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Influence of dietary fat intake on acute changes in postprandial lipid and lipoprotein expression in children and adolescents with nonalcoholic fatty liver disease (NAFLD)

by

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is a chronic liver disease that typically occurs in obese children and adolescents with hyperinsulinemia/insulin resistance and dyslipidemia. While treatment is aimed at lifestyle modification (diet and physical activity), very little is known regarding the contribution of diet to underlying metabolic processes leading to hepatic damage and how modification of meal patterning might contribute to improved patient outcomes. We hypothesized that children and adolescents with biopsy proven NAFLD will be characterized by different body fat distribution, prolonged postprandial hyperinsulinemia, lipemia and altered lipoprotein and inflammatory marker expression when compared to obese and lean healthy children and adolescents after consumption of two different high saturated fat meal challenges (with varying long chain polyunsaturated fat: 0% LCPUFA vs 1.5% LCPUFA). Children and adolescents with NAFLD had higher fat localized to the visceral region compared to the other groups. Higher ($p>0.05$) trunk to extremity ratio and waist to hip ratio was observed in children with NAFLD. Fasting and postprandial insulin were higher ($p<0.05$) in the NAFLD when compared to lean and obese controls. NEFA postprandial clearance in NAFLD population was significantly lower when compared to obese and lean controls. NAFLD subjects were characterized by higher ($p<0.05$) fasting Apo B-100 and C-III as well as higher postprandial Apo B-48; all suggestive of differences in fat balance across the liver following a meal. Acute changes in LCPUFA intake in a high saturated fat meal resulted in significant lowering of iAUC for NEFA in the NAFLD group and significant increased postprandial changes in Apo C-III, but did not evoke any

other major postprandial changes in lipoprotein expression. In conclusion our study suggests that children and adolescents with NAFLD exhibit delayed postprandial lipid and lipoprotein expression in comparison to lean controls. Acute changes in LCPUFA content in a high saturated meal, reflective of the typical amounts present in a fast food meal, evoke small postprandial changes in children and adolescents with NAFLD. Whether an improvement in postprandial lipid clearance can be obtained through a higher LCPUFA dose or whether responses are mainly determined by the chronic intake should be further investigated.

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ACRONYMS IN ALPHABETICAL ORDER

Acceptable Macronutrient Distribution Range (AMDR)

Air displacement phethysmography (ADP)

Alanine aminotransferase (ALT)

Alberta Health Services (AHS)

Analysis of variance (ANOVA)

Apolipoproteins B-100 (Apo B-100)

Apolipoproteins B-48 (Apo B-48)

Apolipoproteins C-III (Apo C-III)

Arachidonic acid (AA)

Area under the curve (AUC)

Aspartate aminotransferase (AST)

Bioelectrical Impedance Analysis (BIA)

Body fat percentage (BF %)

Body mass index (BMI)

Canadian Nutrient File (CNF)

Cardiovascular disease (CVD)

Center for disease control (CDC)

Chylomicron (CM)

Chylomicron remnant (CMr)

C-reactive protein (CRP)

Diabetes Mellitus (DM)

Docosahexaenoic acid (DHA)

Dual energy x-ray absorptiometry (DEXA)

Eicosapentaenoic acid (EPA)

Enzyme-Linked Immuno-Sorbent Assay (ELISA)

Fat free mass (FFM)

Fat mass (FM)

Gamma-glutamyl transpeptidase (GGT)

Glycemic Load (GL)

Health Research Ethics Board (HREB)

Hepatic lipase (HL)

High density lipoproteins (HDL)

Homeostatic model assessment (HOMA)

Incremental area under the curve (iAUC)

Insulin resistance (IR)

Interleukin-10 (IL-10)

Interleukin-6 (IL-6)

International Obesity Task Force (IOTF)

International Society for the Advancement of Kinanthropometry (ISAK)

Lipoprotein lipase (LPL)

Long chain polyunsaturated fatty acids (LCPUFA)

Low density lipoproteins (LDL)

Magnetic Resonance Imaging (MRI)

Metabolic syndrome (MetS)

Minimum detectable dose (MDD)

Monounsaturated fatty acids (MUFA)

National Health and Nutrition Examination Survey (NHANES III)

Nonalcoholic fatty liver disease (NAFLD)

Nonalcoholic steatohepatitis (NASH)

Non-esterified fatty acids (NEFA)

Nothing per mouth (NPO)

Pediatric Centre for Weight and Health (PCWH)

Phospholipids (PL)

Polyunsaturated fatty acids (PUFA)

Polyunsaturated/Saturated ratio (P/S)

Recommended Dietary Allowances (RDAs)

Saturated fatty acids (SFA)

Simple steatosis (SS)

Standard error of the mean (SEM)

Sterol regulatory element binding protein-1c (SREBP-1c)

Technical error (TE)

Total cholesterol (TC)

Total energy intake (TEI)

Triglycerides (TG)

Trunk to extremity ratio (TER)

Tumor of necrosis factor alpha (TNF-α)

Ultrasound (US)

Underwater weighing (UWW)

USDA (United States Department of Agriculture)

Waist circumference (WC)

Waist to height ratio (WHtR)

Waist to hip ratio (WHR)

World Health Organization (WHO)

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 ROLE OF THE LIVER IN BIOLOGICAL PROCESSES

The liver is the largest organ in the body with a weight that ranges between 1.4-1.6 kg (Guyton, 1995) and is one of the most complex in terms of its impact on the metabolism of nutrients, drugs and other biochemicals (Bowman & Russell, 2001; Guyton, 1995). The liver synthesizes plasma proteins, non-essential amino acids, glycogen, fat and hormones and is an intermediary in the metabolism of macro and micronutrients (Bowman & Russell, 2001; Burt et al., 1998). Within the liver, the nutrient end-products of digestion and absorption are processed for either transport to the systemic circulation, stored within the liver (e.g. glycogen, fat or proteins) or undergo transformation within the liver to produce energy or other important end-products (e.g. urea, albumin, acute phase reactants) (Bowman & Russell, 2001; Guyton, 1995). The hepatic blood supply plays a key role in carrying nutrients from the gastrointestinal tract to the periphery; therefore disturbances in blood flow (as in portal hypertension) can result in significant nutritional and health consequences (Bowman & Russell, 2001; Burt et al., 1998; Guyton, 1995).

1.2 LIVER DISEASE AND NUTRIENT METABOLISM

The presence of liver pathologies typically has a huge impact on metabolic functions within the body (Bowman & Russell, 2001; Lonardo et al., 2006). From a nutrient-metabolism perspective, liver dysfunction can result in aberrant nutrient uptake (e.g. malabsorption of fat and fat soluble vitamins) and nutrient utilization (e.g. hyperlipidemia, hyper or hypoglycemia). The extent to which this occurs

depends upon the type and severity of liver disease (Bowman & Russell, 2001; Lonardo et al., 2006). Typically liver diseases of the biliary tree (extra-hepatic) that interfere with biliary flow (e.g. biliary atresia) result in malabsorption of fat and fat soluble vitamins (A, D, E, and K) causing macronutrient and micronutrient deficiencies (Burt et al., 1998). These disorders can also result in impairments of lipid and glucose metabolism with increasing severity of hepatocellular dysfunction (intrahepatic) (Burt et al., 1998). Intrahepatic diseases cause changes in lipid and glucose metabolism and are one of the major contributing factors to the development of hepatic steatosis (Clouston & Powell, 2004). Steatosis consists of fat accumulation in the hepatocytes and may lead to inflammation, fibrosis and even cirrhosis in some cases (Roberts & Yap, 2006; Tagle-Arrospide, 2003). Steatosis typically occurs in the milder stages of some intrahepatic diseases including alcoholic liver disease, Wilson´s disease, autoimmune, viral hepatitis (e.g. Hepatitis C and B) and non-alcoholic fatty liver disease (NAFLD) (Roberts, 2007; Roberts & Yap, 2006).

1.3 NONALCOHOLIC FATTY LIVER DISEASE

1.3.1 Definition

Nonalcoholic Fatty Liver Disease (NAFLD) in both adults and children is defined as lipid accumulation exceeding the normal range of 5% of liver wet weight (Idrovo & Guevara, 2004; Kang et al., 2006). NAFLD is recognized as one of the most common forms of chronic liver disease in both adults and children (Kang et al., 2006). NAFLD typically presents across a spectrum that includes a

fatty liver alone (simple steatosis) to steatosis with inflammation and/or fibrosis (nonalcoholic steatohepatitis or NASH) to cirrhosis (Farrel et al., 2005; Schwimmer et al., 2005). Although not common in childhood, children and adolescents with NASH have a definite risk of cirrhosis (Adams et al., 2005; Molleston et al., 2002; Roberts, 2007). According to Kinugasa et al. the first child with cirrhosis due to NASH was described in 1984: a 15-year-old obese girl with "maturity-onset" diabetes mellitus (Kinugasa et al., 1984; Schwimmer et al., 2005). Subsequent literature suggests that the risk of developing cirrhosis in childhood is around 1-2% (Day, 2005; Mager et al., 2008) but it could be as high as 9% (Schwimmer et al., 2006). Some evidence suggests that the progression of NAFLD to cirrhosis in childhood may depend upon the age at diagnosis and severity of disease at time of initial presentation (Baldridge et al., 1995; Molleston et al., 2002). Another factor may also be increased susceptibility of the pediatric liver to the oxidative and inflammatory stresses induced by insulin resistance (IR) and obesity (Schwimmer et al., 2003).

NAFLD is typically a diagnosis of exclusion whereby the contributions of other disorders (e.g. Wilson's disease, celiac disease) that are known to cause a fatty liver are ruled out (**Table 1.1**). Conditions known to cause a fatty liver include inherited metabolic inborn errors of metabolism (e.g. galactosemia, urea cycle), drugs (e.g. glucocorticoids, tamoxifen), insulin resistance syndromes (Prader-Willi, Polycystic Ovary Syndrome), parenteral nutrition and alcohol (Lavine & Schwimmer, 2004; Mager & Roberts, 2006; Utzschneider & Kahn, 2006) **(Table 1.1**).

Table 1.1. Disorders known to cause steatosis or steatohepatitis. (Adapted from Lavine & Schwimmer, 2004; Mager & Roberts, 2006)

Liver Disease	Other diseases
• Wilson's disease • Hemochromatosis • Hepatitis B and C, autoimmune hepatitis	• Severe infection • Post jejuno-ileal bypass • Gastric reduction operations • Celiac disease • Nephrotic syndrome. • Type 1 diabetes mellitus • Dorfman-Chanarin syndrome
Metabolic inborn errors:	
• Hypobetalipoproteinemia • Cholesterol ester storage disease • Galactosemia • Hereditary fructose intolerance • Hereditary tyrosinemia type I • Weber Christian disease • Wilson disease	• Urea cycle errors • Fatty acid oxidation errors • Organic acidemia errors • Glycogen storage errors • Deficiency of carnitine • Cystic fibrosis
Insulin Resistance Syndromes:	
• Alström syndrome • Lipodystrophy syndromes • Prader-Willi	• Bardetl-Biedl syndrome • Polycystic Ovary Syndrome • Type 2 diabetes
Parenteral nutrition:	
Mainly observed when there is an excessive glucose amount and an alteration in the lipid metabolism induced by stress	
Drugs:	
• Amiodarone (Corderone, Pacerone) • Glucocorticoids • Tamoxifen (Nolvadex, Tamone) • Steroids (such as prednisone, hydrocortisone) and synthetic estrogens	

1.3.2 Prevalence

The exact prevalence of NAFLD in children is still unknown (Frasier et al., 2007; Papandreou et al., 2009); with varying incidence reported globally (Mager et al., 2008; Papandreou et al., 2009; Rashid & Roberts, 2000). NAFLD is the third most common cause of liver disease in adults in North America preceded only by alcoholic induced liver disease and viral hepatitis (Hepatitis C) (Roberts, 2007). Although NAFLD has been historically considered to be a disease occurring only in adults, it is increasingly prevalent in children in North America (Roberts, 2007). Some case reports indicate that NAFLD has occurred in children as young as 2 years of age (Fishbein & Cox, 2004). The increasing prevalence of NAFLD in childhood has been attributed to the global increase in pediatric obesity (Adam & Angulo, 2005; Flores-Calderón et al., 2005; Papandreau et al., 2009; Schwimmer et al., 2003; Utzschneider & Kahn, 2006). According to the third National Health and Nutrition Examination Survey (NHANES III) and other reports, the prevalence of NAFLD in overweight and obese individuals (adults and children) ranges from 16% to 23% and non-alcoholic steatohepatitis (NASH) prevalence ranges from 2% to 6% (Araujo et al., 1998; Collantes et al., 2004; Fraser et al., 2007; Lonardo et al., 1997; Utzschneider & Kahn, 2006). NAFLD has been reported in 2.6% of the general pediatric population, and in 22.5% to 52.8% of obese children (Baldrige et al., 1995; Clark et al., 2004; Ruhl et al., 2003). Prevalence of NAFLD in childhood in Asia (China, Japan, and Korea) ranges from 1-10% in the general population and to 12-80% in overweight and obese children (Kojima et al., 2003). European countries (Italy and Greece) report

that the incidence in overweight and obese children ranges from 10 to 40% (Papandreou et al., 2009). Latin American countries such as Mexico and Brazil report that the prevalence of NAFLD ranges from 7.1 to 42% in overweight and obese children (Flores Calderón et al., 2005). These data suggest that NAFLD is a prevalent and growing concern in children throughout the world.

1.4 RISK FACTORS FOR NAFLD

The most common risk factors for development of NAFLD in adults and children include ethnic background, gender, obesity (central and total body) and metabolic factors. In childhood the major risk factor for the development of NAFLD is the presence of obesity (central and total body obesity) (Baldrige et al., 1995; Schwimmer et al., 2003) and its associated complications, including hyperinsulinemia in the presence of insulin resistance, hyperlipidemia and type 2 diabetes (Adams & Angulo et al., 2005; Burt et al., 1998; Lavine & Schwimmer, 2004; Medina et al., 2004; Patton et al., 2006; Targher et al., 2007; Utzscheneider & Kahn, 2006). Male children, of Caucasian (non-Hispanic and Hispanic origin) and Asian ethnicities appear to have the highest risks for developing NAFLD in childhood (Baldrige et al., 1995; Kinugasa et al., 1984; Schwimmer et al., 2003).

According to Lebovitz (2001), IR is defined as the "inability of a known quantity of exogenous or endogenous insulin to increase glucose uptake and utilization in an individual as much as it does in a normal population" (Lebovitz, 2001). Effects of insulin resistance are tissue specific. Peripheral insulin resistance promote accumulation of fatty acids in skeletal muscle (Shoelson et al.,

2006; Zoppi et al., 2010). In hepatic cells, IR is characterized by the failure of glucose to suppress glucose production leading to elevated release of glucose into the blood (Shoelson et al., 2006). On the other hand, insulin resistance in fat cells reduces uptake of circulating fatty acids into adipose tissue while increasing hydrolysis of stored TG (Shoelson et al., 2006; Zoppi et al., 2010).

Insulin resistance that occurs as part of healthy adolescent development should not be confused as a risk factor. Children experience temporary insulin resistance at puberty (Moran et al., 2002) which has been suggested to limit body fat changes by promoting an environment characterized by less fat storage (Eckel, 1992; Havers et al., 2002; Moran et al., 2002). According to Suzuki et al., puberty has shown to affect the pathophysiology and severity of NAFLD (Suzuki et al., 2012). The peak of insulin resistance is observed at Tanner stage III in both boys and girls (Moran et al., 1999). During puberty, girls have shown to be more insulin resistant than boys (Brufani et al., 2009; Moran et al., 1999; Pilia et al., 2009; Roemmich et al., 2002). IR has been correlated with total body fat percentage, BMI and waist circumference. Increased values of these anthropometric markers do not explain entirely the IR observed at this age suggesting that there may be other hormonal and also unknown factors affecting the transient IR (Juárez-López et al., 2010; Moran et al., 1999). Nevertheless, insulin resistance is greater in obese children (including children with NAFLD) when compared to normal weight children (Pilia et al., 2009; Suzuki et al., 2012) and some obese insulin resistant children may not return to pre-pubertal insulin concentration (Pilia et al., 2009).

1.4.1 Ethnicity

Hispanic and Asian children and adults have a higher incidence of prevalence risk factors for NAFLD such as obesity and insulin resistance (Mager & Roberts, 2006; Pacheco Torres et al., 2006; Park et al., 2001; Patton et al., 2006; Roberts & Yap, 2007). The higher NAFLD incidence observed in the Asian population could be because individuals of Asian ethnicities tend to have a higher total body fat (Deurenberg et al., 2002; Wang et al., 1994), as well as a greater amount of abdominal and visceral fat (Lear et al., 2007; Park et al., 2001) at a given BMI compared with Caucasians. This could be related to an endomorphic somatotype tendency (Godoy & Barcos, 1995; Godoy & Grifferos, 1994; Godoy et al., 1994). This tendency also appears to be prevalent in individuals of Hispanic ancestry. Males (adults and children) of Hispanic ancestry tend to have higher waist circumference measurement, showing predominantly the android shape when compared to Caucasic ancestry (Browning et al., 2004; Fraser et al., 2007; Manton et al., 2000; Patton et al., 2006; Rashid & Roberts, 2000; Schwimmer et al., 2004). The prevalence of the android shape strongly suggests a higher risk for visceral adiposity (Browning et al., 2004; Fraser et al., 2007; Manton et al., 2000; Patton et al., 2006; Schwimmer et al., 2004). When factoring in the higher prevalence of diabetes/impaired fasting glucose due to insulin resistance, it appears that the risk for NAFLD increases substantially in insulin resistant populations (Browning et al., 2004; Fraser et al., 2007; Manton et al., 2000; Patton et al., 2006; Rashid & Roberts, 2000; Schwimmer et al., 2004). However, it is unclear to what extent the risk for NAFLD due to ethnic background is

influenced by genotype or phenotype because most studies examining risk for NAFLD in children of Hispanic origin have been done in the US, rather than in Latin America. This is important to consider since there is evidence to suggest that acculturation can lead to significant changes in eating patterns (higher intakes of trans and saturated fat, simple sugars, energy in addition to decreased physical activity) that can contribute to obesity and metabolic complications (Ghaddar et al., 2010). More research in Latin America is necessary and the impact of acculturation should be strongly considered.

1.4.2 Gender

Gender as a risk factor for NAFLD appears to change over the life cycle. In childhood, most studies demonstrate that NAFLD occurs predominantly in males (2:1) (Baldrige et al., 1995; Mager & Roberts, 2006; Mager et al., 2008; Manton et al., 2000; Moran et al., 1983; Rashid & Roberts, 2000). It is not clear why male children seem to be more susceptible to this disorder than females. Some evidence suggests that the higher risk for NAFLD in male children is related more to body composition (BMI, visceral and subcutaneous) and that the presence of visceral adiposity may be the predisposing factors leading to liver damage in early childhood (Browning et al., 2004; Ford et al., 2006; Fraser et al., 2007; Schwimmer et al., 2004). In contrast, overweight and obese girls appear to be at higher risk for developing type 2 diabetes than male children and express metabolic deregulations of obesity in the form of pancreatic dysfunction (Dean et al., 1992; Drake, 2002; Freedman, 1997; Kadiki, 1996; Kitagawa et al., 1994;

Scott, 1997). It is possible that metabolic derangements such as IR and higher body fat express different in boys than in girls, type 2 diabetes for girls and NAFLD for boys.

1.4.3 Anthropometric Risk Factors

Body Mass Index (BMI) and body fat percentage have been shown to be higher in subjects with fatty liver when compared to overweight subjects without fatty liver and have shown to be independent predictors of fatty liver in male and female adults (Imamura et al., 2008; Tagle Arrospide, 2003). NAFLD features have been found in lean subjects and this is referred to as "metabolically obese" (Yap et al., 2011). "Metabolically obese" subjects are characterized by insulin resistance, lipid abnormalities (e.g. hypertriglyceridemia), central obesity and higher visceral and subcutaneous fat mass when compared to non-metabolically obese subjects (Kelishadi et al., 2008; Ruderman et al., 1998). This body fat distribution suggests that excess subcutaneous and/or visceral fat could lead to serious metabolic consequences (e.g. chronic diseases such as NAFLD, CVD and diabetes).

1.4.3.1 *Adipose tissue*

Adipose tissue is no longer considered a static organ and it is currently considered a metabolic and endocrine organ (Kershaw & Flier, 2004). Adipose tissue comprises the white adipose tissue (WAT) and brown adipose tissue (BAT). WAT and BAT differ in both function and morphology (Wajchenberg, 2000). WAT is present in the body as subcutaneous and visceral adipose tissue (Gil et al., 2011). Visceral WAT can be categorized as omental, mesenteric and

retroperitoneal fat (Gil et al., 2011; Wajchenberg, 2000; Wronska & Kmiec, 2012). Visceral and subcutaneous adipose tissues have shown to be highly metabolically active but different in their lipolysis (LPL activity) and production of adipocytokines.

1.4.3.2 *Adipose tissue and abdominal fat*

Adipose tissue secretes proteins such as leptin, adiponectin, resistin as well as cytokines such as TNF-α, IL-6 (Bulló et al., 2002; Fried et al., 1998). An important component of adipose tissue is abdominal fat which comprises both subcutaneous and intra-abdominal fat; with the latter including visceral (omental and mesenteric) and retroperitoneal fat (Märin et al., 1992; Wajchenberg, 2000). Both subcutaneous and visceral adipose tissue change in response to total body weight, however weight loss changes are observed more rapidly in the subcutaneous adipose tissue than in the visceral adipose tissue (Mayo-Smith et al., 1989; Zamboni et al., 1994).

1.4.3.3 *Visceral adipose tissue*

Data suggest that NAFLD is the result of fat infiltration in the liver as a consequence of the obesity which may lead to IR. Therefore, the main risk factors associated with NAFLD are obesity and central obesity (adults: male >94 cm in waist circumference/female >80 cm/children: ≥90th percentile for age, gender and race-specific, NHANES III) (Clouston & Powell, 2004; Cruz et al., 2004; Lee et al., 2006; Mager & Roberts, 2006). BMI, waist circumference (WC), waist to hip ratio (WHR) and waist to height ratio (WHtR) have been validated as indirect

markers for visceral adiposity and have been shown to strongly correlate with abdominal fat (Fujita et al., 2011; Hubert et al., 2009; Kahn et al., 2005; Parikh et al., 2007). WHR ratio has a moderate association with the amount of abdominal visceral tissue measured by magnetic resonance imaging (MRI) (Wajtchenberg, 2000; Yan et al., 2007). BMI and WC have shown strong correlations with the body fat percentage (BF%) measured by air displacement plethysmography (ADP), lower correlations have been observed with WHR (Neovius et al., 2005). WHtR has been more recently looked at and it has been suggested to be an anthropometric index to identify metabolic risks; such as coronary factors and fatty liver (Hsieh et al., 2003). Strong correlations have been observed between WHtR and intra-abdominal fat (Wu et al., 2009). Anthropometric markers of obesity such as BMI and BF% have been shown to be higher in subjects with fatty liver when compared to healthy subjects and have been suggested to be independent predictors of fatty liver in male and female adults (Imamura et al., 2008). Studies have shown that central obesity and therefore, fat distribution, is even more important than the total body fat (Clouston & Powell, 2004; Mager & Roberts, 2006). Intra-abdominal fat accumulation is strongly correlated with insulin resistance and central adiposity. Research suggests that even lean individuals with increased intra-abdominal fat have shown higher fasting plasma insulin levels and insulin insensitivity as measured by HOMA-IR (Cnop et al., 2002). The waist circumference (WC) measurement is a validated surrogate for visceral adiposity (Nobili et al., 2006). Children with NAFLD have shown larger WC when compared to children without NAFLD with similar BMI (Clouston &

Powell, 2004; Mager & Roberts, 2006). An increase in the amount of visceral fat in adults and in children may contribute directly to an enhanced free fatty acids (FFA) hepatic delivery (Donelly et al., 2005). Therefore, visceral adipose tissue mass is a predictor of liver fat content (Westerbacka et al., 2004).

1.4.3.4 *Subcutaneous adipose tissue*

Subcutaneous adipose tissue has proved to be highly metabolic (Bouchard et al., 1993; Fried et al., 1993; Montague et al., 1998). According to Montague et al., subcutaneous fat is the major source of leptin and its secretion rate is approximately 2-3 times higher in subcutaneous than in the omental tissue (Montague et al., 1998). Additionally, higher lipoprotein lipase (LPL) activity as well as larger adipocytes has been observed in subcutaneous compared to omental adipose tissue (Bouchard et al., 1993; Fried et al., 1993). Goel et al. suggested that in Asian Indians subcutaneous adipose tissue was a better predictor of the metabolic syndrome (MetS) than intra-abdominal fat determined by dual energy x-ray absorptiometry (DEXA) (Goel et al., 2010). A better understanding of the metabolic effect and consequences of excessive subcutaneous fat and its respective distribution warrants further research.

1.4.3.5 *Methods to determine total body fat and percentage of total body fat mass*

There are several methods used to assess body composition in both the research and clinical settings. Underwater weighing (UWW) and ADP use a two compartment model (fat mass and fat free mass) whereas DEXA uses a three

compartment model (fat, bone mineral and lean soft tissue) (Pietrobelli et al., 1996). UWW, ADP, DEXA and bioelectrical impedance analysis (BIA) have been shown to be accurate methods to predict fat mass (FM) and fat free mass (FFM) when performed carefully and according to the manufacturer instructions. Limitations and advantages are inherent to each method making it difficult to define the method which is the most accurate and precise. ADP has shown to have less intra-subject variability in predicting FM and FFM (Bujko et al., 2006; Miyatake et al., 2005; Sardinha et al., 1998). DEXA and BIA body composition measurements are affected by the subject´s hydration status (Roubenoff et al., 1993; Wells et al., 1999). BIA has been suggested to overestimate fat free mass, but studies evaluating accuracy and precision of BIA have been inconsistent (Fogelholm & van Marken, 1997; Frisard, 2005). Accuracy of UWW body fat values can be affected by difficulties presented during measurements (e.g. water immersion) and bone mineral density (Demerath et al., 2002; Fogelholm & van Marken, 1997; Frisard et al., 2005; Isjwara et al., 2007). Magnetic resonance imaging (MRI) is another technique to assess body composition. Advantages of MRI are the low intra-subject variability and accuracy of the adipose tissue measurement (Heymsfield, 2008). Nevertheless, it is difficult to compare MRI results with other available techniques because MRI measures fat tissue in general but does not give an estimate of total fat mass (Heymsfield, 2008; Wells & Fewtrell, 2006). Finally, multiple skinfolds measurements have shown similar results when compared with UWW (Brandon, 1998) when they are correctly performed. Skinfold measurements are the least expensive methodology

compared to others and in some cases the only tool available in the clinical setting. It is worth mentioning that the accuracy depends on the number, proper measurer technique and sites of skinfolds measured as well as other variables such as sex, race and probably age (Andrade et al., 2002; Arroyo et al., 2004; Bujko et al., 2006; Durnin & Womersley, 1974; Frissard et al., 2005; Gibson, 1990; Weyers et al., 2002).

1.4.4 Metabolic Risk Factors

IR and hyperinsulinemia are the most commonly cited metabolic risks factors for the development of NAFLD in childhood. This may or may not occur in the presence of an overweight or obese body habitus (Clouston & Powell, 2004; Mager & Roberts, 2006; Mager & Yap, 2006). The high incidence of IR and hyperinsulinemia is one of the major reasons that NAFLD is referred to as the hepatic manifestation of the metabolic syndrome (Clouston & Powell, 2004; Mager & Roberts, 2006; Rashid & Roberts, 2000). Studies have shown that type 2 diabetes predisposes to NAFLD. Two different studies done in adults with type 2 diabetes reported that NAFLD was present in 70-75% of the studied population (Medina et al., 2004; Targher et al., 2007). Data suggest that the presence of NAFLD in subjects with type 2 diabetes may also be correlated to increased cardiovascular disease (CVD) risk independently of other features of the metabolic syndrome (Targher et al., 2007; 2006; 2005).

Most pediatric case series report that over 80% of children clinically diagnosed with NAFLD exhibit IR and hyperinsulinemia (Chalasani et al., 2004;

Clouston & Powell, 2004; Musso et al., 2006; Nobili et al., 2006; Patton et al., 2006; Poniachik et al., 2006). Other commonly known diagnostic factors for NAFLD (adults and children) include the presence of hyperlipidemias and elevations of serum liver biochemistries [aspartate aminotransferase (AST) and alanine aminotransferase (ALT)] (Chalasani et al., 2004; Clouston & Powell, 2004; Musso et al., 2006; Nobili et al., 2006; Patton et al., 2006; Poniachik et al., 2006). However, it is important to note that elevations in liver biochemistries are not always evident, particularly in early stages of the disease.

Other metabolic risk factors may include the presence of depressed levels of adipocytokines (e.g. adiponectin) and metabolites of glutathione metabolism (Nobili et al., 2006; Schwimmer et al., 2008; 2003). It is still controversial if the levels of adiponectin are associated with changes in disease expression in NAFLD. Some studies have demonstrated decreased serum levels of adiponectin in NASH, while others have found no correlations between serum adiponectin concentrations and liver histology (Bugianesi, 2005; Hui et al., 2004; Matsubara, 2004; Pagano et al., 2005; Targher et al., 2006; 2004). Adiponectin may play an important role in glucose metabolism by stimulating fatty acid oxidation in the liver. Additionally, lower adiponectin levels have been correlated with IR (Bajaj et al., 2004). Low adiponectin levels could decrease fatty acid oxidation which may consequently promote the amount of fatty acids in the liver (Hui et al., 2004; Pagano et al., 2005; Utszchneider & Kahn, 2006). Yokota et al. (2000) observed that TNF-α and adiponectin antagonize biological actions of each other, therefore adiponectin may prevent the progression of simple steatosis to steatohepatitis and

fibrosis by decreasing TNF-α production in the liver (Diehl, 2002; Kamada et al., 2003; Yokota et al., 2000; Xu et al., 2003).

