<th>White</th>
<th>n</th>
<th>Mixed</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>68</td>
<td>11.6 ± 1.5</td>
<td>51</td>
<td>11.5 ± 1.54</td>
<td>81</td>
<td>11.3 ± 1.29</td>
<td>0.380</td>
</tr>
<tr>
<td>BMI z-score</td>
<td>68</td>
<td>0.17 ± 1.04</td>
<td>51</td>
<td>0.21 ± 0.95</td>
<td>80</td>
<td>0.35 ± 1.24</td>
<td>0.567</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>68</td>
<td>61 (58-66)</td>
<td>51</td>
<td>63 (59-67)</td>
<td>81</td>
<td>63 (58-70)</td>
<td>0.482</td>
</tr>
<tr>
<td>Activity (METs)**</td>
<td>68</td>
<td>27.3 (13.8-44.2)</td>
<td>51</td>
<td>40.0 (27.5-62.7)</td>
<td>81</td>
<td>32.3 (16.1-53.0)</td>
<td>0.008</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>68</td>
<td>1692 (1547-1947)</td>
<td>51</td>
<td>1736 (1497-2020)</td>
<td>81</td>
<td>1737 (1406-1918)</td>
<td>0.922</td>
</tr>
<tr>
<td>Protein (% kcal)*</td>
<td>68</td>
<td>15.6 ± 2.9</td>
<td>51</td>
<td>14.5 ± 3.1</td>
<td>81</td>
<td>14.7 ± 2.8</td>
<td>0.067</td>
</tr>
<tr>
<td>Fat (% kcal)</td>
<td>68</td>
<td>31.4 ± 4.9</td>
<td>51</td>
<td>32.5 ± 6.8</td>
<td>81</td>
<td>33.5 ± 5.7</td>
<td>0.098</td>
</tr>
<tr>
<td>Carbohydrates (% kcal)</td>
<td>68</td>
<td>53.3 ± 6.4</td>
<td>51</td>
<td>54.2 ± 8.8</td>
<td>81</td>
<td>52.5 ± 6.9</td>
<td>0.418</td>
</tr>
<tr>
<td>Fish (oz / wk)*****</td>
<td>68</td>
<td>0.85 (0.00-4.74)</td>
<td>51</td>
<td>0.00 (0.00-0.40)</td>
<td>81</td>
<td>0.00 (0.00-3.11)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fish (≥8 oz/wk)*</td>
<td>68</td>
<td>13/68 (19.1%)</td>
<td>51</td>
<td>2/51 (3.9%)</td>
<td>81</td>
<td>10/81 (12.3%)</td>
<td>0.046</td>
</tr>
<tr>
<td>Frequency (0/1/2/3)**</td>
<td>68</td>
<td>22/29/15/2</td>
<td>51</td>
<td>32/15/3/1</td>
<td>81</td>
<td>46/21/11/3</td>
<td>0.016</td>
</tr>
<tr>
<td>Quantity (oz)*</td>
<td>46</td>
<td>0.97 (0.35-1.85)</td>
<td>19</td>
<td>0.25 (0.16-1.12)</td>
<td>35</td>
<td>1.87 (0.50-2.02)</td>
<td>0.010</td>
</tr>
<tr>
<td>High Omega-3 Fish (%)</td>
<td>46</td>
<td>40.5 ± 37.5</td>
<td>19</td>
<td>41.6 ± 42.5</td>
<td>35</td>
<td>39.1 ± 34.4</td>
<td>0.971</td>
</tr>
<tr>
<td>Tanner Score</td>
<td>68</td>
<td>5 (3-7)</td>
<td>49</td>
<td>4 (3-7)</td>
<td>81</td>
<td>5 (3-6)</td>
<td>0.814</td>
</tr>
</tbody>
</table>

WC = waist circumference, MET = metabolic equivalent

Mixed ethnic group includes subjects with a combination of Asian and White ethnicity, BMI z-score for sex-and-age determined using the lambda, mu and sigma technique based on the 2000 Centers for Disease Control and Prevention growth charts (CDC, 2012), METs determined as sum of reported activities that were performed ≥10 times in the last year based on published MET values (Lee, 1992), Fish Quantity is the consumption day fish intake, High omega-3 fish is the percent of fish that was high in omega-3 fatty acid as per the MyPyramid Food Guidance System Education Framework (Sharma et al., 2003), Tanner Score is the sum of the breast and pubic hair Tanner stages (Tanner, 1962).

Values are presented as the number of subjects in each level for multi-level categorical variables (fish frequency), frequency (percent) for bivariate categorical variables (fish intake ≥8 ounces per week), mean ± standard deviation for parametric continuous variables (age, BMI z-score, percent of energy from protein, fat and carbohydrates, Tanner score) and median (interquartile range) for nonparametric continuous variables (waist circumference, physical activity, energy intake, total and consumption day quantity of fish intake and percent of high omega-3 fatty acid fish). P-values reported comparing Asian, White and Mixed Asian/White ethnic groups using chi-square test, analysis of variance and Kruskal-Wallis test for categorical and parametric and nonparametric continuous variables, respectively. Asian and White ethnicities compared using Chi-square test for categorical variables, independent two-sample t-test for parametric continuous variables and Wilcoxon rank sum test for nonparametric continuous variable (*P <0.05, **P <0.01, ***P <0.001).
Table 5.2 Association of fish intake with two-year changes in measurements of body fatness and fat distribution among Asian and White female adolescents

<table>
<thead>
<tr>
<th>Obesity Parameter</th>
<th>n</th>
<th>Exam 1</th>
<th>Exam 2</th>
<th>Δ Exam 1-2</th>
<th>p-value</th>
<th>Crude</th>
<th>Adjusted¹</th>
<th>Adjusted²</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI z-score</td>
<td>103</td>
<td>0.18 ± 1.03</td>
<td>0.10 ± 0.91</td>
<td>-0.08 ± 0.60</td>
<td>0.575</td>
<td>-0.079 (0.426)</td>
<td>-0.148 (0.253)</td>
<td>-0.157 (0.310)</td>
</tr>
<tr>
<td>WC (cm)</td>
<td>103</td>
<td>62 (57-66)</td>
<td>65 (62-69)</td>
<td>3.6 ± 4.0</td>
<td>&lt;0.0001</td>
<td>-0.153 (0.123)</td>
<td><strong>-0.013 (0.026)</strong></td>
<td>-0.874 (0.408)</td>
</tr>
<tr>
<td>Skinfold Thickness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measurements</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Fat (mm)</td>
<td>103</td>
<td>40 (30-54)</td>
<td>54 (43-71)</td>
<td>13.9 ± 14.1</td>
<td>&lt;0.0001</td>
<td>-0.091 (0.361)</td>
<td>-4.463 (0.243)</td>
<td>-1.344 (0.763)</td>
</tr>
<tr>
<td>Trunk Fat (mm)</td>
<td>103</td>
<td>20 (14-27)</td>
<td>29 (23-28)</td>
<td>9.9 ± 9.1</td>
<td>&lt;0.0001</td>
<td>-0.102 (0.303)</td>
<td>-3.069 (0.209)</td>
<td>-1.819 (0.533)</td>
</tr>
<tr>
<td>Trunk: Peripheral</td>
<td>103</td>
<td>0.98 ± 0.24</td>
<td>1.19 ± 0.25</td>
<td>0.21 ± 0.23</td>
<td>&lt;0.0001</td>
<td>-0.062 (0.536)</td>
<td>-0.067 (0.225)</td>
<td>-0.087 (0.203)</td>
</tr>
<tr>
<td>DXA Measurements</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Fat (%)</td>
<td>103</td>
<td>-</td>
<td>28.9 ± 8.1</td>
<td></td>
<td></td>
<td>-0.180 (0.069)</td>
<td>-1.511 (0.280)</td>
<td>-1.287 (0.450)</td>
</tr>
<tr>
<td>Trunk Fat (%)</td>
<td>102</td>
<td>-</td>
<td>13.3 ± 4.4</td>
<td></td>
<td></td>
<td>-0.115 (0.249)</td>
<td>-0.477 (0.509)</td>
<td>-0.294 (0.736)</td>
</tr>
<tr>
<td>Trunk: Peripheral</td>
<td>103</td>
<td>-</td>
<td>0.96 ± 0.17</td>
<td></td>
<td></td>
<td>0.124 (0.212)</td>
<td>+0.013 (0.708)</td>
<td>+0.038 (0.395)</td>
</tr>
</tbody>
</table>

DXA = dual x-ray absorptiometry, WC = waist circumference, Δ Exam 1-2 = change in obesity parameters between Exam 1 and Exam 2

Fish classified as high or low in omega-3 fatty acids based on the MyPyramid Food Guidance System Education Framework (Sharma et al., 2003)

Obesity parameters for Exam 1 and Exam 2 presented as mean ± standard deviation and median (interquartile range), and compared using independent two-sample t-test and Wilcoxon rank sum test for parametric (BMI z-score, trunk-to-peripheral fat ratio for skinfold thickness, and percent body fat, percent trunk fat and trunk-to-peripheral fat mass for DXA) and nonparametric variables (waist circumference, and body fat and trunk fat for skinfold thickness), respectively.

The change in obesity parameters between Exam 1 and Exam 2 are presented as mean ± standard deviation.

Crude relationship between fish intake and obesity parameters assessed using Spearman’s rank correlation coefficient, and reported as Spearman’s Rho (p-value).

Adjusted¹ was a linear regression analysis of fish intake and obesity parameters, which adjusted for data collected during Exam 1 (age, percent Asian ethnicity, physical activity, energy intake, Tanner score and anthropometric value corresponding to the outcome of interest), and the change in Tanner score between Exam 1 and Exam 2. For DXA measurements, skinfold thickness estimates of total, trunk and trunk-to-peripheral ratio of fat mass from Exam 1 were included because the baseline anthropometric values corresponding to evaluation of percent body fat, percent trunk fat and trunk-to-peripheral fat mass ratio, respectively, because DXA measurement were not collected during Exam 1.

Adjusted² was a linear regression analysis of fish intake and obesity parameters, which controlled for variables from Adjusted¹, as well as percent of calories from protein, fat, carbohydrates, saturated fatty acids, polyunsaturated fatty acids and discretionary fat, added sugar, iron and fiber density of the diet, and soda consumption.

For both linear regression analyses, nonparametric parameters were log transformed, and values are presented as the regression coefficient (p-value).
Table 5.3 Summary of demographic, anthropometric, dietary and development characteristics collected during Exam 1 among subjects participating in Exam 1 and 2, or Exam 1 only

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
<th>Exam 1 and 2</th>
<th>n</th>
<th>Exam 1 Only</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>103</td>
<td>11.4 ± 1.4</td>
<td>97</td>
<td>11.5 ± 1.5</td>
<td>0.858</td>
</tr>
<tr>
<td>Ethnicity (Asian/White/Mixed)</td>
<td>103</td>
<td>39/26/38</td>
<td>97</td>
<td>29/25/43</td>
<td>0.445</td>
</tr>
<tr>
<td>BMI z-score</td>
<td>103</td>
<td>0.18 ± 1.03</td>
<td>96</td>
<td>0.34 ± 1.17</td>
<td>0.301</td>
</tr>
<tr>
<td>Waist Circumference (cm)</td>
<td>103</td>
<td>62 (57-66)</td>
<td>97</td>
<td>63 (58-69)</td>
<td>0.219</td>
</tr>
<tr>
<td>Activity (METs)</td>
<td>103</td>
<td>31.0 (15.3-51.5)</td>
<td>97</td>
<td>33.2 (18.5-55.5)</td>
<td>0.781</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>103</td>
<td>1748 (1515-2053)</td>
<td>97</td>
<td>1699 (1461-1937)</td>
<td>0.208</td>
</tr>
<tr>
<td>Protein (% kcal)</td>
<td>103</td>
<td>15.0 ± 2.7</td>
<td>97</td>
<td>14.9 ± 3.2</td>
<td>0.897</td>
</tr>
<tr>
<td>Fat (% kcal)</td>
<td>103</td>
<td>32.9 ± 6.0</td>
<td>97</td>
<td>32.2 ± 5.6</td>
<td>0.384</td>
</tr>
<tr>
<td>Carbohydrates (% kcal)</td>
<td>103</td>
<td>52.9 ± 7.2</td>
<td>97</td>
<td>53.6 ± 7.4</td>
<td>0.457</td>
</tr>
<tr>
<td>Fish (oz / wk)</td>
<td>103</td>
<td>0.02 (0.00-2.91)</td>
<td>97</td>
<td>0.00 (0.00-2.63)</td>
<td>0.768</td>
</tr>
<tr>
<td>Fish (≥8 oz/wk)</td>
<td>103</td>
<td>13/103 (12.6%)</td>
<td>97</td>
<td>12/97 (12.4%)</td>
<td>0.957</td>
</tr>
<tr>
<td>Frequency (0/1/2/3)</td>
<td>103</td>
<td>51/33/16/3</td>
<td>97</td>
<td>49/32/13/3</td>
<td>0.980</td>
</tr>
<tr>
<td>Quantity (oz)</td>
<td>52</td>
<td>1.01 (0.37-1.86)</td>
<td>48</td>
<td>0.89 (0.21-2.02)</td>
<td>0.801</td>
</tr>
<tr>
<td>High Omega-3 Fish (%)</td>
<td>52</td>
<td>34.4 ± 34.2</td>
<td>48</td>
<td>46.6 ± 39.4</td>
<td>0.101</td>
</tr>
<tr>
<td>Tanner Score</td>
<td>103</td>
<td>4 (3-7)</td>
<td>95</td>
<td>5 (3-6)</td>
<td>0.911</td>
</tr>
</tbody>
</table>

WC = waist circumference, MET = metabolic equivalent
Mixed ethnic groups includes subjects with a combination of Asian and White ethnicity, BMI z-score for sex-and-age determined using the lambda, mu and sigma technique based on the 2000 Centers for Disease Control and Prevention growth charts (CDC, 2012), METs determined as sum of reported activities that were performed ≥10 times in the last year based on published MET values (Lee, 1992), Fish Quantity is the consumption day fish intake, High omega-3 fish is the percent of fish that were high in omega-3 fatty acids as per the MyPyramid Food Guidance System Education Framework (Sharma et al., 2003), Tanner Score is the sum of the breast and pubic hair Tanner stages (Tanner, 1962).
Values are presented as the number of subjects in each level for multi-level categorical variables (ethnicity and fish frequency), frequency (percent) for bivariate categorical variables (fish intake ≥8 ounces per week), mean ± standard deviation for parametric continuous variables (age, BMI z-score, percent of energy from protein, fat and carbohydrates, Tanner score) and median (interquartile range) for nonparametric continuous variables (waist circumference, physical activity, energy intake, total and consumption day quantity of fish intake and percent of high omega-3 fatty acid fish). P-values reported comparing subjects that participated in FAM 1 and 2, and subjects that were in FAM 1 only using Chi-square test, analysis of variance and Kruskal-Wallis test for categorical and parameteric and nonparametric continuous variables, respectively.
CHAPTER 6: ESTIMATION OF FISH AND OMEGA-3 FATTY ACID INTAKE IN PEDIATRIC NONALCOHOLIC FATTY LIVER DISEASE

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\textsuperscript{1}University of Hawaii at Manoa; \textsuperscript{2}Washington University; \textsuperscript{3}Johns Hopkkins University, Bloomberg School of Public Health, \textsuperscript{4}Columbia University

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Abstract

Introduction: Fish and omega-3 fatty acids are reported to be beneficial in pediatric nonalcoholic fatty liver disease (NAFLD), but no studies have assessed their relation to histological severity. The objectives of this study were to evaluate the dietary intake of fish and omega-3 fatty acids in children with biopsy-proven NAFLD, and examine their association with serological and histological indicators of disease.

Materials and Methods: This was a cross-sectional analysis of 223 children (6-18 years) that participated in the Treatment of Nonalcoholic Fatty Liver Disease in Children trial, or the NAFLD Database study conducted by the Nonalcoholic Steatohepatitis Clinical Research Network. The distribution of fish and omega-3 fatty acid intake were determined from responses to the Block Brief 2000 Food Frequency Questionnaire, and analyzed for associations with serum alanine aminotransferase, histological features of fatty liver disease, and diagnosis of steatohepatitis after adjusting for demographic, anthropometric and dietary variables.

Results: The minority of subjects consumed the recommended 8 ounces of fish per week (22/223 (10%)) and 200 mg of long-chain omega-3 fatty acids per day (12/223 (5%)). Lack of fish and long-chain omega-3 fatty acid intake was associated with greater portal (p=0.03 and p=0.10, respectively) and lobular inflammation (p=0.09 and p=0.004, respectively) after controlling for potential confounders.

Discussion: Fish and omega-3 fatty acid intake were insufficient in children with NAFLD, which may increase susceptibility to hepatic inflammation. Patients with pediatric NAFLD should be encouraged to consume the recommended amount of fish per week.
Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common complication of pediatric obesity, which is characterized by altered lipid metabolism resulting in macrovesicular liver steatosis (1). Many children with NAFLD have concomitant inflammation and/or fibrosis of the liver termed nonalcoholic steatohepatitis (NASH), which can progress to cirrhosis (2-3). There is emerging evidence that ectopic fat deposition in the liver may be a risk factor for development of other metabolic disorders (4). Similar to other obesity-related conditions, successful weight loss attempts are effective at treating NAFLD in the short-term, but generally fail beyond one year, resulting in recrudescence (5). Consequently, there is considerable interest in identifying dietary factors that affect NAFLD pathogenesis independently of weight loss.