Another adipocytokine that has suggested being involved with NAFLD pathogenesis is leptin. Studies have shown that a lack of response to the adipocytokine leptin (leptin resistance) may be an important factor in the pathogenesis of hepatic steatosis (Kaplan, 1998). Leptin seems to regulate insulin secretion and sensitivity. In the hepatocytes, leptin has complex effects on insulin response. It stimulates glucose transport and turnover (Kaplan, 1998; Mantzoros et al., 1997; Targher, 2004). It has been proposed that leptin could induce insulin resistance because it inhibits insulin-stimulated phosphorylation of several intracellular signaling proteins, including IRS-1 (Anderwald et al., 2002; Havel, 2002; Kaplan, 1998; Mantzoros et al., 1997; Nobili et al., 2006; Poordad, 2004).

Studies in adults and children have shown that older age, obesity and specifically central adiposity, IR, diabetes and increased levels of circulating FFA are associated with liver fibrosis (Angulo, 2006; García Monzon et al., 2000; Harrison et al., 2002; Ratziu et al., 2000; Roberts, 2007; Wanless et al., 1990; Willner et al., 2001). A recent study showed that both IR and systemic hypertension were independently associated with advanced stages of fibrosis (Dixon et al., 2001; Harrison et al., 2002). Other studies have shown that AST/ALT ratio higher than 1 and ALT levels 2 times higher than normal are correlated with increasing fibrosis stage in patients with steatohepatitis (Angulo, 2006; Dixon et al., 2001; Lavine & Schwimmer, 2004; Patton et al., 2006; Roberts, 2007; Sorbi et al., 1999; Utzscheneider & Kahn, 2006).

1.5 CLINICAL DIAGNOSIS OF NAFLD

A presumptive diagnosis of NAFLD should be done after a very careful physical and laboratory evaluation. A clinical diagnosis of NAFLD must then be made upon ultrasonography (CT, MR) evidence of a bright echogenic liver, consistent with the finding of fatty infiltration in the liver necessitating further in depth investigation (Mager et al., 2008; Patton et al., 2006; Roberts, 2007; Tagle Arrospide, 2003). Typically this is done in an overweight/obese child who presents with mild elevations in serum ALT and AST with central obesity and IR (Chitturi et al., 2002). Children may also present vague abdominal pain and clinical evidence of insulin resistance in the form of Acanthosis Nigricans (AN) which is the darkening of the skin around the neck (Chitturi et al., 2002). AN is most often associated with obesity and insulin resistance. IR will cause an increased binding to insulin like growth factor receptor, which may lead to an excessive stimulation of keratinocytes and fibroblasts, causing the over-pigmentation observed in AN (Cruz & Hud, 1992). Many children have clinical evidence of hyperlipidemia (hypertriglyceridemia and hypercholesterolemia) and depressed serum concentrations of adiponectin and anti-oxidant status (Lavine & Schwimmer, 2004; Patton et al., 2006; Utzscheneider & Kahn, 2006). Although, serum AST/ALT ratios are usually less than one, it is important to point out that a clinical diagnosis of NAFLD may be made in the absence of elevations in serum amino transferases (Chalasani et al., 2012; Lavine & Schwimmer, 2004; Mager et al., 2010; Patton et al., 2006; Utzscheneider & Kahn, 2006).

The first step in the clinical diagnosis of NAFLD in children is to rule out other liver pathologies that are known to cause steatosis (**Table 1.1**). These are usually ruled out by standard blood tests (serum auto antibodies, viral tests, ceruloplasmin/copper levels, etc.) and with a careful medical history (Roberts, 2007). The second step in the diagnosis of NAFLD is to conduct a liver biopsy (gold standard for diagnosis), as this is the only method that can definitely rule out other metabolic conditions (Chalasani et al., 2012; Roberts, 2007). Criteria for liver biopsy in suspected NAFLD in childhood is typically based upon any of ONE the following variables: young age (<10 years old), hepatosplenomegaly, very elevated serum AST or ALT, severe insulin resistance (by HOMA-IR), detectable non-specific auto-antibodies, inconclusive results from biochemical tests related to Wilson´s disease, viral hepatitis or α_1-antitrypsin deficiency (Chalasani et al., 2012; Roberts, 2007; Vajro et al., 2012). It is worth to mention that as of 2012 the American Association for the Study of Liver Diseases has stated that a liver biopsy for NAFLD diagnosis "cannot be recommended in patients with unsuspected hepatic steatosis detected on imaging who are asymptomatic and have normal liver biochemistries" (Chalasani et al., 2012).

1.6 HISTOLOGICAL SPECTRUM OF NAFLD

A typical histological presentation of NAFLD in adults is a liver that is characterized by macrovesicular steatosis with degenerative ballooning, Mallory Hyaline bodies and/or perisinusoidal fibrosis (in the absence of portal features) (Clouston & Powell, 2004; Patton et al., 2006; Schwimmner et al., 2005). When liver histology is predominantly characterized by fat deposits, this condition is

referred to as simple steatosis or hepatic steatosis. If inflammation and fibrosis are also present in addition to steatosis, a diagnosis of nonalcoholic steatohepatitis (NASH) may be confirmed (Patton et al., 2006; Roberts, 2007). This type of disorder is referred to as Type 1 NASH (Patton et al., 2006). A careful evaluation of liver histology and a careful medical history is also needed to rule out the potential for alcohol induced liver disease, particularly in adults. In children, NASH typically presents with histological evidence of steatosis with portal inflammation, portal fibrosis with little or no hepato-cellular ballooning, reflecting minimal steatosis with this level of disease (Patton et al., 2006) (**Figure 1.1**). This type of NASH is often referred to as Type 2 NASH and is commonly observed in younger children (51%) that are overweight and obese (Patton et al., 2006). Clinically children with simple steatosis often present with normal or mild elevations in serum ALT and AST which may result in a missed diagnosis of NAFLD (Chan et al., 2004; Patton et al., 2006). It also highlights the need in this population to screen children with a high risk for NAFLD (male children with central and total body obesity, insulin resistance and dyslipidemia) as both BMI and serum ALT concentrations are independent predictors of liver steatosis (Chan et al., 2004).

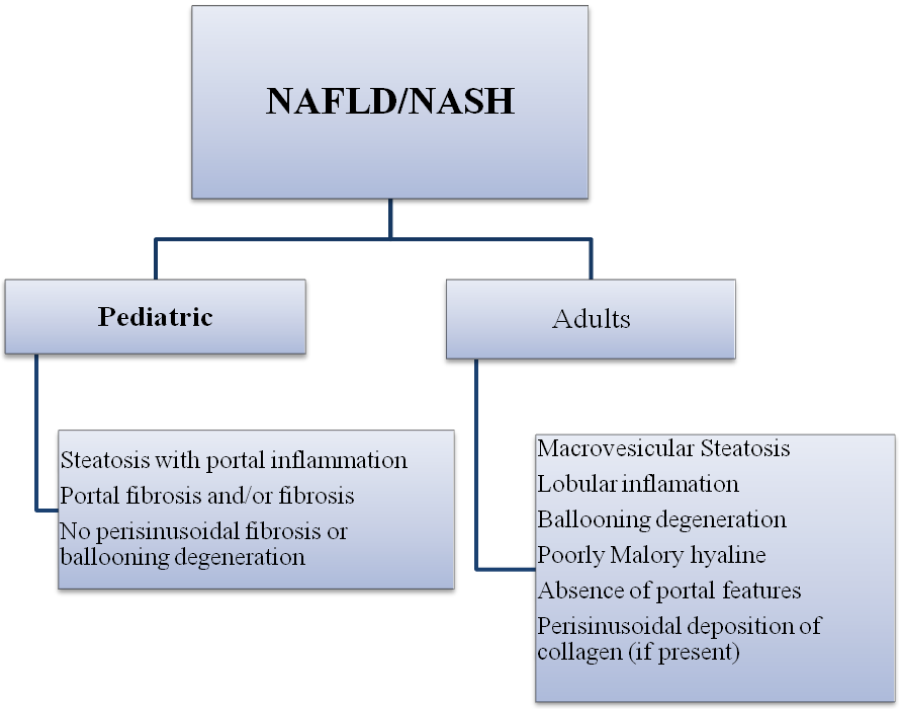
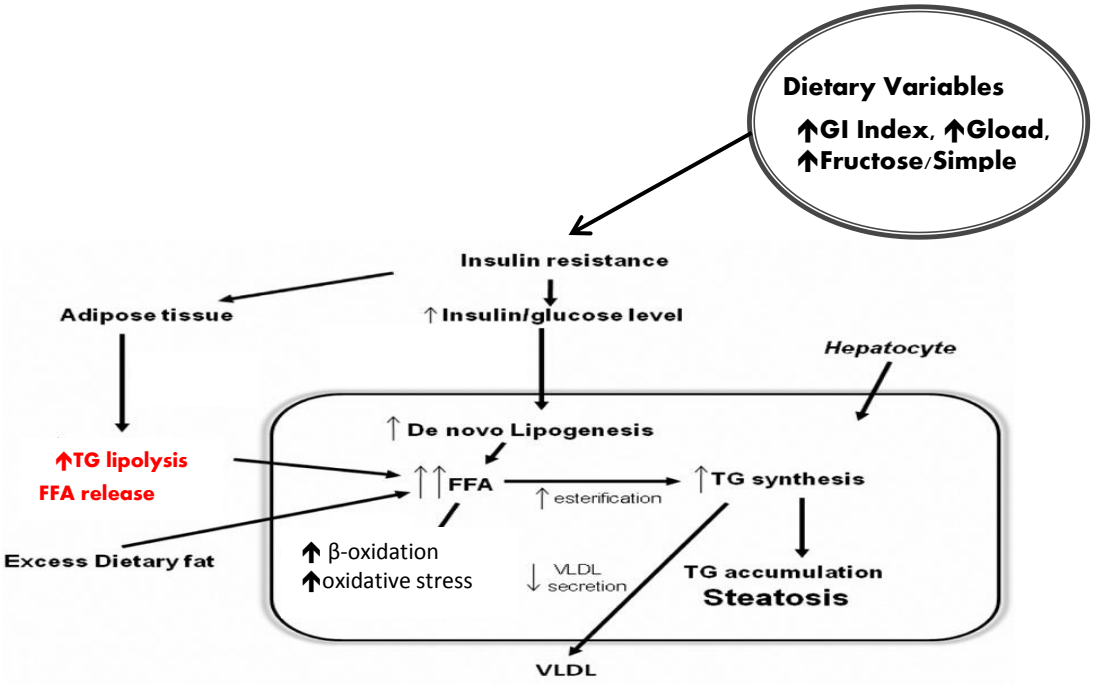


Figure 1.1. Adult and pediatric NAFLD/NASH (Adapted from Patton et al., 2006)

1.7 PATHOPHYSIOLOGY OF NAFLD

The triggering mechanism of NAFLD is still unclear. Up until recently pathogenesis of NAFLD was explained by the "two hit hypothesis". The first hit included the accumulation of triglyceride in the liver probably as a consequence of the hyperinsulinemia and/or IR and the second hit was thought to comprise oxidative stress and cytokine leading to liver injury (Anderson & Borlak, 2008; Day & James, 1998) See **Figure 1.2**.



Adapted from Paschos P & Paletas K. HIPPOKRATIA. 2009;13(1): 9-19

Figure 1.2. Pathophysiology of NAFLD explained by the "two hit hypothesis"

Currently, several concurrent hits have been proposed as the exact sequences of the events are not completely understood (Smith & Adams, 2011; Tilg et al., 2010). Whether NAFLD pathology starts with IR per se or an increased release of free fatty acids (FFA) from the adipose tissue, abnormalities in insulin sensitivity are typically present (Clouston & Powell, 2004; Day & James, 1998; Kang et al., 2006; Krawczyk et al., 2010; Tilg et al., 2010). IR and/or hyperinsulinemia will evoke a series of events leading to hepatic damage (oxidative stress, inflammation, altered cytokine function) It has been suggested that the metabolic events that occur in NAFLD are triggered by the release of FFA from the periphery (adipocyte) caused by IR (Musso et al., 2009; Tessari et al., 2009) In the presence of IR, the mitochondria may reach its capacity for β-oxidation (Larter et al., 2010; Tessari et al., 2009) An increase in microsomal

oxidation will be observed leading to an over expression of the cytochrome P450 which will promote oxidative stress (**Figure 1.3**) (Larter et al., 2010; Musso et al., 2009; Tessari et al., 2009). Consequently, reactive oxidative species (ROS) are produced which may provide the second hit and subsequent develop of NAFLD (Caldwell & Crespo, 2004; Larter et al., 2010; Tagle Arrospide, 2003). Increased oxidative stress would be then considered the second hit in the NAFLD pathology (Caldwell & Crespo, 2004; Tagle Arrospide, 2003). Insulin resistance causes a positive balance in the amount of FFA stored in the liver, which may contribute to the development of hepatic steatosis (Caldwell & Crespo, 2004) (**Figure 1.3**).

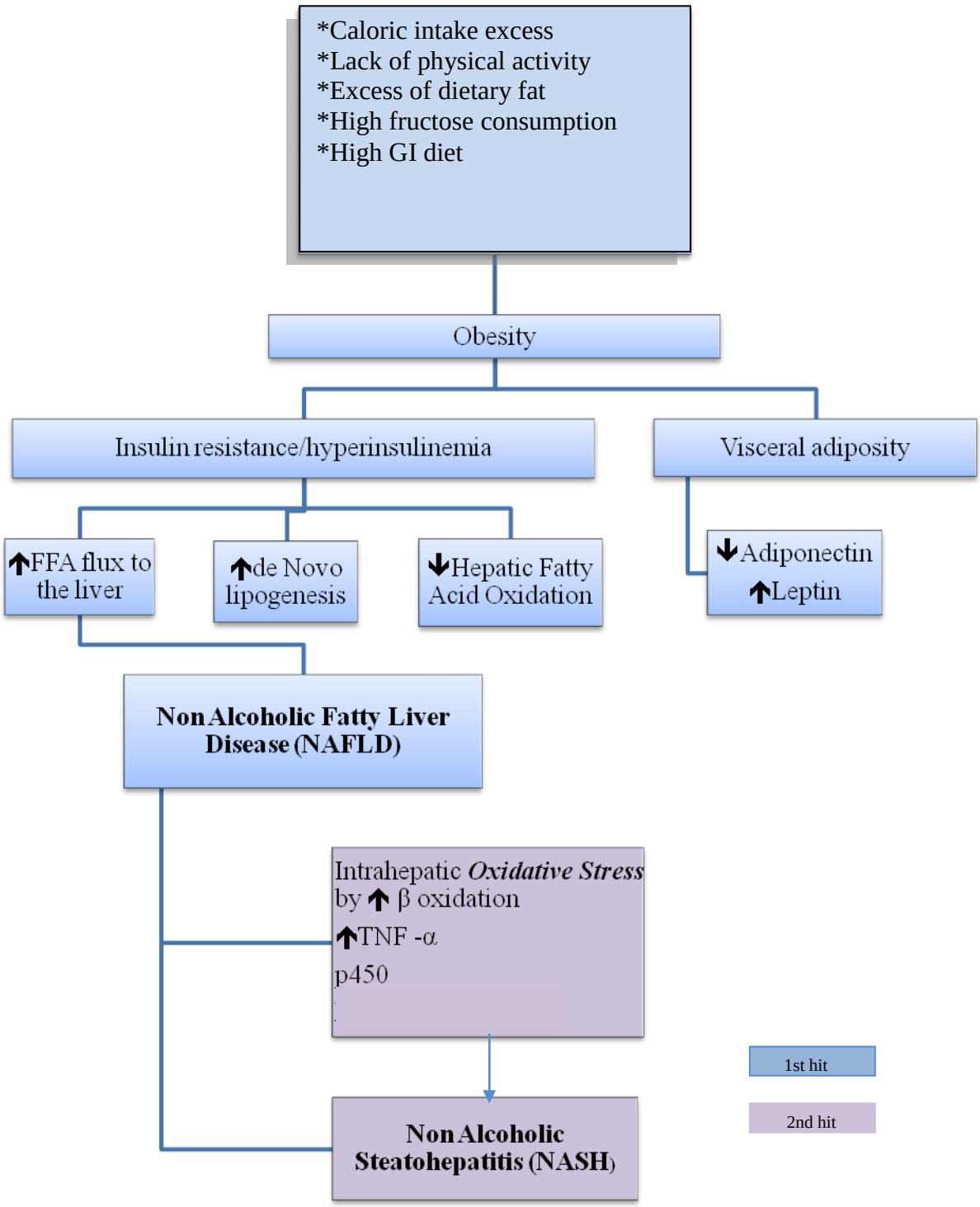


Figure 1.3. Pathophysiology of nonalcoholic fatty liver disease

1.8 IMPACT OF INSULIN RESISTANCE AND HYPERINSULINEMIA ON APOLIPOPROTEINS B-48, B-100 AND C-III IN FASTING AND POSTPRANDIAL STATES

Insulin resistance may induce an increment of FFA flux from the adipose tissue to the liver and intestine (Nzekwu et al., 2007). It has been suggested that the increase of FFA flux from the adipose tissue to the intestines will lead to increased chylomicron (CM) assembly and production (Nzekwu et al., 2007). A positive correlation between IR and serum apolipoprotein B-48 (Apo B-48) levels (Nzekwu et al., 2007) has been observed. Since there is only one Apo B-48 for each CM, the measurement of Apo B-48 levels is reflective of CM production (Phillips et al., 1997) and therefore postprandial intestinal clearance.

Increased accumulation of CM and chylomicron remnant (CMr) has been observed in IR states (Harbis et al., 2001; Xiao & Lewis, 2012). Insulin resistant and type 2 diabetic subjects have increased levels of Apo B-48 in fasted and postprandial states which is one of the reasons why insulin resistance has been strongly correlated with delayed postprandial clearance (Duez et al., 2008; Hsieh et al., 2008). Additionally, fasting plasma insulin has been reported to have an effect on very low density lipoproteins (VLDL) metabolism (Carpentier et al., 2002). According to Aarsland et al, hyperinsulinemia caused by excessive carbohydrate ingestion (>65% of total energy) is associated with increased VLDL-TG production independent of the concomitant increase in FFA levels (Aarsland et al., 1996). Delayed clearance of CM remnants may be partially

affected by the impaired VLDL hepatic secretion observed in insulin resistant states (Carpentier et al., 2002).

Hyperinsulinemia stimulates glycolysis and inhibits gluconeogenesis causing an increase of Acetyl CoA consequently resulting in FFA being more available inside the cell (Caldwell et al. 2007; Cortez-Pinto, 2006; Patton et al., 2006). An increase in the esterification of FFA stimulates the synthesis of triglycerides (TG) inside the hepatocyte and increased apopolipoprotein B-100 (Apo B-100) synthesis might be observed (Caldwell et al. 2007; Cortez-Pinto, 2006; Patton et al., 2006). Apo B-100 is a large protein involved in the transport of triglycerides and cholesterol from the liver to peripheral tissues (Su et al., 2009). Abnormalities in the metabolism of Apo B lipoproteins are associated with increased risk of developing coronary heart disease and hyperlipidemia (Adiels et al., 2008). Acute hyperinsulinemia (during a hyperinsulinemic clamp) has been associated with diminished hepatic production of VLDL Apo B-100 in subjects with type 2 diabetes (Cummings, 1995; Lewis et al., 1993) while chronic hyperinsulinemia has been associated with increased VLDL ApoB-100 synthesis (Cummings, 1995; Ginsberg, 2000). In subjects with elevated fasting TG levels; higher serum and plasma levels of postprandial Apo B-100 have been observed (Mekki et al., 1999). Charlton and Musso et al. suggested that Apo B-100 synthesis is altered in patients with NASH (Charlton et al., 2002; Musso et al., 2003) but studies supporting this hypothesis are limited. Impairment in Apo B-100 synthesis would affect the ability of the hepatocyte to export TG and

cholesterol ester which may lead to steatosis (predominantly triglycerides accumulation) (Charlton et al., 2002; Dashti et al., 1989; Pullinger et al., 1989).

Apo C-III is synthesized in the liver and intestine and inhibits LPL activity and therefore hydrolysis of lipoproteins (Chan et al., 2008). VLDL lipolysis is delayed by Apo C-III; therefore, high fasting Apo C-III levels are indicative of hepatic VLDL Apo C-III overproduction and possibly reflective of delayed clearance (Chan et al., 2008) as it has been observed in obese and hyperinsulinemic subjects (Nguyen et al., 2006; Ooi et al., 2008). During VLDL hydrolysis, Apo C-III exchanges between HDL and VLDL; it transfers from hepatic secreted VLDL to HDL and vice versa (Nguyen et al., 2006). It has been suggested that in subjects without lipid abnormalities, most of the Apo C-III is associated with HDL, while in subjects with lipid abnormalities Apo C-III is mostly associated with triglyceride rich lipoproteins such as CM and VLDL (Chan et al., 2008; Fredenrich et al., 1997). Therefore, fasting Apo C-III levels have been suggested to be strong predictors of cardiovascular disease (Chan et al., 2008). High fasting Apo C-III levels have been correlated with an increase of risk to develop NAFLD, but the information is still scarce (Lee et al., 2011; Petersen et al., 2010). The implications of postprandial Apo C-III levels in children with NAFLD have not been explored.

1.9 POSTPRANDIAL METABOLISM

The postprandial state is the metabolic response following the ingestion of a specific meal (Gill et al., 2004; Ortega et al., 2012). During the postprandial

state, dietary fat is emulsified, hydrolyzed and absorbed from the small intestine into mucosal cells, followed by a re-esterification to form triglycerides and cholesterol esters (Mamo & Proctor, 1999; Ortega et al., 2012; Ricardi et al., 2006). These newly re-esterified particles in addition to several apolipoproteins (A-IV and Apo B-48) will be combined and incorporated into CM, which are secreted into the lymphatic system and finally released into the blood through the thoracic duct (Mamo & Proctor, 1999; Ortega et al., 2012; Ricardi et al., 2006). LPL hydrolyzes the CM which results in release of FFA and a smaller particle called CM remnant (CMr) (Mamo & Proctor, 1999; Ortega et al., 2012; Ricardi et al., 2006). Finally, CMr will be removed from the blood by the liver (Mamo & Proctor, 1999; Ortega et al., 2012; Ricardi et al., 2006).

Postprandial lipemia is a common feature of the metabolic syndrome (MetS). Delayed insulin and triacylglycerol rich lipoproteins clearance has been observed in subjects with type 2 diabetes and hypertriglyceridemia (Couch et al., 2000; Karpe, 1999; Karpe et al., 1998; 1998; 1993; Umpaichitra et al., 2004; van Hees et al., 2008). Altered postprandial clearance (insulin and lipid response) is currently considered a risk factor for NAFLD in adults (Umpaichitra et al., 2004). However, no data is available in childhood NAFLD.

Typically, a human´s postprandial TG will peak between 3 and 4 hours (Parks, 2001). In the fasted state TG are carried by hepatically-derived VLDL particles and postprandial TG will be carried by VLDL and CM (Parks, 2001). Fasting TG concentrations can change daily. Intra-individual variation in healthy pre-pubertal children has shown to be approximately 3.5% for HDL and up to

25% for TG (Tolfrey et al., 1999), therefore the postprandial TG response has been suggested to be a better predictor of cardiovascular disease (López-Miranda et al., 2007).

In healthy subjects, the postprandial insulin rise will suppress lipolysis and VLDL (hepatic) production. Post prandial responses in an insulin resistant/hyperinsulinemic state are not completely understood (Harbis et al., 2001). It has been suggested that IR/hyperinsulinemia elevates blood levels of TG rich lipoproteins (TGrL wouldn't these be VLDLs ?) (López-Miranda et al., 2007). Subjects (adults) with insulin resistance and poor glycemic control have higher fasting values of Apo B-48 and B-100 with more than four fold higher levels of at the peak of the postprandial period (Phillips et al., 2000).

Habitual dietary intake, the amount and type of fat present in a test meal, fiber, glucose, fructose as well as presence or absence of alcohol, age, gender, physical activity, present or absence of certain pathology (e.g. obesity and/or type 2 diabetes, hypertriglyceridemia) are factors that may affect a postprandial response (López-Miranda et al., 2007; Parks, 2001; Sanders, 2003).

Long term changes in dietary intake, such as meals high in saturated fat may influence the postprandial metabolic environment and contribute to an increased risk for postprandial lipemia, hyperinsulinemia and insulin resistance, particularly in overweight and obese individuals (Westerbacka et al., 2005). On the contrary, chronic consumption of omega-3 PUFA; especially long chain PUFA (LCPUFA) have been associated with an increase in the HDL cholesterol

(Sanders et al., 2003), reduction of fasting TG, VLDL and apolipoprotein B concentration (Nestel et al., 1984) in healthy and hypertriglyceridemic subjects (Harris & Muzio, 1993; Harris et al., 1988; Jackson et al., 2005; Weintraub et al., 1988).

Meal challenges rich in carbohydrates (>65%) have been shown to increase insulin levels but have shown no major effects in the postprandial lipid response (Kapur et al., 2010). Postprandial responses comparing meals with different fatty acids composition have been performed but data are still contradictory. Short and medium fatty acids enter the portal circulation and do not enter the general circulation through CM, therefore meals rich in short chain fatty acids will show a lower TG rise (López-Miranda et al., 2007). Studies regarding postprandial responses following a single LCPUFA dose are scarce as most of the postprandial studies have been done after a certain time of supplementation (at least 4-6 weeks). Additionally in most of the postprandial studies, chronic intake was not assessed and only the intakes 2-3 days prior to the postprandial tests were recorded. Food records of 2-3 days may not always represent chronic intake accurately (Yang et al., 2010). Higher postprandial TG levels and CM fractions were observed in a butter (rich in SFA) predominant meal when compared to an olive oil (MUFA) meal (Thomsen et al., 1999). Masson & Mensink observed that the substitution of 50 g SFA for omega-6 PUFA decreased postprandial TG iAUC, IL-6 and TNF-α in overweight men (Masson & Mensink, 2011). Similarly, meals enriched with olive oil and rapeseed oil have shown lower TG postprandial responses when compared to meal rich in saturated fat (Nielsen et al., 2002)

nevertheless subjects participating in this study followed an energy controlled diet for three weeks prior to the meal challenge (Nielsen et al., 2002). The three week standardization of the type and amount of fat intake may have impacted the study findings by avoiding the potential confounding factor of the chronic dietary intake.

Altered postprandial TG fatty acid profiles, insulin resistance and lower clearance of fatty acids have been observed in obese pre-pubertal children after consuming a standardized breakfast (32% lipids and 59% carbohydrates) (Gil-Campos et al., 2008; Larqué et al., 2006). Addition of omega-3 LCPUFA to a fat challenge has been suggested to reduce the postprandial TG response when compared to a meal without LCPUFA supplementation (Yahiah et al., 1996) by greater activation of lipoprotein lipase (LPL) (Jackson et al., 2005; Williams, 1998; Zampelas et al., 1994).

Comparisons of postprandial meals by modifying the LCPUFA ratios have also been studied but information is scarce regarding whether the addition of LCPUFA in a single meal improves postprandial lipid clearance and if so what amount of omega-3 would be recommended. Tulk & Robinson modified the omega-6/omega-3 LCPUFA on a high saturated fat challenge; no improvement of postprandial TG levels and inflammatory markers in overweight subjects with metabolic syndrome were observed (Tulk & Robinson, 2009). Similarly, Monteggard et al. compared two meals one high in LCPUFA vs low LCPUFA; no significant differences in postprandial clearance in TG and glucose levels were observed (Monteggard et al., 2010). Overgaard et al. compared two meals rich in

SFA; one was enriched with 352 mg of fish oil. No significant differences in the postprandial plasma fatty acid composition, with the exception of a decrease in TG at 2 hours post meal, were observed. The incremental area under the curve (iAUC) for TG was not significantly different between groups (Overgaard et al., 2008). Finally, Hanwell et al. compared two high fat, high fructose meals, one of them rich in fish oil (2.8 g EPA and 1.4g DHA). The authors concluded that fish oil did not modify the TG postprandial response or oxidative stress (Hanwell et al., 2009). Nevertheless, it is possible that the pure effect of fish oil was blunted by the high fructose meal. All these studies suggest that more research is necessary in order to determine whether or not a single dose of LCPUFA improves the postprandial lipid clearance response.

1.10 LONG TERM INTAKE OF FAT AND ITS POTENTIAL METABOLIC CONSEQUENCES

Very little is known about the postprandial response to dietary intake in children and adolescents with NAFLD (Musso et al., 2005). Whether the postprandial response depends mostly on meal composition per se or chronic intake is still unclear.

Dietary fats are classified based on their structural and chemical characteristics into saturated (SFA) and unsaturated; which are subdivided into monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA). High fat diets have been associated with overweight and obesity (Mensink et al., 2003; Papandreou et al., 2009; Ramírez-Silva et al., 2006; Westerbacka et al., 2005).

The effect of dietary fat on body fat and weight has been questioned in recent years due to the impact of changing fat intake on macronutrient distribution in the diet; specifically increasing intakes of carbohydrates in response to decreasing fat intakes (Mensink et al., 2003; Papandreou et al., 2009; Westerbacka et al., 2005). High carbohydrates/lower fat diets are associated with hyperglycemia, hyperinsulinemia, hyperlipidemia (specifically increases in serum TG, VLDL) (Anderson et al., 1980; Clouston, 1989; Parks et al., 2000; 1999). Overweight and insulin resistant subjects seem to be more susceptible to these effects (Anderson et al., 1980; Clouston, 1989; Dreon et al., 1994; Griel et al., 2006; Kane et al., 1965; Parks et al., 2000; 1999; Reaven et al., 1965; Roust et al., 1994; Ullman et al., 1991). On the other hand, a higher fat intake (>40%) especially rich in saturated fat, increases the risk of overweight and obesity and cardiovascular diseases (Griel et al., 2006; Musso et al., 2003; Simopoulos, 2008).

Clinical evidence has shown that a consumption of SFA less than 7% of the total energy intake or less than one third of the total fat intake may may not offer any further improvements in blood lipids levels, at least in patients with IR (Capanni et al., 2006; Zivkovic et al., 2007). Hence, when it comes to dietary intake of fat, it is important to consider the amount (no more than 35% of total energy consumption) and type of fat (SFA vs PUFA vs MUFA) that is being consumed in NAFLD patients. Studies in children and adults with NAFLD indicate that dietary intake patterns of fat are characterized by higher SFA (9-14% of energy intake) and MUFA intake (14-15% of total energy intake) and lower PUFA (4-5% total energy intake) (Mager et al., 2010; Mager & Roberts, 2008;

2006; Musso et al., 2003; Cortez-Pinto et al., 2006; Roberts, 2007; Toshimitsu et al., 2006). As a consequence, the dietary SFA to PUFA ratio is high in patients with NASH (de Piano et al., 2007; Janssen et al., 2005; Mager & Roberts, 2007; Molnar et al., 2004; Musso et al., 2003; Cortez-Pinto et al., 2006). With regards to the omega-3/omega-6 ratio (n-3/n-6), evidence that is available indicates that dietary intakes of omega-3 PUFA are low (0.25-1.65 g/d) in children and adults with NAFLD, resulting in diets characterized by high n-6/n-3 ratios (8.8-15:1) (Araya et al., 2004; Mager et al., 2010; Mager & Roberts, 2006; Musso et al., 2003; Cortez- Pinto et al., 2006).

The importance of the PUFA consumption in the diet appears to be related to their ability to reduce sterol regulatory element binding protein-1 (SREBP-1) (Videla et al., 2004). A reduction in the SREBP-1 causes a decrease in lipogenesis and TG storage (Videla et al., 2004). Additionally, PUFA increase the peroxisome proliferative-activated receptor-α (PPAR-α) to cause an increase in β-oxidation (Videla et al., 2004). Supplemental PUFA has been associated with significant decreases in AST, ALT, TG and fasting glucose; laboratory markers that are strongly correlated with disease expression in NAFLD (Capanni et al., 2006).

High MUFA diets have a protective effect against cardiovascular disease (Hussein et al., 2007; Keys et al., 1986; Sanderson, 2002). Data has shown that MUFA´s atheroprotective effects are mostly through a lowering of total and LDL cholesterol (Gardner et al., 1995; Mensink et al., 2003; 1992) or through a decrease in coronary risk factors such as hypertension, diabetes, or obesity (Bosello et al., 1991; Carluccio et al., 1999; Garg et al., 1992; Griffin et al., 1996;

Hannah et al., 1994; Paniagua et al., 2007; Sanderson, 2002). Jenkins et al. showed that Mediterranean based diets (rich in MUFA) not only decrease LDL cholesterol levels but also CRP concentrations (Ambring et al., 2006; Jenkins et al., 2004; 2003). CRP has shown to be strongly correlated with inflammation and cardiovascular disease (Ridker, 2005). The fact that MUFA decrease CRP and improve fasting glycemic levels suggests they may be beneficial for people with NAFLD (Ambring et al., 2006; Jenkins et al., 2004; 2003). Each of these effects would be beneficial to address the first (by improving glycemic levels) and second hits (by decreasing inflammation). The appropriate balance between dietary MUFA and PUFA for the treatment of NAFLD is still unknown. It has been suggested that 1:1 MUFA/PUFA ratio may optimize the anti-atherogenic and anti-inflammatory properties of PUFA and MUFA (Videla et al., 2004; Moussavi et al., 2008). Whether or not this represents the optimal ratio for balanced lipogenesis and oxidation in NAFLD remains unclear.

1.11 OMEGA-6 AND OMEGA-3 POLYUNSATURATED FATTY ACIDS: RELATIONSHIPS TO INFLAMMATION AND OTHER METABOLIC PARAMETERS

Omega-3 (n-3) and omega-6 (n-6) PUFA must be either obtained from the diet such as α-linolenic (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6) or obtained through elongation and desaturation such as eicosapentaenoic (EPA, 20:5n-3), docosahexaenoic (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) (Ortega et al., 2011). Conversion of omega-6 PUFA (e.g. AA) in mammals is usually very efficient; on the other hand, omega-3 PUFA (e.g. EPA and DHA)

conversion is less efficient (Brenna et al., 2009; Ortega et al., 2011). Additionally, it has been suggested that ALA supplementation is more effective in increasing EPA than DHA (Brenna et al., 2009).

Competition for enzymes, transport systems and incorporation into the tissues between omega-3 and omega-6 PUFA has been suggested (Brenna et al., 2009). Special interest has been placed in LA as it is one of the major constituents in western diet and its consumption has increased, which would lead to higher AA levels (Simopoulos, 2008; 2002). On the other hand, omega-3 PUFA consumption has remained stable. Because omega-3 and omega-6 PUFA effects are antagonistic it is of huge importance to achieve an adequate ratio (Simopoulos, 2008; 2002).

Deficits/excessive intakes of omega-3 and omega-6 PUFA have shown to influence the pathogenesis of several disorders (cardiovascular disease, cancer, autoimmune disorders) (Araya et al., 2004; Simopoulos, 2008; 2002). Historically, the ratio of omega-6/omega-3 PUFA intake was 0.70, but consumption patterns in the western diet have changed and ratios now typically range from 15-16:1 to 38:1 (Simopoulos & Cleland, 2003). According to Ferrucci et al. increased dietary intake of omega-3 PUFA is independently associated with lower levels of pro-inflammatory markers and higher anti-inflammatory markers (Ferrucci et al., 2006). Other studies have proposed that fish oil, which is rich in omega-3 (n-3) fatty acids, reduces TG levels (Benatti et al., 2004; Etherton et al., 2001; Lee et al., 2007; Nestel, 1986; Skulas-Ray et al., 2008). In particular, increased dietary intakes of omega-3 PUFA have been associated with the up-

regulation of anti-inflammatory prostaglandins, thromboxanes and series 5 leukotrienes (Simopoulos & Cleland, 2003; Simopoulos, 2008). These benefits could be helpful when aiming treatment strategies to variables that are thought to contribute to disease pathogenesis in NAFLD (Jenkins et al., 2003).

1.12 DIETARY FAT INTAKE IN CHILDREN AND ADULTS WITH NAFLD: INTERRELATIONSHIPS TO THE METABOLIC ENVIRONMENT

Currently there are limited data on dietary intakes of omega-6 and omega-3 PUFA in children and adults with NAFLD. Araya et al. demonstrated that adults with NASH and simple steatosis had higher omega-6/omega-3 ratios in the diet (7.66:1 and 15:1 respectively) when compared to healthy subjects (Araya et al., 2004). In contrast the limited data in children with NAFLD indicate that dietary intakes of omega-6 PUFA (α-linoleic acid mean: 9.1 g/d; range: 2.2 -21.9 g/d) were within recommended ranges, but intakes of omega-3 PUFA (α-linolenic: mean: 0.77 g/d; range 0.25 -1.65 g/d) were below recommended levels of intakes (omega-6/omega-3 PUFA ratios of 11:1) (Araya et al., 2004; Cortez-Pinto et al., 2006; Mager et al., 2010; 2008; Musso et al., 2003). Low dietary intakes of omega-3 PUFA (C20:5n-3 and C22:6n-3) in children with NAFLD were associated with increased serum ALT and HOMA-IR values, while intakes of omega-6 PUFA (C20:4n-6 and C18:2n-6) in the high end of normal were correlated with increased serum concentrations of TNF-α, CRP and LDL-cholesterol (Cortez-Pinto et al., 2006; Mager et al., 2008).

With regard to the effect of chronic PUFA consumption and the postprandial response, fish oil chronic supplementation was shown to lower postprandial TG, VLDL, LDL and Apo B concentrations (Nestel et al., 1984; Jiménez-Gómez et al., 2010; Kelley et al., 2007; Volek et al., 2000; Westphal et al., 2000) as well as decreased CM and CMr levels in the fasted state (Demacker et al., 1991) probably by increasing the LPL activity (Harris & Muzio, 1993; Weintraub et al., 1988). Nevertheless it was suggested that the primary factor affecting the postprandial response was the chronic intake and not the meal challenge composition (Harris & Muzio, 1993; Weintraub et al., 1988). It has been suggested that chronic intake of PUFA could probably change the fatty acid composition of VLDL and CM, increasing their susceptibility to lipolysis (Harris & Muzio, 1993; Weintraub et al., 1988; Westphal et al., 2000). In addition CM may be more susceptible to LPL lipolysis (Weintraub et al., 1998; Westphal et al., 2000). Another mechanism proposed is that chronic fish oil supplementation may slow down the chylomicron entry into the blood, delaying fat absorption (Harris & Muzio, 1993) as well as decreasing secretion of VLDL and/or CM (Harris et al., 1988). The latter effect was independent of the type of fat ingested during the meal challenge (Harris et al., 1988). Additionally, diet composition has been shown to affect plasma fatty acid composition. Fatty acid compositions of the phospholipids of plasma, red blood cells (RBCs), and platelets reflect the major dietary fatty acids (Dougherty et al., 1987). TG and phospholipids (PL) plasma lipid fractions show the most predominant fats in the diet (Vega-López et al., 2006). After a 28-day intervention Raatz et al. observed that a low fat high PUFA

diet was associated with higher total omega-3 fatty acids such as EPA (20:5n-3) and DHA (22:6n-3) and reduced LA (18:2n-6) in the phospholipids and cholesterol ester fractions (Raatz et al., 2000). This suggests that dietary intake patterns of omega-3 and omega-6 PUFA may contribute to a metabolic milieu that enhances hepatic inflammation and oxidative damage in childhood NAFLD (Ferruci et al., 2006; Mager & Roberts, 2007; Simopoulos, 2008). How this may be translated into treatment approaches is unknown, but clearly optimizing intakes of omega-3 PUFA is of importance in order to promote up regulation of anti-inflammatory pathways (Ferruci et al., 2006; Simopoulos, 2008).

1.13 NAFLD IN CHILDREN: INTERRELATIONSHIP OF METABOLISM WITH DISEASE PATHOGENESIS

The damage and consequences of NAFLD observed in adults may be different than in children. Most of the inflammatory and fibrotic changes that occur in pediatric NAFLD occur within the periportal region of the liver which has a high concentration of gluconeogenic enzymes (Friedman & Artur, 1989). In adults, damage occurs in the perivenular zone (Roberts & Yap, 2006) where there is a lower concentration of these enzymes. This suggests that children with NAFLD may be more prone to changes in nutrient metabolism that contribute to the development of NAFLD. An excessive activation of the Kupfer cells may lead to increases fibrogenesis which may exacerbate the transition from a simple fatty liver to NASH (Friedman & Artur, 1989). The fact that NAFLD in children affects more the gluconeogenic region suggests that intervention in children should be done as early as possible to avoid the metabolic complications

associated with inhibition of gluconeogenesis and prevent the stimulation of TG synthesis inside the hepatocyte (Anderson & Borlak, 2009). All of these factors indicate that treatment strategies aimed at the underlying causes of NAFLD are critical to prevent disease onset and progression. In childhood, this is particularly important, as there is evidence that lack of treatment may lead to more serious liver disease.

1.14 FAST FOOD INTAKE IN OBESE CHILDREN AND THEIR POTENTIAL CONTRIBUTION TO DIETARY FAT INTAKE.

There is no consensus about the exact definition of fast food. According to Currie et al. fast food could be defined as "inexpensive food, cooked in bulk and in advance and kept warm, and served quickly" (Currie et al., 2010). Fast foods are characterized for being high nutrient dense and usually rich in saturated fat (Van Zyl et al., 2010). Increasing evidence suggests that consumption of goods that are hyper-caloric, rich in simple sugars, fructose, saturated fat and low in fiber, which are all characteristics present in fast food meals, (Bowman & Vinyard, 2004) contribute to weight gain, high body fat and increased waist circumference (Araya et al., 2004; Cortes-Pinto et al., 2005; Musso et al., 2003; Papandreou et al., 2007; Pereira et al. 2004; Valtueña et al., 2006). These variables have also been associated with an increased risk of insulin resistance and hyperinsulinemia (Kang et al., 2006; Segal et al., 2007; Wei et al., 2007), all known risk factors for the development of NAFLD. Research suggests that the consumption of fast food in children leads to higher weekly energy intakes by approximately 40% (St-Onge et al., 2003). Unhealthy eating patterns have been

associated with an increase in the prevalence of type 2 diabetes and glucose impairment; higher insulin and BMI, as well as increased subcutaneous and visceral fat have been observed (St-Onge et al., 2003). The types of foods associated with these consumption patterns include a high intake of baked goods rich in saturated fat and trans fatty acids (e.g. cakes, pastries, etc), deep fried foods (e.g. eggs, bacon, sausage, fried potatoes, etc) and excessive consumption of saturated (e.g. butter) and unsaturated fat (avocado, canola oil, etc). All of these types of foods are present in many commercial outlets that sell breakfast and other meal items in their menu (Fernández San Juan, 2000). It should be noted that natural sources of fat such as butter, avocado and canola oil if consumed on moderation are not detrimental for the human being.

1.15 CONCLUSIONS AND CLINICAL IMPLICATIONS

Nonalcoholic Fatty Liver Disease (NAFLD) in both adults and children typically presents across a spectrum of liver disease that ranges from simple steatosis to steatosis with inflammation and/or fibrosis (nonalcoholic steatohepatitis or NASH) to cirrhosis (Farrel et al., 2005; Schwimmer et al., 2005). The exact prevalence of NAFLD is still unknown; but it has been related to global increase in pediatric obesity (Frasier et al., 2007; Papandreou et al., 2009).

The etiology of childhood NAFLD seems to be multifactorial, but definitively IR and abnormalities on the lipid metabolism in both the fasted and postprandial state have been observed (Musso et al., 2003). Since NAFLD is the liver manifestation of the metabolic syndrome variables influencing NAFLD development include quantity and quality of food intake as well as the amount and

type of physical activity (Mager et al., 2008). Chronic and acute intake of diets rich in simple sugar, saturated fat and low PUFA have been correlated with lipid abnormalities such as delayed postprandial clearance which has been suggested to be altered in subjects with NAFLD including children and adolescents (Musso et al., 2003; Gil-Campos et al., 2008). Meal composition may evoke different postprandial responses and potentially lead to an increase in disease susceptibility. Whether a postprandial response is mainly affected by a single meal or by an accumulative effect is still not clear and therefore needs to be studied. Children with NAFLD have been shown to eat diets rich in simple sugar, saturated fat and low PUFA (Mager et al., 2010). Currently, there are no evidenced based guidelines on how to treat NAFLD in either children and adolescents or adults. Clinical implications of studying the postprandial response to a high saturated meal would help to develop new diet strategies to avoid or discourage the consumption of certain types of food that could be harmful for children and adolescents in general but be potentially worse in children and adolescents with NAFLD.

The focus of the current thesis was to describe potential anthropometric variables that could be used to screen for NAFLD disease in children and adolescents and to study how feeding two different types of fast food meals (both high in saturated fat) that are representative of the types of fast food meals that children and adolescents consume influence the postprandial metabolic environment. Understanding this information is important for future studies focused on dietary intervention strategies with specific and easy to measure

anthropometric and biochemical measures to monitor success of these interventions within the clinical setting.

CHAPTER 2. RESEARCH PLAN

2.1 RATIONALE

Adult and childhood obesity have become a global health problem. The causes of the global increase in pediatric obesity are multifactorial but are largely attributed to lack of physical activity, consumption of hyper-caloric fast foods and large food portion sizes. Up until recently, the main long term health risks considered for children and adolescents with overweight or obesity included cardiovascular disease or type 2 diabetes (Fishbein & Cox, 2004; Kang et al., 2006). Current research and clinical evidence has illuminated a new health risk called non-alcoholic fatty liver disease (NAFLD) which is currently considered the liver expression of the metabolic syndrome (Mager & Roberts, 2006). NAFLD in both adults and children typically presents across a spectrum of liver disease that spans from simple steatosis to steatosis with inflammation and/or fibrosis (nonalcoholic steatohepatitis or NASH) to cirrhosis (Farrell & Larter, 2006; Idrovo & Guevara, 2004; Schwimmer et al., 2005). The etiology of NAFLD has been suggested to be multifactorial. Both fasted and postprandial changes are observed with IR and hyperinsulinemia (Fishbein & Cox, 2004; Idrovo & Guevara, 2004; Schwimmer et al., 2005; Utzschneider & Steven, 2006). NAFLD diagnosis can be challenging due to the invasive techniques that are required for a proper diagnosis (e.g. liver biopsy and ultrasound). Therefore there is a need to develop simple and easy to use tools that could assist in the screening of NAFLD.

There is not a single treatment for NAFLD per se; treatment has been focused on lifestyle modification and pharmacotherapy directed at antioxidant

functions and improvements in insulin sensitivity. Obesity and overweight, particularly visceral obesity, have been recognized as independent risk factors for the progression of fibrosis in other chronic liver diseases, and hence lifestyle changes such as a healthier diet and increase of physical activity are encouraged to treat NAFLD (Cortez- Pinto et al., 2006; Kang et al., 2006; Mager & Roberts, 2006; Nobili et al., 2006; Schwimmer et al., 2005).

Research has shown that when it comes to a healthier diet, the focus should not be only about the quantity of food consumed but also on dietary quality. There is strong evidence to suggest that hyper-caloric diets, rich in simple sugars, saturated fat and low in fiber predispose an individual to obesity, high body fat, increased waist circumference and metabolic dysregulation including, but not limited to effects on glucose, insulin sensitivity and lipid metabolism (Adams & Angulo, 2006; Cortez-Pinto et al., 2006; Fraser et al., 2007; Pereira et al., 2002; Schwimmer et al., 2005; 2003). On the other hand, diets with a higher content of long chain polyunsaturated fatty acids (LCPUFA), low in fructose, glycemic index and glycemic load evoke improved lipid clearance and insulin sensitivity as well as better glycemic control, all factors known to favorably influence metabolic dysregulation in obesity (Jackson et al., 2005; Larqué et al., 2006; Nobili et al., 2011; Segal et al., 2007). Additionally, long term intake of PUFA, specially omega-3 LCPUFA has shown to improve insulin response and lipid profile (e.g. higher HDL, lower TG, and lower total cholesterol) (Benatti et al., 2004; Kris-Etherton et al., 2000; Moussavi et al., 2008; Musso et al., 2003; Simopoulos, 2002; Skulas-Ray et al., 2008). Both short and long term changes in

diet composition can contribute directly or indirectly to metabolic impairments associated with obesity, type 2 diabetes and NAFLD (e.g. increase or decrease of insulin sensitivity, lipogenesis, and oxidation) (Araya et al., 2004; Chitturi et al., 2002; Cortez-Pinto et al., 2006; Wei et al., 2007).

Poor food choices, high in simple sugars and glycemic load and low LCPUFA, can evoke impaired synthesis of fat in the liver (Malaguarnera et al., 2009) as well as potentially influence immediate effects such as excessive postprandial insulin surges and delayed lipid clearance leading to prolonged lipemia in both lean and obese subjects. These damaging consequences have been shown to be exacerbated in obese subjects and in adults with NAFLD (Araya et al., 2004), but to date no data are available in children and adolescents with NAFLD. The repercussions of repeated consumption of fast foods high in fat (mostly saturated fat), simple sugars, glycemic index and glycemic load and how this may affect lipid clearance, lipid and chylomicron expression, insulin sensitivity and inflammation of a high fat load (typically present in fast food meals) in a single meal in children and adolescents with NAFLD have not been explored. Additionally, the consequences of a meal with the same fat content but with more LCPUFA in children and adolescents with NAFLD have not been characterized. Changes in the omega-3 (n-3) fatty acid content of a meal have shown to decrease the production and/or secretion of chylomicrons in the enterocyte which could lead to improved lipid clearance in children with NAFLD (Demacker et al., 1991; Harris et al., 1988; Harris & Muzio, 1993).

There are no evidence-based guidelines for the treatment and prevention of NAFLD in childhood. Therefore, in order to suggest and develop effective lifestyle interventions focused on dietary intake it is important to understand the contribution of different food intake patterns on the underlying mechanisms that lead to NAFLD pathology. The main goals of this research project were **a)** to examine what simple body composition measures have potential to be used to screen for risk of NAFLD, **b)** to understand and characterize the postprandial metabolic response (change in insulin sensitivity, lipid, lipoprotein and inflammation) to acute consumption of high saturated fat meals representative of typical fast foods consumed in children and adolescents with NAFLD and to compare these responses to responses in lean healthy and obese children and adolescents without NAFLD and; once the postprandial response in children and adolescents with NAFLD has been understood, **c)** to determine if small changes in the LCPUFA content of a high fat/high saturated meal will improve and ameliorate the postprandial metabolic response. Understanding these key points would provide important information regarding the effects of acute intakes of foods; which can inform dietary guidelines for treatment and prevention of childhood NAFLD and potentially future clinical trials examining the efficacy of n-3 LCPUFA supplementation.

2.2 RESEARCH OBJECTIVES AND HYPOTHESES

Primary objective: To identify simple and easy to use body composition tools to identify the risk for NAFLD in children.

Secondary objective: To determine the impact of a high saturated meal (reflective of typical fast food breakfast meals consumed by children in North America) with varying long chain polyunsaturated fatty acids (LCPUFA) content in the fasted and postprandial state.

Primary hypothesis: Simple and easy to use body composition tools such as skinfolds may help to determine if a child is at higher risk to develop NAFLD as compared to less available methods.

Secondary hypothesis: Alterations in postprandial responses such as delayed insulin and lipid clearance will be observed after two meals high in saturated fat with varying contents of LCPUFA.

2.2.1 Specific objectives and hypotheses:

Objective 1 (**Investigated in chapter 3**). To describe the somatotype and compare body composition in children and adolescents with NAFLD using air displacement plethysmography and anthropometric measures (circumferences, skinfolds and diameters) as well as to study the potential interrelationships between body somatotype, fat distribution and biomarkers of liver disease such as ALT, AST and insulin.

Hypothesis 1. Somatotype and body composition of children with NAFLD will be different from children without NAFLD and characterized by higher body fat percentage and different body fat distribution. We expect that visceral and subcutaneous fat will be associated with fasting hyperinsulinemia, insulin resistance and increased ALT and AST.

Objective 2 (**Investigated in chapter 4**). To define the impact of acute intake of a high saturated fat meal/LCPUFA content on postprandial lipid, insulin, lipoproteins, apolipoproteins (apolipoprotein B-48, B-100 and C-III) and cytokines (IL-10, IL-6 and TNF-α) in children and adolescents who are lean, obese or overweight controls and those with NAFLD.

Hypothesis 2. Prolonged postprandial hyperinsulinemia induced by acute intakes of a high saturated fat will result in delayed lipid, apolipoprotein (specifically: apolipoprotein B-48, B-100 and C-III) and cytokine expression [specifically: IL-10 (decreased), IL-6 and TNF-α (increased)] in childhood and adolescents with NAFLD.

Objective 3 (**Investigated in chapter 5**). To determine if changing the LCPUFA content of a similar high SFA meal as studied in objective 2 evokes changes in insulin, lipid, apolipoprotein and cytokine expression in lean and NAFLD children and adolescents.

Hypothesis 3. Higher LCPUFA amounts in a high SFA meal will ameliorate postprandial hyperinsulinemia, lipemia and altered lipoprotein and apolipoprotein expression in lean and NAFLD children and adolescents.

The body composition study (**Chapter 3**) was part of a larger study designed to study the effects of chronic dietary intake on hepatic fat metabolism. Additionally, 31 subjects were recruited for the postprandial study number 1 (**Chapter 4**); from the 31 subjects recruited, 6 subjects agreed to participate in the second postprandial study (**Chapter 5**) and 11 new children and adolescents were recruited.

FLOW CHART

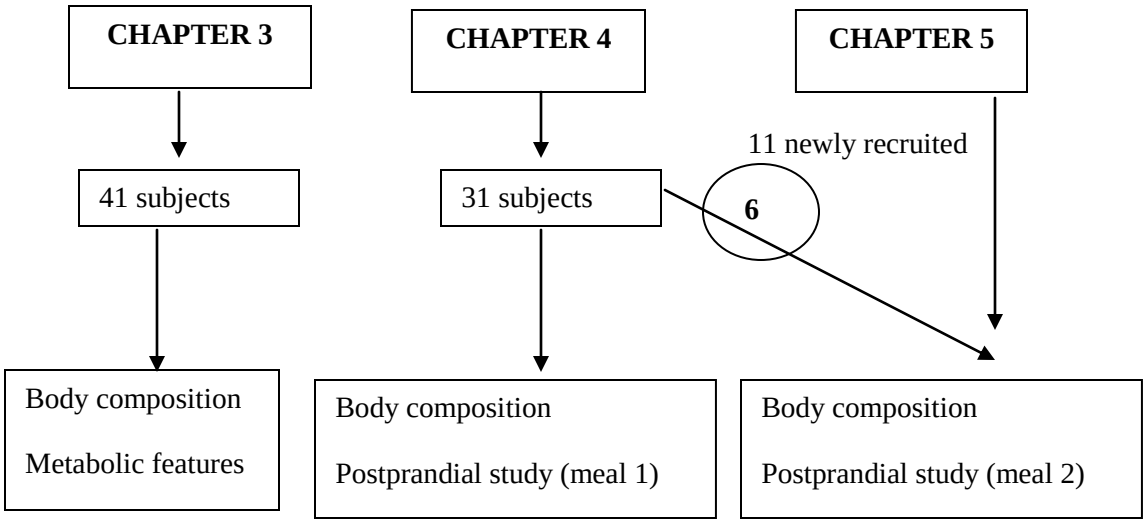


Figure 2.1. Flow chart

CHAPTER 3. SOMATOTYPE AND BODY COMPOSITION IN CHILDREN AND ADOLESCENTS WITH NAFLD

3.1 INTRODUCTION

Obesity has been increasing rapidly all around the world (Cole et al., 2000). The increase in the prevalence of obesity has been observed not only in adults but also in children. This increase is worrisome since obesity in children usually leads to adult obesity and an increase in obesity related complications (Whitaker et al., 1997).

Obesity and more specifically central adiposity, have been strongly correlated with an increased prevalence in cardiovascular diseases (CVD), type 2 diabetes, hyperlipidemia, hypertension and more recently non-alcoholic fatty liver disease or NAFLD (Imamura et al., 2008; Kahn et al., 2005; Parikh et al., 2007; Ponder & Anderson, 2007). Higgins et al. observed that children with body composition characterized by total fat percentages higher than 33 percent were fifteen times more likely to have an adverse profile (high serum triglycerides, hypertension, low serum HDL-cholesterol levels) placing them at risk for future CVD in comparison to children whose body fat percentage was lower than 20% (Higgins et al., 2001).

Anthropometric markers of obesity such as BMI and body fat percentage have been shown to be higher in subjects with fatty liver when compared to healthy (lean and overweight) subjects and were found to be independent predictors of fatty liver in male and female adults (Imamura et al., 2008). Nevertheless, it is possible for lean subjects but with “metabolically obese”

characteristics to present NAFLD (Ruderman et al., 1998; Yap et al., 2011). These "metabolically obese" subjects are characterized by the presence of insulin resistance, hypertriglyceridemia, central obesity and higher visceral and subcutaneous fat mass when compared to non-metabolically obese subjects (Kelishadi et al., 2008; Ruderman et al., 1998). This body fat topography suggests that excess subcutaneous and/or visceral fat in someone with a BMI within a healthy range could lead to serious metabolic consequences (e.g. chronic diseases such as NAFLD, CVD and diabetes) and hence are important clinical markers to monitor risk for these consequences.

3.1.1 Fat distribution and its metabolic outcomes

There is an increasing amount of research suggesting that body fat distribution may be as important as total body fat. Goodpaster et al. observed that subcutaneous abdominal fat was as strongly correlated with insulin resistance as visceral fat (Goodpaster et al., 1997). According to Eguchi et al. visceral fat area correlates positively with the severity of fatty liver and insulin resistance (Dâmaso et al., 2008; Eguchi et al., 2006; Petta et al., 2012). In adults, abdominal obesity has been strongly correlated with glucose intolerance, decreased insulin sensitivity, insulin resistance as well as hepatic steatosis and alterations in the plasma lipoprotein levels such as high triglycerides and low HDL levels (Ascaso et al., 2003; Carr & Brunzel., 2004; Mirza, 2011; Santi et al., 2005). Based on these metabolic disturbances, it has been suggested that visceral and/or abdominal obesity should both be considered as factors that could potentiate and aggravate

the genetic susceptibility to the diverse derangements observed in the metabolic syndrome (Wajchenberg, 2000).