The long-chain omega-3 fatty acids found in fish, eicosapentaenoic acid (EPA; 20:5 ω-3) and docosahexaenoic acid (DHA; 22:6 ω-3) are thought to have a protective role in the development and progression of NAFLD (6-7). This is most clearly demonstrated in animal models of obesity where EPA and DHA are able to prevent and reverse liver disease (6). In humans, obesity and NAFLD are negatively associated with the long-chain omega-3 fatty acid content of cell membranes, which has been linked to altered hepatic lipid metabolism (8-9). Moreover, supplementation with long-chain omega-3 fatty acids has been shown to improve serological biomarkers of NAFLD and radiological measures of liver steatosis in several clinical trials, including one study in children, which found a marked reduction in ultrasound liver steatosis grade in subjects that received DHA supplements (10-11).
There is a paucity of research looking at the dietary intake of fish and omega-3 fatty acids in pediatric NAFLD. One study reported a low intake of omega-3 fatty acids, and a significant negative correlation between EPA and DHA intake and serum alanine aminotransferase (ALT) in 35 children with NAFLD (12). A more robust analysis with liver biopsy data would provide important insight into the role of dietary fish and omega-3 fatty acids in attenuating the progression of NAFLD. The purpose of this study was to evaluate the dietary intake of fish and omega-3 fatty acids, and their relation to serum ALT and histological features of liver disease in pediatric NAFLD. We hypothesized that most pediatric NAFLD patients would report fish and omega-3 fatty acids intakes that were below the recommended levels for children, and that lower intakes of fish and omega-3 fatty acids would be associated with higher serum ALT values and more severe histological indicators of liver disease.

Methods

This study was a cross-sectional analysis of data that was collected as part of the Treatment of Nonalcoholic Fatty Liver Disease in Children (TONIC) trial and the NAFLD Database study (13-14). The design of the TONIC trial has been described previously (13,15). Briefly, children (8-17 years) with biopsy-proven NAFLD were recruited amongst unsolicited referrals from September 2005 to September 2007 to eight clinical centers of the Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN, n = 229) including the University of California, San Diego, University of California, San Francisco, University of Washington, St. Louis University, Duke University Medical Center, Indiana University, Case Western Reserve University and Virginia Commonwealth University. Subjects that had liver cirrhosis, diabetes mellitus,
other liver diseases, or who were pregnant were excluded from the study (n = 56) (15). The NAFLD Database study included NAFLD patients age 2 years or older who were being treated at one of the NASH CRN clinical centers (n = 218) from October 2004 to February 2008, and contained 23 patients who participated in the TONIC trial.

Of the 368 children enrolled in TONIC or the NAFLD Database, 238 completed the Block Brief 2000 food frequency questionnaire (FFQ, 16) within six months of the liver biopsy. Three subjects did not meet the definition of NAFLD because they had a liver steatosis grade indicating <5% macrovesicular steatosis, and were excluded from the study. Additional subjects were excluded because they were missing one or more variables of interest (n = 7), or did not report intake of fried and/or non-fried fish (n = 4). Finally, there was a two-year-old that was identified as an outlier with respect to age, and was therefore not included in the study sample. Consequently, 223 (61%) children from the initial study population were deemed eligible and included in the study sample. Sensitivity analysis was performed for demographic and histological characteristics, and compared to children that were excluded, study subjects were more likely to be male, and have more severe steatosis and hepatocyte ballooning (p <0.05).

Participants completed the FFQ at baseline and annually for the TONIC trial and NAFLD Database study. This semi-quantitative FFQ approximates usual dietary intake based on reported consumption of 77 food items in the previous year. The food items are a composite of food lists developed for Whites, Hispanics and African Americans based on 24-hour dietary recall data from the National Health and Nutrition Examination Survey III (NHANES III) (16). Visual displays of portion sizes were included in the FFQ to assist in estimation of quantity (16). This FFQ has been used previously to assess the
relationship between other dietary variables and histological features of pediatric NAFLD (17).

As part of the Block Brief 2000 FFQ, subjects reported their intake of fried and non-fried fish across nine frequencies ranging from never to daily, and four serving sizes ranging from 1/4 cup to 2 cups. The responses for fried and non-fried fish were pooled together to estimate the overall frequency and amount of fish consumed. The nutrient content of the diet was calculated by NutritionQuest using the USDA Food and Nutrient Database for Dietary Studies, which provide a population-weighted nutrient composition for each food item based on national dietary intake data (16). Total omega-3 fatty acid consumption was analyzed as a nutrient density (g / 1,000 kcal) and in relation to omega-6 fatty acid intake. The ratio of omega-6 fatty acids to omega-3 fatty acids was used because the synthesis of long-chain omega-3 fatty acids (EPA and DHA) from alpha-linolenic acid (ALA; 18:3 ω-3), which makes up approximately 95% of total omega-3 fatty acids in the diet of children and adolescents in the US, is thought to be dependent on omega-6 fatty acid intake due to shared metabolic pathways (18-19). Although nutrient density is often preferred over absolute intakes when using FFQ data, absolute intakes of EPA, DHA and long-chain omega-3 fatty acids were used in statistical analyses since they are concentrated in a limited number of sporadically consumed foods that are represented in the Block Brief 2000 FFQ (20).

Liver disease was evaluated using serum alanine aminotransferase (ALT) and histological features of NAFLD. For the histological parameters, liver biopsies were graded centrally by NASH CRN pathologists for steatosis (<5%, 5% to 33%, >33% to 66% or >66% macrovesicular steatosis), portal inflammation (none, mild, more than
mild), lobular inflammation (<2 foci, 2-4 foci or >4 foci at 20x field), hepatocellular ballooning (none, few, many), and fibrosis (none [0], zone 3 perisinusoidal, delicate [1A], zone 3 perisinusoidal, dense [1B], portal/periportal [1C], perisinusoidal and portal/periportal [stage 2], bridging fibrosis [stage 3] or cirrhosis [stage 4]) using standard scoring criteria (21). Zone 3 perisinusoidal [1A] and [1B], and portal/periportal [1C] fibrosis were collapsed for analysis as stage 1, as were bridging and cirrhosis, as stage 3-4. Finally, subjects biopsies were diagnosed as "not NASH", "borderline zone 1 pattern", "borderline zone 3 pattern", or "definite NASH", as described previously (14).

Demographic and anthropometric data were obtained from participants in the TONIC trial and NAFLD Database study through structured interviews and questionnaires conducted at each of the NASH CRN clinical centers. For ethnicity, subjects were asked to identify themselves as American Indian, Asian, Pacific Islander, Black or White, and as Hispanic or non-Hispanic. After examining the ethnicity distribution, responses were assigned as Hispanic, non-Hispanic White and Other due to a limited representation from most ethnic groups. Height and weight were measured in duplicate to the nearest 0.1 cm and 0.1 kg, respectively, with subjects wearing lightweight clothing and no shoes. The average of height and weight were used to calculate BMI (kg/m²), and these values were converted in z-scores for age and gender based on the Center for Disease Control and Prevention (CDC) growth charts (22). Laboratory data including serum triglycerides, total-, LDL- and HDL-cholesterol, glucose, insulin and c-reactive protein (CRP) were available for a subset of subjects. The homeostatic model of insulin resistance (HOMA-IR) index was calculated as fasting
glucose (mmol/L) x fasting insulin (µU/mL) / 22.5, to provide an estimate of insulin resistance (23).

Demographic and dietary characteristics of subjects were summarized as frequency and percentage for categorical variables (gender and ethnicity), mean ± standard deviation for normally distributed continuous variables (age), and median and interquartile range for non-normally distributed continuous variables (energy intake, total, fried and non-fried fish consumption, omega-3 fatty acid density and omega-6 to omega-3 fatty acid ratio of the diet, and EPA, DHA and long-chain omega-3 fatty acid intake). Additionally, the proportion of subjects consuming the recommended amount of fish (≥ 2 serving and ≥ 8 oz / week), and long-chain omega-3 fatty acids (≥ 200 mg / d) was determined and reported as frequency and percentages (24-25). Continuous variables were designated as normally distributed based on Shapiro-Wilk test, and visual analysis of frequency distribution graphs. The association between age and dietary fish and omega-3 fatty acid intake variables were analyzed using Spearman’s rank correlation coefficient. Differences in subject characteristics by gender and ethnicity were evaluated using chi-square analysis, independent two-sample t-test and Wilcoxon rank sum test for categorical, and normally and non-normally distributed continuous variables, respectively.

A positive correlation has been observed between how often a food is consumed, and the serving size that is eaten (26). To determine whether this pattern was present for dietary fish in this population, the relationship between the frequency and the quantity of fried and non-fried fish intake was assessed. Subjects were grouped according to the reported frequency of fried and non-fried fish intake as a few times a year, monthly (1x
per month and 2-3x per month), and weekly (1x per week, 2x per week, 3-4x per week, 5-6x per week and daily), and the corresponding portion size of fried and non-fried fish were compared across the frequency groups using the Mantel-Haenszel test for trend. Only a few subjects reported eating 2-cup servings of fried (n = 3) and non-fried (n = 4) fish, so a combined 1-2 cups serving was used in this analysis.

The association of serum ALT with total, fried and non-fried fish consumption, omega-3 fatty acid density and omega-6 to omega-3 fatty acid ratio of the diet, and EPA, DHA and long-chain omega-3 fatty acid intake was assessed using Spearman's rank correlation coefficient, and linear regression analysis adjusting for age, gender, BMI z-score, and daily intake of energy, carbohydrates, protein, total, saturated, monounsaturated and polyunsaturated fat, sugar, fiber, cholesterol, vitamins A, C and E, β-carotene, betaine and choline. For the histological features of NAFLD, the differences in fish, long-chain omega-3 fatty acid and omega-6 to omega-3 fatty acid ratio were examined across histology levels using the Kruskal-Wallis test for steatosis, portal and lobular inflammation, hepatocyte ballooning and fibrosis, and the Wilcoxon’s rank-sum test to compare subjects diagnosed with isolated steatosis to those with definite NASH. The associations with histological features and NASH diagnosis were also assessed using multivariate analysis of variance (MANOVA) adjusting for age, gender, ethnicity, BMI z-score, and daily intake of energy, carbohydrates, protein, total, saturated, monounsaturated and polyunsaturated fat, sugar, fiber, cholesterol, vitamins A, C and E, β-carotene, betaine and choline. In the linear regression and MANOVA, serum ALT and all dietary variables were log-transformed because they were non-normally distributed as per Shapiro-Wilk test and analysis of frequency distribution graphs. Total, fried and non-
fried fish consumption were entered as log (fish intake + 1) prior to transformation to accommodate zero values.

All statistical tests were carried out using SAS version 9.2, and graphs were constructed using Microsoft Excel for Mac 2011 v. 14.2.5. The University of Hawaii Committee on Human Studies approved this research study.

**Results**

The 223 subjects included in the study were mostly male (171 (77%)), and Hispanic (128 (57%)) or non-Hispanic White (79 (35%)) with a mean age of 12.6 ± 2.6 years old (Table 6.1). Consistent with the diagnosis of pediatric NAFLD, both BMI z-score (2.31 ± 0.36) and serum ALT (85 U/L (IQR 64-130 U/L)) were well above the normal ranges. The dietary variables of interest are summarized in Table 6.1. The median daily intake of 1616 kcal (IQR 1185-2351 kcal) was lower than would be expected based on the energy needs for this population, which is a common issue of dietary assessment using FFQs with moderately sized food lists. However, the macronutrient distribution appears probable (50.4 ± 8.3% carbohydrates, 16.2 ± 3.4% protein, 35.3 ± 6.9% fat), and was similar to what has been reported in other pediatric NAFLD studies (12,27).

Although there is no specific recommendation for fish intake in pediatric NAFLD patients, most guidelines suggest consuming at least two servings, or eight ounces of fish per week (25). Only 33 / 223 (15%) of subjects reported consumption of fish two or more times per week, and less than 10% (22 / 223) achieved the target of eight ounces per week (Table 6.1). Furthermore, nearly one-quarter (52 / 223, 23%) indicated that they never ate fish (Figure 6.1). As seen in Figure 1, the distribution of fish consumption was typical of sporadically consumed foods with the mode located at the lowest level of
intake, and a large right skew. This pattern can be partly attributed to the larger portion sizes consumed by regular fish eaters (Figures 6.2 & 6.3). Among subjects that reported eating fish (n = 171), there was no correlation between fried and non-fried sources (Spearman’s rho = -0.05, p = 0.55), suggesting that the two food items were distinct in the mind of the respondents (data not shown). As expected, fish intake was a major determinant of the long-chain omega-3 fatty acid content of the diet (Spearman’s rho 0.73, p<0.0001, data not shown). Given the relatively limited amount of fish consumed, it is not surprising that the diets were low in long-chain omega-3 fatty acids with only 12 / 223 (5%) of subjects consuming more than 200 mg / d (Table 6.1).

The omega-3 fatty acid density of the diet was largely reflective of alpha-linolenic acid (ALA) intake, as ALA constituted 96% (IQR 94-98%) of omega-3 fatty acid intake (data not shown). According to the dietary reference intakes (DRIs), the acceptable macronutrient distribution range (AMDR) for ALA in children and adolescents is 0.6-1.2% of energy, which equates to 0.67-1.33 g per 1,000 kcal of omega-3 fatty acids when applying an Atwater factor of 9 kcal per g of ALA (25). The omega-3 fatty acid density of the diets observed here (0.72 g / 1,000 kcal (IQR 0.59-0.93 g / 1,000 kcal)) was concentrated around the lower end of the AMDR (Figure 6.2, Table 6.1). The omega-3 fatty acid intake was also low in relation to omega-6 fatty acids (median omega-6 to omega-3 ratio = 9.0 (IQR 7.4-10.2) (Table 6.1). While a desirable omega-6 to omega-3 fatty acid ratio has not been determined for pediatric NAFLD, the observed ratio of 9:1 greatly exceeds most references for disease prevention, which range from approximately 3:1 to 6:1 (19).
Despite greater reported energy intake in boys (1772 kcal (IQR 1369-2406 kcal)) compared to girls (1220 kcal (IQR 949-1860 kcal)) (p <0.001), there were no significant differences in total, fried and non-fried fish consumption by gender (Table 6.2). Girls appeared to consume a more omega-3 fatty acid rich diet than boys in relation to energy intake (0.77 g / 1,000 kcal (0.61-0.98 g / 1,000 kcal vs 0.71 g / 1,000 kcal (0.58-0.88 g / 1,000 kcal), p = 0.05), and omega-6 fatty acid intake (omega-6 to omega-3 ratio 8.6 (7.1-10.0) vs 9.1 (7.6-10.3), p = 0.05) (Table 6.2). Larger disparities in fish and omega-3 fatty acid intake were observed between ethnic groups than between genders (Table 6.3). Subjects from the “Other” ethnicity group consumed greater amount of fish (12.8 oz / month (3.7-31.3 oz / month)) than Hispanics (4.4 oz / month (0.0-13.0 oz / month), p = 0.03) and non-Hispanic Whites (3.3 oz / month (0.7-13.6 oz / month), p = 0.02). Additionally, the non-Hispanic Whites reported diets that were lower in omega-3 fatty acid content compared to subjects in the Hispanic and “Other” ethnicity groups (Table 6.3). Age was not associated with any of the fish or omega-3 fatty acid parameters measured (data not shown).

Higher fish and omega-3 fatty acid intakes were generally associated with lower ALT values, although none of the correlations were strong or statistically significant (p >0.05). However, when additional factors including demographic, anthropometric and dietary variables were accounted for in a linear regression model, the relationships between serum ALT and long-chain omega-3 fatty acid intake tended towards significance (p = 0.08, data not shown).

The dietary fish and long-chain omega-3 fatty acid intake, and omega-6 to omega-3 fatty acid intake ratio were also examined in relation to histological features of
NAFLD. The main significant findings of these analyses were related to inflammation (Table 6.4). There appeared to be a protective effect of fish intake on hepatic inflammation that was significant for portal (p = 0.02), and tended towards significance for lobular inflammation (p = 0.08) (Table 6.4). Fish and long-chain omega-3 fatty acid consumption, and omega-6 to omega-3 fatty acid ratio were not associated with the other histological parameters or a diagnosis of definite NASH (data not shown). Adjustment for demographic, anthropometric and dietary variables did not influence the findings for fish intake and hepatic inflammation, but resulted in a stronger association between long-chain omega-3 fatty acid intake and lobular inflammation (p = 0.004, Table 6.4).

Discussion

There is emerging evidence that long-chain omega-3 fatty acids may be important mediators of NAFLD pathogenesis (7). The findings from this registry-based study offer insight into the dietary intake of fish and omega-3 fatty acids in children with documented NAFLD in the United States. This provides valuable contextual information, and a useful perspective from which to examine the diet-disease relationship.

Most of the subjects in this sample were not consuming the recommended amounts of fish and omega-3 fatty acids (Table 6.1) (24-25). The observed low intake of omega-3 fatty acids are consistent with the results of 3-day food records collected from 35 pediatric NAFLD patients in Toronto, which reported average intakes of ALA that were less than two-thirds the adequate intake level (12). Together, these findings indicate that diet may be contributing to the low EPA and DHA content of cell membranes that has been observed in patients with NAFLD (9). Although this study did not have a control group for comparison, the frequency of fish consumption was less than that
reported from a sample of more than 1,000 adolescents from five public schools in Rhode Island, which had 36% of subjects who indicated eating fish at least once per week (vs 26%), and 17% who reported never eating fish (vs 23%) (28). Of interest, the “Other” ethnicity group reported approximately three times greater fish consumption than the Hispanic and non-Hispanic White subjects; however, this was a diverse group of non-Hispanic Indian, Asian, Pacific Islander, Black and Mixed ethnicities that contained only 16 subjects, which precluded meaningful subgroup analysis (Table 6.3).

Although NAFLD risk could not be determined from this analysis, it was possible to explore the effect of fish and omega-3 fatty acid consumption on serological and histological indicators of disease. The Toronto study noted a strong inverse relationship between EPA and DHA intake, and ALT (12). A similar, though non-significant, inverse association between fish and long-chain omega-3 fatty acid intake and ALT was observed in this sample. More importantly, lack of fish and long-chain omega-3 fatty acid consumption was correlated with greater portal and lobular inflammation (Table 6.4). Although fish and long-chain omega-3 fatty acid intake were not associated with a diagnosis of definite NASH, inflammation is known to predispose to fibrosis and progressive liver disease (29). The failure to detect a relationship between fish intake and definite NASH may be related to the fact that histologic parameters for steatohepatitis in pediatric biopsies are not as well understood as they are in adults (30).