3.1.2 Methods to determine total body fat and percentage of total body fat mass

A variety of anthropometric markers and methods have been proposed to determine the relative fat percentage and visceral distribution present in the body. One of the most popular and widely used markers due to its ease of measurement is body mass index (kg/m^2) or BMI. BMI is an indicator of relative weight and/or body composition (Kahn et al., 2005) but it does not distinguish between distribution of body fat and/or type of body tissue (fat versus lean body mass). The need of accurate, easy and more specific anthropometric markers to determine total body fat percentage and fat distribution are required.

3.1.3 Somatotype

Somatotype, also known as biotype, is a method that helps to determine individual´s morphology in order to distinguish the body shape in an easy and quantitative way (Sillero Quintana, 2004). Currently, the most common somatotype system used is the one described by Heath and Carter (Sillero Quintana, 2004; Singh, 2000). The Heath and Carter somatotype method has three components; endomorphy, mesomorphy and ectomorphy. "The endomorphy component refers to the relative fatness and leanness present in the individual, the mesomorphy component refers to the musculo-skeletal development and the ectomorphy refers to relative linearity present in an individual" (Heath & Carter, 1967). The Heath and Carter anthropometric method requires the following

anthropometric measurements in order to be able to calculate the somatotype: weight, height, triceps, subscapular, supraspinale and calf skinfold, calf circumference as well as the humerus and femur bi-epicondylar diameter (Heath & Carter, 1967; Carter, 1996; 1990; 1980; Ross & Marfell-Jones, 1991). Somatotyping is common in the athletic and clinical fields. The potential relationship between somatotype and disease was first explored by Sheldon et al. who identified correlations between schizophrenic patients and somatotype values (Sheldon et al., 1960; 1954; 1940) Many years later, Williams et al. observed that the endomorphic component was a good indicator of abdominal obesity (Williams et al., 2000). According to a study performed by Herrera et al. (Herrera et al., 2004) a negative correlation between blood pressure (systolic and diastolic) and the ectomorphic component was observed (Herrera et al., 2004). Malina et al. suggested that subjects with a higher risk of CVD tended to have higher levels of endo and mesomorphic and less ectomorphic values (Malina et al., 1997). Additionally, Koleva et al. observed that subjects with high blood pressure and liver disease were usually those with higher endo and mesomorphic values (Koleva et al., 2002).

The potential use of somatotype and multiple skinfold measures, specifically in children and adolescents with NAFLD has not been well explored. The use of these simple assessments as potential additional screening tools used to identify liver disease risk is important to study; particularly if interrelationships with metabolic variables related to liver, insulin and other parameters of metabolic dysfunction can be identified. It is possible that, if well performed,

assessing somatotype along with other laboratory markers (e.g. AST, ALT, lipid profile, glucose and insulin), may be a useful tool to determine which subjects (especially children and adolescents) have the potential to develop NAFLD and therefore, be identified earlier for interventions. Additionally, the somatotype analysis concurrent with laboratory analysis might be useful to monitor as part of the overall clinical response to lifestyle interventions.

The purposes of the study were a) to describe the somatotype and compare body composition in children and adolescents with NAFLD using air displacement plethysmography and anthropometric measures (circumferences, skinfolds and diameters) b) to study the potential interrelationships between body somatotype, fat distribution and biomarkers of liver disease such as ALT, AST and insulin. This information is critically needed to ensure that effective screening methods to determine relative risk for NAFLD in obese children and adolescents are identified and to ensure that easy to use tools are available in the clinical setting to determine disease risk and overall effectiveness of dietary and lifestyle interventions for treatment of this disease.

For clinical relevance, a comparison and assessment of what predictive equations might be useful to determine total body fat in this population when other methods such as ADP are not available was performed.

3.2 SUBJECTS and METHODS

Subjects were recruited under the auspices of a larger prospective study examining the influence of dietary intakes and hepatic markers of fat metabolism.

Data presented in this study represent measures performed on body composition and the interrelationships with biomarkers of disease risk.

3.2.1 Subject recruitment

We prospectively studied 41 obese and lean children and adolescents (n=15 NAFLD, Non-alcoholic steatohepatitis [NASH] =3, Simple steatosis [SS] =12; n=9 age-and-BMI matched healthy obese controls; n=17 healthy lean controls) between the ages of 7-18 years. Participants were recruited from the University of Alberta Hospital Liver Clinics (NAFLD) and Pediatric Centre for Weight and Health (PCWH) at the Stollery Children's Hospital, Edmonton, Alberta. Lean controls were recruited from local advertisements within the University of Alberta and Alberta Health Services.

3.2.2 Inclusion and exclusion criteria

To minimize the potential for inclusion of children and adolescents with other conditions known to cause fatty liver, liver patients, once eligible, underwent full metabolic and serological screening to rule out other causes of liver steatosis (exclusion criteria) prior to study entry. This included serological testing for chronic hepatitis B & C, serum immunoglobulins, anti-nuclear, anti-smooth muscle and anti-liver/kidney microsomal antibodies for testing of autoimmune hepatitis and serum copper and ceruloplasmin for testing of Wilson Disease. Of the children and adolescents clinically diagnosed with NAFLD 9 had biopsy-proven disease in addition to ultrasonic evidence of fat in the liver and serum blood testing that included ALT, AST, GGT, CRP, hepatitis B & C, immunoglobulins, testing of autoimmune hepatitis, serum copper and

ceruloplasmin for testing of Wilson Disease to rule out the potential for other liver diseases that might result in a fatty liver. The remaining children and adolescents diagnosed with NAFLD had ultrasonography and blood testing as they did not meet the clinical criteria for liver biopsy (Kleiner et al., 2005). Criteria for liver biopsy included any one of the following variables: >10 years old, hepatosplenomegaly, one and a half times normal aspartate aminotransferase (AST) and alanine aminotransferase (ALT), severe insulin resistance (based on HOMA-IR, abnormal when >3), detectable non-specific auto antibodies, inconclusive results from biochemical tests related to Wilson disease, viral hepatitis or deficiency or α_1-antitrypsin deficiency (Burt et al., 1998; Kleiner et al., 2005; Mager & Roberts, 2008). Radiological imaging (ultrasonography, computed tomography and MRI) has sensitivity that ranges from 93% to 100% (Saadeh et al., 2002) but disadvantages of these methods are that they do not distinguish simple steatosis from steatohepatitis (Saadeh et al., 2002).

Obese controls underwent screening blood work and/or abdominal ultrasound (US) and a review of medical history to rule out NAFLD (6 obese controls underwent screening blood work and abdominal US, the remaining subjects underwent screening blood work and medical history only); lean subjects underwent screening blood work and a review of medical history to rule out any potential for NAFLD. Family history for NAFLD and diabetes was also reviewed. Written informed consent/assent was obtained from the patient or the responsible caregiver of the patient prior to study entry. The study was approved by the University of Alberta Health Research Ethics Board (HREB); (Appendix1

FormsA-H). Operational Approval and Administrative Approval was obtained from the Stollery Children's Hospital, Alberta Health Services (AHS) and the Northern Alberta Clinical Trials Centre, University of Alberta/AHS/Caritas, Edmonton, Alberta Canada.

3.2.3 Anthropometric and Body Composition Assessment

3.2.4 Subjects

Subjects were asked to fast for a minimum of 10-12 hours overnight prior to each study day at the Clinical Research Unit, University of Alberta. Weight was measured using the Air displacement plethysmography (Bod Pod, COSMED Chicago, IL, USA, Inc.) calibrated scale, which has been validated for body composition measures in children and adolescents (Lockner et al., 2000; McCrory et al., 1995). Subjects were encouraged to void before the anthropometrical measurements. Subjects were weighed in the fasting without shoes and wearing minimum clothing. Standing height was measured without shoes to the nearest 0.5 cm with the use of a commercial stadiometer (Charder HM200PW, Medical Supplies, North Blend, WA, USA). BMI was calculated as weight in kilograms to height-squared in square meter ratio (kg/m^2).

Subjects were classified as 'normal weight', 'overweight' and 'obese', according to the International Obesity Task Force (IOTF), World Health Organization (WHO) and Center of Disease Control (CDC) criteria for age and sex-specific BMI cut-off points (overweight: >85th percentile, obese: >95th percentile) (Cole et al., 2000; de Onis et al., 2007; WHO Multicentre Growth Reference Study Group, 2006). WC was measured using the WHO criteria at the

midpoint between the lower border of the rib cage and the iliac crest (Rudolf et al., 2007).

3.2.5 Skinfold Measures and Body Somatotype

Arm, hip and thigh circumferences were measured using a steel flexible tape (Calibres Argentinos, Rosario, Argentina). Skinfolds (triceps, biceps, subscapular, supraspinale, iliac-crest, abdominal and calf) were measured using a Lange skinfold caliper (Beta technology, Santa Cruz, CA, USA). Humerus and femur diameter were measured using a small bone caliper (Calibres Argentinos, Rosario, Argentina). The International Society for the Advancement of Kinanthropometry (ISAK) methodology was used for all the previous measurements using standardized approaches (ISAK, 2001; Marfell Jones et al., 2006). Measurement sites included five skinfolds: biceps, triceps, subscapular, supraspinale and medial calf. All measurements were taken twice by the same investigator and the technical error was calculated. A technical error of 5% for skinfolds and 2% for circumferences was accepted (Marfell-Jones et al., 2006; Sillero Quintana, 2005). Somatotype analysis (Carter & Heath, 2003) was performed using the Somatotype software (Sweat Technologies, Australia). Briefly, subjects are rated on each of the three components (endomorphy, mesomorphy & ectomorphy). A score of 0.5 to 2.5 is considered low, 3 to 5 moderate and above 7.5 is considered very high for each of the categories (Carter & Heath, 2003; Sillero Quintana, 2005).

In order to determine the distribution between the truncal and extremity area a ratio between the trunk and extremity was used. There is no universal

consensus about the best way to determine the trunk to extremity ratio (TER) (de Jongh et al., 2006; Jelenkovic et al., 2011; Katzmaryk et al., 2004; Leppik et al., 2004; Tresaco et al., 2009). We chose the method of Leppik et al. (2004) because this one includes more skinfolds in the trunk area. TER was calculated using the following formula: Σskinfolds (Supraspinale + abdominal + subscapular + iliac crest) / Σ(biceps + triceps + calf) (Leppik et al., 2004). Total arm fat and muscle area were calculated according to the Frisancho formulas (Frisancho, 1981) and were classified into their respective percentiles. Triceps and subscapular skinfold thickness were classified into their respective percentiles (Addo & Himes, 2009; Frisancho, 1981).

3.2.6 Determination of Total Body Fat and Body Fat Percentage

Total body fat was estimated using Air Displacement Plethysmography (ADP) using the Bod Pod (Bod Pod, COSMED Chicago, IL, USA, Inc.) and by the use of a variety of predictive equations (Siri, 1961). The Bod Pod uses the Siri equation to determine body fat. Comparison to other body fat percentage equations (Brozek, Slaughter, Deurenberg (adapted for children) and Durnin & Womersley) was performed (Brozek et al., 1963; L'Abée et al., 2010, Slaughter et al., 1988) to assess what predictive equation might be more appropriate for use in the clinical setting when Bod Pod is not available. These formulas have been used and validated in pediatric populations, in both genders as well as in lean and overweight and obese children (Eisenmann et al., 2004; Kim et al., 1993, Yoshinaga et al., 2002). **See Appendix 2. Table 1.**

3.2.7 Biochemical Parameters

Metabolic variables indicative of liver dysfunction and metabolic deregulation were measured (see below).

3.2.8 Liver Biochemistries and C - reactive protein

Fasting blood was collected for measurement of ALT, AST and C-reactive protein (CRP). These were analyzed by Laboratory Services, Alberta Health Services (AHS) using standardized methodologies.

3.2.9 Insulin and Glucose

Fasting glucose and insulin were analyzed in Laboratory Services, AHS using standardized methods (Synchron LX® Systems analyzer, Beckman, Coulter, Fullerton, CA for glucose and Roche Diagnostics Elecsys 2010 System for insulin). Insulin resistance was assessed using the homeostasis model of assessment of insulin resistance (HOMA-IR). HOMA-IR is a model that relates fasting levels of plasma insulin and glucose and has been validated for use in children. A value of > 3 was used to classify subjects as insulin resistant (Duncan et al., 2001; Gungor et al., 2004; Hrebicek et al., 2002; Keskin et al., 2005; Matthews et al., 1985).

3.2.10 Lipids and Cholesterol, Non-esterified fatty acids (NEFA)

Fasting blood samples were used to assess concentrations of total cholesterol (TC), low density lipoproteins (LDL), triglycerides (TG), high density lipoproteins (HDL) and non-esterified fatty acids (NEFA). TC, LDL, TG and HDL were analyzed in Laboratory Services, AHS using standardized methods (Synchron LX® Systems analyzer, Beckman, Coulter, Fullerton, CA). Plasma

concentrations of NEFA were analyzed using commercial available Enzyme-Linked Immuno-Sorbent Assay (ELISA) kits (WAKO Pure Chemical Industries, Ltd, Richmond, USA). For NEFA, the minimum detectable level or minimum detectable dose (MDD) was estimated to be 0.0014mEq/L in a 10µl sample size and a range of 0.01 to 4mEq/L. Samples were diluted 1 in 2.5 for obese patients and 1 in 2 for lean patients.

3.2.11 Inflammatory mediators and leptin

Fasting blood samples were used to assess leptin and adiponectin (leptin, adiponectin; Millipore-Biomanufacturing and Life Science Research Products, Missouri, USA), inflammatory markers such as tumor of necrosis factor alpha (TNF-α; R&D Systems, Minneapolis, USA), interleukin-6 (IL-6; R&D systems, Minneapolis, USA) and anti-inflammatory markers such as interleukin-10 (IL-10; Invitrogen by Life technologies, California, USA). For leptin, the kit has a MDD of 0.5ng/mL in a 25µl sample size and a range of 0.5 to 100ng/mL. For adiponectin, the kit has a MDD of 0.78ng/mL in a 20µl sample size and a range of 1.56 to 100ng/mL. For TNF-α, the kit has a MDD of 0.038pg/mL in a 200µl sample size and a range of 0.038 to 0.0191pg/mL. For IL-6, the kit has a MDD of 0.016pg/mL in a 100µl sample size and a range of 0.016 to 0.110pg/mL. For IL-10, the kit has a MDD of <0.2pg/mL in a 50 µl sample size and a range of 0 to 35 pg/mL.

3.2.12 Statistical analysis

Data are expressed as mean $\pm$ SEM unless otherwise specified. Differences between groups (NAFLD vs obese vs lean) were analyzed by repeated measures

analysis of variance (one way ANOVA) for normally distributed variables. Tests for deviations from a Gaussian distribution were performed using the D'Agostino-Pearson omnibus and Shapiro-Wilk normality tests (Graph Pad PRISM Software. version 5.0 La Joya California USA). Kruskal Wallis test was utilized for variables with skewed distributions. In order to normalize the data, HOMA-IR, ALT, AST, GGT, insulin and TG values were transformed into natural logarithms (base e). When a significant interaction was found between factors, differences across groups were analyzed by repeated measures one way ANOVA followed by a Bonferroni's correction if variables were normally distributed (Graph Pad PRISM Software. version 5.0 La Joya California USA). Subjects who were missing the Bod Pod body fat measurement and/or who did not have at least another body fat measurement (calculated by Brozek, Slaughter, Deurenberg and/or Durnin & Womersley) were excluded. Pearson correlations were performed in order to determine the strength and direction of linear relationships between body composition variables and laboratory work (HOMA-IR, insulin fasted, TG, ALT, AST, CRP, HDL, LDL, leptin, adiponectin, IL-6, IL-10 and TNF-α). Differences were considered statistically significant if $p < 0.05$. A post-hoc statistical power calculator (Free Online Software http://www.danielsoper.com/statcalc3) was used to compute the power calculation. Power calculations between groups were performed for insulin, ALT, BMI, abdominal and subscapular skinfolds. Power calculations between and within groups were performed for important laboratory variables (ALT, AST,

insulin, TG, HDL and apolipoproteins), anthropometric skinfolds (biceps, triceps, subscapular, supraspinale, ileact crest) and somatotype components.

Bod Pod measurement was considered the point of comparison for body fat percentage and different normative equations were compared to this. Bland Altman analysis was performed to assess potential bias for the different methods to calculate body fat percentage and fat free mass (Bland & Altman, 1986).

3.3 RESULTS

3.3.1 Anthropometric and demographic data

Detailed anthropometric analysis (**Table 3.1**) including the three somatotype components of the subjects is presented in **Table 3.2**. All the NAFLD subjects except one were >95th percentile (for BMI), with one NAFLD child being between the 85th and 95th percentile. All subjects in the obese control group were >95th percentile. All the lean subjects were within normal ranges (below the 85th percentile for BMI) (Reilly et al., 2010). All the NAFLD and healthy obese control subjects exhibited a WC >90th ile according to their age and gender (Fernandez et al., 2004). Total body fat percentage as determined by ADP (Bod Pod) was significantly higher in both NAFLD and obese group when compared to lean controls (**Table 3.1**). Consequently, the percentage of fat free mass (FFM) was significantly lower in the NAFLD and obese subjects when compared to leans (**Table 3.1**).

Table 3.1. Anthropometric characteristics of the subjects

Anthropometric data	Lean Control (n=17) 8F, 9M	Obese (n=9) 1F, 8M	NAFLD (n=15) 2F, 13M
Age (y)	13.7±0.5	14.3±0.4	13.3±0.7
Weight (kg)	47.1±2.9[a]	96.4±9.2[b]	91.8±7.8[b]
Weight z-scores CDC	-0.2±0.2[a]	2.4±0.2[b]	2.6±0.1[b]
Height (m)	1.5±0.0	1.6±0.0	1.6±0.0
Height z-scores CDC	0.08±0.2	0.3±0.2	0.8±0.3
BMI (kg/m^2)	18.6±0.5[a]	35.3±3.0[b]	34.0+2.0[b]
BMI z-scores CDC	-0.3 ± 0.2[a]	2.2 ± 0.1[b]	2.3 ± 0.1[b]
BMI z-scores WHO	-0.3±0.2[a]	3.2±0.4[b]	3.5±0.3[b]
BMI percentile IOTF	40.9±6.6[a]	98.2±0.5[b]	98.3±0.6[b]
BMI percentile CDC	41.2±6.6[a]	98.2±0.5[b]	98.3±0.6[b]
Waist circumference(cm)[1]	63.5±1.5[a]	101.8±6.0[b]	98.9± 4.0[b]
Waist/height (cm)[1]	0.4±0.0[a]	0.6±0.0[b]	0.6±0.0[b]
Waist/hip (cm)[1]	0.7±0.0[a]	0.8±0.0[b]	0.9±0.0[b]
Body fat (%); Bod Pod*	14.0 ± 1.5[a]	39.8± 3.5[b]	36.2 ± 2.3[b]
Fat free mass (%), Bod Pod*	86.0±1.5[a]	60.1±3.5[b]	63.7±2.3[b]

Values are mean ± SEM. Different superscripts indicate significant differences between groups by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons.[1]Waist circumference; NAFLD=14, Obese control=9; Lean controls=14. *Bod Pod analysis; NAFLD n=14, Obese control=7, Lean controls=16. Center of Disease Control (CDC), World Health Organization (WHO), International Obesity Task Force (IOTF)

3.3.2 Skinfold Measures, Areas and Somatotype Analysis

3.3.2.1 *Skinfolds and somatotype analysis*

Skinfolds were performed in NAFLD n=13, Obese controls n=9, Lean controls n=13. Subjects with NAFLD exhibited several significant differences in body composition when compared to lean subjects (**Table 3.2**). When comparing NAFLD to the obese group a higher (p=0.1) abdominal subcutaneous skinfold was observed in the NAFLD group. Additionally, differences in subcutaneous fat

distribution in the abdominal area (Supraspinale, abdominal and iliac crest or ileocristale) were observed (**Table 3.2**). With regard to the triceps percentiles, 9 NAFLD, 6 obese were above the 95th percentile. None of the lean subjects were above the 95th percentile. All NAFLD and obese subjects were above the 95th percentile for subscapular skinfolds, none of the lean subjects were above the 95th percentile. Somatotype components of NAFLD and obese were similar and were all significantly different when compared to the lean group (**Figure 3.1**). No significant somatotype differences were observed between the NAFLD and obese group.

Table 3.2. Skinfolds and somatotype components of the subjects

Anthropometric data	Lean (n=13)	Obese (n=9)	NAFLD (n=13)
Skinfolds (mm)			
Biceps	7.5±1.2^{a}	30.1±2.6^{b}	23.2±2.3^{b}
Triceps	13.8±1.3^{a}	39.7±4.4^{b}	33.8±2.9^{b}
Subscapular	10.6±1.3^{a}	44.6±3.5^{b}	44.6±3.8^{b}
Supraspinale	11.3±1.4^{a}	44.2±2.9^{b}	41.9±3.1^{b}
Iliac crest	9.2±1.5^{a}	44.8±3.0^{b}	40.2±3.1^{b}
Abdominal	13.4±1.9^{a}	40.3±2.0^{b}	43.6±2.5^{b}
Calf	12.2±1.1^{a}	33.6±3.7^{b}	28.8±2.9^{b}
TER*	1.3±0.1^{a}	1.7±0.0^{a,b}	2.0±0.1^{b}
Diameters (cm)			
Humerus	5.7±0.1^{a}	6.6±0.2^{b}	6.7±0.2^{b}
Femur	5.8±0.1^{a}	8.5±0.5^{b}	8.1±0.3^{b}
Somatotype components			
Endomorphic component	3.5±0.3^{a}	9.4±0.4^{b}	9.2±0.4^{b}
Mesomorphic component	1.3±0.3^{a}	5.5±0.7^{b}	5.1±0.5^{b}
Ectomorphic component	3.6±0.3^{a}	0.2±0.1^{b}	0.3±0.1^{b}

Values are mean ± SEM. Different superscripts indicate significant differences between groups by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. *Trunk-extremity ratio (TER): Σ 4 trunk (Subscapular, supraspinale, iliac crest and abdominal) / Σ 3 extremity skinfolds.

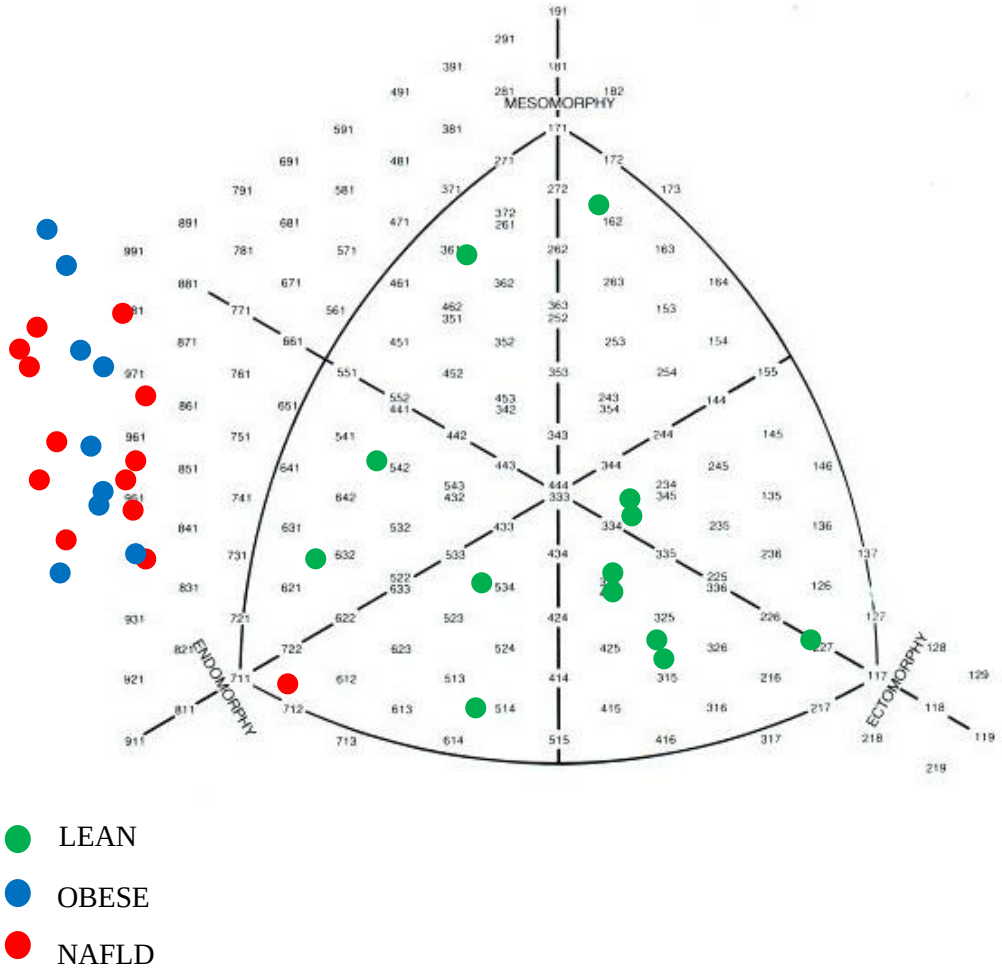


Figure 3.1. Somatochart image showing the comparisons per group between somatotype children and adolescents with nonalcoholic fatty liver disease (n=13), healthy obese-gender-and aged matched (n=9) and healthy lean-age, gender matched (n=13) controls. Somatochart was computed by using Somatotype software (Carter & Heath, 2003)

3.3.2.2 *Mid arm muscle and fat area determination*

Total fat and muscular area of the arm were calculated according to the Frisancho formulas (Frisancho, 1981) and were classified into their respective percentiles. In the NAFLD group, 9 subjects were at or below the 75th percentile for arm´s total muscle area. On the contrary, when analyzing the arm´s total fat area, 12 NAFLD subjects were above the 95th percentile. In the obese group 6 subjects were at or below the 75th percentile for the arm´s total muscle. When analyzing the total fat area, all the obese subjects (NAFLD and non-NAFLD) were at or above the 95th percentile. The arm´s total muscle in the lean group was at or below the 50th percentile for all subjects (**Table 3.3**).

Table 3.3. Medium arm muscle and fat area of the subjects

Anthropometric data	**Lean (n=13)**	**Obese (n=9)**	**NAFLD (n=13)**
Mid arm circumference (mm)	215.2±5.5^{b}	349.7±22.5^{a}	330.9±15.4^{a}
Total area (mm^2)	3715±188.6^{b}	10054±1352^{a}	8945±836.5^{a}
Total arm muscle area (mm^2)**	2391±195.4^{b}	4135±476.2^{a}	4121±376.4^{a}
Total arm fat area (mm^2)**	1324±125.7^{b}	5918±1030^{a}	4824±568.9^{a}
Total muscle arm mass (kg)	12.8±1.2^{b}	23.5±2.9^{a}	23.3±2.5^{a}

Values are mean ± SEM. Different superscripts indicate significant differences between groups by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

**Total arm muscle area 90-95th percentile: 4 NAFLD, 3 obese, 0 lean. >95th percentile: 0 NAFLD, 2 obese, 0 lean.
**Total arm fat area >95th percentile: 12 NAFLD, 8 obese, 0 lean.

3.3.3 Biochemical analysis

Complete laboratory data are presented in **Table 3.4**.

Table 3.4. Fasting metabolic characteristics of the subjects

Laboratory variable	Lean (n=17)	Obese (n=9)	NAFLD (n=15)	Fasting normal values
Insulin (mU/L)	9.5±1.3[a]	26.0±6.7[b]	28.7±4.1[b]	5.0-20.0
Glucose (mmol/L)	4.5±0.1[a]	4.9±0.1[a,b]	5.1±0.1[b]	3.3-11.0
HOMA-IR *	1.9±0.2[a]	5.6±1.4[b]	6.6±1.1[b]	<3
ALT (U/L)	16.0±1.0[a]	22.0±2.0[a]	77.0+14.0[b]	<50
AST (U/L)	24.0±1.0[a]	23.0±1.0[a]	48.0+8.0[b]	<40
CRP (mg/L)	0.6±0.2[a]	2.4±0.8[a,b]	3.1±0.8[b]	<8.0
GGT (U/L)	6.0±1.0	18.0±6.0	41.0±17.0	<55
Triglyceride (mmol/L)	0.6±0.0[a]	1.4±0.1[b]	1.4±0.2[b]	<1.5
Total cholesterol (mmol/L)	3.9±0.1	4.2±0.2	4.0±0.3	<4.40
HDL (mmol/L)	1.3±0.0[a]	0.8±0.0[b]	0.9±0.0[b]	>1.00
Adiponectin (ng/mL)	11.5±1.0[a]	7.8±1.0[a,b]	7.8±0.9[b]	♂: 9.8 ±1.3 ♀:10.7±1.2
Leptin (ng/mL)	0.4±0.0[a]	4.5±1.4[b]	2.1±0.2[c]	♂: 3.8 ±1.8 ♀:7.4±3.7
TNF-α (pg/mL)**	1.3±0.1[a]	1.3±0.1[a]	1.9±0.1[b]	--
IL-6 (pg/mL)	1.2±0.4	0.6±0.1	0.5±0.0	--
IL-10 (pg/mL)	4.0±0.0[a]	3.2±0.0[b]	3.5±0.1[c]	--
Apo B-48 (μg/mL)	4.4±0.4	4.7±0.5	5.1±0.3	--
Apo B-100 (μg/mL)	308.5±33.0[a]	586.4±98.7[b]	652.4±89.1[b]	--
Apo C-III (μg/dl)	9.8±1.5[a]	9.2±0.8[a]	19.0±3.0[b]	--

Values are mean ± SEM. Different superscripts indicate significant differences between groups by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

*HOMA-IR: (mmol/L x μU/ml) = fasting glucose (mmol/L) x fasting insulin (μU/ml)/22.5 (Mathew et al., 1985)

**Tumor of Necrosis alpha (TNF-α) limits of detection: 0.5-32 pg/mL
Interleukin 6 (IL-6) limits of detection: 0.156-10 pg/mL
Interleukin 10 (IL-10) limits of detection: 0.78-50 pg/mL
Apolipoprotein B-48 (Apo B-48) limits of detection: 0.25-16 μg/mL
Apolipoprotein B-100 (Apo B-100) limits of detection: 7.5-1000 μg/mL
Apolipoprotein C-III (Apo C-III) limits of detection: 0.2-200 μg/dL

3.3.4 Comparison of body fat percentage and fat free mass using different methods and normative equations

Subjects who were missing the Bod Pod body fat measurement and/or who did not have at least another body fat measurement (calculated by Brozek, Slaughter, Deurenberg and/or Durnin & Womersley) were excluded. Data from 37 children and adolescents was analyzed (NAFLD=14, healthy obese controls=7, healthy lean controls=16) using Bland Altman analysis to determine potential bias for the different methods to calculate body fat percentage and fat free mass (Bland & Altman, 1986)

3.3.5 Predictive Equations

A variety of predictive equations were examined to determine what predictive equation would be appropriate to determine body composition in children and adolescents with NAFLD, when other techniques such as DXA, BIA or ADP were not routinely available. All of these predictive equations (Brozek, Slaughter, Deurenberg and Durnin & Womersley) have been validated in pediatric population against DXA, BIA and ADP, in both genders as well as in lean and overweight and obese children (Eisenmann et al., 2004; Kim et al., 1993, Yoshinaga et al., 2002). **Table 1 in Appendix 2** illustrates the populations in which these equations have been used and what criteria were used to validate the use of these equations to predict fat mass. It also identifies what variables are present in the equations.