There are several anti-inflammatory and pro-resolution mechanisms of EPA and DHA in fish that would support the observation of protective effects on both portal and lobular inflammation. Historically, the effects of long-chain omega-3 fatty acids on inflammation have been largely attributed to the shift from omega-6 to omega-3 fatty
acid-derived eicosanoids (19). In recent years, additional EPA and DHA-derived lipid mediators, including protectins and resolvins, have been identified, and are thought to be important anti-inflammatory mediators (31). A series of experiments by Oh et al. (2010) also demonstrated that DHA suppresses activation of the nuclear factor-kappaB (NF-κB) inflammatory pathway in macrophages through a G-protein coupled receptor (GPR 120) (32). C-reactive protein (CRP), an indicator of systemic inflammation, was measured in a subset of subjects in this study (n = 151). While CRP was not associated with fish consumption, there was a weak inverse correlation with long-chain omega-3 fatty acid intake (Spearman’s rho = -0.155, p = 0.058, data not shown).

Subjects consuming greater amounts of fish and long-chain omega-3 fatty acids did not have less hepatic steatosis (Table 6.4). The profound effects of EPA and DHA on lipid metabolism that have been reported in experimental animal models of obesity have been given as a major rationale for their importance in NAFLD (6). Moreover, this finding conflicts with the results of a double-blind, randomized trial of pediatric NAFLD patients in Italy, which noted dramatic reductions in ultrasound liver steatosis grade among subjects that were receiving DHA compared to germ oil (11). One possible explanation for this discrepancy may be the dose of long-chain omega-3 fatty acids required to induce an effect. The DHA supplements in the Italian study were 5-times greater than the median intake of long-chain omega-3 fatty acids that was reported by our subjects (11). Alternatively, the effect of omega-3 fatty acids on hepatic steatosis may have been confounded by genetic factors. Recently, a study of obese adolescents noted that hepatic fat fraction detected by magnetic resonance imaging was associated with the omega-6 to omega-3 fatty acid ratio of the diet, but only among participants that were
homozygous for the G allele of rs738409 in the patatin-like phospholipase 3 gene, which codes for adiponutrin (33).

In interpreting the results of this study, there are limitations that should be considered. The diet was assessed using a FFQ that was administered after subjects were diagnosed with NAFLD. The relatively low intake of sugar-sweetened beverages observed in the analysis of children in the NASH CRN database by Vos et al. (2012) suggests that respondents may have already modified their diets prior to filling out the FFQ, and/or were misreporting their intake to appear healthier (17). Although the NASH CRN Standards of Care for Pediatric Patients with Fatty Liver Disorders makes no reference to fish consumption, there are recommendations to limit fried food, which may have prompted subjects to reduce the amount of fried fish they were eating or reporting. At the same time, most adolescents consider fish to be healthy, so overall consumption of fish may have actually increased (28). The validity of the Block Brief FFQ has not been evaluated in adolescents, but a moderate amount of error can be inferred based on the subject’s low calorie intakes (Table 1). However, for the size and purpose of this study, a FFQ may be superior to short-term dietary assessment instruments such as 24-hour dietary recalls or food diaries for episodically consumed foods such as fish. While data were collected on both fried and non-fried fish intake, the long-chain omega-3 fatty acid content varies considerably by species (34). Furthermore, no data were available on the use of supplements containing omega-3 fatty acids. Finally, care must be taken when attempting to extrapolate these findings to other pediatric NAFLD populations, as there appeared to be some selection bias based on the sensitivity analysis.
The results of this study show that pediatric NAFLD patients consume less than the recommended amount of fish and omega-3 fatty acids (24-25). Promoting the intake of fish may help to reduce both portal and lobular inflammation, but further research is needed to test this hypothesis and to determine the necessary amount and best sources. Based on the current fish consumption, dietary supplements may be a good option for increasing long-chain omega-3 fatty acid intake to recommended levels (25). Advances in food biotechnology may offer opportunities for alternative sources of omega-3 fatty acids in the future, but this remains to be seen (35). Until additional clinical trials evaluating the effectiveness of long-chain omega-3 fatty acid supplements on pediatric NAFLD are conducted, patients should be encouraged to increase fish intake to meet general health recommendations.

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References


Table 6.1 Demographic, anthropometric and dietary characteristics of subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>12.6 ± 2.5</td>
</tr>
<tr>
<td>Gender (M)</td>
<td>171 / 223 (77%)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
</tr>
<tr>
<td>White, non-Hispanic</td>
<td>79 / 223 (35%)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>128 / 223 (57%)</td>
</tr>
<tr>
<td>Other</td>
<td>16 / 223 (7%)</td>
</tr>
<tr>
<td>Energy (kcal / d)</td>
<td>1616 (1186-2367)</td>
</tr>
<tr>
<td>Fish (oz / mo)</td>
<td>4.4 (0.7-13.6)</td>
</tr>
<tr>
<td>(≥ 8 oz / wk)</td>
<td>22 / 223 (10%)</td>
</tr>
<tr>
<td>(≥ 2 times / wk)</td>
<td>33 / 223 (15%)</td>
</tr>
<tr>
<td>Fish, not fried (oz / mo)</td>
<td>1.4 (0.0-12.1)</td>
</tr>
<tr>
<td>Fish, fried (oz / mo)</td>
<td>1.5 (0.0-3.0)</td>
</tr>
<tr>
<td>Omega-3 Fatty Acid (g / 1,000 kcal)</td>
<td>0.71 (0.59-0.93)</td>
</tr>
<tr>
<td>Omgea-6: Omega-3 Fatty Acid</td>
<td>9.0 (7.4-10.2)</td>
</tr>
<tr>
<td>Eicosapentaenoic Acid (mg / d)</td>
<td>9 (5-22)</td>
</tr>
<tr>
<td>Docosahexaenoic Acid (mg / d)</td>
<td>27 (16-48)</td>
</tr>
<tr>
<td>Long-Chain Omega-3 Fatty Acid (mg / d)</td>
<td>43 (26-80)</td>
</tr>
<tr>
<td>(≥ 200 mg / d)</td>
<td>12 / 223 (5%)</td>
</tr>
</tbody>
</table>

Other ethnicities include non-Hispanic American Indian, Asian, Pacific Islander, Black and Mixed.

Categorical variables (gender, ethnicity, and proportion of subjects consuming ≥8 oz and ≥2 servings of fish per week, and ≥200 mg of long-chain omega-3 fatty acids per day) are presented as frequency (percent), normally distributed continuous variables (age, BMI, and proportion of calories from carbohydrates, protein and fat) are presented as mean ± standard deviation, non-normally distributed continuous variables (energy intake, total, fried and non-fried fish consumption, omega-3 fatty acid density and omega-6 to omega-3 fatty acid ratio of the diet, and eicosapentaenoic acid, docosahexaenoic acid and long-chain omega-3 fatty acid intake) are presented as median (interquartile range). Continuous variables determined to be normally distributed based on Shapiro-Wilk test and analysis of frequency distribution graph.
Table 6.2 Fish and omega-3 fatty acid intake of subjects by gender

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Males</th>
<th>Females</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>171</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Energy (kcal / d)</td>
<td>1772 (1369-2406)</td>
<td>1220 (949-1860)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fish (oz / mo)</td>
<td>4.4 (0.7-14.0)</td>
<td>4.4 (1.5-12.2)</td>
<td>0.90</td>
</tr>
<tr>
<td>(≥ 8 oz / wk)</td>
<td>16 / 171 (9%)</td>
<td>6 (12%)</td>
<td>0.56</td>
</tr>
<tr>
<td>(≥ 2 times / wk)</td>
<td>24 / 171 (14%)</td>
<td>9 (17%)</td>
<td>0.64</td>
</tr>
<tr>
<td>Fish, not fried (oz / mo)</td>
<td>1.4 (0.0-12.1)</td>
<td>1.4 (0.0-6.5)</td>
<td>0.62</td>
</tr>
<tr>
<td>Fish, fried (oz / mo)</td>
<td>0.7 (0.0-3.0)</td>
<td>1.5 (0.0-3.0)</td>
<td>0.37</td>
</tr>
<tr>
<td>Omega-3 Fatty Acid (g / 1,000 kcal)</td>
<td>0.71 (0.58-0.88)</td>
<td>0.77 (0.61-0.98)</td>
<td>0.05</td>
</tr>
<tr>
<td>Omega-6: Omega-3</td>
<td>9.1 (7.6-10.3)</td>
<td>8.6 (7.1-10.0)</td>
<td>0.05</td>
</tr>
<tr>
<td>Eicosapentaenoic Acid (mg / d)</td>
<td>10 (5-23)</td>
<td>9 (5-18)</td>
<td>0.62</td>
</tr>
<tr>
<td>Docosahexaenoic Acid (mg / d)</td>
<td>27 (16-48)</td>
<td>25 (17-40)</td>
<td>0.57</td>
</tr>
<tr>
<td>Long-Chain Omega-3 Fatty Acid (mg / d)</td>
<td>46 (26-81)</td>
<td>41 (25-71)</td>
<td>0.48</td>
</tr>
<tr>
<td>(≥ 200 mg / d)</td>
<td>9 / 171 (5%)</td>
<td>3 (6%)</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Categorical variables (proportion of subjects consuming ≥8 oz and ≥2 servings of fish per week, and ≥200 mg of long-chain omega-3 fatty acids per day) are presented as frequency (percent), and continuous variables (energy intake, total, fried and non-fried fish consumption, omega-3 fatty acid density and omega-6 to omega-3 fatty acid ratio of the diet, and eicosapentaenoic acid, docosahexaenoic acid and long-chain omega-3 fatty acid intake) are presented as median (interquartile range). Values for males and females compared using Chi-square test, independent two-sample t-test and Wilcoxon rank sum test for categorical, and continuous variables, respectively. Continuous variables were determined to be non-normally distributed based on Shapiro-Wilk test and analysis of frequency distribution graph.
Table 6.3 Fish and omega-3 fatty acid intake of subjects by ethnicity

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hispanic</th>
<th>White, non-Hispanic</th>
<th>Other</th>
<th>Group Comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>128</td>
<td>79</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Energy (kcal / d)</td>
<td>1669 (1194-2378)</td>
<td>1578 (1181-2262)</td>
<td>1760 (1230-2742)</td>
<td>0.21 0.68 0.46</td>
</tr>
<tr>
<td>Fish (oz / mo)</td>
<td>4.4 (0.0-13.0)</td>
<td>3.3 (0.7-13.6)</td>
<td>12.8 (3.7-31.3)</td>
<td>0.40 0.03 0.02</td>
</tr>
<tr>
<td>(≥ 8 oz / wk)</td>
<td>15 / 128 (12%)</td>
<td>4 / 79 (5%)</td>
<td>3 (19%)</td>
<td>0.11 0.42 0.06</td>
</tr>
<tr>
<td>(≥ 2 times / wk)</td>
<td>22 / 128 (17%)</td>
<td>7 / 79 (9%)</td>
<td>4 (25%)</td>
<td>0.09 0.44 0.07</td>
</tr>
<tr>
<td>Fish, not fried (oz / mo)</td>
<td>1.4 (0.0-6.5)</td>
<td>2.8 (0.0-12.1)</td>
<td>6.3 (1.1-24.3)</td>
<td>0.20 0.04 0.08</td>
</tr>
<tr>
<td>Fish, fried (oz / mo)</td>
<td>1.5 (0.0-3.0)</td>
<td>0.7 (0.0-3.0)</td>
<td>3.0 (0.0-7.0)</td>
<td>0.15 0.28 0.12</td>
</tr>
<tr>
<td>Omega-3 Fatty Acid (g / 1,000 kcal)</td>
<td>0.73 (0.59-1.01)</td>
<td>0.68 (0.58-0.83)</td>
<td>0.73 (0.64-1.19)</td>
<td>0.02 0.52 0.09</td>
</tr>
<tr>
<td>Omega-6: Omega-3 Fatty Acid</td>
<td>8.7 (7.0-10.1)</td>
<td>9.3 (8.5-10.6)</td>
<td>8.5 (7.9-9.6)</td>
<td>&lt;0.001 0.95 0.014</td>
</tr>
<tr>
<td>Eicosapentaenoic Acid (mg / d)</td>
<td>9 (5-19)</td>
<td>8 (4-22)</td>
<td>17 (8-42)</td>
<td>0.24 0.07 0.04</td>
</tr>
<tr>
<td>Docosahexaenoic Acid (mg / d)</td>
<td>27 (15-47)</td>
<td>25 (16-48)</td>
<td>32 (24-68)</td>
<td>0.36 0.16 0.11</td>
</tr>
<tr>
<td>Long-Chain Omega-3 Fatty Acid (mg / d)</td>
<td>41 (26-78)</td>
<td>40 (26-80)</td>
<td>55 (36-126)</td>
<td>0.41 0.09 0.09</td>
</tr>
<tr>
<td>(≥ 200 mg / d)</td>
<td>8 / 128 (6%)</td>
<td>1 / 79 (1%)</td>
<td>3 (19%)</td>
<td>0.09 0.08 0.001</td>
</tr>
</tbody>
</table>

Other ethnic groups include non-Hispanic American Indian, Asian, Pacific Islander, Black and Mixed.
Categorical variables (proportion of subjects consuming ≥8 oz and ≥2 servings of fish per week, and ≥200 mg of long-chain omega-3 fatty acids per day) are presented as frequency (percent), parametric continuous variables (proportion of calories from carbohydrates, protein and fat) are presented as mean ± standard deviation, nonparametric continuous variables (energy intake, total, fried and non-fried fish consumption, omega-3 fatty acid density and omega-6 to omega-3 fatty acid ratio of the diet, and eicosapentaenoic acid, docosahexaenoic acid and long-chain omega-3 fatty acid intake) are presented as median (interquartile range). Values compared across ethnicities use Chi-square test for categorical variables, independent two-sample t-test for parametric continuous variables, and Wilcoxon rank sum test for nonparametric continuous variables, and presented as p-values. Continuous variables determined to be parametric based on Shapiro-Wilk test and analysis of frequency distribution graph.
H-W = Hispanic versus non-Hispanic White, H-O = Hispanic versus other ethnic groups, W-O = non-Hispanic White versus other ethnic groups.
Table 6.4 The relationship between dietary fish and long-chain omega-3 fatty acid intake, and histological features of nonalcoholic fatty liver disease

<table>
<thead>
<tr>
<th>Histological Parameter</th>
<th>n</th>
<th>Fish (oz / mo)</th>
<th>LCn-3 (mg / d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steatosis</td>
<td>55</td>
<td>4.4 (0.7-13.6)</td>
<td>45 (23-83)</td>
</tr>
<tr>
<td>Borderline, Zone 1</td>
<td>42</td>
<td>3.3 (0.0-8.0)</td>
<td>48 (27-72)</td>
</tr>
<tr>
<td>Borderline, Zone 3</td>
<td>50</td>
<td>3.0 (0.0-25.0)</td>
<td>40 (27-117)</td>
</tr>
<tr>
<td>Definite NASH</td>
<td>76</td>
<td>6.0 (1.1-14.4)</td>
<td>40 (26-80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Crude</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p = 0.39</td>
<td>p = 0.90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adjusted</td>
<td>p = 0.38</td>
</tr>
<tr>
<td>Portal Inflammation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>20</td>
<td>11.7 (4.7-19.8)</td>
<td>57 (36-110)</td>
</tr>
<tr>
<td>Mild</td>
<td>181</td>
<td>4.3 (0.7-13.1)</td>
<td>44 (26-78)</td>
</tr>
<tr>
<td>More than Mild</td>
<td>22</td>
<td>2.9 (0.0-5.9)</td>
<td>33 (23-102)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>p = 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adjusted</td>
<td>p = 0.03</td>
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<tr>
<td>Lobular Inflammation</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>48 (26-81)</td>
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<tr>
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<td>5.3 (1.4-13.0)</td>
<td>43 (28-80)</td>
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</tr>
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<td></td>
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<td>Adjusted</td>
<td>p = 0.09</td>
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Zones 1 and 3 refer to the periportal and centrilobular zones of the liver, respectively. Intake of fish and long-chain omega-3 fatty acids are reported for each level of the histological parameters as a median with interquartile range in parentheses. Crude analysis using Kruskal-Wallis test to assess differences in dietary variables across levels of portal and lobular inflammation, and Wilcoxon’s rank-sum test to compare dietary variables of subjects diagnosed with steatosis and definite nonalcoholic steatohepatitis. Multivariate analysis of variance (MANOVA) controlling for age, gender, ethnicity, BMI z-score, and intake of energy, carbohydrates, protein, total, saturated, monounsaturated and polyunsaturated fat, sugar, fiber, cholesterol, vitamins A, C and E, β-carotene, betaine and choline was used for the adjusted analysis. Dietary variables were determined to be non-normally distributed using Shapiro-Wilk test and analysis of frequency distribution graphs, and were log-transformed for the MANOVA. Fish intake was entered as log (fish intake + 1) to accommodate zero values. LCn3 = long-chain omega-3 fatty acids, NASH = nonalcoholic steatohepatitis.
**Figure 6.1** Frequency distribution of fish intake among fish consumers (n = 171)

Fish intake is sum of fried and non-fried fish intake calculated from semi-quantitative food frequency questionnaire responses. An additional 52 (23.3%) subjects reported never consuming fish.
Figure 6.2 Frequency distribution of omega-3 fatty acid intake density (g / 1,000 kcal)
Figure 6.3 Portion size of fried fish consumed by frequency of intake

Weekly fried fish intake includes once per week, twice per week, 3-4 times per week, 5-6 times per week and daily. Monthly fried fish intake includes once per month and 2-3 times per month. More frequency consumption of fried fish was associated with larger serving sizes ($p = 0.06$) as per Mantel-Haenszel test for trend.
Weekly non-fried fish intake includes once per week, twice per week, 3-4 times per week, 5-6 times per week and daily. Monthly non-fried fish intake includes once per month and 2-3 times per month. More frequency consumption of non-fried fish was associated with larger serving sizes \((p = 0.007)\) as per Mantel-Haenszel test for trend.
CHAPTER 7: THE USE OF FISH OIL-BASED LIPID EMULSIONS IN INFANTS WITH PARENTERAL NUTRITION-ASSOCIATED LIVER DISEASE: A CASE SERIES

Authors: David E St-Jules, RD¹, Corilee A Watters, MSc, RD, PhD, CNSC¹, Lynn M Iwamoto, MD²

¹University of Hawaii at Manoa; ²John A. Burns School of Medicine and Kapi‘olani Medical Center for Women and Children
Abstract

Background: Parenteral nutrition-associated liver disease (PNALD) is a common and serious complication of parenteral nutrition use in infants with short bowel syndrome. The use of fish oil-based lipid emulsions (FOLE) in the treatment of PNALD remains investigational. Additional evidence for safety and efficacy, particularly in the neonatal and pediatric populations, is needed.

Materials and Methods: Retrospective chart review was conducted on ten infants with short bowel syndrome at Kapi‘olani Medical Center for Women and Children who received FOLE for PNALD.

Results: Direct bilirubin concentrations normalized in surviving subjects within 4.1 to 22.7 weeks of starting treatment. Time until direct bilirubin normalization directly correlated with length of hospital stay (p=0.015). Although earlier initiation of FOLE was not associated with more rapid normalization of direct bilirubin concentrations, it trended towards a significant correlation with reduced length of hospital stay (p = 0.058). The reduction in direct bilirubin levels and transition from parenteral to enteral feeding were statistically significant within six weeks of initiating the FOLE. Subjects did not have impaired growth and did not develop an essential fatty acid deficiency. These infants were discharged from the hospital 7.9 to 42.3 weeks since starting FOLE treatment, and two had transitioned completely off parenteral nutrition at discharge.

Conclusion: In this study, FOLE appeared to be a safe and effective treatment for PNALD in infants with short bowel syndrome. Future studies are necessary to determine whether FOLE can help to prevent or shorten the duration of cholestasis.


**Introduction**

Parenteral-nutrition associated liver disease (PNALD) is a common disorder in parenteral nutrition (PN) dependent infants with short bowel syndrome, and can result in potentially life-threatening liver cirrhosis if untreated (1-3). Although the etiology of PNALD is not completely understood, the high omega-6:omega-3 fatty acid ratio and phytosterol content of conventional vegetable oil-based lipid emulsions are thought to be contributing factors (4-6). Restricting intravenous lipid infusion may help to prevent or treat PNALD; however this practice can be problematic because lipids are thought to be an important source of non-protein calories, and essential fatty acids (7). In a landmark case report by Gura et al. (2006), two infants with intestinal failure and severe PNALD were provided Omegaven® (Fresenius Kabi, AG, Bad Homburg, Germany), an intravenous fish-oil based lipid emulsion (FOLE) that is phytosterol-free and rich in long-chain omega-3 fatty acids. These infants had reversal of their cholestasis and improvement in hepatic function as evidenced by normalization of direct bilirubin and aminotransferase concentrations (8). These and other findings prompted the U.S. Food and Drug Administration (FDA) to permit the compassionate use of Omegaven® in infants with PNALD who have not responded to conventional therapies.

Since this time, several case reports (9-15), case series (16-18), and case-control studies (19-21), have reported resolution of cholestasis in PNALD patients receiving Omegaven®. However there is a need for additional evidence regarding FOLE administration in the neonatal and pediatric populations, as currently available reports generally focus on a limited number of outcomes apart from cholestasis. A recent systematic review concluded that additional studies with long-term, clinically relevant outcomes are needed (22). In this case series, we provide a detailed evaluation of our
experience with FOLE administration in ten infants with short bowel syndrome and PNALD.

Methods
Since December 2009, patients at Kapi’olani Medical Center for Women and Children (KMCWC) with PNALD were provided with Omegaven® in accordance with an FDA open-label protocol. The conventional lipid emulsion at this facility is soybean oil-based Intralipid® (Fresenius Kabi, AG, Bad Homburg, Germany). Under the open-label protocol, infants with short bowel syndrome who are PN-dependent and weigh at least three kilograms are eligible to receive 1 g/kg/d of FOLE if they have two consecutive direct bilirubin levels ≥2.0 mg/dL indicative of PNALD and are thought to be at risk for significant hepatic injury due to prolonged need for PN (>30 days). Before beginning FOLE treatment, other causes of liver disease including hepatitis C, cystic fibrosis, biliary atresia, and α-1 anti-trypsin deficiency are excluded and standard therapies were attempted, including serial transverse enteroplasty (STEP) procedure, PN cycling, PN restriction of copper and manganese content and ursodiol treatment. Cystic fibrosis and biliary atresia were ruled out using immunoractive trypsinogen assay and ultrasound, respectively. Subjects for this report were consecutive patients who received FOLE for PNALD. The Western Institutional Review Board and the University of Hawaii Human Studies Program approved this retrospective review.

Demographic, anthropometric, biochemical, clinical and dietary information were collected retrospectively from the inpatient electronic medical record. Subject characteristics and clinical course prior to receiving treatment included the primary diagnoses resulting in PN-dependence, bowel available for enteral feeding, age at
diagnosis of PNALD, and standard PNALD therapies implemented. Cyclic PN was defined as a daily break from PN of greater than four hours.

Anthropometric, biochemical, clinical and dietary outcomes were monitored from FOLE treatment through discharge from the hospital. The hospital laboratory conducted all biochemical tests, except for serum fatty acid analyses, which were done by the Mayo Clinic Department of Laboratory Medicine and Pathology (Rochester, MN). Direct bilirubin concentration was determined using a timed endpoint diazo method (SYNCHRON® systems, Beckman Coulter). Enteral and parenteral intakes were analyzed per kilogram body weight for each subject on a median of three days around at each time point to accommodate for fluctuations due to the clinical course. Body weight, length and head circumference were converted into z-scores for age-and-sex using the lambda, mu and sigma technique based on the 2000 Centers for Disease Control and Prevention growth charts (23). Most of the subjects were born prematurely, so the expected date of delivery was used to establish their adjusted age for this calculation. An episode of line sepsis was defined as the occurrence of two positive blood cultures with the provision of antibiotics for 5 days or more.

The proportion of subjects with abnormal values at baseline and 12 weeks was determined based on hospital laboratory reference ranges for biochemical data and the 3rd and 97th percentiles for age-and-sex for anthropometric parameters. Baseline and follow up values for biochemical, nutritional and anthropometric data were compared using the Wilcoxon rank sum test. The relationship between direct bilirubin concentration normalization and length of stay, and the effect of timely initiation of FOLE treatment on these outcomes were assessed using Spearman’s rank correlation coefficient. For these
analyses, the diagnosis of PNALD was used as the starting point. All statistical tests were conducted using Statistical Analysis Software (SAS), version 9.2, and graphs were prepared using Microsoft Excel for Mac 2011, version 14.2.5. Statistically significant differences were based on a p-value of <0.05. This study is registered with the U.S. National Institutes of Health clinical trials registry (NCT01194063) (24).

**Results**

All subjects required PN from the time of birth, and 90% developed PNALD before nine weeks of age (Table 7.1). As seen in Table 7.1, the underlying etiologies of short bowel syndrome and remaining bowel anatomy varied substantially. Subjects had PNALD for a median of 6.1 (3.0 to 12.3) weeks before starting treatment with FOLE except for two subjects who had PNALD for 30 or more weeks when the Omegaven® protocol was introduced. The requirement for subjects to weigh at least three kilograms was the most common cause of treatment delays among subjects who developed PNALD once the Omegaven® protocol was in place. During the interim, several approaches to reduce cholestasis and prevent further liver injury were used including cycling of PN (90%), restriction of copper and manganese (90%), ursodiol administration (30%) and STEP surgery (20%).

During the treatment follow up period, the four subjects with diverting jejunostomies had surgery to restore bowel continuity at weeks two, three, eight and twenty. Another subject underwent repeat STEP surgery at week 10 that was estimated to increase the small bowel length from 50 to 68 cm long. Finally, the subject with a duodenum to colon anastomosis was transferred temporarily to another institution for intestinal transplantation evaluation, and received Intralipid® for nearly six weeks. Of
the ten subjects in this study, one expired at 31 weeks of life due to complications that were unrelated to gastrointestinal or liver disease. The surviving nine subjects were discharged from the hospital 7.9 to 42.3 weeks after starting treatment. During this time, the number of episodes of sepsis was generally quite minimal.

Table 7.2 summarizes changes that occurred during the first 12 weeks of treatment. The laboratory values at baseline were consistent with the diagnosis of liver disease with elevated concentration of liver enzymes in many of the patients. By week six of treatment, direct bilirubin concentrations were reduced significantly \( (p = 0.037) \), and values continued to decrease to a low of 3.1 (0.1 to 6.3) mg/dL by week 12 with one-third of subjects having normal direct bilirubin values (Figure 7.1). None of the other liver disease biomarkers parameters changed significantly after 12 weeks of treatment including prothrombin time and international normalized ratio which were measured at various times during the FOLE course \( (p< 0.05, \text{ data not shown}) \).

Direct bilirubin concentrations normalized 4.1 to 22.7 weeks after starting treatment (Table 7.1). A quicker resolution of cholestasis was associated with a decreased length of stay (Spearman’s rho = 0.810, \( p = 0.015 \)). Although direct bilirubin concentrations decreased after starting treatment (Figure 7.1), earlier initiation of FOLE following PNALD diagnosis was not associated with more rapid normalization of direct bilirubin (Spearman’s rho = 0.571, \( p = 0.139 \)). However, a longer delay in FOLE treatment did trend towards a significant correlation with an increased length of hospital stay (Spearman’s rho = 0.690, \( p = 0.058 \)). The subject who expired and the subject who received soybean oil based-lipid emulsion for six weeks during the treatment period were not included, leaving only eight subjects for these analyses.
The summary of fatty acid profile changes is shown in Table 7.3. FOLE treatment was associated with a significant increase in omega-3 fatty acids and decrease in omega-6 fatty acids (p <0.05) within 4.0 to 13.0 weeks of starting treatment. However, there was no evidence of essential fatty acid deficiency and the mead acid concentrations and triene: tetraene ratios were relatively stable during this time.

As seen in Figure 7.2, the 12-week period following initiation of treatment was marked by a dramatic shift in enteral and parenteral intake. Enteral intake increased significantly whereas parenteral intake tended to decrease, albeit to a lesser extent, resulting in a net increase in energy intake. At the end of the 12 weeks, most subjects (80%) had reduced requirements for PN as a result of increasing enteral tolerance. Moreover, two of the surviving subjects (22%) were no longer PN-dependent at discharge (Table 7.1). Although the dose of FOLE was set at 1 g/kg/d, the transition from soybean oil-based lipid emulsion did not result in a significant reduction in intravenous lipids (p >0.05). This appears to be related to a decrease in PN lipid of ≥1 g/kg/d after diagnosis of PNALD that occurred for all subjects (data not shown).

The nutrition provided during FOLE treatment was sufficient to support normal growth. At baseline, even after adjusting for prematurity, subjects tended to have lower than normal weight, length and head circumference for age-and-sex with 40% or more falling below the 3rd percentiles for each parameter (Table 7.2). After 12 weeks of treatment, the median weight, length and head circumference z-scores for age-and-sex increased and the proportion of subjects below the third percentile of weight, length and head circumference for age-and-sex decreased (Table 7.2). While these improvements in
anthropometric measurements suggest catch up growth, none of these changes were statistically significant (p >0.05).

**Discussion**

The results of this study contribute to the growing literature on the use of FOLE in infants with PNALD. This is one of the larger case series, and includes clinically relevant outcomes not reported in previous reports 16-21). The summary of findings provides important information on the clinical progression of patients with PNALD treated with FOLE.

As expected, within six weeks of initiating treatment, direct bilirubin concentrations had significantly decreased, indicating resolution of cholestasis. Factors other than FOLE treatment may have contributed to the normalization of direct bilirubin concentrations in these subjects. Most notably, there was a concomitant transition from parenteral to enteral feeding, which has been found to promote direct bilirubin normalization (16,25). However it has been proposed that the improvements in enteral tolerance occur secondary to cholestasis resolution resulting from the change to FOLE treatment from vegetable oil-based lipid emulsions (16,19). Supporting this hypothesis, fish oils contain none of the phytosterols found in conventional vegetable oils that have been shown to inhibit bile acid production resulting in reduced bile flow (26). In this study, a temporal relationship between direct bilirubin and enteral intake could not be discerned as the changes in both variables began on average at week four, and became significant at week six. Although reanastomosis and STEP procedures also have the potential for improving enteral tolerance and bile absorption, only two of these procedures preceded the normalization of direct bilirubin, limiting the effect that this
might have had on the resolution of cholestasis (27). Finally, it is possible that improvements in direct bilirubin concentration and enteral feeding reflect normal disease progression or other ongoing PNALD treatments (e.g., PN cycling, PN restriction of copper and manganese), and would have occurred without FOLE treatment. While this factor could be tested by comparison to a historical cohort of PNALD patients, this approach was not used here because of the variable delay in initiating FOLE treatment, in addition to changes in disease management over time that would bias such comparisons.

Historically the fatty acid composition and dose restriction associated with the use of FOLE were thought to present risks for essential fatty acid deficiency and growth impairment (28-30). Since this time, numerous case series and case-control studies have been published demonstrating that essential fatty acid levels (17-21) and anthropometric measurements of growth (17,19) remain normal in patients receiving FOLE. The results of this study support these earlier findings in a multiethnic cohort of formerly preterm infants with short bowel syndrome. Importantly, all but one subject received some enteral nutrition support which provided omega-6 fatty acid intake. To date there are no reports of clinically significant fatty acid deficiencies associated with the use of FOLE in infants, even among patients who received no enteral feeding (18).

Length of hospital stay is an important clinical outcome that had not been considered in prior case series or case-control studies (16-21). In this study, subjects who had a more rapid return of direct bilirubin concentrations to normal levels were discharged from the hospital earlier. Unlike previous reports (15), earlier initiation of FOLE treatment after the diagnosis of PNALD did not appear to reduce the duration of cholestasis, but may have contributed to a decrease in length of stay. The requirement for
subjects to weigh at least three kilograms was the most common delay in starting FOLE and was set by the FDA based on the quantity of blood obtained from patients for monitoring of biochemical indices as per the Omegaven® treatment protocol at KMCWC.

The information in this case series add to the growing body of evidence supporting the safety and positive outcomes in infants with short bowel syndrome and PNALD treated with FOLE. A reduction in length of hospital stay may be an important clinical benefit of early FOLE intervention, although this association may have been confounded by external factors that caused the delay in FOLE treatment. Some of these patients were able to transition off PN to full enteral feeds and all surviving patients have been discharged from the hospital with improved growth and without developing essential fatty acid deficiency. A concomitant reduction in direct bilirubin levels and enteral nutrition tolerance occurred after four weeks of treatment, but the exact relationship between these outcomes in relation to the use of FOLE is still unclear. Many questions remain, particularly in smaller and younger infants who are at risk for PNALD due to intestinal complications and immature bowel function. Additional studies on safety, dosage, timing and potential combination therapy are needed to achieve optimal intestinal and growth outcomes especially in the high risk infant populations.
References


17. de Meijer VE, Le HD, Meisel JA, Gura KM, Puder M. Parenteral fish oil as monotherapy prevents essential fatty acid deficiency in parenteral nutrition-dependent patients. JPGN. 2010;50:212-218.