When analyzing the different body fat percentage equations, Brozek´s formula showed the most similar values to the ones obtained by ADP in obese and

non-obese subjects (**Table 3.5).** Brozek´s formula overestimated BF% in NAFLD and obese by 2.7 and 0.3% respectively. Additionally, it underestimated BF% in lean subjects by 0.14% when compared to ADP. Bland Altman analysis performed in the 37 subjects corroborated the ADP (Bod Pod; Siri´s equation) and Brozek´s equation similar results **(Table 3.6, Figure 3.2).** It is worth to mention, that Slaughter´s formula overestimated and underestimated the total fat and fat free mass percentage, respectively in lean and obese (NAFLD and non-NAFLD) when compared to estimates determined using ADP (Table 5).Slaughter´s formula overestimated BF% in NAFLD, obese control and lean control by 84.5, 80.2 and 33.0% respectively. Comparisons for each group (intragroup) between ADP estimates of total body fat percentage and values obtained using other equations are presented in **Figure 3.3**. Comparisons between different groups (intergroup) are presented in **Figure 3.4**. Additionally, data for fat free mass (FFM) between ADP determinations and between different groups is shown in **Figure 3.5** and **Figure 3.6**, respectively.

Table 3.5. Body fat and fat free mass percentages

	Lean (n=14) 8F, 6M	Obese (n=7) 1F, 6M	NAFLD (n=16) 2F, 14M	p value
Body fat percentage				
ADP (Bod Pod)	14.0 ± 1.5^{a}	39.8 ± 3.5^{b}	36.2 ± 2.3^{b}	<0.0001
Brozek et al., 1963	13.9 ± 1.2^{a}	40.0 ± 2.7^{b}	36.6 ± 2.4^{b}	<0.0001
Slaughter et al., 1988	18.6 ± 1.4^{a}	71.8 ± 6.9^{b}	60.9 ± 5.2^{b}	<0.0001
Deurenberg et al., 1990	18.2 ± 1.1^{a}	43.6 ± 4.9^{b}	41.3 ± 3.0^{b}	<0.0001
Durnin & Womersley, 1974	21.5 ± 2.1^{a}	34.0 ± 1.9^{b}	30.9 ± 1.4^{b}	0.0007
Fat free mass percentage				
ADP (Bod Pod)	86.0 ± 1.5^{a}	60.1 ± 3.5^{b}	63.7 ± 2.5^{b}	<0.0001
Brozek et al., 1963	86.0 ± 1.2^{a}	59.9 ± 2.7^{b}	63.3 ± 2.4^{b}	<0.0001
Slaughter et al., 1968	79.0 ± 1.5^{a}	32.8 ± 6.0^{b}	39.0 ± 5.2^{b}	<0.0001
Deurenberg et al., 1990	81.7 ± 1.1^{a}	57.2 ± 5.6^{b}	58.6 ± 3.0^{b}	<0.0001
Durnin & Womersley, 1974	78.4 ± 2.1^{a}	65.9 ± 1.9^{b}	69.5 ± 1.5^{b}	0.001

Values are mean $\pm$ SEM. Different superscripts indicate significant differences between groups by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

Table 3.6. Bland Altman Analysis

Equation	Difference vs average			Ratio vs average			%Difference vs average		
	Bias	SD of bias	95% limit of agreement	Bias	SD of bias	95% limit of agreement	Bias	SD of bias	95% limit of agreement
Brozek[1]	-0.63	5.29	From -11.01 to 9.75	0.97	0.25	From 0.47 To 1.46	-7.00	34.41	From -74.45 to 60.43
Slaughter[2]	-20.15	14.63	From - 48.48 to 8.53	0.59	0.16	From 0.26 To 0.93	-53.24	30.53	From -113.09 to 6.60
Deurenberg[3]	-4.34	7.10	From -18.26 to 9.57	0.85	0.25	From 0.35 To 1.35	-20.24	36.01	From -92.82 to 50.34
D & W[4]	-2.60	7.76	From -17.81 to 12.60	0.85	0.31	From 0.24 To 1.47	-22.23	44.03	From -108.53 to 64.06

Comparisons of the body fat percentage obtained with the ADP (Bod Pod; Siri´s equation) vs Brozek, Slaughter, Deurenberg and Durnin & Womersley´s (D&W) equations.[1]Brozek et al., 1963, [2]Slaughter et al., 1988, [3]Deurenberg et al., 1990, [4]Durnin & Womersley, 1974

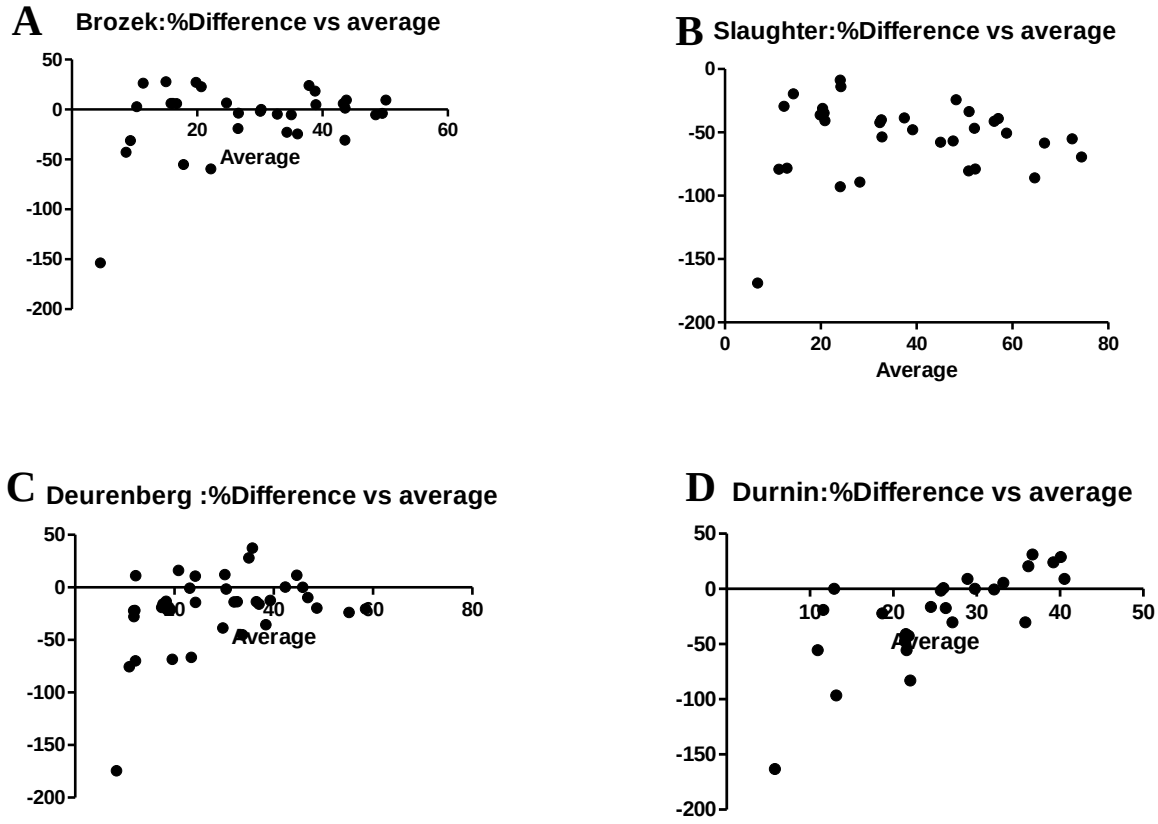


Figure 3.2. Bland-Altman body fat percentage comparisons. **A**. ADP vs Brozek, **B**. ADP vs Slaughter, **C.** ADP vs Deurenberg, **D.** ADP vs Durnin & Womersley. Results are shown %Difference vs average.

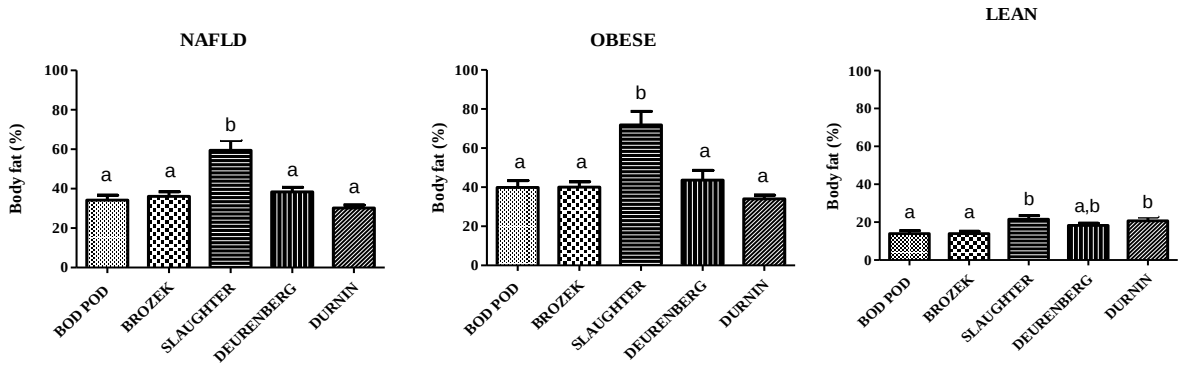


Figure 3.3. Comparisons per group (intragroup) between body fat percentages obtained with ADP (Bod Pod; Siri´s equation) and body fat percentages obtained with Brozek, Slaughter, Deurenberg and Durnin & Womersley´s formulas in children and adolescents with nonalcoholic fatty liver disease (n=16), healthy obese-gender-and aged matched (n=7) and healthy lean-age, gender matched (n=14) controls. Values with different superscripts are significantly different between groups by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

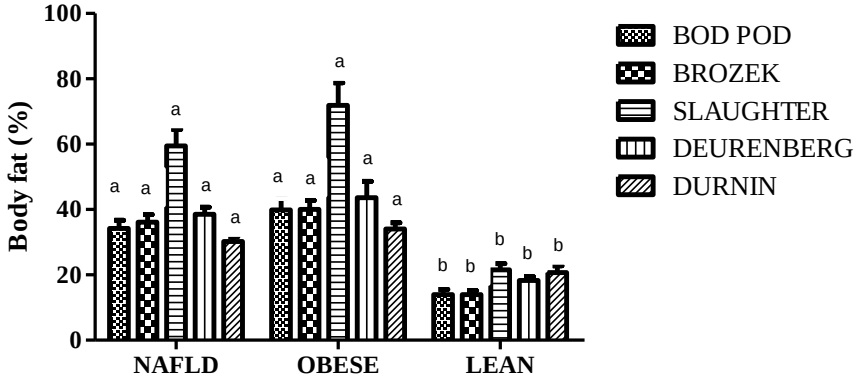


Figure 3.4. Comparison of body fat percentage between groups in children and adolescents with nonalcoholic fatty liver disease (n=16), healthy obese-gender-and aged matched (n=7) and healthy lean-age, gender matched (n=14) controls. Values with different superscripts are significantly different between groups by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

Fat Free Mass (%) intragroup comparisons

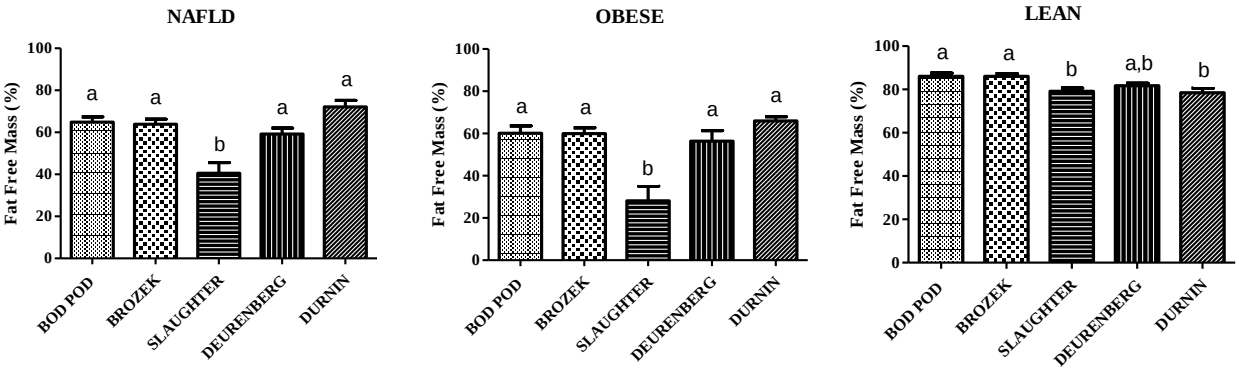


Figure 3.5. Comparisons per group between Fat free mass (FFM) percentages obtained with ADP (Bod Pod; Siri´s equation) and body fat percentages obtained with Brozek, Slaughter, Deurenberg and Durnin & Womersley´s formulas in children and adolescents with nonalcoholic fatty liver disease (n=16), healthy obese-gender-and aged matched (n=7) and healthy lean-age, gender matched (n=14) controls. Values with different superscripts are significantly different between groups by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

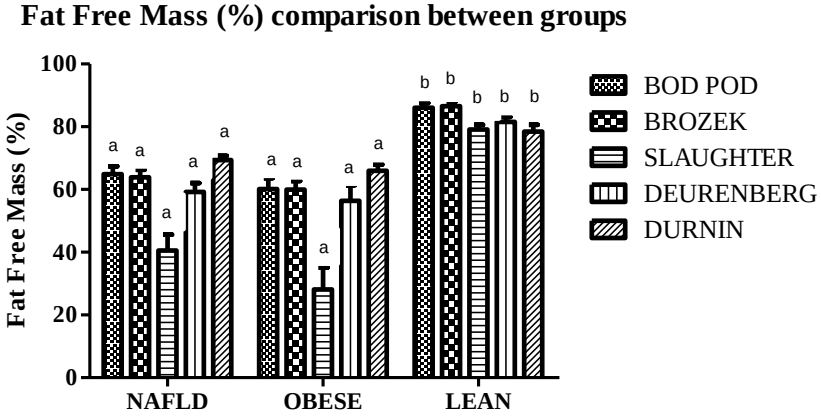


Figure 3.6. Comparison of Fat Free Mass (FFM) percentage between groups in children and adolescents with nonalcoholic fatty liver disease (n=16), healthy obese-gender-and aged matched (n=7) and healthy lean-age, gender matched (n=14) controls. Values with different superscripts are significantly different different between groups by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

3.3.6 Relationships between BMI, waist circumference (WC), waist to height ratio (WHtR), waist to hip ratio (WHR) with insulin, liver enzymes, cytokines and lipid profile.

Individuals with missing measurements for body fat percentage and somatotype were excluded. Individuals were included if they had one variable (Bod Pod or somatotype). Natural log transformation (base e) was performed for HOMA-IR, insulin, ALT, AST, GGT and TG. Significant ($p<0.05$) correlations between BMI and HOMA-IR, insulin, ALT and AST were observed (r^2=0.54, 0.55, 0.30, 0.12; respectively). WC showed also significant ($p<0.01$) correlations with HOMA-IR, insulin, ALT, AST, TG and IL-10 (r^2=0.52, 0.54, 0.40, 0.25, 0.38, (-) 0.44). A significant group effect was observed between ALT and WC ($p<0.0001$).

WHtR showed a stronger correlation with HOMA-IR, insulin, ALT, AST, TG and CRP (<0.001; r^2=0.54, 0.55, 0.47, 0.31, 0.46, 0.39) than WHR ($p<0.05$; r^2=0.38, 0.36, 0.45, 0.24, 0.12, 0.14). A significant ($p<0.001$) group effect between WHtR and liver enzymes (ALT and AST) was observed. When performing a categorical analysis for adiponectin (below and above the mean); BMI correlated significantly ($p<0.05$) with IL-10 and adiponectin above the mean (r^2=-0.42, -0.22). See **Appendix 2. Table 2**.

Weak correlations were observed between BMI, WHR and CRP (see **Appendix 2. Table 2**). No significant correlation was observed between TNF-α and BMI. Additionally, a weak correlation between TNF-α and WC, WHtR and

WHR was observed (see **Appendix 2. Table 2**). No significant correlations were observed between BMI, waist, WHtR, WHR and IL-6 and leptin (see **Appendix 2. Table 2**).

3.3.7 Relationships between total body fat (BF) and fat free mass (FFM) with insulin, and lipid profile.

Individuals with both laboratory data and body composition as measured by ADP were included. BF percentage correlated with insulin (r^2= 0.45; $p<0.0001$). Significant correlation ($p<0.05$) was observed between BF and TG and HDL (r^2= 0.30, (-) 0.44; respectively). BF correlated significantly with IL-10 (r^2= (-) 0.50; p=<0.0001). FM (kg) correlated significantly with HOMA-IR and insulin (r^2= 0.45, 0.48 respectively; $p<0.0001$ (see **Appendix 2. Table 3)**. No significant correlation between body fat, FFM percentage and AST, adiponectin, leptin, TNF-α and IL-6 was observed (see **Appendix 2. Table 3**).

3.3.8 Relationships between skinfold measures, trunk-extremity ratio and somatotype with insulin, cytokines and lipid profile.

Individuals with missing somatotype data (endo-meso-ectomorphic values) were excluded to match the laboratory data. Subscapular and abdominal skinfolds (**Figure 3.7A, 3.7B**), followed by supraspinale and triceps skinfolds showed the strongest correlation with HOMA-IR values (r^2=0.52, 0.49, 0.46, 0.45 respectively; $p<0.0001$). The sum of 4 trunk skinfolds (supraspinale, ileac crest, abdominal and subscapular) showed significant correlation with HOMA-IR and insulin (r^2=0.49, 0.51, respectively; $p<0.0001$) **Figure 3.7C**. Supraspinale, ileac

crest and abdominal skinfolds showed the strongest correlations with TG (r^2=0.46, 0.45, 0.41, respectively $p<0.0001$). Subscapular, supraspinale and abdominal skinfolds showed the strongest correlation with HDL (r^2= (-) 0.56, (-) 0.57, (-) 0.53, respectively; $p<0.0001$). A significant correlation between ALT and the abdominal, subscapular and sum of trunk skinfolds was observed ($p<0.001$; r^2=0.50. 0.47, 0.44) **Figure 3.7D, 3.7E and 3.7F**. Biceps and ileac crest skinfolds showed the strongest correlation with IL-10 (r^2=0.68, 0.63; $p<0.0001$).

Within the somatotype analysis; the endomorphic component showed the strongest correlations with the laboratory markers. Significant positive correlations were observed between the endomorphic somatotype component and important liver enzymes such as AST and ALT ($p<0.01$; r^2=0.19, 0.40; respectively) as well as with HOMA-IR and insulin ($p<0.0001$; r^2=0.49, 0.52; respectively). With regard to the lipid profile significant correlations were observed endomorphic and TG ($p<0.0001$, r^2=0.45) and HDL ($p<0.0001$, r^2= (-) 0.54). A categorical analysis was performed for comparison of the strength relationship between adiponectin levels. Subjects were categorized below and above the mean $\pm$ SEM (8.9$\pm$0.58ng/mL). No significant correlations ($p>0.05$) were observed between the endomorphic somatotype component and the adiponectin levels. No significant correlation was observed between any of the somatotypes components and IL-6. See **Appendix 2. Table 4** for a complete correlation table.

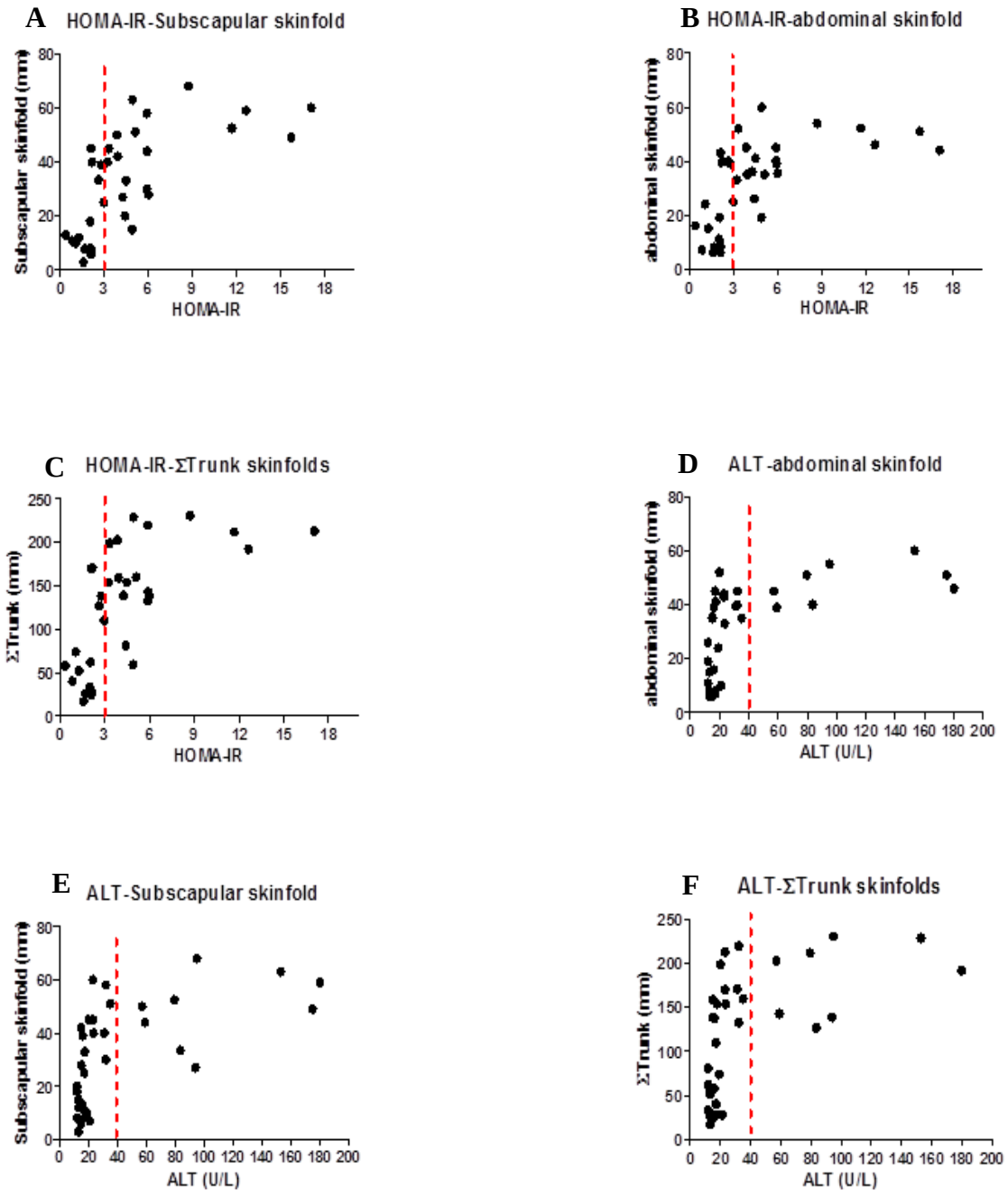


Figure 3.7. A. HOMA-IR-subscapular skinfold (r^2=0.49, p<0.0001), B. HOMA-IR-abdominal skinfold (r^2=0.50, p<0.0001); C. HOMA-IR-ΣTrunk skinfolds (r^2=0.49, p<0.0001); D. ALT-abdominal skinfold (r^2=0.50, p<0.0001); E. ALT-subscapular skinfold (r^2=0.47, p<0.0001); F. ALT-ΣTrunk skinfolds (r^2=0.44, p<0.0001). Data was transformed by using the natural logarithm (base e) to normalize the data and then treated as categorical variables. ALT above and below 40 U/L and HOMA-IR above and below 3. - - - - cut off values

3.4 DISCUSSION

Body composition could be influenced by several factors such as gender, age and physical activity but it is also a reflective of the nutritional status of a human being. Correlations between a subject´s somatotype and disease have been observed (Herrera et al., 2004; Koleva et al., 2002; Singh, 2007; Williams et al., 2000). Higher numbers for endomorphic and mesomorphic components have been correlated with a higher risk of CVD, and higher blood pressure (Malina et al., 1997; Koleva et al., 2002). Somatotype and body fat topography in the pediatric NAFLD population have not been explored and it is possible that it may be different to what is observed in non-NAFLD subjects. If true, such analyses could be used in the clinical setting in order to identify who is at higher risk to develop NAFLD. To our knowledge this is the first study that describes in detail the body somatotype analysis in children and adolescents diagnosed with NAFLD and compares this with other measures of body composition. The main purposes of the study were to describe and compare the somatotype and measures of body fat observed in obese (NAFLD and non NAFLD) and lean children and adolescents as well as to study potential interrelationships between body topography and biomarkers of liver disease risk and inflammation as these may provide useful and important surrogate markers of disease risk for NAFLD.

Skinfold measurements were performed because they are relatively simple to perform when the individual is trained. These measurements are inexpensive and more likely to be readily available when compared to more complex techniques such as MRI. Additionally, we wanted to compare different predictive

equations and suggest which might be most accurate and simple to use in the clinical setting when more complex methods are not available.

Subcutaneous body fat distribution was different between obese subjects with and without NAFLD. Children and adolescents with NAFLD had higher subcutaneous measures of abdominal/central adiposity than obese in the presence of a constant hip or extremity measure. These results are an interesting clinical finding suggestive of different subcutaneous adipose tissue distribution in the body and abdominal area in NAFLD children and adolescents. This finding is similar to what other researchers have observed in adults with NAFLD (Jun et al., 2008). Higher proportion of subcutaneous fat assessed by computer tomography has been observed in adults (women and men) with NAFLD when compared to non NAFLD subjects (Jun et al., 2008). Fishbein et al. observed an association between elevated ALT and subcutaneous adipose tissue measured by MRI in children and adolescents with NAFLD (Fishbein et al., 2006) but the exact subcutaneous topography (as per skinfolds measurements) was not explored.

Marfell-Jones mentioned that the "somatotype is a classification of physique based on the concept of shape, disregarding size" (Marfell-Jones, 2006). Therefore, the slight ($p>0.05$) numerical somatotype differences (higher meso and endomorphic values) observed in NAFLD subjects, suggest that they have a different type of body shape and therefore different fat distribution. The somatotype is the result of a three number expression and somatotype sub classifications (e.g. highly endomorphic-highly ectomorphic) are very likely to

occur in individuals. Body classification subtypes change with time. Therefore, the somatotype is more descriptive in nature of overall body habitus. All of NAFLD subjects fell within the meso-endomoprhic area. Higher mesomorphic and endomorphic values have been observed in subjects with CVD (Malina et al., 1997; Koleva et al., 2002). Therefore, plotting a subject in a somatochart (somatotype analysis) could be used in the clinical setting to complement the skinfold measurements. A larger sample size is likely needed to distinguish between the obese NAFLD and non-NAFLD.

Biological development can only be determined with hand-wrist radiographs (Greulich & Pyle, 1959) for assessment of bone age or based on sex maturation (Tanner stage) (Tanner, 1962). Pubertal development in humans ranges between 9 to 13 years of age in girls, and 10 to 14 years in boys (Bitar et al., 2000; Rogol et al., 2000). Obesity has been associated with early sexual maturation (Bitar et al., 2000; Epstein et al., 1985; Forbes, 1987; Rogol et al., 2000). Therefore, since our population includes children and adolescents and since the three somatotype components take into account the height, somatotype changes would be expected over the time and somatotype analysis should be always accompanied by other anthropometric measurements such as BMI and waist circumference.