<table>
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<th>ID (sex, race)</th>
<th>Gest Age</th>
<th>Diagnoses (Intact Bowel)</th>
<th>Dx</th>
<th>Age (wks)</th>
<th>N Bili</th>
<th>D/C</th>
<th>Clinical Outcomes After Tx</th>
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<td>1 (M, PI)*</td>
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<td>Gs/At (Jej: Colon)</td>
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<td>32.6</td>
<td>55.3</td>
<td>65.6</td>
<td>4</td>
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<tr>
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<td>34.9 wks</td>
<td>Gs/At (Jej: Colon)</td>
<td>2.0</td>
<td>36.3</td>
<td>55.6</td>
<td>67.9</td>
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</tr>
<tr>
<td>3 (F, As)</td>
<td>38.6 wks</td>
<td>Vo/Om/At/NEC (Jej: Colon)</td>
<td>15.3</td>
<td>21.7</td>
<td>28.3</td>
<td>47.4</td>
<td>1</td>
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<td>At (Jejunum: Colon)</td>
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<td>15.1</td>
<td>27.1</td>
<td>49.0</td>
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<td>Gs/At (Jejunostomy)</td>
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<td>9.9</td>
<td>29.4</td>
<td>52.1</td>
<td>3</td>
</tr>
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<td>36.3 wks</td>
<td>Gs/At (Jejunostomy)</td>
<td>7.9</td>
<td>10.9</td>
<td>15.0</td>
<td>24.6</td>
<td>1</td>
</tr>
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<td>8 (F, Asian)</td>
<td>29.0 wks</td>
<td>Perf/At (Jejunostomy)</td>
<td>5.0</td>
<td>10.9</td>
<td>18.4</td>
<td>18.7</td>
<td>0</td>
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<tr>
<td>9 (M, As/PI/Wt)</td>
<td>31.0 wks</td>
<td>Perf (Duod: Colon)</td>
<td>3.9</td>
<td>9.4</td>
<td>25.3</td>
<td>40.4</td>
<td>1</td>
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<td>10 (F, As/Wt)</td>
<td>27.6 wks</td>
<td>Vo/NEC (Jejunostomy)</td>
<td>8.1</td>
<td>16.7</td>
<td>27.9</td>
<td>28.4</td>
<td>0</td>
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</tbody>
</table>

As = Asian, At = Intestinal atresia, D/C = Discharge from hospital, Duod = Duodenum, Dx = Diagnosis with PNALD, Gest = Gestation, Gs = Gastrochisis, Hi = Hispanic, Jej = Jejunum, N Bili = Normalization of direct bilirubin, NEC = Necrotizing enterocolitis, Om = Omphalocele, Perf = Intestinal perforation, PI = Pacific Islander, PN = Parenteral nutrition, Tx = Treatment with fish oil-based lipid emulsion, Vo = Volvulus, Wt = White

*Had pre-existing PNALD when Omegaven® protocol was introduced
Table 7.2 Anthropometric, and biochemical characteristics at baseline and after 12 weeks of fish oil-based lipid emulsion treatment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normal</th>
<th>Abnormal</th>
<th>Baseline Value</th>
<th>Abnormal</th>
<th>Week 12 Value</th>
<th>Abnormal</th>
<th>p-value</th>
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<tr>
<td>Weight (z-score)</td>
<td>-1.88 to 1.88</td>
<td>10</td>
<td>-1.64 (-4.48 to 0.21)</td>
<td>4/10 (40%)</td>
<td>-1.11 (-2.42 to 0.22)</td>
<td>2/10 (20%)</td>
<td>0.174</td>
</tr>
<tr>
<td>Length (z-score)</td>
<td>-1.88 to 1.88</td>
<td>10</td>
<td>-1.82 (-4.41 to 0.18)</td>
<td>5/10 (50%)</td>
<td>-1.55 (-4.96 to 0.15)</td>
<td>3/8 (38%)</td>
<td>0.790</td>
</tr>
<tr>
<td>HC (z-score)</td>
<td>-1.88 to 1.88</td>
<td>9</td>
<td>-1.91 (-4.13 to -0.72)</td>
<td>5/9 (56%)</td>
<td>-1.69 (-3.30 to 0.65)</td>
<td>2/7 (29%)</td>
<td>0.491</td>
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<tr>
<td>Albumin (g/dL)</td>
<td>4.0 to 5.1</td>
<td>10</td>
<td>3.5 (2.8 to 4.7)</td>
<td>8/10 (80%)</td>
<td>3.6 (2.8 to 4.4)</td>
<td>7/9 (78%)</td>
<td>0.566</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>7 to 51</td>
<td>10</td>
<td>145 (38 to 362)</td>
<td>9/10 (90%)</td>
<td>145 (18 to 561)</td>
<td>7/9 (78%)</td>
<td>0.870</td>
</tr>
<tr>
<td>GGTP (U/L)</td>
<td>12 to 72</td>
<td>10</td>
<td>131 (32 to 281)</td>
<td>7/10 (70%)</td>
<td>219 (49 to 366)</td>
<td>7/8 (88%)</td>
<td>0.110</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>82 to 383</td>
<td>10</td>
<td>337 (216 to 661)</td>
<td>4/10 (40%)</td>
<td>316 (188 to 562)</td>
<td>3/9 (33%)</td>
<td>0.870</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>&lt; 150</td>
<td>10</td>
<td>187 (76 to 501)</td>
<td>7/10 (70%)</td>
<td>129 (54 to 241)</td>
<td>3/9 (33%)</td>
<td>0.060</td>
</tr>
<tr>
<td>Platelet Count (x10⁹)</td>
<td>≤ 100</td>
<td>9</td>
<td>173 (81 to 498)</td>
<td>2/9 (22%)</td>
<td>193 (112 to 339)</td>
<td>0/7 (0%)</td>
<td>0.560</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>&lt; 1.0</td>
<td>9</td>
<td>8.0 (3.9 to 36.4)</td>
<td>9/9 (100%)</td>
<td>3.4 (0.4 to 49.1)</td>
<td>4/5 (80%)</td>
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</tr>
</tbody>
</table>

HC = head circumference, ALT = alanine aminotransferase, GGTP = γ-glutamyl transpeptidase, ALP = alkaline phosphatase, CRP = c-reactive protein

Weight, length and head circumference z-scores for age-and-sex determined using lambda, mu and sigma method based on 2000 Centers for Disease Control and Prevention growth charts and prematurity adjusted age (23). The normal range of z-scores corresponds to the 3rd and 97th percentiles for age-and-sex. The normal ranges for laboratory values were based on reference values provided by the hospital laboratory.

Values are presented as median (range) for continuous variables.
Table 7.3 Comparison of serum lipid profiles before and after treatment with fish oil-based lipid emulsion

<table>
<thead>
<tr>
<th>Variable</th>
<th>Before</th>
<th>After</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>Variable</td>
<td>Before</td>
<td>After</td>
<td>p-value</td>
</tr>
<tr>
<td>Timing of Blood Sample (d)</td>
<td>-3 (-19 to 1)</td>
<td>31 (30 to 91)</td>
<td></td>
</tr>
<tr>
<td>Omega-3 (mmol/L)</td>
<td>0.7 (0.2 to 1.0)</td>
<td>3.3 (1.2 to 7.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Omega-6 (mmol/L)</td>
<td>5.4 (2.2 to 6.7)</td>
<td>3.1 (1.7 to 4.6)</td>
<td>0.004</td>
</tr>
<tr>
<td>Omega-6: Omega-3 Ratio</td>
<td>8.7 (6.6 to 13.5)</td>
<td>0.8 (0.5 to 3.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>α-Linolenic Acid (µmol/L)</td>
<td>4429 (1111 to 5600)</td>
<td>2383 (1282 to 3662)</td>
<td>0.016</td>
</tr>
<tr>
<td>Linoleic Acid (µmol/L)</td>
<td>219 (38 to 295)</td>
<td>77 (30 to 184)</td>
<td>0.027</td>
</tr>
<tr>
<td>Eicosapentaenoic Acid (µmol/L)</td>
<td>76 (33 to 150)</td>
<td>1631 (516 to 3706)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Docosahexaenoic Acid (µmol/L)</td>
<td>265 (59 to 421)</td>
<td>1346 (442 to 3205)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Arachidonic Acid (µmol/L)</td>
<td>890 (463 to 1641)</td>
<td>564 (333 to 1075)</td>
<td>0.034</td>
</tr>
<tr>
<td>Mead Acid (µmol/L)</td>
<td>11.5 (10.0 to 99.0)</td>
<td>10.0 (4.0 to 24.0)</td>
<td>0.135</td>
</tr>
<tr>
<td>Triene: Tetraene Ratio</td>
<td>0.014 (0.011 to 0.124)</td>
<td>0.016 (0.011 to 0.046)</td>
<td>0.528</td>
</tr>
</tbody>
</table>

Timing of blood sample is in relation to the initiation of treatment.
Values are presented as median (range) for continuous variables. Lipid profile before and after treatment were compared using Wilcoxon rank sum test.
Figure 7.1 Biweekly direct bilirubin concentrations in subjects in relation to fish oil-based lipid emulsion treatment

Data points indicate medians; error bars indicate interquartile ranges. Changes in direct bilirubin concentrations were assessed using Kruskal-Wallis test (p=0.001), and each time point was compared to baseline using the Wilcoxon rank sum test (*p-value <0.05).
Values missing for one subject at weeks four, six and eight. Each time point was compared to baseline using Wilcoxon rank sum test (\*p-value <0.05 enteral intake; \(^\#\)p-value <0.05 parenteral intake; \(^\dagger\)p-value <0.05 enteral: parenteral ratio; \(^\ddagger\)p-value <0.05 total intake).
CHAPTER 8: CONCLUSION

Research Summary

Nonalcoholic fatty liver disease (NAFLD) and parenteral nutrition-associated liver disease (PNALD) are two liver diseases afflicting pediatric populations. Despite growing awareness of the burden of NAFLD and PNALD, they have yet to be examined in Hawai‘i, and there is a paucity of effective treatments (1-2). Both of these liver diseases are theorized to benefit from the long-chain omega-3 fatty acids (LCω3s) found in fish, although the role of LCω3s in pediatric patients with NAFLD and PNALD remains investigational (3-4).

This dissertation provides information pertaining to three research questions:

1. Is nonalcoholic fatty liver disease a health concern in Hawai‘i adolescents?
2. Does intake of long-chain omega-3 fatty acids affect pediatric nonalcoholic fatty liver disease?
3. Are fish oil-based lipid emulsions safe and effective in the treatment of parenteral nutrition-associated liver disease in infants?

Five research studies were conducted to address these questions. The first study, “Liver disease among children in Hawaii diagnosed with metabolic syndrome” (Chapter 3), was a cross-sectional analysis of 195 patients referred to a pediatric endocrinologist at Kapi‘olani Medical Center for Women and Children (KMCWC) with a diagnosis of metabolic syndrome that examined the relative prevalence of NAFLD as an obesity-related morbidity. The second study, “The effect of weight loss on pediatric nonalcoholic fatty liver disease” (Chapter 4), included a cross-sectional analysis of 81 overweight pediatric NAFLD patients referred to two pediatric
gastroenterologists at KMCWC to determine its clinical presentation, as well as a prospective evaluation of the relationship between changes in body weight and serum alanine aminotransferase (ALT) concentrations in these patients to assess the conventional recommendation of weight loss as a treatment for NAFLD in children (5). The third study, “Estimation of fish intake in Asian and White female adolescents, and association with two-year changes in body fatness and body fat distribution: The Female Adolescent Maturation (FAM) study” (Chapter 5), examined intake of fish based on three-day food records in 200 Asian and White female adolescents recruited from the Kaiser Permanente Oahu membership database, and assessed the relationship between fish consumption and two-year changes in anthropometric parameters in those with follow up data (n=103). The fourth study, “Estimation of fish and omega-3 fatty acid intake in pediatric nonalcoholic fatty liver disease” (Chapter 6), was a cross-sectional analysis of fish and LCω3s intake reported on a semi-quantitative food frequency questionnaire, and association with histological features of NAFLD in 223 children who were attending eight clinical centers of the Nonalcoholic Steatohepatitis Clinical Research Network. The fifth study, “The use of fish oil-based lipid emulsions in infants with parenteral nutrition-associated liver disease: A case series” (Chapter 7), followed ten infants with PNALD at KMCWC who were receiving fish oil-based lipid emulsions to evaluate its safety and effectiveness in this population. The results of these studies in relation to the dissertation research questions, as well as gaps in the literature that still need to be addressed are outlined in the sections below.
Although pediatric NAFLD had not yet been studied in Hawai`i, there appeared to be relatively good awareness of it locally as 86% of patients being referred to the pediatric endocrinologist with metabolic syndrome had serum ALT measured compared to only 10% at other children’s hospitals (6). Similar to what has been reported elsewhere (7), NAFLD was found to be one of the most common pathologies in children diagnosed with metabolic syndrome, affecting an estimated 63% of these patients. As expected, NAFLD tended to be more common in boys (68%) than in girls (57%), and in older children, although these differences did not reach statistical significance (p >0.05) (8). Interestingly, children with Pacific Islander surnames were more likely to present with NAFLD (88%, p=0.07), indicating that Pacific Islanders may represent a previously unrecognized ethnic group that is at-risk for pediatric NAFLD.

Comparison of serum ALT concentrations of the children with metabolic syndrome who were suspected of having NAFLD (41 U/L (IQR 29-63 U/L) with overweight NAFLD patients seen by pediatric gastroenterologists (105 U/L (IQR 78-153 U/L) suggested that only a small fraction of pediatric NAFLD patients who had quite high serum ALT concentrations were being appropriately referred for evaluation by a pediatric gastroenterologist. Much of this issue may be related to the local reference used to define the upper limit of normal for serum ALT concentrations, which were higher than the serum ALT concentration cutoffs recommended for NAFLD screening in boys (51 U/L vs 25.8 U/L) and girls (31 U/L vs 22.1 U/L) (9). Using these local reference cutoffs, more than one-half (54%) of the children with metabolic syndrome who were suspected of having NAFLD would be missed.
Examination of this latter patient cohort showed that children with NAFLD who were seen by the pediatric gastroenterologist were generally overweight (98%), and usually suffered from other obesity-related conditions. Dyslipidemias, including elevated cholesterol (62%) and triglycerides (63%), and low HDL-cholesterol (52%), were particularly common comorbidities in these patients. In addition to presenting with many other metabolic disorders, liver biopsy samples obtained in a subgroup of these patients (n=13, 16%) found that features of steatohepatitis such as hepatic inflammation (100%) and fibrosis (77%) were frequently present. Although the serum ALT concentrations of patients with liver biopsy were higher (147 U/L (IQR 109-200 U/L)) than those without (102 U/L (75-150 U/L), p = 0.01), this liver disease biomarker is not associated with hepatic fibrosis (10-13), and was found to be associated with hepatic inflammation one (10), but not other studies (11-13). Consequently, the majority of the NAFLD patients being referred to the pediatric gastroenterologist at KMCWC are likely to have some features of steatohepatitis, which is thought to have a worse prognosis (13).

The standard treatment for overweight pediatric NAFLD patients is weight reduction through diet and exercise (5). Despite the fact that children with NAFLD followed by pediatric gastroenterologists at KMCWC for more than one year (n=19) had an average body weight gain of 7.1 ± 9.1 kg, there was a significant reduction in serum ALT concentration -45 ± 69 (p=0.01). Moreover, unlike the findings from clinical trials (14-16), changes in body weight and BMI z-score were not associated with changes in serum ALT concentration in this sample. This suggests that while weight reduction may be efficacious in a controlled setting, this approach may not be an effective in clinical
practice where weight gain is the norm over time. Moreover, this highlights the need for additional evidence-based therapies to treat these patients.

**Influence of fish and long-chain omega-3 fatty acid intake on pediatric nonalcoholic fatty liver disease, and its risk factors**

As previously mentioned, the LCω3s found in fish are thought to have a protective effect on pediatric NAFLD (3). It is currently recommended that adolescents consume eight or more ounces of fish per week (17-19). The findings from the cross-sectional analysis of children with NAFLD on the mainland US indicated high rates of fish inadequacy (85.2%), which corresponded to 95% of patients consuming less than 200 milligrams of LCω3 per day. There is a limited number of published studies reporting on fish intake in the general population; however, the frequency of fish consumption in these patients was lower than that reported in a sample of over 1,000 adolescents attending public schools in Rhode Island (20), suggesting that low fish intake may be a risk factor for pediatric NAFLD.

One possible means by which fish intake could decrease pediatric NAFLD risk is by reducing adiposity in adolescents (21-24). This hypothesis was tested in 103 Asian and White female adolescents who participated in the Female Adolescent Maturation (FAM) study. The findings from this study suggest that greater fish intake may be associated with a reduced two-year change in waist circumference (p=0.03), a measure of visceral adiposity and major risk factor for pediatric NAFLD (22). In addition to potentially reducing pediatric NAFLD risk, it appears that fish and LCω3 intake may have a role in slowing liver disease progression. In the aforementioned multi-center study of children with NAFLD on the mainland US, higher intakes of fish and omega-3 fatty acids were associated with less severe portal and lobular inflammation.
Use of fish oil-based lipid emulsions in infants with parenteral nutrition-associated liver disease

The effect of FOLE in infants with PNALD has been reported previously in case series (25-28) and case-control studies (29-31); however, there is a need for addition research, as many clinically relevant outcomes such as length of stay have not been evaluated (32). In line with previous reports (25-31), infants with PNALD at KMCWC had significant reductions in direct bilirubin concentrations after receiving FOLE treatment, which reached normal levels (<2.0 mg/dL) within 4.1 to 22.7 weeks. Additionally, there was a concurrent transition for parenteral to enteral feeding that mirrored the drop in direct bilirubin concentration, and may represent an additional benefit of FOLE treatment. Despite concerns regarding the nutritional adequacy of FOLEs (33), these patients appeared to grow normally, and none exhibited biochemical evidence of essential fatty acid deficiency. All of the surviving patients had been safely discharged, and earlier initiation of FOLE treatment tended to be associated with a reduction in length of hospital stay following diagnosis with PNALD (p=0.06).

Future Research

The findings presented in this dissertation provide important information regarding the present burden of pediatric NAFLD in Hawai‘i, and the role of LCω3 intake in pediatric NAFLD and PNALD. In addition to these direct contributions, this research has identified potential areas for future investigation.

Given the knowledge that NAFLD is a relatively common obesity-related comorbidity, future studies looking at clinical-pathological correlates of childhood obesity in Hawai‘i should include liver disease biomarkers such as serum ALT. If feasible, it would be useful to obtain liver biopsies from willing participants who present
with elevated serum ALT concentrations indicative of NAFLD to confirm the high amounts of hepatic inflammation and fibrosis that were observed herein. One of the more interesting findings from this preliminary look at pediatric NAFLD in Hawai’i was the influence of ethnicity on disease risk, in particular the apparent increased prevalence of NAFLD among children with Pacific Islander surnames. While this result needs to be replicated in a larger, population-based sample, study of the environmental and genetic factors that underlie such differences may provide useful insights into NAFLD pathogenesis and identify additional modifiable risk factors that contribute to disease risk.

Presented findings also suggested that weight loss might not be a useful outcome for overweight pediatric NAFLD patients in clinical practice. This appears to have been the first study to have directly compared changes in body weight and BMI z-score with liver disease biomarkers in a non-experimental setting, and has important implications for the management of these patients as weight reduction is currently the primary treatment approach (5). Consequently, this study should be replicated in a more robust sample of pediatric NAFLD patients, which includes lifestyle and histological measurements to help identify modifiable behaviors that can help to reduce liver disease severity (33). Given the generally inadequate consumption of fish and LCω3s in children with NAFLD, and their possible connections to visceral adiposity and hepatic inflammation, a clinical trial that includes histological outcomes should be conducted to examine the effect of increasing fish and/or LCω3 intake on pediatric NAFLD.