Interesting correlations between some anthropometric measures and biochemical markers were observed. High body fat percentage especially central adiposity has been correlated with insulin sensitivity, hyperinsulinemia and high

levels of HOMA-IR (Gonzalez-Ortiz et al., 2002; Goodpaster et al., 1997; Kahn & Flier, 2000; Smith et al., 2001; Wajchenberg, 2000). In our study WC, WHtR, BMI in addition to the subscapular skinfold were the anthropometric markers with the strongest correlation with HOMA-IR. WC and WHtR have been suggested to be good surrogates of insulin sensitivity and dyslipidemia (Ashwell et al., 1996; Tabata et al., 2009; Wang, 2003). Positive correlations between insulin and skinfolds such as subscapular and triceps have been observed by others (El-Koofy et al., 2012; Freedman et al., 1999; Gan et al., 2011; Kahn & Flier, 2000; Lambert et al., 2004; Legido et al., 1989; Vikram et al., 2006). Ayonrinde et al. observed significant higher subscapular and triceps skinfolds in subjects with NAFLD when compared to non-NAFLD adolescents (Ayonrinde et al., 2011). The fact that not all the skinfolds exhibited the same coefficient of determination (r^2) in the correlations with the same laboratory variables suggests that body fat distribution is more critical than total body fat in terms of metabolic deregulation. In addition suprailiac, subscapular and abdominal skinfolds showed the strongest correlations with TG and HDL. Therefore, monitoring trunk skinfolds might be important especially in the obese population. Our results with regard to the lipid profile are similar to the correlations between skinfolds and TG and HDL observed by others (Botton et al., 2007; Tresaco et al., 2009).

No significant correlation between subcutaneous adipose tissue and adiponectin was observed. Literature available about adiponectin and subcutaneous adipose tissue is contradictory. Some researchers have observed absence of correlation between subcutaneous adipose tissue and adiponectin

(Matsuzawa et al., 2004; Nakamura et al., 2009; Staiger et al., 2003). Others have found positive correlation between SAT and adiponectin (Hanley et al., 2007). van der Poorter et al., observed significant positive correlation between SAT and adiponectin in NAFLD subjects while some others have found a negative correlation between adiponectin and subcutaneous adipose tissue but not with visceral adipose tissue (Cnop et al., 2003; Frederiksen et al., 2009; van der Poorter et al., 2008). A very interesting correlation found in our study was that between IL-10 and amount of subcutaneous adipose tissue. Research suggests that visceral adipose tissue releases more IL-10 than does abdominal subcutaneous adipose tissue (Fain, 2006; Tam et al., 2011). More research is necessary in order to determine the link between subcutaneous adipose tissue and IL-10. Overall our results suggest that subcutaneous adipose tissue especially the trunk skinfolds are reflective of the metabolic deregulations present in the body. It is worth to mention that the majority of our NAFLD and obese subjects were male. The lean group was not gender matched to the NAFLD and non NAFLD subjects. Body fat percentage and skinfolds averages might be different that their female counterparts. Even though the somatotype values are not affected per se by gender, research has suggested that males show higher ALT and insulin values when compared to females (Wiegand et al., 2010; di Bonito et al., 2009). On the other hand, Spinneker et al. observed that even when lipid concentrations tend to be higher in girls than in males the main factors affecting lipid levels were body fat percentage and BMI (Spinneker et al., 2012). In our study an interaction between gender and HOMA-IR was observed but an ALT gender effect with the

skinfold analysis was not. These changes suggest that males are metabolically more sensitive to subcutaneous fat changes when compared to females. Additionally the HOMA-IR gender interaction may be affecting our correlations and therefore showing stronger values (r^2) than what we would have seen if the majority of our subjects were females.

With regard to the different body fat percentage equations, there is no consensus about what type of formula should be used in children and adolescents, especially when it comes to measure the body fat percentage with skinfolds in obese subjects. Anthropometric studies require larger samples to ascertain which formula should be used in a specific type of population (e.g. obese children and adolescents). In our study Brozek´s formula showed the closest body fat percentages in both lean and obese subjects (NAFLD and non-NAFLD) when compared to Bod Pod values measured by ADP. Our results are similar to what Frissard et al., observed (Frissard et al., 2005). Potential explanations are that both ADP (Bod Pod; Siri´s formula) and Brozek´s formula take into account body density calculations. Additionally, Brozek´s formula factorizes key body distribution skinfolds (Triceps, subscapular and abdominal) which is potentially another reason of the similarities observed between the ADP and Brozek´s calculations. Durnin & Womersley, Deurenberg and Slaughter formulas overestimated the values obtained by the Bod Pod, especially in the overweight population (NAFLD and obese control); this is similar to what other researchers have observed (Bujko et al., 2006; Chan et al., 2009; Valizadeh et al., 2007). Based on our results Brozek´s formula would be the most appropriate equation for

obese children and adolescents as it takes into account key body distribution skinfolds (triceps, subscapular and abdominal).

3.4.1 Strengths and limitations

Some of the strengths of this study are the multiple measures of body composition using simple and inexpensive methods typically used in the clinical setting and the biochemical fasted markers performed. These allowed us to infer potential interrelationships between the different subcutaneous adipose tissue measurement and important laboratory biomarkers that reflect disease risk for NAFLD. Although skinfold measures require trained personnel, an additional strength of this study was that a one highly trained individual took all measurements, thus limiting the variability in measures of body composition. Our study findings uniquely point to the fact that the use of these skinfold measures in clinical practice could be used for screening purposes to assess potential disease risk for NAFLD in obese children and adolescents.

Potential limitations related to our study included the relatively small sample size in our overall cohort and the inability to determine if pubertal development and gender differences between groups influenced study findings. A post hoc power analysis indicates that sample size was not a primary factor in determining the interrelationships between body composition (using skinfold measures and ADP) between primary outcome measures (insulin resistance and biomarkers of liver disease and HDL); as all had a power in excess of 0.8. Although the majority of obese children and adolescents (both NAFLD and non-

NAFLD) were male, both genders were equally represented in the lean group, which may have limited the ability to compare differences due to gender between the three groups. In addition, although the average age of the study participants was not different between groups, the differences in gender may have resulted in varying influences of pubertal development on study outcomes, particularly in relation to insulin sensitivity. To examine this potential we adjusted the data for these potential gender differences and found significant interactions between gender and values of HOMA-IR and plasma insulin levels across all groups; with males having the highest HOMA-IR and plasma insulin levels. However, we did not find any impact of gender influences relating to the interrelationships between the markers of subcutaneous and visceral adiposity and markers of liver dysfunction and/or altered lipid or inflammation. All suggesting that while gender differences may have influenced IR, it did not appear to largely predict liver disease in the obese children and adolescents with and without NAFLD. Future studies including radiological assessment of bone maturity and Tanner staging by trained medical personnel would confer additional strength to the study design.

3.4.2 Conclusions and clinical implications

In conclusion, our results suggest that especially in obese subjects (but not exclusively) the measurement of BMI, waist circumference, body fat percentage, subcutaneous skinfolds (e.g. abdominal, subscapular) and the somatotype calculation may be used as an extra clinical tool (in addition to lab work) to screen for disease risk for NAFLD in the clinical setting. In our study we have concluded

that the most appropriate equation for obese children and adolescents is Brozek´s formula. A complete anthropometric assessment may help to determine a) who is at higher risk to develop metabolic disturbances (e.g. higher liver enzymes, hyperinsulinemia, hypertriglyceridemia and hypercholesterolemia and inflammation) and therefore allow prompt interventions and b) once the metabolic disturbances are already present an anthropometric analysis may be useful to monitor the clinical response to lifestyle interventions from a body composition and metabolic deregulation perspective. Last but not least skinfold measurements when performed by trained individuals are an accurate, inexpensive and available technique when compared to more sophisticated and complex techniques (e.g. ADP, MRI, UWW) so its use should be encouraged in the clinical setting.

CHAPTER 4. INFLUENCE OF ACUTE CHANGES IN DIETARY INTAKE IN POSTPRANDIAL LIPID AND LIPOPROTEIN METABOLISM IN CHILDREN AND ADOLESCENTS WITH NONALCOHOLIC FATTY LIVER DISEASE (NAFLD)

4.1 INTRODUCTION

Postprandial lipemia is a common feature of the metabolic syndrome (MetS) and it is currently considered a risk factor for nonalcoholic fatty liver disease (NAFLD) in adults (Umpaichitra et al., 2004). Development and progression of cardiovascular disease (CVD) has been observed in adults with delayed clearance of triacylglycerol-rich lipoproteins after consumption of a high-fat meal (acute meal intake) compared with control subjects (Couch et al., 2000; Karpe & Hamsten, 1995; Karpe et al., 1994; Patsch et al., 1992; Simpson et al., 1990; Umpaichitra et al., 2004). Delayed fat clearance is also observed in patients with diabetes mellitus (DM), particularly in the presence of obesity (Couch et al., 2000; Umpaichitra et al., 2004). Fat clearance might be influenced in adults with NAFLD, but very little is known about whether this is the case for children and adolescents. Adult and childhood obesity has become a health problem worldwide (Flores-Calderón et al., 2005; Papandreau et al., 2007). The increase of the obesity rates has caused a higher incidence in the MetS not just in adults, but also in children and adolescents. (Adam & Angulo, 2006; Flores-Calderón et al., 2005; Papandreau et al., 2007; Schwimmer et al., 2003; Utzschneider et al., 2006).

Diet composition could potentially contribute to metabolic impairments associated with obesity, type 2 diabetes and NAFLD (e.g. increase or decrease of

insulin sensitivity, lipogenesis, and oxidation) (Araya et al., 2004; Chitturi et al., 2002; Cortes Pinto et al., 2006; Wei et al., 2007). Lack of physical activity as well as chronic intake of hyper-caloric diets, rich in simple sugar, fructose (e.g. sugar sweetened beverages, artificial juice, pop, etc), high in saturated fat (e.g. deep fried foods such as potatoes, pizza, etc) and low in fiber (e.g. baked goods) and polyunsaturated fatty acids (PUFA) predispose an individual to obesity, high body fat and increased waist circumference (Adams & Angulo, 2006; Fraser et al., 2007; Pereira et al., 2002; Schwimmer et al., 2005; 2003). These variables have been associated with an increased risk for IR, hyperinsulinemia, hyperlipemia and altered lipoprotein metabolism in obesity, which suggest, that these factors could promote NAFLD development or contribute to increasing severity of disease (Chitturi, 2002; Tomkin & Owens, 2001).

Dietary effects on liver metabolism may be related to chronic intakes and potentially to meal composition. According to Westerbacka et al. the amount of fat in the liver of IR overweight non-diabetic adults was associated with the amount of dietary fat intake consumed over a 2 week period of time (Westerbacka et al., 2005). This group reported that a decrease in the amount of saturated fat and an increase in the LCPUFA content, over a 2 week period were followed by diminished fat in the liver and improved insulin sensitivity (Westerbacka et al., 2005). In a study performed by Musso et al. a 7 day food records of NAFLD and non-NAFLD subjects (all adults), revealed an association between high saturated fat intake and increased fat in the liver (Musso et al., 2005; 2003). The same associations were observed by Solga et al. with regards to the saturated fat and

simple sugar intake (Solga et al., 2004). Additionally, it has been observed that acute intakes of meals high in fat (>50g), are followed by prolonged postprandial hyperlipemia and a higher postprandial state of oxidative stress (higher TNF-α, IL-6) in obese adults (Berry et al., 2008; Hyson et al., 2003).

Dietary patterns have changed all around the world. A worldwide increase in consumption of hyper-caloric foods, high in fat (especially saturated fat) and high in simple sugar foods has been observed (Marchesini et al., 2008; Pachuki, 2011). It is possible that chronic changes in dietary intake may influence the postprandial metabolic environment and contribute to an increased risk for postprandial lipemia, hyperinsulinemia and insulin resistance, particularly in overweight and obese individuals (Westerbacka et al., 2005). Delayed postprandial insulin, triglyceride (TG) and non-esterified fatty acid (NEFA) responses were observed in obese adult subjects when compared to lean adults after a fat challenge (60% fat) (van Hees et al., 2008). Additionally, altered postprandial TG fatty acid profiles, IR and lower clearance of fatty acids have been observed in obese pre-pubertal children after consuming a standardized breakfast (32% lipids and 59% carbohydrates) (Gil-Campos et al., 2008; Larqué et al., 2006). Very little is known about the postprandial response to dietary intake in children and adolescents with NAFLD (Muso et al., 2005) and whether or not meal composition may contribute to changes in the metabolic environment that adversely influence the liver.

Currently, there are no evidence-based guidelines for the treatment and prevention of NAFLD in childhood. It is important to understand the underlying

contribution of dietary intake (particularly foods with high fat/high saturated fat) to disease mechanisms that lead to NAFLD pathology and how modulation of dietary intake (acute and chronic) may contribute to or protect from disease pathology in childhood NAFLD to aid in development of interventions to prevent and treat NAFLD.

The purpose of this study was to determine how high intakes of saturated fat concurrent with low intakes of LCPUFA within a meal may contribute to postprandial insulin, lipid and lipoprotein expression as well as to markers of inflammation in children and adolescents with NAFLD. We hypothesized that a meal characterized by a high saturated/LCPUFA free (0% LCPUFA) intake would evoke a postprandial metabolic environment of prolonged lipemia, hyperinsulinemia, increased inflammation, and altered lipoprotein expression in overweight and obese children and adolescents with NAFLD when compared to age and gender-matched obese and lean healthy controls.

4.2 SUBJECTS and METHODS

Subjects recruited for this study were recruited from a larger study examining the impact of chronic dietary intake and hepatic fat metabolism in children and adolescents with NAFLD. While many subjects from the larger study consented to participate in this study, additional subjects (NAFLD and lean healthy controls) were recruited for this study (Chapter 4, see flow chart in chapter 2. Figure 2.1 page 50).

4.2.1 Subject recruitment

We prospectively studied 31 obese and lean children and adolescents (n=11 NAFLD; Non-alcoholic steatohepatitis [NASH] =3, Simple steatosis [SS] =8; n=9 age-and-BMI matched obese controls; n=11 lean controls). Overweight and obese children and adolescents were recruited from the University of Alberta Hospital Liver Clinics (NAFLD) and Pediatric Centre for Weight and Health (PCWH) (obese/overweight controls) at the Stollery Children's Hospital, Edmonton, Alberta. Lean controls were recruited from local advertisements. Some, but not all, of these subjects also participated in the second postprandial study (Chapter 5).

4.2.2 Inclusion and exclusion criteria

From the total of children and adolescents diagnosed with NAFLD, 7 had biopsy-proven disease in addition to ultrasonic evidence of fat in the liver and serum blood testing that included ALT, AST, GGT, CRP, hepatitis B & C, immunoglobulins, testing of autoimmune hepatitis, serum copper and

ceruloplasmin for testing of Wilson Disease. Criteria for liver biopsy included subjects >10 years old in addition to any one of the following variables: hepatosplenomegaly, one and a half times normal aspartate aminotransferase (AST) and alanine aminotransferase (ALT), severe IR (based on HOMA-IR, abnormal when >3), detectable non-specific auto antibodies, inconclusive results from biochemical tests related to Wilson disease, viral hepatitis or deficiency or α_1-antitrypsin deficiency (Mager & Roberts, 2008). Four children diagnosed with NAFLD had only ultrasonography and serum blood testing performed as they did not meet the criteria for liver biopsy (Mager & Roberts, 2008).

Sensitivity of the radiological imaging (ultrasonography, computed tomography and MRI) ranges from 93% to 100% (Saadeh et al., 2002); these methods do not distinguish simple steatosis from steatohepatitis (Saadeh et al., 2002). To minimize the potential for inclusion of children and adolescents with other conditions known to cause fatty liver, liver patients, once eligible, underwent full metabolic and serological screening to rule out other causes of liver steatosis (exclusion criteria) prior to study entry. This included serological testing for chronic hepatitis B & C, serum immunoglobulins, anti-nuclear, anti-smooth muscle and anti-liver/kidney microsomal antibodies for testing of autoimmune hepatitis and serum copper and ceruloplasmin for testing of Wilson Disease. This is part of standard care within this patient population.

Children and adolescents who had BMI classified as obese or overweight (obese controls) underwent screening blood work and/or abdominal ultrasound (US) and a review of medical history to rule out NAFLD, diabetes and other

related syndromes such as polycystic ovary syndrome (4 subjects underwent screening blood work, medical history and abdominal US; 5 subjects underwent screening blood work and medical history only). A subsequent statistical analysis of the biochemical data in the obese children and adolescents (with and without US) was performed to determine if there were any metabolic differences between these subjects (**Appendix 2. Figure N-O**).

Subjects who had BMI classified in the healthy range (lean controls) underwent screening blood work and a review of medical history to rule out any potential for NAFLD. Family history for NAFLD and diabetes was also reviewed. Written informed consent/assent was obtained from the responsible caregiver and the patient prior to study entry. The study was approved by the University of Alberta Health Research Ethics Board. (**Appendix 1. Forms A-I**). Operational Approval from Alberta Health Services (AHS) and Administrative Approval from the Northern Alberta Clinical Trials Centre, University of Alberta/AHS/Caritas was obtained prior to subject recruitment.

4.2.3 Anthropometric and Body Composition Assessment

4.2.4 Subjects

Subjects were asked to fast for a minimum of 10 hours overnight prior to each study day. Weight was measured using air displacement plethysmography (ADP; Bod Pod, COSMED Chicago, IL, USA, Inc.) calibrated scale. ADP has been validated for body composition measures in children and adolescents (Lockner et al., 2000; McCrory et al., 1995). The child's total body fat percentage was estimated from ADP using normative equations (Siri, 1961). Subjects were

encouraged to void before the anthropometrical measurements. Subjects were weighed in the fasting state without shoes and wearing the minimum clothing possible. Standing height was measured without shoes to the nearest 0.5 cm with the use of a commercial stadiometer (Charder HM200PW, Medical Supplies, North Blend, WA, USA). BMI was calculated as weight in kilograms to height-squared in square meter ratio (kg/m^2). Subjects were classified as 'normal weight', 'overweight' and 'obese', according to the IOTF age and sex-specific BMI cut-off points (overweight: $\geq 85^{th}$ percentile, obese: $\geq 95^{th}$ percentile) (Cole et al., 2000). Waist circumference was measured at the midpoint between the lower border of the rib cage and the iliac crest (Rudolf et al., 2007)

4.2.5 Habitual Food Intake

Three day food records were used to determine subjects' habitual intake and to assess dietary fat intake prior to the study days (Food Processor SQL, version 10.4.0, ESHA Research, Salem, OR, 2008). Canadian Nutrient File (CNF) was the database used for macronutrient composition. For fructose analysis, only when not available in the CNF the USDA (United States Department of Agriculture) nutrient database was used and when necessary commercial food label information from the individual brand types was used. Glycemic Index (GI) values were obtained from Foster-Powell et al. (2002). Glycemic Load (GL) was calculated using the mixed meal approach (Collier et al., 1988; Ebbeling et al., 2004; Foster-Powell et al., 2002).

4.2.6 Composition of Test Meal

A standardized meal reflective of a typical fast food breakfast consumed by children and adolescents in North America (Pachuki, 2011) was given to all participants after taking the first blood sample (0 hours or study baseline; see fasting and postprandial blood work section). The meal consisted of chocolate milk (120ml), butter (15g), mozzarella-cheddar cheese (40g) and white bread (50g); foods commonly consumed in fast food breakfast meals served at a variety of commercial outlets in North America (Pachuki, 2011). Participants were given 10-15 minutes to finish their meal. The macronutrient distribution of the standardized meal was a **high SFA/LCPUFA free (0% LCPUFA)** reflective of a typical fast food breakfast meal consumed by children in North America (Pachuki, 2011). Fructose content was less that 1% of the total energy intake (kcal). This amount has shown to have no adverse effects in humans and limited or no effects on de novo lipogenesis (Elliott et al., 2002). GL and GI were at the lower end in order to avoid excessive postprandial glucose raising (Foster-Powell et al., 2002). Meal total energy content was 421.7 kilocalories, macronutrient distribution was as follows protein: 17.35%, carbohydrates: 39.04% (Fructose content: 568mg, GL: 20), fat: 43.34% and cholesterol (52.2mg). Percentages are based on the total amount of kilocalories present in the meal and were analyzed using the Food Processor SQL (version 10.4.0, ESHA Research, Salem, OR, 2008). CNF, the USDA nutrient database and commercial food label information from the individual brand types were used to calculate the fructose content of the meal challenge. Mixed meal approach was used to calculate the GL (Collier et al., 1998; Ebbeling et al., 2004). Since dietary TG represent above the 90% of the

total fat intake (Cohn et al., 2010) only the fatty acid composition of total TG was analyzed. Fatty acid (FA) composition of total TG of the meal was assessed using gas liquid chromatography (GC) (**Table 4.1**). The lipid components were extracted using the chloroform-methanol process (Folch et al., 1957). Prior to saponification and methylation an internal standard was added (5mg of C15:0) to all samples. Samples were saponified (using KOH in methanol) and methylated using Boron trifluoride (BF3) in methanol and hexane (Murphy et al., 2011).

Table 4.1. Fatty acid composition of triglycerides* in the test meal

Fatty Acid	Percentage (%)
Saturated	
C10:0	0.56
C12:0	2.63
C13:0	0.56
C14:0	8.19
C16:0	25.22
C18:0	6.24
Monounsaturated	
C14:1	0.79
C16:1	1.30
C18:1	35.51
Polyunsaturated	
C18:2n6	15.23
C18:3n3	3.76
Long chain polyunsaturated	
C20:2n6	ND
C20:3n6	ND
C20:4n6	ND
C20:5n3	ND
C22:6n3	ND
SFA/MUFA from total kcal	1.14
SFA/PUFA from total kcal	2.28
n-3/n-6 g of fat as per GC	0.23

Since dietary TG represent above the 90% of the total fat intake (Cohn et al., 2010) only the fatty acid composition of total TG was analyzed.

4.2.7 Baseline and Postprandial Blood work

A catheter was placed in an antecubital vein by a registered nurse for collection of blood samples after a 10 hour, overnight fast prior to meal consumption (baseline) and at 1, 3 and 6 hours post meal consumption. Two tubes [one lavender top (K3 EDTA) and one serum separator tube (SST)] were collected at each time point. Blood was centrifuged (Jouan® model CR422) at 1500 rpm for 15 minutes. Plasma was separated and stored at -80°C until time of assay. When samples were analyzed in different batches an internal plasma control was used for all the assays in order to determine that intra-assay and inter-assay variability. All results from this assay were within acceptable ranges according to the manufactures' recommendations.

4.2.8 Fasting Blood work: Liver Biochemistries, C- reactive protein and Leptin

Fasting blood (baseline or prior to meal consumption) was collected for measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and C-reactive protein (CRP). These were analyzed by Laboratory Services, Alberta Health Services (AHS) using standardized methodologies. Fasting plasma leptin was analyzed using a commercially available Enzyme-Linked Immuno-Sorbent Assay (ELISA) kit (Millipore-Biomanufacturing and Life Science Research Products, Missouri, USA). For leptin, the kit has a minimum detectable dose (MDD) of 0.5ng/mL in a 25µl sample size and a range of 0.5 to 100ng/mL.

4.2.9 Postprandial Blood work: Insulin and Glucose, Lipids, Cholesterol and NEFA, Apolipoproteins B-48, B-100 and C-III.

Blood samples were collected at baseline, 1, 3 and 6 hours post meal consumption for assessment of insulin, and glucose, triglycerides (TG), HDL, LDL, total cholesterol, NEFA, Apo B-48, Apo B-100, Apo C-III. Glucose and insulin were analyzed in Laboratory Services, AHS using standardized methods (Synchron LX® Systems analyzer, Beckman, Coulter, Fullerton, CA for glucose and Roche Diagnostics Elecsys 2010 System for insulin. Coefficient of variation < 5%) IR was assessed using the homeostasis model of assessment of IR (HOMA-IR). HOMA-IR is a model that relates fasting levels of plasma insulin and glucose and has been validated for use in children and adolescents. A value of > 3 was used to classify subjects as insulin resistant (Duncan et al., 2001; Gungor et al., 2004; Hrebicek et al., 2002; Keskin et al., 2005; Matthews et al., 1985).

Plasma concentrations of NEFA were analyzed using commercially available ELISA kits (WAKO Pure Chemical Industries, Ltd, Richmond, USA). For NEFA, the minimum detectable level was estimated to be 0.0014mEq/L in a 10µl sample size and a range of 0.01 to 4mEq/L. Samples were diluted 1 in 2.5 for obese patients and 1 in 2 for lean patients. Plasma concentrations of apolipoproteins were analyzed using commercially available ELISA kits (Apo B-48 (Shibayagi Co. Ltd; Gunma, Japan), Apo B-100 (Kamiya Biomedical Company, Seattle, USA), Apo C-III (Abnova, California, USA). For Apo B-48, the kit has a minimum detectable dose (MDD) of 2.5 ng/ml in a 10µl sample size and a range of 2.5 to 160 ng/ml; samples were diluted 1/200. For Apo B-100, the kit has a MDD of <6.1 ng/mL in a 100µl diluted sample size and a range of 13.7 to 10 000 ng/mL, samples were diluted 1/4000 for obese and 1/1000 for lean

subjects. Apo C-III has a MDD of 0.002 µg/ml in a 50 µl diluted sample size and a range of 0.002 to 2µg/ml.

4.2.10 Plasma Fatty Acid profile of Triglyceride (TG) and Phospholipid (PL) fractions

Blood samples were taken at baseline, 1, 3 and 6 hours post meal consumption for analysis of the plasma fatty acid composition of individual lipid components (triglycerides, phospholipids). Plasma fatty acid composition was determined quantitatively in order to identify changes in marker pools of fatty acids. The lipid component of plasma was extracted using chloroform-methanol and 0.8mL of 0.025% $CaCl_2$ (Folch et al., 1957). Samples were left overnight to separate at 4°C. The bottom layer was extracted and washed with 1mL chloroform/methanol/water (86:14:1 by volume) solution. Samples were dried under nitrogen gas. 120 µL chloroform / methanol (2:1 by volume) was added to each tube. The extracted lipid samples were spotted in duplicate onto thin layer chromatography "G" plates. Plates were put into a tank for about 30 to 40 min with petroleum ether (PE)/diethyl/ethyl ether (DE)/acetic acid (glacial; HAC) 80:20:1 by volume). Plates were sprayed with 0.1% aniline naphthalene sulfonic acid (ANSA). The following fatty acid classes were identified under UV light: PL, cholesterol, TG and cholesterol ester. An internal standard was added; C17:0 for PL and C15:0 for TG. TG were saponified (KOH in methanol) and methylated using boron trifluoride (BF3) in methanol and hexane. PL were methylated using BF3 and hexane. Fatty acids were quantified using gas liquid chromatography (Murphy et al., 2011). The differences between the TG analysis by GC and TG

analysis performed by laboratory services using standardized methods, include that the former includes TG rich lipoproteins (e.g. chylomicron and VLDL) while the latter does not.

4.2.11 Inflammatory mediators and adiponectin

Blood samples were taken 0, 1, 3 and 6 hours post meal consumption for adiponectin (Millipore-Biomanufacturing and Life Science Research Products, Missouri, USA), tumor of necrosis factor alpha (TNF-α; R&D Systems, Minneapolis, USA), interleukin-6 (IL-6; R&D systems, Minneapolis, USA) and interleukin-10 (IL-10; Invitrogen by Life technologies, California, USA). For adiponectin, the kit has a MDD of 0.78ng/mL in a 20µl sample size and a range of 1.56 to 100ng/mL. For TNF-α, the kit has a MDD of 0.038pg/mL in a 200µl sample size and a range of 0.038 to 0.0191pg/mL. For IL-6, the kit has a MDD of 0.016pg/mL in a 100µl sample size and a range of 0.016 to 0.110pg/mL. For IL-10, the kit has a MDD of <0.2pg/mL in a 50 µl sample size and a range of 0 to 35 pg/mL.

4.2.12 Statistical analysis

Data are expressed as mean $\pm$ SEM unless otherwise specified. The area under the curve (AUC) and incremental area under the curve (iAUC) of different metabolites during the meal test were calculated by the trapezoidal method (Graph Pad PRISM Software. version 5.0 La Joya California USA). Differences between groups for iAUC and AUC were analyzed by repeated measures one way analysis of variance (ANOVA) followed by Bonferroni correction pairwise comparison for normally distributed variables. Tests for deviations from a Gaussian distribution

were performed using the D'Agostino-Pearson omnibus and Shapiro-Wilk normality tests (Graph Pad PRISM Software. version 5.0 La Joya California USA). Kruskal Wallis test was utilized for variables with skewed distributions. Repeated measures two-way ANOVA was used to test the interaction between time and group. When a significant interaction was found between factors, analyses were followed by Bonferroni's correction and pairwise comparisons (Graph Pad PRISM Software. version 5.0 La Joya California USA). Multiple regression was performed to examine the relationship between several independent (predictor) variables and a dependent variable (IBM SPSS statistics. version 19.0 Chicago IL USA). Pearson correlation and linear regression were performed to determine the strength and direction of linear relationships between variables in the different groups (Graph Pad InStat 3 and Graph Pad PRISM Software. version 5.0 La Joya California USA). Differences were considered statistically significant if $p < 0.05$.

4.3 RESULTS

4.3.1 Anthropometric and Demographic Variables

Anthropometric and metabolic characteristics of the subjects are presented in **Table 4.2**. There were no significant differences in age between the three groups. Both NAFLD and obese controls were obese (BMI-z: 2.3 ± 0.1; range 3-1.2) with waist circumference > the 97th percentile for their respective age (Cole et al., 2000). Body fat percentage, z-scores, waist circumference, waist to hip and height ratio between NAFLD and obese subjects were not significantly different (**Table 4.2**). All the subjects except one in the NAFLD group had a percentile for

weight > the 95th percentile, all the subjects from the obese control group had a percentile > the 95th. A complete description of the body composition analysis was presented in the Chapter 3 entitled "Somatotype and body composition in children and adolescents with NAFLD".

Table 4.2. Anthropometric characteristics of the patients

Anthropometric data	Lean Control (n=11) 5F, 6M	Obese (n=9) 1F, 8M	NAFLD (n=11) 1F, 10M	p value
Age (y)	13.0±0.8	14.0±0.4	12.5±0.9	0.4
BMI z-scores	0.1±0.3^{a}	2.2±0.1^{b}	2.3±0.1^{b}	<0.0001
Weight z-score	-0.05±0.3^{a}	2.4±0.2^{b}	2.5±0.2^{b}	<0.0001
Height z-score	-0.06±0.3	0.3±0.2	0.8±0.4	0.2
Waist circumference(cm)	63.8±2.6^{a}	101.8±6.0^{b}	98.2±5.0^{b}	<0.0001
waist/hip ratio (cm)	0.7±0.0^{a}	0.8±0.0^{b}	0.9±0.0^{b}	0.003
Waist to Height Ratio (WHR)	0.4±0.0^{a}	0.6±0.0^{b}	0.6±0.0^{b}	<0.0001
Body fat (%); Bod Pod	15.3±1.7^{a}	39.8±3.5^{b}	33.9±3.8^{b}	<0.0001
Fat mass, kg (FM)	7.5±1.3^{a}	41.7±8.0^{b}	28.5±1.5^{b}	0.0005

Values are mean ± SEM. Different superscripts indicate significant differences between groups by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

4.3.2 Fasting Laboratory Parameters

At baseline, children and adolescents with NAFLD had significant elevations in ALT and AST compared to obese and lean controls ($p<0.0001$, $p<0.002$ respectively). CRP was significantly higher in NAFLD ($p=0.05$) when compared to lean controls. Fasting insulin was significantly higher ($p=0.005$) in the NAFLD subjects when compared to the lean group; but not when compared to obese controls ($p=0.5$). No significant differences were observed between groups

for fasting concentrations of glucose, TG, LDL and total cholesterol. HDL-cholesterol was significantly lower in all obese subjects (NAFLD and non-NAFLD) when compared to healthy controls ($p<0.0001$). Leptin was significantly higher in the obese control group when compared to lean subjects ($p=0.004$) (**Table 4.3**).

Table 4.3. Fasted metabolic characteristics of the patients

	Lean Control (n=11) 5F, 6M	**Obese (n=9) 1F, 8M**	**NAFLD (n=11) 1F, 10M**	**p value**
ALT (U/L)[1]	17.0±1.5[a] (12-28)	22.0±2.3[a] (15-35)	87.0 ±16.1[b] (17-175)	<0.0001
AST(U/L)[2]	24.0±1.9[a] (17-35)	23.0±1.1[a] (19-29)	52.0±9.9[b] (20-132)	0.002
CRP (mg/L)[3]	0.6±0.2[a] (0.2-3.5)	2.4±0.8[ab] (0.2-8.1)	4.1±1.4[b] (0.5-15)	0.049
GGT (U/L)[4]	7.0±4.9 (5.0-21.0)	17.3±6.4 (6.0-67.0)	47.1±21.8 (10.0-256.0)	0.1
Glucose (mmol/L)[5]	4.6±0.1 (3.8-5.1)	4.9±0.1 (4-5.5)	5.1±0.2 (4.3-6.5)	0.12
Insulin (mU/L)[6]	8.6±1.4[a] (4.1-20.6)	26.0±6.7[ab] (8.5-76.6)	30.8±5.2[b] (11.8-56.1)	0.005
Triglyceride (mmol/L)[7]	0.7±0.0 (0.3-1.2)	1.3±0.1 (0.6-2.2)	1.3±0.2 (0.5-3.3)	0.05
Total cholesterol (mmol/L)[8]	3.9±0.2 (3.1-4.9)	4.2±0.2 (3.1-5.0)	4.2±0.4 (2.7-6.8)	0.75
HDL cholesterol (mmol/L)[9]	1. 4±0.0[a] (0.9-2.0)	0.8±0.0[b] (0.6-1.05)	0.8±0.0[b] (0.5-1.1)	< 0.0001
LDL cholesterol (mmol/L)[10]	2.2±0.1 (1.2-3.0)	2.8±0.1 (1.9-3.3)	2.7±0.3 (1.5-4.5)	0.18

Values are mean ± SEM (except otherwise mentioned). Different superscripts indicate significant differences between groups by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM. Ranges (minimum-maximum) are expressed within the parentheses

Table 4.3 continues…

	Lean Control (n=11) 5F, 6M	Obese (n=9) 1F, 8M	NAFLD (n=11) 1F, 10M	p value
Leptin (ng/mL)[11]	0.7±0.1[a] (0.02-1.7)	3.2±0.9[b] (0.8-8.5)	2.2±0.3[ab] (0.6-4.3)	0.006
HOMA-IR[12]	1.8±0.3[a] (0.8-4.3)	5.6±1.4[ab] (2.0-17.0)	7.3±1.4[b] (2.7-15.7)	0.007
# subjects (TG >1.5mmol/L)	0	5	3	
# subjects (Insulin >20mU/L)	1	5	6	
# subjects (HDL >1mmol/L)	9	1	2	

Values are mean ± SEM (except otherwise mentioned). Different superscripts indicate significant differences between groups by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM. Ranges (minimum-maximum) are expressed within the parentheses

Normal values:
[1] ALT <50(U/L), [2] AST<40 (U/L), [3] CRP <8.0 (mg/L), [4] GGT <70 (U/L), [5] Glucose: 3.3-11.0 (mmol/L), [6] Insulin 5.0-20.0 (mU/L), [7] Triglycerides<1.50 (mmol/L), [8] Total cholesterol <4.40 (mmol/L), [9] HDL >1.00 (mmol/L), [10] LDL <2.80 (mmol/L),), [11] Leptin: lean subjects with a body fat % of 16.2%: 2.74 mg/L in girls, 1.59 mg/L in boys (Blum et al., 1997), [12] HOMA-IR<3.

4.3.3 Postprandial responses for laboratory variables

Data are presented in time course series for postprandial meal response (at times 0, 1, 3 & 6 hrs). Incremental area under the curve (iAUC) for most of the study variables (unless a significant difference was observed) are presented in **Appendix 2.** Areas under the curve (AUC) are presented in **Appendix 2.**

4.3.4 Postprandial insulin and glucose responses

Absolute and incremental postprandial changes in plasma insulin concentrations are shown in **Figure 4.1a and 4.1b.** As expected, insulin levels in the three groups increased post meal, reaching the highest value at 1 hour. NAFLD subjects exhibited significantly higher values at 1 and 3 hrs post meal ($p<0.001$, $p<0.05$ respectively) when compared to lean controls. In addition,

NAFLD subjects were significantly different at 1 hour post meal when compared to obese ($p<0.001$). No other differences in insulin were observed in the other time points. A significant interaction between group and time was observed ($p=0.003$). iAUC ($p=0.007$) and AUC for insulin were significantly higher ($p=0.001$) in children and adolescents with NAFLD when compared to lean controls, but did not differ from obese controls (**Figure 4.1b, Appendix 2. Figure A**). Postprandial changes exclusively for NAFLD subjects comparing NASH (n=3) vs simple steatosis (n=8) are shown in **figure 4.1c**. Glucose values were significantly higher in the NAFLD population at one hour post meal (5.4 $\pm$ 0.2mmol/L) when compared to the lean controls ($p<0.001$) (**Figure 4.2**). Significant interaction between group and time was observed ($p=0.01$). No significant differences in iAUC and AUC for glucose ($p>0.05$) were observed between groups **(Appendix 2. Figure A)**. All the patients were within the expected normal ranges (3.3-11 mmol/L range for random glucose values and 3.3-6.1 mmol/L for fasted) at each time point (**Figure 4.2).**

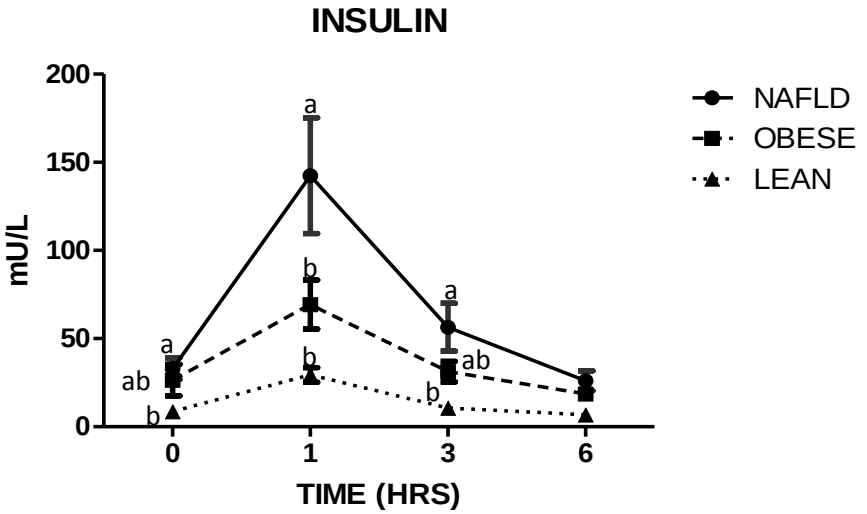


Figure 4.1a. Insulin concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender- and aged matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

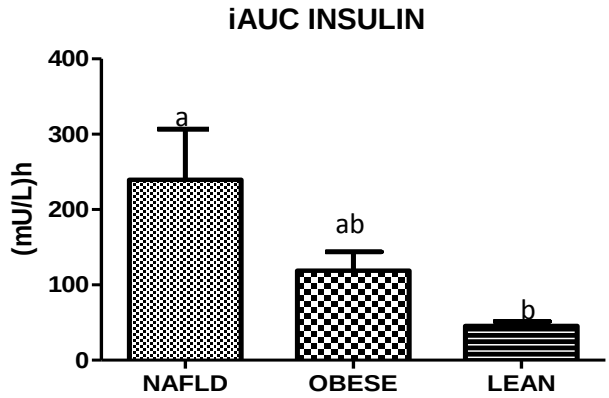


Figure 4.1b. Postprandial incremental area under the curve (iAUC) for insulin in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and lean healthy controls (n=11). Variables with different superscripts are significantly different by repeated measures one way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

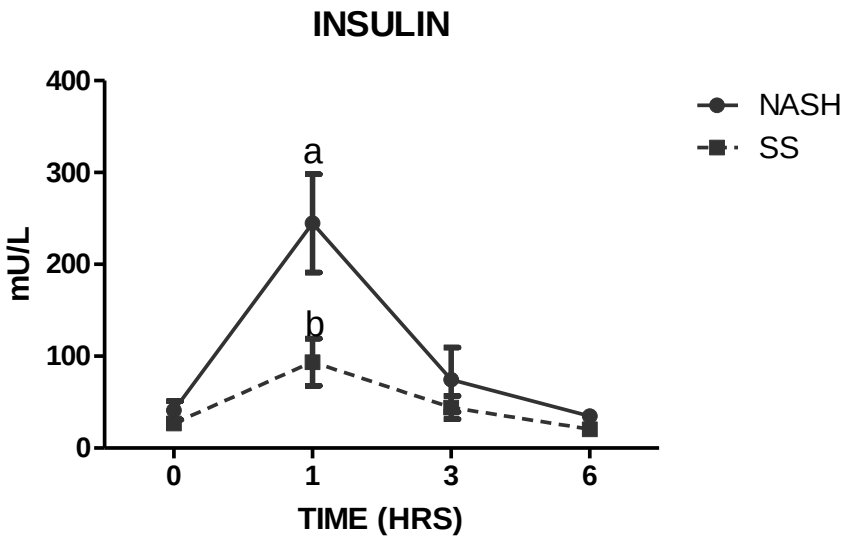


Figure 4.1c. Insulin concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with NASH (n=3) and simple steatosis (n=8). Variables with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

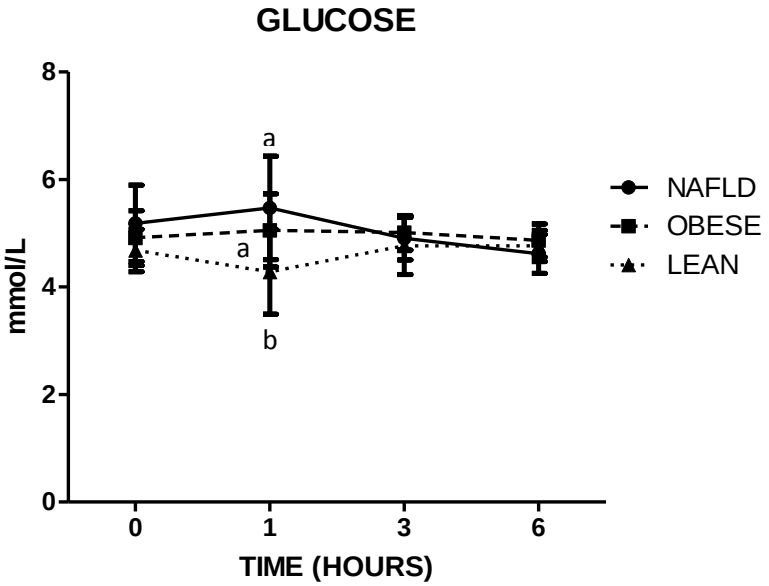


Figure 4.2. Glucose concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender- and aged matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

4.3.5 Postprandial TG, NEFA, LDL, HDL and Cholesterol Profile

4.3.5.1 *Triglycerides (TG)*

Peak plasma values for TG were observed at 3 hours post meal in all the groups. At this time point 5 NAFLD, 7 obese controls and 3 lean subjects exhibited blood levels higher than 1.5 mmol/L (cut-off for normal values) (**Figure 4.3)**. At 6hrs post meal NAFLD subjects exhibited higher (p=0.03) TG values when compared to lean subjects (1.7±0.3 vs. 0.9±0.08 mmol/L). No significant interaction between group and time was observed (p>0.05). No significant differences between groups were observed when calculating iAUC and AUC (p=0.3, 0.08; respectively) (**Appendix 2. Figure B)**.

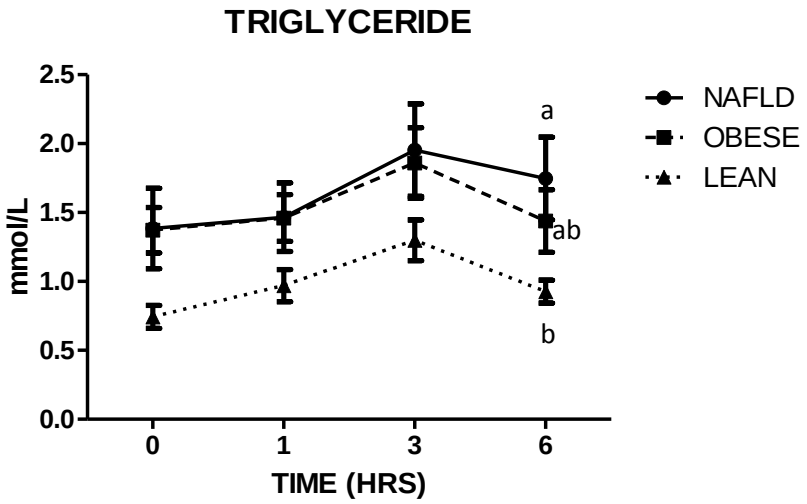


Figure 4.3. Triglyceride concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-age and gender (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA p<0.05 followed by Bonferroni correction and pairwise comparisons**.** Results are shown mean ± SEM.

4.3.5.2 *Non-esterified Fatty Acids (NEFA)*

The pattern of postprandial NEFA differed between groups. Significantly higher NEFA values were observed in NAFLD subjects in the fasted ($p<0.05$) and 1 hour post meal ($p<0.01$) when compared to lean controls (**Figure 4.4**). On the other hand, significantly lower values were exhibited in the NAFLD subjects at 6 hours post meal when compared to obese population ($p=0.02$). Significant interaction between group and time was observed ($p=0.003$). When comparing the iAUC and AUC no significant differences between groups were observed ($p=0.2$) (**Appendix 2. Figure B**).

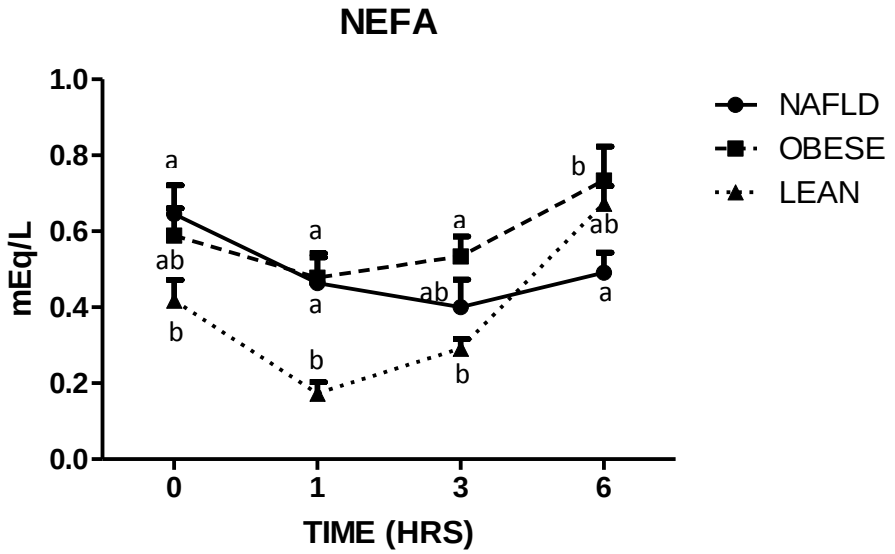


Figure 4.4. Non-esterified fatty acids concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-age and gender (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

4.3.5.3 *LDL, HDL and cholesterol profile*

No significant differences in AUC, iAUC (**Appendix 2. Figure C**) or time effects in LDL-cholesterol and total cholesterol plasma profile was seen between the three groups (p>0.05) **(Figure 4.5)**. HDL levels were significantly lower in both fasted and postprandial states in NAFLD and obese subjects when compared to lean (p<0.001) (**Figure 4.6)**. No meal effects were observed over the 6 hour postprandial study period **(Appendix 2. Figure C**). No significant differences were observed in total cholesterol postprandial values between groups (p>0.05) (**Figure 4.7, Appendix 2. Figure C).** No significant interactions between group and time were observed for LDL, HDL and total cholesterol (p>0.05). AUC and iAUC for LDL, HDL and total cholesterol are shown in **Appendix 2. Figure C.**

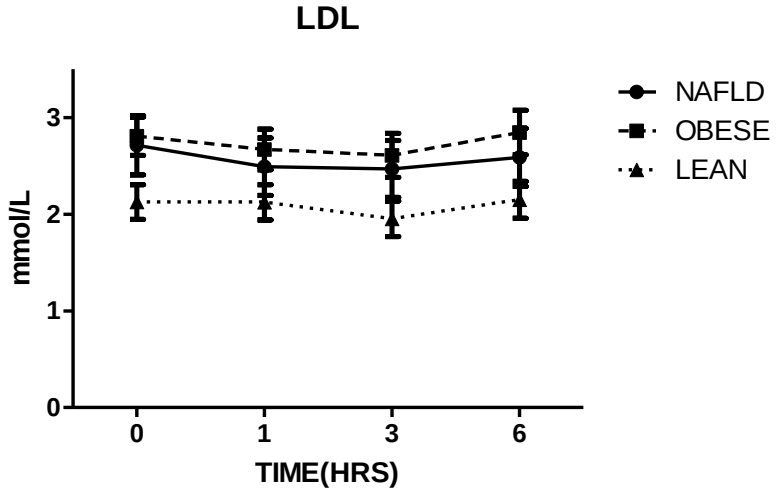


Figure 4.5. LDL concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA p<0.05 followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

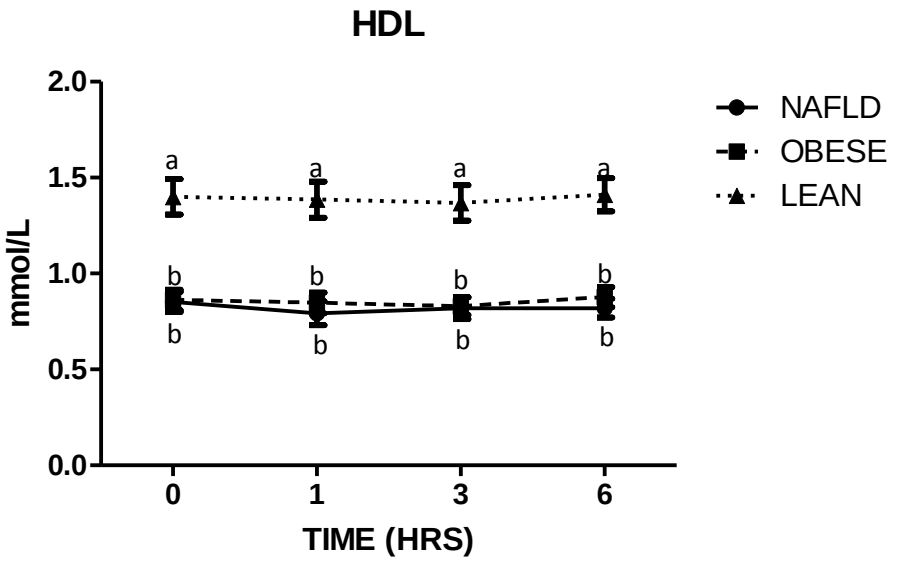


Figure 4.6. HDL concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

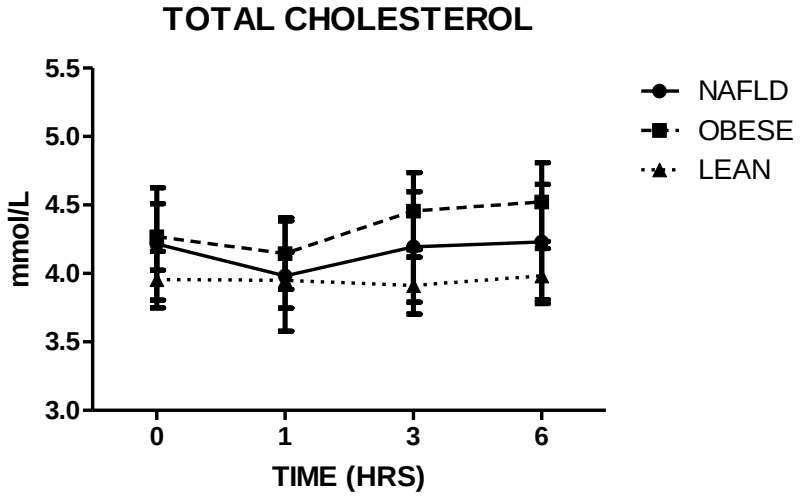


Figure 4.7. Total cholesterol concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

4.3.6 Fatty Acid Profile of Plasma Triglyceride (TG) Fraction

The complete fatty acid (FA) profiles (in percentages) and concentrations (μg/ml) are presented in **Tables 4.4a and 4.4b** .Time course data are presented in **Figure 4.8a** for relevant SFA [myristic (C14:0) and palmitic (C16:0)], **8b** for total (sum) of SFA. Total (sum) of time course data for MUFA is presented in **Figure 4.9** and **Figures 4.10a and 4.10b** for polyunsaturated fatty acids (PUFA). Only linoleic (C18:2n6) fatty acid time course data is presented as it is the only PUFA that showed significant differences ($p<0.05$). AUC data for highly prevalent in nature fatty acids [C14:0, C16:0, palmitoleic (C16:1), C18:2n6 and arachidonic (C20:4n6)] are presented in **Appendix 2. Figure D**. iAUC data for highly prevalent saturated fatty acids [C14:0, C16:0 and stearic (C18:0)] are presented in **Appendix 2. Figure E and F**. **Figure G** in **Appendix 2** represents the iAUC sum for MUFA, SFA, omega-6 (n-6), omega-3 (n-3) and total fatty acids.

Table 4.4a. Postprandial Plasma TG fatty acid composition (percentages) at different time points

Fatty Acid	TG(%)LEAN				TG(%)OBESE				TG (%) NAFLD			
	Fasted	**1hr**	**3hr**	**6hr**	**Fasted**	**1hr**	**3hr**	**6hr**	**Fasted**	**1hr**	**3hr**	**6hr**
14:0	2.8±0.4[a]	2.8±0.3	4.5±0.5[a]	4.4±0.6[a]	1.2±0.3[b]	1.7±0.3	1.7±0.4[b]	1.8±0.4[b]	1.7±0.3[a,b]	1.8±0.3	2.4±0.4[b]	2.1±0.4[b]
16:0	26.2±1.2	26.9±1.1[a]	32.1±0.8	31.7±1.6	29.4±1.1	31.3±1.0[b]	28.7±1.2	28.3±0.9	29.3±1.0	29.2±1.0[ab]	29.5±1.0	29.6±1.3
16:1	3.1±0.4	3.0±0.3	3.5±0.3	3.3±0.3	2.8±0.6	2.9±0.6	2.5±0.6	2.4±0.4	3.8±0.6	3.6±0.5	3.5±0.4	3.7±0.5
18:0	5.0±0.4	5.4±0.3	6.5±0.3	6.4±0.3	4.3±0.5	4.5±0.5	5.2±0.6	4.9±0.5	4.2±0.8	4.8±0.8	5.3±0.6	5.9±0.5
18:1	40.7±1.7	40.9±1.3	37.2±1.2	36.3±1.3	42.3±1.4	40.7±1.5	42.0±1.5	41.2±1.3	38.7±2.1	41.0±1.2	40.4±0.9	41.1±1.1
18:2n6	14.2±0.8	15.2±0.9	11.9±0.7[a]	11.3±0.8[a]	17.7±1.3	16.5±0.8	17.4±1.2[b]	18.9±1.3[b]	17.6±2.2	16.3±1.4	14.6±1.1[ab]	14.6±1.1[ab]
18:3n3	1.3±0.5	1.0±0.3	0.3±0.1	0.4±0.1	0.3±0.2	0.4±0.2	0.4±0.2	0.6±0.3	0.5±0.2	0.7±0.2	0.7±0.3	0.5±0.2
20:4n6	1.3±0.2	0.8±0.2	0.5±0.1	0.8±0.2	0.8±0.1	0.9±0.1	0.8±0.1	0.9±0.1	0.8±0.2	0.9±0.2	0.8±0.2	0.9±0.3
20:5n3	0.0±0.0	0.1±0.1	0.0±0.0	0.0±0.0	0.1±0.1	0.2±0.1	0.1±0.1	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.0±0.0
22:6n3	0.1±0.1	0.2±0.1	0.1±0.1	0.1±0.1	0.0±0.0	0.1±0.1	0.1±0.1	0.1±0.0	0.1±0.1	0.2±0.1	0.1±0.1	0.2±0.1
Σ n-3	1.5±0.5	1.3±0.4	0.6±0.2	0.6±0.2	0.6±0.2	0.7±0.2	0.6±0.3	0.7±0.4	0.8±0.2	1.0±0.3	1.0±0.3	0.8±0.2
Σ n-6	15.9±0.9	16.2±0.9	12.6±0.7[a]	12.2±0.8[a]	18.7±1.4	17.7±0.9	18.3±1.3[b]	20.0±1.4[b]	18.6±2.1	17.3±1.4	15.4±1.1[ab]	15.6±1.2[ab]
ΣMUFA	43.8±1.5	43.9±1.2	40.7±1.1	39.6±1.2[a]	45.1±1.0	43.6±1.5	44.5±1.2	43.7±1.1[a,b]	42.5±2.2	44.7±1.2	43.9±0.9	44.8±0.8[b]
Σ SFA	34.0±1.6	35.1±1.5	43.1±1.0[a]	42.6±2.0[a]	35.0±1.4	37.5±1.2	35.6±1.7[b]	35.0±1.2[b]	35.2±1.2	35.8±1.2	37.3±1.3[b]	37.5±1.5[ab]
Σμg/ml	729.1±178.5	923.8±229.6	746.2±168.5	590.7±104.7	524.4±49.7	524.6±73.8	693.2±98.2	662±131.9	529.5±108.5	627.3±137.2	783.9±152.5	833.0±211.6

Values are mean ± SEM (except otherwise mentioned). Variables with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Total Fatty acids sum is expressed in μg/ml

Table 4.4b. Postprandial Plasma TG fatty acid composition (µg/ml) at different time points

Fatty Acid	TG(µg/ml)LEAN				TG(µg/ml)OBESE				TG(µg/ml) NAFLD			
	Fasted	1hr	3hr	6hr	Fasted	1hr	3hr	6hr	Fasted	1hr	3hr	6hr
14:0	20.2±5.8	20.6±5.4	31.9±7.6[a]	23.1±3.9	7.2±2.0	8.4±2.3	13.9±4.5[b]	13.1±4.6	9.2±3.0	11.3±2.6	18.3±3.6	14.8±3.0
16:0	205.0±58.5	247.8±67.5	240.0±53.2	186.0±30.8	155.9±18.3	160.7±24.3	209.9±33.2	197.1±44.1	163.4±35.6	186.0±40.4	234.7±45.0	227.6±44.1
16:1	25.9±8.0	30.9±10.9	26.7±7.3	21.7±5.6	16.1±3.5	17.8±4.3	19.7±5.2	17.7±5.7	24.1±7.9	26.1±7.8	31.4±7.7	32.2±7.5
18:0	38.0±11.3	50.2±13.6	49.5±11.7	38.9±6.7	27.4±4.8	26.2±7.1	35.9±8.1	35.6±11.3	21.4±4.7	30.8±7.6	42.1±8.5	48.5±13.1
18:1	299.4±69.9	389.6±94.4	287.1±64.9	229.4±42.3	219.1±19.1	219.1±28.9	292.1±44.7	270.3±50.4	208.6±45.0	260.7±59.1	323.5±65.0	361.3±103.1
18:2n6	103.2±21.2	147.5±35.9	96.9±22.4	77.1±16.7	89.6±7.6	83.9±8.5	109.5±11.0	116.9±15.6	89.8±17.8	95.6±18.1	113.9±23.1	126.7±40.2
18:3n3	11.5±4.5	13.3±5.7[a]	2.9±1.5	3.2±1.1	2.5±1.2	3.9±1.4	4.3±1.6	5.2±2.4	2.7±0.9	3.3±1.2[b]	4.5±1.7	3.0±1.3
20:4n6	9.9±2.7	9.5±3.0	5.1±1.8	5.1±2.3	3.6±0.5	3.7±0.6	5.8±1.0	5.0±1.4	4.8±1.1	6.9±3.0	6.1±1.6	11.8±6.7
20:5n3	0.4±0.3	0.3±0.3	0.4±0.3	0.2±0.1	0.2±0.2	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.5±0.4	0.8±0.4	0.0±0.0
22:6n3	1.4±0.6	2.5±1.1	1.0±0.7	0.6±0.4	0.4±0.3	0.2±0.2	0.5±0.3	0.2±0.2	0.9±0.4	2.1±1.3	1.8±1.0	1.4±0.8
Σ n-3	19.5±6.3	23.3±8.5	6.4±2.3	8.0±3.0	4.1±1.8	4.3±1.3	6.0±1.8	6.0±2.4	6.5±1.6	7.9±2.3	11.8±3.4	8.4±3.0
Σ n-6	119.2±24.1	161.1±39.4	104.3±25.0	83.2±18.2	94.2±7.9	87.9±8.8	115.44±11.8	122.1±16.8	95.7±19.0	104.0±21.8	121.5±25.1	139.8±46.9
ΣMUFA	325.3±76.9	420.6±103.0	313.8±71.6	251.2±46.9	235.2±20.8	236.9±31.8	311.8±46.9	288.1±55.2	232.8±52.3	286.9±66.4	355±72.0	393.7±109.8
Σ SFA	263.7±75.6	318.7±85.2	321.5±71.7	248.2±40.0	190.7±23.4	195.3±32.9	259.9±43.4	245.9±59.3	194.4±42.2	228.4±49.2	295.5±55.4	291.0±58.0

Values are mean ± SEM (except otherwise mentioned). Variables with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

4.3.6.1 *Saturated FA in TG Fractions*

NAFLD subjects exhibited lower proportion levels ($p<0.01$) of myristic acid (C14:0) at 3 and 6 hrs post meal when compared to lean subjects (**Figure 4.8a**). A significant increase ($p<0.05$) over time (1hour post meal to 3 hrs. and 6 hrs. post meal) was observed in lean subjects only. No other significant changes in postprandial (C14:0) were observed. Children and adolescents with NAFLD exhibited a trend of higher proportions of palmitic acid (C16:0) values at 1 hour post meal when compared to the lean subjects; the highest levels were observed in the obese subjects. Significant interaction between group and time was observed ($p=0.004$). A significant increase in C16:0 ($p<0.01$) over the postprandial period was observed in the lean subjects (fasted to 3hrs. post meal, fasted to 6 hrs. post meal (**Figure 4.8a**). Stearic acid (C18:0) proportions were not significantly different between groups ($p>0.05$). In addition, although not significant, C14:0, C16:0 and C18:0 acid iAUC were lower ($p=0.09$, $p=0.2$, $p=0.2$ respectively) in the NAFLD group when compared to lean subjects (**Appendix 2. Figure E**). Total SFA proportions were lower at 3 hours post meal in the NAFLD group when compared to lean subjects ($p<0.05$). Significant interaction between group and time was observed ($p=0.005$) (See **Figure 4.8b)**. Although iAUC for the total SFA proportions tended to be lower in the children and adolescents with NAFLD when compared to leans, no significant differences were observed between the three groups ($p=0.2$) (**Appendix 2. Figure G**).