Lastly, despite the apparent benefit of FOLEs in infants with PNALD, additional questions remain regarding the appropriate clinical utility of this treatment approach. Future investigators should attempt to determine the optimal timing for FOLE treatment.
In particular, researchers need to assess the risk-benefit of using FOLE prophylactically in infants with intestinal failure who are at high risk of developing PNALD. Additionally, if possible, a double blind trial comparing clinically relevant outcomes of infants with PNALD who are randomized to receive either conventional lipid emulsions or FOLE would offer more solid evidence of its efficacy than is currently being provided by observational studies.

References
5. Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of non-alcoholic fatty liver disease: Practice guidelines by the American Association for the Study of Liver Diseases, American College of Gastroenterology, and the


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<th>Energy / Macronutrients</th>
<th>Micronutrients</th>
<th>Methods / Comments</th>
</tr>
</thead>
</table>
| **[1]** United States, Hispanic | 2133 kcal (Pro 15%, CHO 52%, Fat 34%) | | Dx: ALT >97.5th for age/sex  
Diet: 2-d multiple-pass 24-hr diet recall by RD; subjects <7 y assisted by caregiver; analyzed using Nutrition Data System software |
| No NAFLD (n=210M), 11.2 y, BMI z-score 2.3, ALT 21 U/L | | | Findings: No differences in macronutrient intake by gender or NAFLD status |
| No NAFLD (n=181F), 10.8 y, BMI z-score 2.2, ALT 18 U/L | 1921 kcal (Pro 14%, CHO 53%, Fat 34%) | | |
| NAFLD (n=68M), 11.6 y, BMI z-score 2.6, ALT 75 U/L | 2317 kcal (Pro 15%, CHO 52%, Fat 34%) | | |
| NAFLD (n=58F), 11.1 y, BMI z-score 2.3, ALT 62 U/L | 1873 kcal (Pro 14%, CHO 54%, Fat 34%) | | |
| **[2]** Brazil (n=19M:24F), 17.2 y (15-19 y) | | | Dx: Liver ultrasound  
Diet: 3-d food record assisted by caregiver (RD education provided); analyzed using Nutwin software for Windows (v.1.5) |
| No NAFLD (n=30), BMI 34 kg/m² | 1867 kcal (Pro 22%, CHO 52%, Fat 26%) | Cholesterol 245mg | Findings: No differences in macronutrient intake by gender or NAFLD status |
| NAFLD (n=13), BMI 36 kg/m² | 1621 kcal (Pro 24%, CHO 53%, Fat 24%) | Cholesterol 253mg | |
| **[3]** Greece | | | Dx: Liver ultrasound  
Diet: 3-d (2 x weekday, 1 x weekend) 24-hr dietary recall by RD assisted by caregiver; analyzed using Sciencetech Diet 200A |
| No NAFLD (n=13M/12F), 11.0 y, BMI 27 kg/m², ALT 24 U/L | 2716 kcal (Pro 106g, CHO 242g, Fat 144g) | vitamin B₁ 2.6mg, vitamin B₂ 2.9mg, vitamin B₃ 5.1mg, vitamin B₅ 2.5mg, vitamin B₆ 6.3µg, folate 471µg, vitamin A 5696IU, vitamin D 223IU, vitamin E 11mg, vitamin K 170µg, vitamin C 119mg | Findings: Significant differences (p<0.05) denoted as:  
a₁, 2nd/3rd  
b₁, 2nd/3rd/3rd  
c₁, 1st/3rd |
| SFA 44g, Fiber 19g, Sugar 12g | % of kcal as CHO and SFA | | |
| SFA 44g, Fiber 19g, Sugar 12g | % of kcal as CHO and SFA | | |
| Mild NAFLD (n=5M/4F), 11.5 y, BMI 26 kg/m², ALT 28 U/L | 2435 kcal (Pro 95g, CHO 269g, Fat 123g) | vitamin B₁ 2.2mg, vitamin B₂ 2.7mg, vitamin B₃ 4.8mg, vitamin B₅ 2.5mg, vitamin B₆ 4.9µg, folate 336µg, vitamin A 3145IU, vitamin D 204IU, vitamin E 8.2mg, vitamin K 109mg, vitamin C 112mg | | |
| SFA 55g, Fiber 16g, Sugar 19g | | | |
| Mod/Sev NAFLD (n=7M/2F), 11.8 y, BMI 37 kg/m², ALT 24 U/L | 2815 kcal (Pro 116g, CHO 278g, Fat 140g) | vitamin B₁ 2.7mg, vitamin B₂ 3.1mg, vitamin B₃ 4.9mg, vitamin B₅ 2.4mg, vitamin B₆ 6.3µg, folate 435µg, vitamin A 4765IU, vitamin D 224IU, vitamin E 11mg, vitamin K 121mg, vitamin C 118mg | Only SFA α steatosis grade after adjusting for age, sex and diet |
Table I. Summary of studies reporting on nutrient intake in pediatric nonalcoholic fatty liver disease (cont.)

<table>
<thead>
<tr>
<th>Study Population</th>
<th>Energy / Macronutrients</th>
<th>Micronutrients</th>
<th>Methods / Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>[4] Canada (n=26M/12F and 18Wht/11Hisp/8As/10oth), 14.1 y (5.5-11.8 y), BMI z-score 2.0 (0.1-3.2), ALT 111 U/L</td>
<td>1774 kcal (Pro 19%, CHO 52%, Fat 31%) SFA 11%, PUFA 5%, MUFA 9% LA 9.1g, AA 0.14g, ALA 0.77g, EPA 0.22g Sucrose 23g, Fructose 22g, Fiber 14g Sugars (Sucrose+Fructose) = 24% CHO GI 59, GL 122</td>
<td>Cholesterol 278mg, vit B1 1.4mg, vit B2 1.5mg, vit B3 21mg, vit B6 1.5mg, vit B12 4.9µg, folate 359µg, vit A 920RE, vit C 89mg, vit E 4.6IU, Se 98µg</td>
<td>Dx: Liver ultrasound Diet: 3-d (2xW/E, 1xW/D) food record assisted by caregiver (RD education provided); analyzed using Diet Analysis+ (v.4), USDA NDSR (for sucrose, fructose and Se), KIM-2 software (for ω-3 and ω-6 fatty acids) &amp; published values (for GI/GL based on glucose ref.) Findings: EPA/DHA intake 1/α ALT in multivariate model</td>
</tr>
<tr>
<td>[5] Brazil (n=22M/28F), 16.7 y (15-19 y)</td>
<td>1914 kcal (Pro 19%, CHO 51%, Fat 30%) (Pro 86g, CHO 246g, Fat 66g) SFA 11%, MUFA 8%, PUFA 3%</td>
<td>Cholesterol 232mg</td>
<td>Dx: Liver ultrasound Diet: 3-d food record assisted by caregiver (RD education provided); analyzed using Nutwin software for Windows (v.1.5) Findings: No differences in macronutrient or cholesterol intake by NAFLD status</td>
</tr>
<tr>
<td>No NAFLD (n=25), BMI 33 kg/m², ALT 30 U/L</td>
<td>2185 kcal (Pro 20%, CHO 49%, Fat 31%) (Pro 111g, CHO 266g, Fat 79g) SFA 10%, MUFA 9%, PUFA 3%</td>
<td>Cholesterol 302mg</td>
<td></td>
</tr>
<tr>
<td>NAFLD (n=25), BMI 38 kg/m², ALT 48 U/L</td>
<td>1526 kcal (Pro 16%, CHO 51%, Fat 35%) Fiber 13g, SSB 4.6/wk, Desserts 7.4% kcal</td>
<td>Cholesterol 205mg, folate 275µg, vit A 954RE, vit C 100mg, vit D 122 IU, vit E 6.6IU</td>
<td></td>
</tr>
<tr>
<td>[6] United States*</td>
<td>1502 kcal (Pro 17%, CHO 49%, Fat 35%) SFA 21g, MUFA 24g, PUFA 11g Fiber 15g, SSB 4.2/wk, Desserts 5.2% kcal</td>
<td>Cholesterol 191mg, folate 329µg, vit A 907RE, vit C 109mg, vit D 119 IU, vit E 6.7IU</td>
<td>Dx: Liver biopsy Diet: 77-item semi-quantitative FFQ (2000 Block Brief) of intake in previous year; analyzed by NutritionQuest proprietary software; SSB includes fruit juices, Kool Aid and similar drinks, fruit pouches and soda Findings: No differences in NAFLD diagnosis by macronutrient or cholesterol intake; vit E 1/α steatosis, fiber 1/α lobular inflammation, vitamin C 1/α ballooning degeneration</td>
</tr>
</tbody>
</table>
### Table I. Summary of studies reporting on nutrient intake in pediatric nonalcoholic fatty liver disease (cont.)

<table>
<thead>
<tr>
<th>Study Population</th>
<th>Energy / Macronutrients</th>
<th>Micronutrients</th>
<th>Methods / Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>[7] United States* (n=114 and 39Wht/67Hisp/7Oth), 12 y (9-13 y), BMI z-score 2.4, ALT 83 U/L</td>
<td>1789 kcal (Pro 72g, CHO 234g, Fat 66g)</td>
<td>Choline 292mg, folate 416µg</td>
<td>Dx: Liver biopsy. Diet: 77-item semi-quantitative FFQ (2000 Block Brief) of intake in previous year; analyzed by NutritionQuest proprietary software &amp; USDA Database for Choline Content of Common Foods (Release 2) for choline. Findings: No differences in ALT, NAFLD diagnosis or histological features of NASH by choline intake.</td>
</tr>
</tbody>
</table>

Abbr: ALT=Alanine aminotransferase, BZ3=Borderline zone 3 NASH, BZ1=Borderline zone 1 NASH, Diet (Pro=protein, CHO=carbohydrate, SFA=saturated fatty acid, MUFA=monounsaturated fatty acid, PUFA=polysaturated fatty acid, LA=linoleic acid, AA=arachidonic acid, ALA=α-linolenic acid, EPA=eicosapentaenoic acid, DHA=docosahexaenoic acid, GI=glycemic index, GL=glycemic load, SSB=sugar-sweetened beverages, vit=vitamin, Se=selenium), Dx=Method of diagnosing NAFLD, Ethnicity (Mex=Mexican, Centr=Central, Wht=White, Hisp=Hispanic, Oth=Other, As=Asian), FFQ=Food frequency questionnaire, Mod/Sev=Moderate to severe, NAFLD=Nonalcoholic fatty liver disease, NASH=Nonalcoholic steatohepatitis, RD=Registered dietitian, USDA NDSR=USDA Nutrient Database for Standard Reference, W/D=weekday, W/E=weekend

*Studies [6] and [7] were both conducted through the Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN), and contained overlapping data. The study by Vos et al., 2012 [6] included participants in the NAFLD database study, whereas the study by Guerrerio et al., 2012 [7] included these subjects as well as those enrolled in the Treatment of NAFLD in Children trial.

**References:**


<table>
<thead>
<tr>
<th>Study Population</th>
<th>Design</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1] Italy (6M/3F), 8.7 y, Wt z-score 4.6, ALT 84 U/L, high ALT/AST x 14mo</td>
<td>Dx: ALT; Duration: 6-12 mo Diet/PA: Balanced, individualized, hypocaloric diet and exercise for goal of -500 g/wk Wt loss</td>
<td>If ↓ ≥10% Wt z-score (78%), then ↓ ALT (100% N) (p&lt;0.01), and 4/5 (80%) ↓ US Fat If ↑ Wt (22%), then no Δ in ALT or US Fat</td>
</tr>
<tr>
<td>[2] Italy (n=17M/21F), 8.2 y, 163% IBW, 32% high ALT/AST</td>
<td>Dx: Liver ultrasound; Duration: 3-6 mo Diet: 1200-1400 kcal (35 kcal/kg/d), Pro 12%, CHO 65%, Fat 23% PA: ≥6 hr/wk aerobic + lifestyle change</td>
<td>If ↓ ≥10% IBW (85%), then 100% ↓ US Fat (85% N) If ↓ &lt;10% IBW (15%), then 0% ↓ US Fat If ↓ Wt, then 100% N ALT at 3 mo</td>
</tr>
<tr>
<td>[3] Italy (n=59M/25F), 11.7 y, BMI z-score 1.9, ALT 76 U/L</td>
<td>Dx: ALT, liver ultrasound and biopsy; Duration 12 mo Diet: Hypocaloric (25-30 kcal/kg/d) 15-20% Pro, 50-60% CHO, 23-30% Fat 2/3 SFA, 1/3 MUFA/PUFA, 4:1 ω-6:ω-3 fatty acids RD counseling x 1hr with subject/caregiver PA: 3x/wk aerobic (30-45 mins/x)</td>
<td>If ↓ &gt;10% Wt (33%), then 100% ↓ US Fat (29% N) If ↓ 5-10% Wt (33%), then 100% ↓ US Fat If ↓ &lt;5% Wt (35%), then 67% ↓ US Fat If ↓ Wt, then 100% N ALT at 3 mo</td>
</tr>
<tr>
<td>[4] Brazil (n=24M/49F), 17.0 y, BMI 36.5 kg/m²</td>
<td>Dx: Liver ultrasound; Duration: 12 wks Diet: Told to reduce intake &amp; follow balanced diet, nutrition training q 3wk (e.g., food pyramid, weight-loss diets, diet v. light, fat/cholesterol) PA: 2x/wk activity sessions (60 min/x) + encouraged spontaneous activity</td>
<td>Wt ↓ 102 to 98 kg (p=0.0001) (↓ ≥4.5 kg (48%), ↓&lt;4.5 kg (48%) and ↑ &gt;0 kg (4%)) US steatosis ↓ to 29/29% (R/L side) (p&lt;0.05) Energy and fat intake ↓, and protein intake ↑ before/after intervention</td>
</tr>
<tr>
<td>[5] Brazil (n=19M/24F), 15-19 y, BMI 34.2 kg/m²</td>
<td>Dx: Liver ultrasound; Duration: 12 wks Diet: Told to reduce intake &amp; follow balanced diet, dietetics classes q wk (e.g., food pyramid, weight-loss diets, diet v. light, fat/cholesterol) PA: 3x/wk aerobic (60 min/x @ 50-70% O₂ max.) + encouraged spontaneous activity</td>
<td>NAFLD: Wt ↓ 103 kg (n=13) to 96 kg (n=10) (NS) Total: US steatosis ↓ to 10/23 (18.6%) Energy intake ↓ before/after intervention</td>
</tr>
<tr>
<td>[6] China</td>
<td>Dx: ALT + Liver ultrasound; Duration: 1 mo Control Group (n=26M/12F), 14.0 y, BMI z-score 3.5, ALT 145 U/L</td>
<td>No treatment No Δ in BMI z-score or ALT</td>
</tr>
<tr>
<td>Treatment Group (n=13M/6F), 13.4 y, BMI z-score 3.0, ALT 152 U/L</td>
<td>Diet: 1300-1600 kcal/d (goal of -250 kcal/d) 40% Pro, 50% CHO, 10% Fat; H₂O to drink PA: 3hrs aerobic q d; ≤1hr TV/d (in Summer camp)</td>
<td>BMI z-score ↓ to 2.2 (p&lt;0.05) ALT ↓ to 64 U/L (53% N ALT) (p=0.001)</td>
</tr>
</tbody>
</table>
Table II. Summary of studies investigating the effect of lifestyle intervention for pediatric nonalcoholic fatty liver disease (cont...)

<table>
<thead>
<tr>
<th>Study Population</th>
<th>Design</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>[7] Germany (n=187 eligible), 6-16 y</td>
<td>Dx: Liver ultrasound; Duration: 1 and 2 yrs</td>
<td>Subject/caregiver consultation on suitable diet, physical activity and behavior (15 mins); handouts with recipes given</td>
</tr>
<tr>
<td>Control Group (n=51), unable to do program (live too far away); 43/51 (84%) (n=26M/17F) with f/u data; BMI z-score 2.3, ALT 47 U/L</td>
<td></td>
<td>BMI z-score ↓ to 2.3 (yr 1) and 2.2 (yr 2) (p=0.001) ALT ↓ to 38 U/L (yr 1) and 39 U/L (yr 2) (p = 0.002) US steatosis ↓ to 50% (yr 1) and 54% (yr 2) (p&lt;0.001)</td>
</tr>
<tr>
<td>Treatment Group (n=58M/51F); BMI z-score 2.5, ALT 48 U/L</td>
<td></td>
<td>BMI z-score ↓ to 2.3 (yr 1) and 2.2 (yr 2) (p=0.001) ALT ↓ to 38 U/L (yr 1) and 39 U/L (yr 2) (p = 0.002) US steatosis ↓ to 50% (yr 1) and 54% (yr 2) (p&lt;0.001)</td>
</tr>
<tr>
<td>Note – 8 dropouts, but had complete data</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[8] Italy (n=11M/15F), 6-15 y, BMI z-score 2.3, Hepatic fat 7.8%</td>
<td>Dx: Liver MRI; Duration: 1 yr Diet: Normocaloric; 12-15% Pro, 55-60% CHO (&lt;10% high GI), 25-30% Fat, &lt;10% SFA, Fiber based on age; RD consult x 1hr (diet guidelines provided) PA: Moderate aerobic exercise program (30-45 min/d)</td>
<td>MRI steatosis: MRI steatosis ↓ to 2/9 (22%)</td>
</tr>
<tr>
<td>Note – 9/26 (35%) MRI steatosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[9] Netherlands (n=180) 144/180 (80%) (n=54M/90F) with f/u data, 14.1 y, BMI z-score 3.4, ALT 28 U/L</td>
<td>Dx: ALT or liver ultrasound; Duration: 6 mo Diet: Education on diet quality and eating behaviors to subject/caregivers (separately); Subjects had 12x sessions with homework (if outpatient) or sessions 5d/wk (if inpatient)</td>
<td>MRI z-score ↓ to 2.9 (p&lt;0.001) ALT ↓ to 23 U/L and 16/144 (11%) high ALT (p=0.001) US steatosis ↓ to 17/144 (12%) (p&lt;0.001)</td>
</tr>
<tr>
<td>Note – 37/144 (26%) with high ALT and steatosis; subject with f/u data were less likely to have liver high ALT or steatosis</td>
<td>PA: Exercise 3x/wk x 1hr, Promote self-initiated PA</td>
<td></td>
</tr>
<tr>
<td>Note – program facilitated by PE teachers, RD and child psychologist</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table II. Summary of studies investigating the effect of lifestyle intervention for pediatric nonalcoholic fatty liver disease (cont…)

<table>
<thead>
<tr>
<th>Study Population</th>
<th>Design</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>[10] Denmark (n=51M/66F), 12.1 y, BMI z-score 2.9</td>
<td>Dx: ALT or liver ultrasound</td>
<td>At 10 wk: BMI z-score ↓ to 2.3 (p&lt;0.05), ALT ↓ (p&lt;0.05), US steatosis ↓ to 33/109 (30%) (p&lt;0.05)</td>
</tr>
<tr>
<td>Note – 58/117 (50%) with high ALT and 49/114 (43%) with steatosis; 71/117 (61%) with f/u data at 12 mo</td>
<td>Note – 58/117 (50%) with high ALT and 49/114 (43%) with steatosis; 71/117 (61%) with f/u data at 12 mo</td>
<td>At 12 mo: BMI z-score ↓ to 2.5 (p&lt;0.05), but ↑ from 10 wk (p&lt;0.05), no ∆ in ALT, US steatosis ↓ to 16/62 (26%) (p&lt;0.05)</td>
</tr>
<tr>
<td>Diet: 1550 kcal (15% Pro, 60% CHO, 24% Fat)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 g Fiber, 3 meals + 3 snacks, no soft drinks, candy in small amounts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA: Moderate exercise 1hr/d</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ALT=Alanine aminotransferase, AST=Aspartate aminotransferase, CHO=Carbohydrates, Dx=Diagnosis, H2O=Water, IBW=Ideal body weight, f/u=Follow up, L=Left, MRI=Magnetic resonance imaging, MUFA=Monounsaturated fatty acids, N=Normal, NS=Non-significant difference, O2=Oxygen, PA=Physical activity, PE=Physical education, Pro=Protein, PUFA=Polyunsaturated fatty acids, q=Every, R=Right, RD=Registered dietitian, SFA=Saturated fatty acids, Tx=Treatment, US Fat=Ultrasound fat grade, US Steatosis=Ultrasound steatosis diagnosis, Wt=Weight

References:
Table III. Summary of trials investigating the effect of long-chain omega-3 fatty acids for pediatric nonalcoholic fatty liver disease

<table>
<thead>
<tr>
<th>Study Population</th>
<th>Methods</th>
<th>Liver Disease</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>[1] Greece (n=9M/14F), 52 y, ALT 110 U/L</td>
<td>Design: Open-label trial; Duration: 24 wk Tx: 2.3 g DHA, 1.6 g EPA</td>
<td>↓ US fat, ALT, AST, GGTP</td>
<td>↓ TG, Chol, LDL-C; No Δ BMI, HDL-C</td>
</tr>
<tr>
<td>[2] Italy Tx: (n=23M/19F), 58 y, ALT 34 U/L, US grade (0/8/19/15) Ctr: (9M:5F), 62 y, ALT 38 U/L, US grade (0/3/5/6)</td>
<td>Design: Open-label trial; Duration: 12 mo Tx: 1 g LCo3 (0.9: 1.5 ratio EPA: DHA) Ctr: Refused Tx</td>
<td>↓ US fat, DPI, ALT, AST, GGTP</td>
<td>↓ TG, FBG</td>
</tr>
<tr>
<td>[3] Italy Tx: (11M/7F), 50.2 y, ALT 57 U/L, US grade (0/0/7/11) Ctr: (8M/10F), 51.3 y, ALT 60 U/L, US grade (0/0/8/10)</td>
<td>Design: Randomized, double blind, placebo-controlled trial; Duration: 6 mo Tx: 1 g LCω3 bid</td>
<td>↓ US fat, ALT, GGTP; No Δ AST</td>
<td>↓ BMI, TG, Chol, HDL-C, HOMAIR, TNF-α; ↑ HDL-C</td>
</tr>
<tr>
<td>[4] Japan (n=14M9F), 56 y, ALT 79 U/L, US grade (0/7/5/11) Note – 7/23 had repeat Bx</td>
<td>Design: Open-label trial; Duration: 12 mo Tx: 2.7 g EPA</td>
<td>↓ US fat, ALT, AST, Bx fat (5/7), Lob Inf (4/7), Hep Ball (5/7), Fibr (6/7); No Δ GGTP, ALP</td>
<td>↓ Chol, FFA, sTNF-R1/R2; No Δ Wt, TG, HDL-C, HOMAIR, TNF-α, Adiponectin</td>
</tr>
<tr>
<td>[5] China Tx: (47M/19F), 45 y, ALT 63 U/L, US grade (0/30/56/14) Ctr: (50M/18F), 44y, ALT 80 U/L, US grade (0/37/48/15)</td>
<td>Design: Randomized, double blind, placebo-controlled trial; Duration: 24 wk Tx: 2 g seal oil tid</td>
<td>↓ US fat*, ALT*, AST, GGTP</td>
<td>↓ Sx*, TG*, Chol, LDL-C; ↑ HDL-C</td>
</tr>
<tr>
<td>[6] United States (n=4M/12), 50 y, ALT 23 U/L</td>
<td>Design: Placebo-controlled trial; Duration: 4 wk Ctr, 8 wk Tx Tx: 9 g fish oil (51% EPA, 24% DHA)</td>
<td>Tx not compared to Ctr</td>
<td>↓ TG, VLDL, IDL-C, ApoB; No Δ LDL-C, HDL-C</td>
</tr>
<tr>
<td>[7] Australia (n=12), 35.3 y, ALT 30 U/L Note – All subjects had PCOS</td>
<td>Design: Randomized, double blind, placebo-controlled crossover trial; Duration: 8 wk Tx/WO/Ctr Tx: 4 g LCo3 (27% EPA, 56% DHA)</td>
<td>Tx compared to Ctr</td>
<td>↓ MRS fat; No Δ ALT</td>
</tr>
<tr>
<td>[8] Italy Tx: (n=4M/2F), 55 y, ALT 87 U/L, US grade (0/1/2/3) Ctr: (n=5M), 54 y, ALT 59 U/L, US grade (0/1/2/2)</td>
<td>Design: Randomized, double blind, placebo-controlled trial; Duration: 12 mo Tx: 6.5 mL olive oil with LCo3 (0.83 g ω-3, 0.47 g EPA, 0.24 g DHA)</td>
<td>↓ US fat, DPI, ALT*, AST*, GGTP*</td>
<td>No Δ TG*, Chol, LDL-C, HOMAIR, ROS; ↑ HDL-C*, adiponectin*</td>
</tr>
</tbody>
</table>

Note – All subjects had PCOS
Table III. Summary of trials investigating the effect of long-chain omega-3 fatty acids for pediatric nonalcoholic fatty liver disease (cont…)

<table>
<thead>
<tr>
<th>Study Population</th>
<th>Methods</th>
<th>Liver Disease</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>[9] Italy</td>
<td>Design: Randomized, double blind, placebo-controlled trial; Duration: 6 mo</td>
<td>↓ US fat; No Δ ALT</td>
<td>No Δ BMI; ↑ ISI</td>
</tr>
<tr>
<td>Tx1: (n=8M/12F), 11 y, ALT 70 U/L, US grade (0/0/8/12)</td>
<td>Tx1: 250 mg DHA</td>
<td>No Δ BMI; ↑ ISI</td>
<td></td>
</tr>
<tr>
<td>Tx2: (n=9M/11F), 11 y, ALT 57 U/L, US grade (0/0/8/12)</td>
<td>Tx2: 500 mg DHA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctr: (n=8M/12F), 13 y, ALT 78 U/L, US grade (0/0/8/12)</td>
<td>Ctr: Control</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ALP=Alkaline phosphatase, ALT=Alanine aminotransferase, ApoB=Apolipoprotein B, AST=Aspartate aminotransferase, bid=Twice daily, BMI=Body mass index, Bx=Biopsy, Chol=Cholesterol, CRP=C-reactive protein, Ctr=Control, DHA=Docosahexaenoic acid, DPI=Doppler perfusion index, EPA=Eicosapentaenoic acid, FBG=Fasting blood glucose, Fibr=Fibrosis, FFA=Free fatty acids, GGTP=γ-glutamyl transpeptidase, HDL-C=HDL-cholesterol, Hep Bal=Hepatocyte ballooning, HOMA_IR=Homeostatic model of insulin resistance, ISI=Insulin sensitivity index, LCω-3=Long-chain ω-3, LDL-C=LDL-cholesterol, Lob Infl=Lobular inflammation, MRS=Magnetic resonance spectroscopy, PCOS=Polycystic ovary syndrome, ROS=Reactive oxygen species, sTNF-R1/R2=Soluble tumor necrosis factor-receptors 1 and 2, Sx=symptoms, tid=Thrice daily, TG=Triglycerides, TNF-α=Tumor necrosis factor-α, Tx=Treatment, US=Ultrasound, WO=Washout, Wt=Weight

References:
## Table IV. Summary of case series and case-control studies investigating the use of fish oil-based intravenous lipid emulsions in infants with parenteral nutrition-associated liver disease

<table>
<thead>
<tr>
<th>Study Population</th>
<th>Protocol</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Case-Series Studies</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| [1] Toronto, Canada (n=7M/5F), 7.5 (3.6-46.0) mo, DBili 8.1 (3.2-11.9) mg/dL | PNALD: DBili >5.9 mg/dL or >2.9 mg/dL + other evidence of liver disease  
1 g/kg FOLE with 1 g/kg VOLE (↓ VOLE no Δ or worse PNALD) | 9/12 DBili N in 24 (7-37) wks;  
EN ↑ 10% (3/9 not PN-dependent);  
↓ ALT, AST; no Δ in GGTP, INR; ↑ Alb |
| [2] Boston, United States (n=7M/3F), 3.5 mo (0.8-37 mo), DBili 6.8 (2.5-12.8) mg/dL | PNALD: DBili ≥ 2.0 mg/dL  
1 g/kg FOLE  
Duration: 3.8 (1.2-9.3) mo | DBili ↓ to 0.9 (0.1-9.6) mg/dL (6/10 DBili N);  
MA and T:T N in 100%; ↓ ω-6; ↑ ω-3, No s/s of EFAD;  
no Δ in Wt, LG, or HCir z-score |
| [3] Boston, United States (n=49M/30F), 3.0 (1.8-6.2) mo, DBili 5.4 (3.5-8.5) mg/dL | PNALD: DBili ≥ 2.0 mg/dL  
1 g/kg FOLE  
Duration: 3.9 (IQR 2.0-8.9) mo | DBili N in 18.6 (16.4-19.3) wks;  
No s/s of EFAD;  
↓ ω-6, MA, T:T (100% T:T N); ↑ ω-3;  
↓ TG, VLDL, Chol, LDL-C, CRP; no Δ in HDL-C |
| [4] London, Canada (n=4M/4F), 2.2 (0.7-7.0) mo, DBili 3.4 (2.6-4.2) mg/dL | PNALD: DBili >2.0 mg/dL  
FOLE: 1 g/kg  
Duration: 4.6-8.4 mo | DBili N in 9.7 (8.1-15.9) wks |
| **Case-Control Studies** | | |
| [5] Boston, United States (n=12M/6F), 14±7 wk, DBili 5.4 (3.4-7.7) mg/dL | PNALD: DBili ≥2.0 mg/dL  
Duration: 3.9 (1.9-7.8) mo | *↓ in Tx compared to Ctr,  
DBili N in 11/18 (61%) at 9.4 (7.6-10.9) wks*;  
↓ Plt Cnt; no Δ in Wt z-score, infection, TG; 1/18 EFAD;  
0 expired r/t liver, 0 liver TX, d/c PN 13.8 (7.6-36.4) wks |
| Ctr: (n=10M11F), 14±20 wk, DBili 3.5 (3.2-5.5) mg/dL | 1-4 g/kg VOLE | DBili N in 7/21 (33%) at 44.1 (10.9-45.6) wks;  
6 expired r/t liver, 2 liver TX, d/c PN 22.9 (12.6-76.8) wks |
| [6] Boston, United States (n=28M/6F), 12 (IQR 8-25) wk, DBili 5.5 (IQR 3.7-8.5) mg/dL | PNALD: DBili ≥2.0 mg/dL  
Duration: 1.5 (IQR 0.9-3.0) mo | *↓ in Tx compared to Ctr, ↑† in Tx compared to Ctr  
DBili N in 19/42† at 11.7 (IQR 7.7-13.9) wks;  
T:T ↑ in 2/42, ↓ TG†, ↑ Plt Cnt†, no Δ ALT†, INR;  
3 expired, 1 liver TX, 4 expired or TX†, d/c PN 20 (IQR 9-29) wks† |
| Ctr: (n=31M/18F), 7 (IQR 5-12) wk, DBili 3.3 (IQR 2.9-4.4) mg/dL | 1-4 g/kg VOLE | DBili N in 2/49;  
12 expired, 6 liver TX, 17 expired or TX, d/c PN 4 (IQR 3-10) wks |

Note: PN sole source of lipid x >1 mo
Table IV. Summary of case series and case-control studies investigating the use of fish oil-based intravenous lipid emulsions in infants with parenteral nutrition-associated liver disease (cont…)

<table>
<thead>
<tr>
<th>Study Population</th>
<th>Protocol</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>[7] Boston, United States</td>
<td>PNALD: DBili &gt;2.0 mg/dL Duration: max 4.1 mo</td>
<td>*↓ in Tx compared to Ctr</td>
</tr>
<tr>
<td>Tx: (n=12M/6F), 13 (IQR 8=18) wk, DBili 5.4 (IQR 3.4-6.9) mg/dL</td>
<td>1 g/kg FOLE Duration: 3.0 (IQR 2.4-3.9) mo</td>
<td>16/18 (89%) DBili N; ↓ in TG*, 2 expired, d/c PN 8</td>
</tr>
<tr>
<td>Ctr: (n=35M/24F), 7 (IQR 5-12) wks, DBili 4.3 (IQR 3.3-6.6) mg/dL</td>
<td>1-4 g/kg VOLE Duration: 3.2 (IQR 1.5-4.1) mo</td>
<td>28/59 (47%) DBili N; no Δ in TG 12 expired, 6 liver TX, d/c PN 40</td>
</tr>
</tbody>
</table>

ω-3=Omega-3 fatty acid, ω-6=Omega-6 fatty acid, Alb=Albumin, ALT=Alanine aminotransferase, AST=Aspartate aminotransferase, Chol=Cholesterol, Ctr=Control, DBili=Direct bilirubin, d/c=Discontinue, EFAD=Essential fatty acid deficiency, EN=Enteral nutrition, FOLE=Fish oil-based lipid emulsion, GGTP=γ-glutamyl transpeptidase, Hcirc=Head circumference, HDL-C=High density lipoprotein cholesterol, INR=International normalized ratio, IQR=Interquartile range, LDL-C=Low density lipoprotein cholesterol, LG=Length, MA=Mead acid, N=Normal, Plt Cnt=Platelet Count, PN=Parenteral nutrition, PNALD=Parenteral nutrition-associated liver disease, r/t=Related to, s/s=Sign or symptom, TG=Triglyceride, T:T=Triene:Tetraene ratio, Tx=Treatment, TX=Transplant, VLDL=Very low density lipoprotein, VOLE=Vegetable oil-based lipid emulsion, Wt=Weight

References:
January 24, 2011

TO: David St-Jules, R.D.
    Principal Investigator
    Human Nutrition, Food and Animal Sciences

FROM: Nancy R. King
    Director

Re: CHS #18834- "Epidemiology of Pediatric Nonalcoholic Fatty Liver Disease in Children with Metabolic Syndrome"

This letter is your record of CHS approval of this study as exempt.

On January 24, 2011, the University of Hawai‘i (UH) Committee on Human Studies (CHS) approved this study as exempt from federal regulations pertaining to the protection of human research participants. The authority for the exemption applicable to your study is documented in the Code of Federal Regulations at 45 CRF 46 (4).

Exempt studies are subject to the ethical principles articulated in The Belmont Report, found at http://www.hawaii.edu/irb/html/manual/appendices/A/belmont.html

Exempt studies do not require regular continuing review by the Committee on Human Studies. However, if you propose to modify your study, you must receive approval from CHS prior to implementing any changes. You can submit your proposed changes via email at uhirb@hawaii.edu. (The subject line should read: Exempt Study Modification.) CHS may review the exempt status at that time and request an application for approval as non-exempt research.

In order to protect the confidentiality of research participants, we encourage you to destroy private information which can be linked to the identities of individuals as soon as it is reasonable to do so. Signed consent forms, as applicable to your study, should be maintained for at least the duration of your project.

This approval does not expire. However, please notify CHS when your study is complete. Upon notification, we will close our files pertaining to your study.

If you have any questions relating to the protection of human research participants, please contact CHS at 956-5007 or uhirb@hawaii.edu. We wish you success in carrying out your research project.
January 28, 2011

Sorrell Waxman, MD
1319 Punahou Street
Suite 1100
Honolulu, HI 96826

Dear Dr. Waxman:

SUBJECT: ACKNOWLEDGEMENT OF STUDY
Principal Investigator: Sorrell Waxman, MD
Protocol Title: Epidemiology of Pediatric Nonalcoholic Fatty Liver Disease in Children with Metabolic Syndrome
HPHRI Study No.: 2011-005

This letter serves as your acknowledgement that Hawai‘i Pacific Health is aware that your study is being conducted and has been approved by the University of Hawai‘i Committee on Human Subjects. Hawai‘i Pacific Health Research Institute has determined that your study does not require our approval because it will be conducted in the private office of a non-employed physician and will not access Hawai‘i Pacific Health medical records.

As the principal investigator please submit any changes to the protocol, including closure of the study to the Hawai‘i Pacific Health Research Institute.

Although your study will not be overseen by Hawai‘i Pacific Health Research Institute, we will maintain a study file.

Sincerely,

Christine Nelson
Director
Hawai‘i Pacific Health Research Institute
August 14, 2012

TO: David St-Jules
    Principal Investigator
    Human Nutrition, Food and Animal Science

FROM: Ching Yuan Hu, Ph.D.
    Interim Director
    Human Studies Program
    Office of Research Compliance
    University of Hawaii, Manoa

Re: CHS #20438 - "Natural History of Pediatric Nonalcoholic Fatty Liver Disease"

This letter is your record of the Human Studies Program approval of this study as exempt.

On August 14, 2012, the University of Hawai‘i (UH) Human Studies Program approved this study as exempt from federal regulations pertaining to the protection of human research participants. The authority for the exemption applicable to your study is documented in the Code of Federal Regulations at 45 CFR 46.101(b) (4).

Exempt studies are subject to the ethical principles articulated in The Belmont Report, found at http://www.hawaii.edu/irb/html/manual/appendices/A/belmont.html

Exempt studies do not require regular continuing review by the Human Studies Program. However, if you propose to modify your study, you must receive approval from the Human Studies Program prior to implementing any changes. You can submit your proposed changes via email at uhirb@hawaii.edu. (The subject line should read: Exempt Study Modification.) The Human Studies Program may review the exempt status at that time and request an application for approval as non-exempt research.

In order to protect the confidentiality of research participants, we encourage you to destroy private information which can be linked to the identities of individuals as soon as it is reasonable to do so. Signed consent forms, as applicable to your study, should be maintained for at least the duration of your project.

This approval does not expire. However, please notify the Human Studies Program when your study is complete. Upon notification, we will close our files pertaining to your study.

If you have any questions relating to the protection of human research participants, please contact the Human Studies Program at 956-5007 or uhirb@hawaii.edu. We wish you success in carrying out your research project.
January 18, 2011

TO: David St-Jules, R.D.
    Principal Investigator
    Human Nutrition, Food and Animal Science

FROM: Nancy R. King
    Director

Re: CHS #18835- “Natural History of Pediatric Nonalcoholic Fatty Liver Disease”

This letter is your record of CHS approval of this study as exempt.

On January 14, 2011, the University of Hawai‘i (UH) Committee on Human Studies (CHS) approved this study as exempt from federal regulations pertaining to the protection of human research participants. The authority for the exemption applicable to your study is documented in the Code of Federal Regulations at 45 CFR 46 (4).

Exempt studies are subject to the ethical principles articulated in The Belmont Report, found at http://www.hawaii.edu/irb/html/manual/appendices/A/belmont.html

Exempt studies do not require regular continuing review by the Committee on Human Studies. However, if you propose to modify your study, you must receive approval from CHS prior to implementing any changes. You can submit your proposed changes via email at uhirb@hawaii.edu. (The subject line should read: Exempt Study Modification.) CHS may review the exempt status at that time and request an application for approval as non-exempt research.

In order to protect the confidentiality of research participants, we encourage you to destroy private information which can be linked to the identities of individuals as soon as it is reasonable to do so. Signed consent forms, as applicable to your study, should be maintained for at least the duration of your project.

This approval does not expire. However, please notify CHS when your study is complete. Upon notification, we will close our files pertaining to your study.

If you have any questions relating to the protection of human research participants, please contact CHS at 956-5007 or uhirb@hawaii.edu. We wish you success in carrying out your research project.
February 15, 2012

Jeremy King, DO
Kapi‘olani Medical Specialists
1319 Punahou Street
Honolulu, HI 96826

Dear Dr. King:

SUBJECT: EXEMPT FROM REGULATIONS
Principal Investigator: Jeremy King, DO
Protocol Title: Natural History of Pediatric Nonalcoholic Fatty Liver Disease
HPHRI Study Number: 2011-065

On February 15, 2012 a designee of the Institutional Official of Hawai‘i Pacific Health determined the above referenced study to be exempt from regulations for category 4 using the guidelines set by the Office of Human Research Protection (45 CFR 46.101(b)).

As the principal investigator you are required to submit any changes to the protocol, including closure of the study, and a progress report to Hawai‘i Pacific Health Research Institute on a biennial basis should the research be conducted for more than two years. Investigators are also required to provide copies of any abstracts, presentations, or publications submitted to outside entities.

As the principal investigator you are required to ensure that data is de-identified and collected for the approved date range of January 1, 2008 to August 31, 2011. No data may be collected after August 31, 2011 without a formal approval to extend the date range.

Hawai‘i Pacific Health Research Institute will maintain files on all studies determined to be exempt from regulations.

Sincerely,

[Signature]

David T. Horio, MD
Hawai‘i Pacific Health Institutional Official Designee

DH/ik
Study: The Effect of Weight Loss on Pediatric Nonalcoholic Fatty Liver Disease

February 15, 2012

Jeremy King, DO
Kapi'olani Medical Specialists
1319 Punahou Street
Honolulu, HI 96826

Dear Dr. King:

SUBJECT: WAIVER OF AUTHORIZATION
Principal Investigator: Jeremy King, DO
Protocol Title: Natural History of Pediatric Nonalcoholic Fatty Liver Disease
HPHRI Study Number: 2011-065

On February 15, 2012 the Hawaii Pacific Health Privacy Board (HPH PB) approved a request for a waiver of authorization for use and disclosure of protected health information (PHI) for the above-referenced research. This review was conducted by expedited review.

HPH PB determined that documentation received from you satisfies the three requirements for a waiver of authorization. These requirements are:

1. The use or disclosure of the PHI involves no more than minimal risk to the individuals, based on the following elements:
   a. An adequate plan to protect identifiers from improper use and disclosure;
   b. An adequate plan to destroy the identifiers at the earliest opportunity consistent with conduct of the research (unless there is a health or research justification for retaining the identifiers, or such retention is otherwise required by law); and
   c. Adequate written assurances that the PHI will not be reused or redisclosed to any other person or entity, except as required by law, for authorized oversight of the research project, or for other research for which the use or disclosure of PHI would be permitted by HIPAA.
Study: The Effect of Weight Loss on Pediatric Nonalcoholic Fatty Liver Disease

2. The research could not be practicably conducted without access to and use of the PHI; and

3. The research could not practicably be conducted without the waiver.

The Board determined that this waiver of authorization covers only the Protected Health Information listed in the protocol or data collection sheet.

No other information may be collected. If additional data is needed, the revised data sheet must be submitted for approval prior to collection of the data.

Sincerely,

David T. Horio, MD
Chairman

DH/Ik

cc: David St-Jules, RD
January 27, 2011

Ken Nagamori, MD
1319 Punahou Street
Suite 1030
Honolulu, HI 96826

Dear Dr. Nagamori:

SUBJECT: ACKNOWLEDGEMENT OF STUDY
Principal Investigator: Ken Nagamori, MD
Protocol Title: Natural History of Pediatric Nonalcoholic Fatty Liver Disease
HPHRI Study No.: 2010-141

This letter serves as your acknowledgement that Hawai‘i Pacific Health is aware that your study is being conducted and has been approved by the University of Hawai‘i Committee on Human Subjects. Hawai‘i Pacific Health Research Institute has determined that your study does not require our approval because it will be conducted in the private office of a non-employed physician and will not access Hawai‘i Pacific Health medical records.

As the principal investigator please submit any changes to the protocol, including closure of the study to the Hawai‘i Pacific Health Research Institute.

Although your study will not be overseen by Hawai‘i Pacific Health Research Institute, we will maintain a study file.

Sincerely,

Christine Nelson
Director
Hawai‘i Pacific Health Research Institute
Study: Estimation of fish intake in Asian and White female adolescents, and association with two-year changes in body fatness and body fat distribution: The Female Adolescent Maturation (FAM) study

January 11, 2013

TO: David St-Jules
Principal Investigator
Human Nutrition, Food and Animal Science

FROM: Denise A. Lin-DeShetler, MPH, MA
Director

Re: CHS #21013- "The Effect of Fish Intake on Obesity in Asian and White Female Adolescents in Hawaii: The Female Adolescent Maturation (FAM) Study"

This letter is your record of the Human Studies Program approval of this study as exempt.

On January 11, 2013, the University of Hawai‘i (UH) Human Studies Program approved this study as exempt from federal regulations pertaining to the protection of human research participants. The authority for the exemption applicable to your study is documented in the Code of Federal Regulations at 45 CFR 46.101(b) (4).

Exempt studies are subject to the ethical principles articulated in The Belmont Report, found at http://www.hawaii.edu/irb/html/manual/appendices/A/belmont.html

Exempt studies do not require regular continuing review by the Human Studies Program. However, if you propose to modify your study, you must receive approval from the Human Studies Program prior to implementing any changes. You can submit your proposed changes via email at uhirb@hawaii.edu. (The subject line should read: Exempt Study Modification.) The Human Studies Program may review the exempt status at that time and request an application for approval as non-exempt research.

In order to protect the confidentiality of research participants, we encourage you to destroy private information which can be linked to the identities of individuals as soon as it is reasonable to do so. Signed consent forms, as applicable to your study, should be maintained for at least the duration of your project.

This approval does not expire. However, please notify the Human Studies Program when your study is complete. Upon notification, we will close our files pertaining to your study.

If you have any questions relating to the protection of human research participants, please contact the Human Studies Program at 956-5007 or uhirb@hawaii.edu. We wish you success in carrying out your research project.
Study: Estimation of fish intake in Asian and White female adolescents, and association with two-year changes in body fatness and body fat distribution: The Female Adolescent Maturation (FAM) study

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NOTIFICATION OF APPROVAL

8/23/2012
To: Rachel Novotny
CC:
Yee Hwa Daida
Rachel Novotny

Re: Study ID: HI-04TVorgt-01
Continuing Review ID: CR00002129
Study Title: Nutritional and Genetic Determinants of Early Puberty (FAM) HI-04TVorgt-01

The continuing review materials submitted with this report, for the above referenced study were reviewed and accepted by the Kaiser Permanente Hawaii Institutional Review Board (KPHI IRB) expedited review procedures on 8/23/2012. Therefore, approval for this study has been extended for one year.

This approval expires on 8/23/2013.

Please use this notification of approval should your funding agency require documentation. Our Federal Wide Assurance number is FWA 00002344 – IRB KPHI: IRB00000402.


Lori A. Jennings, CIP, CHRC
Research Compliance Manager and IRB Administrator
711 Kapioali Blvd
Honolulu, HI 961813
(808) 432-5411

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Study: Estimation of fish and omega-3 fatty acid intake in pediatric nonalcoholic fatty liver disease

April 24, 2012

TO: David St-Jules, RD
    Principal Investigator
    Human Nutrition, Food and Animal Sciences

FROM: Nancy R. King
      Director

Re: CHS #20108- “Omega-3 Fatty Acid in pediatric NAFLD: Correlation with Disease Severity”

This letter is your record of the Human Studies Program approval of this study as exempt.

On April 24, 2012, the University of Hawai‘i (UH) Human Studies Program approved this study as exempt from federal regulations pertaining to the protection of human research participants. The authority for the exemption applicable to your study is documented in the Code of Federal Regulations at 45 CRF 46 (4).

Exempt studies are subject to the ethical principles articulated in The Belmont Report, found at http://www.hawaii.edu/irb/html/manual/appendices/A/belmont.html

Exempt studies do not require regular continuing review by the Human Studies Program. However, if you propose to modify your study, you must receive approval from the Human Studies Program prior to implementing any changes. You can submit your proposed changes via email at uhirb@hawaii.edu. (The subject line should read: Exempt Study Modification.) The Human Studies Program may review the exempt status at that time and request an application for approval as non-exempt research.

In order to protect the confidentiality of research participants, we encourage you to destroy private information which can be linked to the identities of individuals as soon as it is reasonable to do so. Signed consent forms, as applicable to your study, should be maintained for at least the duration of your project.

This approval does not expire. However, please notify the Human Studies Program when your study is complete. Upon notification, we will close our files pertaining to your study.

If you have any questions relating to the protection of human research participants, please contact the Human Studies Program at 956-5007 or uhirb@hawaii.edu. We wish you success in carrying out your research project.
January 14, 2013

Lynn Iwamoto, M.D.
Pediatrics

Subject: CHS #20959 - “Use of Omegaven for Parenteral Nutrition Associated Liver Disease”

Dear Dr. Iwamoto,

The University of Hawaii (UH) Human Studies Program has received notice that an application for human subjects review and approval of the study identified above has been submitted to the Western IRB (WIRB).

Under the IRB Authorization Agreement executed between WIRB and UH on 11-23-08, UH cedes authority to WIRB for human subjects review of your study. Please provide the Human Studies Program with the following:

- A copy of the WIRB-approved consent form(s), if applicable;
- Any unanticipated problems & serious adverse events; and
- Notification of changes in the study status.

You may submit the required information and documents to Human Studies Program via email to uhirb@hawaii.edu.

Please be aware that you are responsible for ensuring compliance with the WIRB’s determinations pertaining to this study as well as its policies and procedures for seeking approval of study modifications, applying for continuing review, reporting unanticipated problems, etc. You should contact WIRB directly if you have questions about these requirements.

Thank you for your cooperation. Please contact our office at 808-956-5007 if you have any questions.

Sincerely,

Denise A. Lin-DeShetler, MPH, MA
Director
Study: The use of fish oil-based lipid emulsions in infants with parenteral nutrition-associated liver disease: A case series

INVESTIGATOR: Lynn Iwamoto M.D.  
Department of Neonatology  
1319 Punahou Street  
Honolulu, Hawaii 96826

SPONSOR: Lynn Iwamoto, M.D.

PROTOCOL NUM: None

AMD. PRO. NUM: None

TITLE: Use of Omegaven for Parenteral Nutrition Associated Liver Disease

APPROVAL INCLUDES:
Amendment 2 (11-07-2012)
Waiver of Authorization

WIRB APPROVAL IS GRANTED SUBJECT TO:
The Board found that this research meets the requirements for a waiver of consent under 45 CFR 46.116(d).

WIRB HAS APPROVED THE FOLLOWING LOCATIONS TO BE USED IN THE RESEARCH:
Kapalolani Medical Center for Women and Children, 1319 Punahou Street, Honolulu, Hawaii 96826

If the PI has an obligation to use another IRB for any site listed above and has not submitted a written statement from the other IRB acknowledging WIRB’s review of this research, please contact WIRB’s Client Services department.

ALL WIRB APPROVED INVESTIGATORS MUST COMPLY WITH THE FOLLOWING:

1. Conduct the research in accordance with the protocol, applicable laws and regulations, and the principles of research ethics as set forth in the Belmont Report.

2. Although a participant is not obliged to give his or her reasons for withdrawing prematurely from the clinical trial, the investigator should make a reasonable effort to ascertain the reason, while fully respecting the participant’s rights.
Study: The use of fish oil-based lipid emulsions in infants with parenteral nutrition-associated liver disease: A case series

3. Unless consent has been waived, conduct the informed consent process without coercion or undue influence, and provide the potential subject sufficient opportunity to consider whether or not to participate. (Due to the unique circumstances of research conducted at international sites outside the United States and Canada where WIRB approved materials are translated into the local language, the following requirements regarding consent forms bearing the WIRB approval stamp and regarding certification of translations are not applicable.)
   a. Use only the most current consent form bearing the WIRB "APPROVED" stamp.
   b. Provide non-English speaking subjects with a certified translation of the approved consent form in the subject’s first language. The translation must be approved by WIRB unless other arrangements have been made and approved by WIRB.
   c. Obtain pre-approval from WIRB for use of recruitment materials and other materials provided to subjects.

4. Obtain pre-approval from WIRB for changes in research.

5. Obtain pre-approval from WIRB for planned deviations and changes in research activity as follows:
   - If the research is federally funded, conducted under an FWA, or is a clinical investigation of a drug or biologic, then all planned protocol deviations must be submitted to WIRB for review and approval prior to implementation except where necessary to eliminate apparent immediate hazards to the human subjects (DHHS 45 CFR § 46.103(b)(4); 21 CFR § 56.108(a)(4); [ICH 3.3.7]).
   - However, if the research is not a clinical investigation of a drug or biologic, then only planned protocol deviations that may adversely affect the rights, safety, or welfare of subjects or the integrity of the research data should be submitted to WIRB for review and approval prior to implementation except where necessary to eliminate apparent immediate hazards to the human subjects (DHHS 45 CFR § 46.103(b)(4); 21 CFR § 56.108(a)(4); [ICH 3.3.7]).

The reason for these different requirements regarding planned protocol deviations is that the Office for Human Research Protections (OHRP) and the Food and Drug Administration (FDA) drug and biologic divisions have adopted the regulatory interpretation that every planned protocol deviation is a change in research that needs prior IRB review and approval before implementation; however, the FDA device division operates under a distinct regulation (21 CFR §512.150(a)(4)).

Deviations necessary to eliminate apparent immediate hazards to the human subjects should be reported within 10 days.

6. Promptly report to WIRB all unanticipated problems (adverse events, protocol deviations and violations and other problems) that meet all of the following criteria:
   a. Unexpected (in terms of nature, severity or frequency);
   b. Related or possibly related to participation in the research, and
   c. Suggests that the research places subjects or others at a greater risk of harm than was previously known or recognized.

Please go to www.wirb.com for complete definitions and forms for reporting.

7. Provide reports to WIRB concerning the progress of the research, when requested.

8. Ensure that prior to performing study-related duties, each member of the research study team has had training in the protection of human subjects appropriate to the processes required in the approved protocol.

Federal regulations require that WIRB conduct continuing review of approved research. You will receive Continuing Review Report forms from WIRB. These reports must be returned even though your study may not have started.

DISTRIBUTION OF COPIES:
Contact Company
Christine Nelson, Hawaii Pacific Health
Lynn Iwamoto M.D., Kapolei Medical Center for Women and Children
Andrea Stu, Hawaii Pacific Health Research Institute
Annette Amiotte, Kapolei Medical Specialists