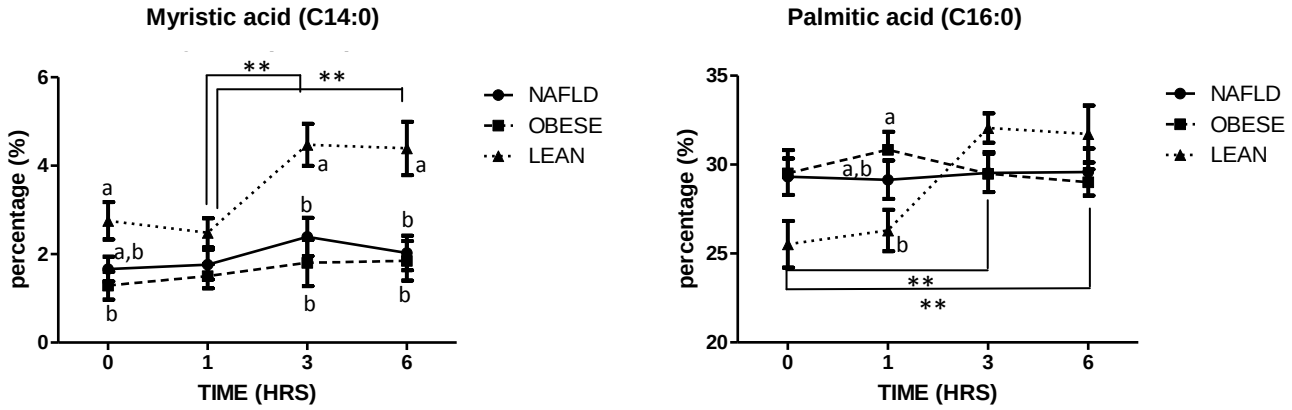


Figure 4.8a. TG fractions for C14:0 and C16:0 concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

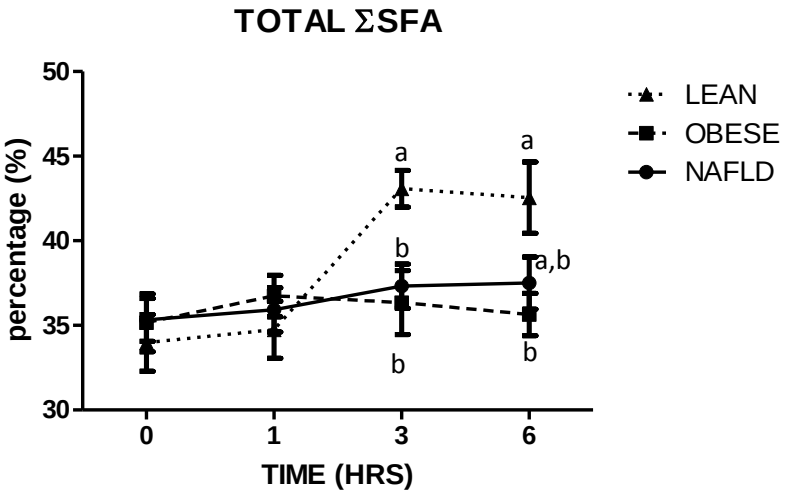


Figure 4.8b. Total sum of SFA concentrations for TG fractions prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

4.3.6.2 *Monounsaturated (MUFA) FA in Plasma TG Fractions*

There were no major differences in the classes of MUFA in the plasma TG proportions studied (palmitoleic and oleic) pre-and-post meal consumption at the time points studied in all three groups. In addition, iAUC values for NAFLD, lean and obese subjects for C16:1 [79.1±25.3 vs 75.8±27.9 vs 29.5±9.0(µg/ml)h, respectively] and C18:1 [761.4±287.0 vs 797.3±225.1 vs 355.4±86.6(µg/ml)h] did not differ significantly between groups. NAFLD subjects exhibited higher total sum of MUFA proportions (**Figure 4.9**) within plasma at 6 hours post meal in comparison to the lean group ($p<0.05$). However, iAUC for total sum of MUFA in the TG fractions did not differ between the three groups (**Appendix 2. Figure G**).

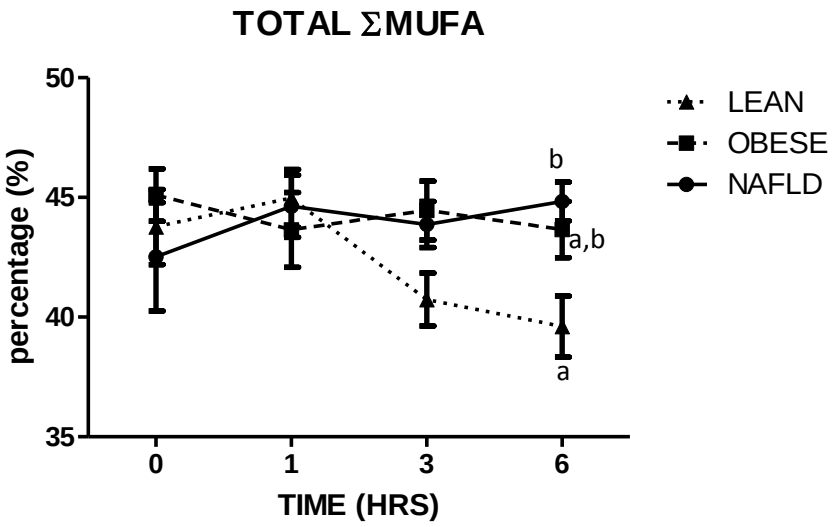


Figure 4.9. Total sum of MUFA concentrations for TG fractions prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

4.3.6.3 *Polyunsaturated FA in TG Fractions*

Proportions of linoleic acid (C18:2n6) were not different between NAFLD and the other two groups at each time point. However, obese controls exhibited higher ($p<0.05$) C18:2n6 acid levels at 3 and 6hrs post meal when compared to lean subjects. Additionally, a significant ($p<0.05$) decrease in the linoleic TG proportions from 1hr to 3 and 1hr to 6hr post meal was observed in the lean group (**Figure 4.10a)**. Proportions of linolenic (C18:3n3), arachidonic (C20:4n6), eicosapentaenoic (C20:5n3) and docosahexaenoic acid (C22:6n3) showed no significant changes between groups and/or time course (**Table 4.4a**).

When analyzing the iAUC, NAFLD subjects exhibited a trend of higher C18:2n6 acid values when compared to the lean and obese control subjects ($p=0.12, 0.058$) (**Figure 4.10b**). C20:4n6 acid iAUC levels showed no significant ($p=0.29$) differences between groups. C20:5n3 acid iAUC higher, but not significant ($p=0.53$), levels were observed in the lean subjects, followed by the NAFLD group. C22:6n3 acid iAUC had the highest ($p=0.54$) levels in the NAFLD, followed by the lean and obese (**Appendix 2. Figure F**). No significant differences between groups in the total omega-3 and omega-6 fatty acids iAUC were observed ($p=0.17, 0.30$, respectively). Total fatty acids (sum) (μg/ml) and iAUC were not significantly different between groups (**Appendix 2. Figure G**).

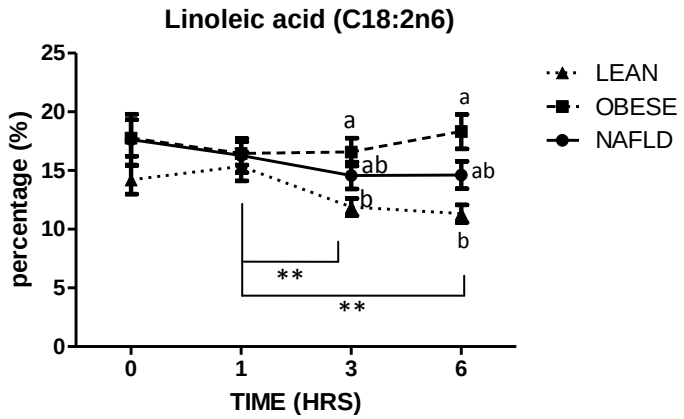


Figure 4.10a. TG fractions for C18:2n6 concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

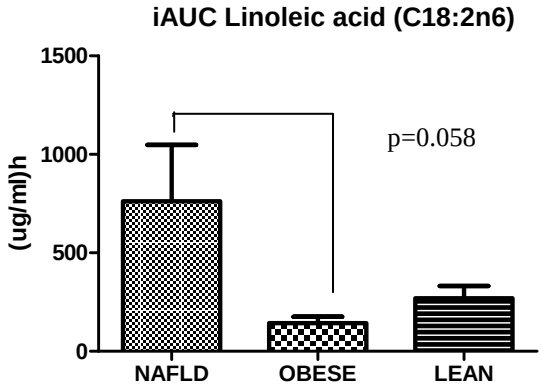


Figure 4.10b. Postprandial incremental area under the curve (iAUC) of TG fractions for C18:2n6 fatty acid in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and lean healthy controls (n=11). Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

4.3.7 Fatty acid profile of plasma phospholipid (PL) Fraction

The complete Fatty acid (FA) profiles (in percentages) and concentrations are presented in **Tables 4.5a and 4.5b**. Time course data and iAUC data for C18:0 acid is presented in **Figure 4.11a** and **Appendix 2. Figure H**; respectively. Total (sum) of SFA time course data is presented in **Figure 4.11b**. Total (sum) of MUFA time course data is presented in figure **11c**. AUC data for highly prevalent in nature fatty acids (C14:0, C16:0, C16:1, C18:2n6 and C20:4n6) is presented in **Appendix 2. Figure I.** PUFA (C18:2n6, C18:3n3, C20:5n3, C22:6n3 and C20:4n6) iAUC data is showed in **Appendix 2. Figure J. Appendix 2. Figure K** represents the iAUC sum for MUFA, SFA, omega-6 (n-6), omega-3 (n-3) and total fatty acids.

Table 4.5a. Postprandial Plasma TPL fatty acid composition (percentages) at different time points

Fatty Acid	TPL(%)LEAN				TPL (%)OBESE				TPL (%)NAFLD			
	Fasted	1hr	3hr	6hr	Fasted	1hr	3hr	6hr	Fasted	1hr	3hr	6hr
14:0	0.3±0.1	0.2±0.1	0.2±0.1	0.1±0.0	0.1±0.1	0.1±0.1	0.1±0.1	0.2±0.1	0.2±0.1	0.1±0.0	0.2±0.1	0.2±0.1
16:0	32.1±0.8	32.1±1.0	31.9±0.7	31.9+0.9	31.7±1.0	31.5±1.5	31.0±1.2	31.6±1.1	32.5±0.6	31.6±0.8	32.4±0.5	32.3±0.5
16:1	0.5±0.1	0.4±0.1	0.4±0.1	0.5±0.1	0.2+0.1	0.2±0.1	0.2±0.1	0.1±0.0	0.6±0.2	0.5±0.1	0.6±0.1	0.6±0.1
18:0	16.5±0.5[a]	17.0±0.6[a]	17.4±0.4	17.5+0.4	17.8±0.5[ab]	18.5±0.5[ab]	18.7±0.6	18.3±0.5	18.8±0.3[b]	18.9±0.3[b]	19.0±0.4	18.9±0.4
18:1	11.6±0.4	11.8±0.4	11.7±0.4	11.8+0.3	10.2±0.9	10.5±0.6	10.3±0.6	10.9±0.7	10.9±0.5	10.8±0.5	10.6±0.6	9.9±0.8
18:2n6	22.0±0.4	22.4±0.4	22.6±0.7	23.0±0.6	22.4±1.1	23.2±1.4	23.5±1.2	21.9±1.6	21.2±0.8	21.4±0.8	21.5±0.7	21.9±0.7
18:3n3	ND	ND	ND	ND	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.0	0.1±0.1	0.1±0.0	0.1±0.0	0.1±0.1
20:4n6	8.8±0.4	9.1±0.5	8.8±0.3	9.2±0.3	7.5±0.7	7.9±0.8	7.4±0.7	7.3±0.7	7.4±0.9	7.7±1.0	7.2±0.8	7.8±1.1
20:5n3	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.4±0.1	0.4±0.1
22:6n3	2.2±0.2	2.0±0.2	2.1±0.3	1.9±0.2	1.3±0.2	1.1±0.3	1.2±0.2	1.3±0.1	1.4±0.3	1.5±0.3	1.4±0.2	1.2±0.2
Σ n-3	2.2±0.2	2.1±0.3	2.2±0.3	2.0±0.3	2.1±0.3	1.7±0.4	1.8±0.3	2.2±0.2	2.3±0.2	2.4±0.2	2.3±0.2	2.2±0.3
Σ n-6	34.1±0.5	34.7±0.5	34.7±0.6	35.5±0.6	32.8±1.4	33.4±1.9	33.4±1.7	31.5±2.3	30.9±1.4	31.5±1.5	31.0±1.2	32.1±1.4
ΣMUFA	13.1±0.4	13.0±0.4	13.1±0.3	12.8±0.3	10.5±0.9	10.8±0.7	10.6±0.6	11.1±0.8	12.5±0.7	12.2±0.7	12.0±0.7	11.3±1.0
Σ SFA	49.2±0.5[a]	49.6±0.6	49.6±0.5[a]	49.8±0.7	52.6±1.5[ab]	52.9±1.7	52.6±1.4[ab]	53.2±1.3	53.9±1.2[b]	53.1±1.4	53.8±1.1[b]	53.7±1.0
Σ(µg/ml)	703.3±44.4	768.1±121.5	823.3±132.3	765.6±130.7	725.2±98.1	808.2±258.0	947.6±351.3	588.7±71.6	677.8±170.5	570.6±115.3	584.8±126.4	608.1±112.3

Values are mean ± SEM (except otherwise mentioned). Variables with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Total Fatty acids sum is expressed in µg/ml

Table 4.5b. Postprandial Plasma TPL fatty acid composition (µg/ml) at different time points

Fatty Acid	TPL(µg/ml)LEAN				TPL(µg/ml)OBESE				TPL(µg/ml) NAFLD			
	Fasted	1hr	3hr	6hr	Fasted	1hr	3hr	6hr	Fasted	1hr	3hr	6hr
14:0	1.6±0.6	1.3±0.7	1.5±0.7	1.2±0.7	2.2±1.8	2.9±2.5	1.4±1.1	4.4±3.8	0.3±0.2	0.3±0.2	0.3±0.2	0.6±0.3
16:0	224.0±15.2	243.1±37.8	271.5±41.2	241.5±39.3	237.8±51.7	266.6±94.6	304.8±114.6	294.6±103.6	215.8±50.2	180.0±36.3	188.9±39.9	194.0±35.1
16:1	3.1±1.4	3.0±1.5	3.4±1.4	3.6±1.7	0.8±0.4	0.7±0.5	0.9±0.6	0.5±0.3	4.4±1.7	3.4±1.4	3.7±1.3	3.1±0.9
18:0	119.1±6.2	135.2±21.7	152.7±24.0	138.7±23.1	106.6±16.3	151.48±50.5	185.4±76.8	171.4±62.0	124.9±31.1	105.0±20.2	107.1±21.5	115.0±20.9
18:1	84.9±6.8	94.3±16.3	103.3±16.5	93.8±16.2	70.6±10.5	88.2±30.3	98.5±37.3	123.2±59.3	74.5±21.7	60.7±14.0	60.8±14.1	56.7±12.4
18:2n6	154.5±7.1	170.4±25.2	194.6±31.1	178.3±30.7	164.0±29.4	180.9±51.5	228.6±87.3	165.9±31.2	145.7±38.7	121.7±23.5	128.1±27.6	132.7±22.9
18:3n3	ND	ND	ND	ND	0.4±0.2	0.4±0.2	0.4±0.3	0.7±0.3	0.4±0.1	0.3±0.1	0.4±0.2	0.3±0.1
20:4n6	64.0±5.7	71.4±11.6	75.3±10.5	68.5±9.9	59.1±14.3	69.5±26.0	70.7±26.0	61.2±15.2	55.8±14.9	51.1±13.3	49.2±14.3	59.2±16.5
20:5n3	0.5±0.0	0.4±0.0	1.9±0.0	1.8±0.2	2.0±0.7	174±0.6	1.7±0.9	1.9±0.6	1.0±0.3	0.9±0.2	0.8±0.2	0.8±0.2
22:6n3	16.7±2.1	15.6±2.9	19.0±3.8	15.8±3.8	9.3±1.9	6.3±1.8	7.6±1.8	10.4±2.1	11.9±3.6	10.9±3.1	11.0±3.6	9.3±2.9
Σ n-3	17.2±2.2	16.0±2.9	17.7±3.8	15.6±3.8	12.3±2.2	8.4±2.1	9.8±1.9	13.3±2.3	13.4±3.4	12.2±2.8	12.3±3.3	10.5±2.7
Σ n-6	243.4±14.5	267±39.6	285.8±44.5	268±44.2	241.1±46.3	268.7±81.7	325.8±124.4	244.2±48.3	225.5±62.7	192.0±42.1	196.2±47.0	212.5±43.7
ΣMUFA	94.7±8.3	102.8±19.3	109.4±19.5	98.7±18.9	74.3±10.9	91.9±31.5	101.3±37.1	127.3±61.3	86.5±25.4	70.1±16.4	70.6±17.0	64.5±14.3
Σ SFA	347.9±21.5	382.1±60.6	410.2±65.2	375.1±62.0	397.4±85.0	439.1±146.3	510.7±191.1	493.3±171.6	352.3±80.5	296.1±56.2	305.6±60.3	320.4±55.5

Values are mean ± SEM (except otherwise mentioned). Variables with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

4.3.7.1 Saturated FA of plasma PL Fractions.

No significant differences were observed between NAFLD and the control groups in the proportions of saturated FA composition, except for the C18:0 acid. Higher proportions of C18:0 acid were observed in the NAFLD population when compared to lean subjects in the fasted state and 1 hr post meal (p=0.004, 0.03; respectively). Nevertheless, C18:0 acid iAUC was very similar between groups (p=0.9). Time course data for C18:0 acid is presented in Figure **4.11a**; iAUC is presented in **Appendix 2. Figure H**. Total sum of SFA proportions were significantly higher (p<0.05) in the NAFLD groups at 0 and 3 hrs post meal when compared to lean subjects (**Figure 4.11b**). Similar values of iAUC were observed between the NAFLD, obese non NAFLD and lean subjects.

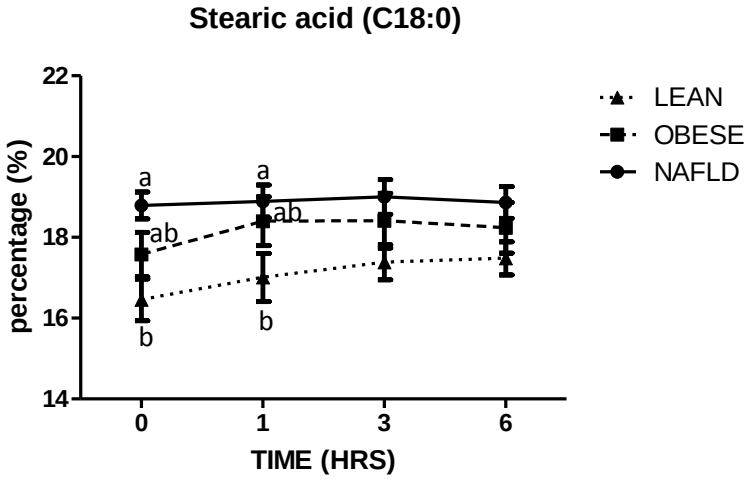


Figure 4.11a. C18:0 concentrations for PL fractions prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA p<0.05 followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

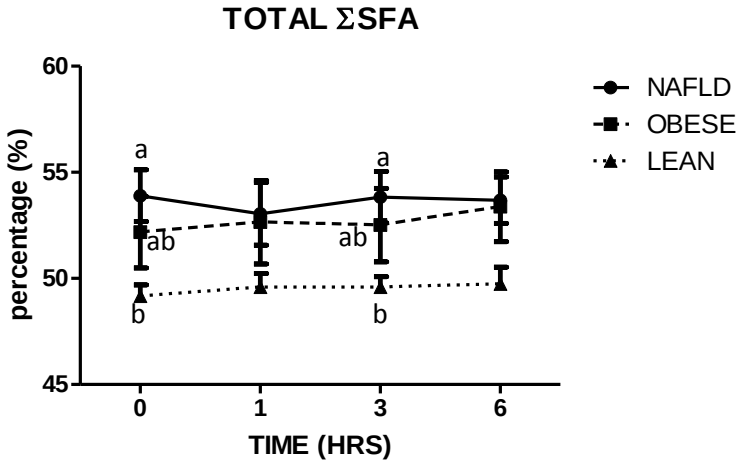


Figure 4.11b. Total sum of SFA concentrations for PL fractions prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

4.3.7.2 *Monounsaturated FA of plasma PL Fractions*

No significant differences were observed in the C16:1 and C18:1n9 proportions and their respective iAUC. No significant differences were observed in the total sum of MUFA proportions (**Figure 4.11c)** and MUFA iAUC (**Appendix 2. Figure K**).

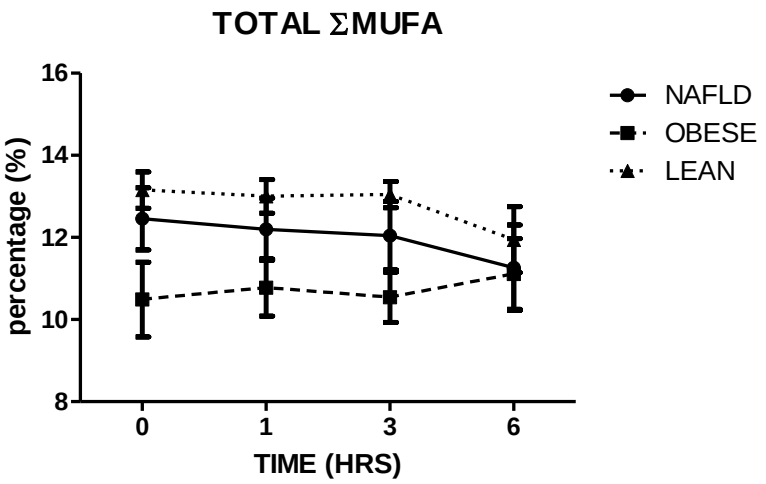


Figure 4.11c. Total sum of MUFA concentrations for PL fractions prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

4.3.7.3 *Polyunsaturated FA of plasma PL Fractions*

No significant differences were observed in each of the PUFA proportions (C18:2n6, C20:4n6, C20:5n3 and C22:6n3) or their respective PUFA iAUC (**Appendix 2. Figure J**). Additionally, the sum of PUFA proportions was not significantly different between groups. No significant differences between groups were observed in the omega-3 and omega-6 PL proportions and their respective iAUC (**Appendix 2. Figure K**). Total fatty acids (μg/ml) were not significantly different between groups.

4.3.8 Apolipoproteins

4.3.8.1 *Apolipoprotein B-100*

NAFLD had higher apolipoprotein B-100 in the fasted state and 3hrs post meal ($p<0.01$, <0.05, respectively) when compared to lean subjects (**Figure 4.12).** No significant interaction between group and time was observed ($p>0.05$)**.** When comparing AUC and iAUC, only AUC was significantly higher in the children and adolescents with NAFLD when compared to the lean controls ($p=0.02$) (**Appendix 2. Figure L**)**.**

4.3.8.2 *Apolipoprotein B-48*

Apolipoprotein B-48 was higher in children and adolescents with NAFLD when compared to lean subjects at 3 ($p<0.05$) and 6 hours ($p<0.001$) post meal consumption (**Figure 4.13**). When compared to obese controls, children and adolescents with NAFLD had higher plasma Apo B-48 values at 6 hours post meal consumption only ($p<0.001$) (**Figure 4.13**). Significant interaction between group and time was observed ($p=0.01$). No other significant differences were observed (**Appendix 2. Figure L**).

4.3.8.3 *Apolipoprotein C-III*

Plasma values of apolipoprotein C-III children and adolescents with NAFLD had significantly higher fasting levels than their obese and/or lean age and gender matched controls ($p<0.01$) (**Figure 4.14)**. No significant interaction between group and time was observed ($p>0.05$). No differences in plasma values of Apolipoprotein C-III were observed between groups over the postprandial period studied (**Appendix 2. Figure L).**

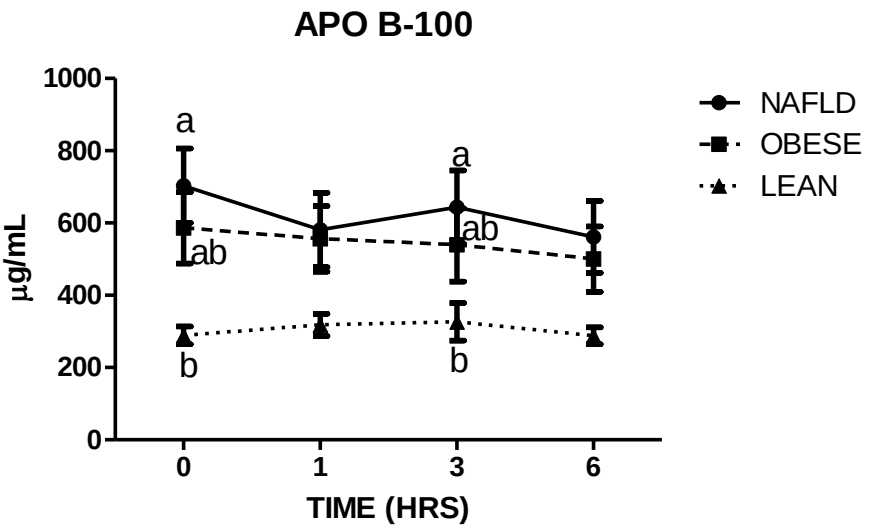


Figure 4.12. Apolipoprotein B-100 concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-age and gender matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons**.** Results are shown mean ± SEM.

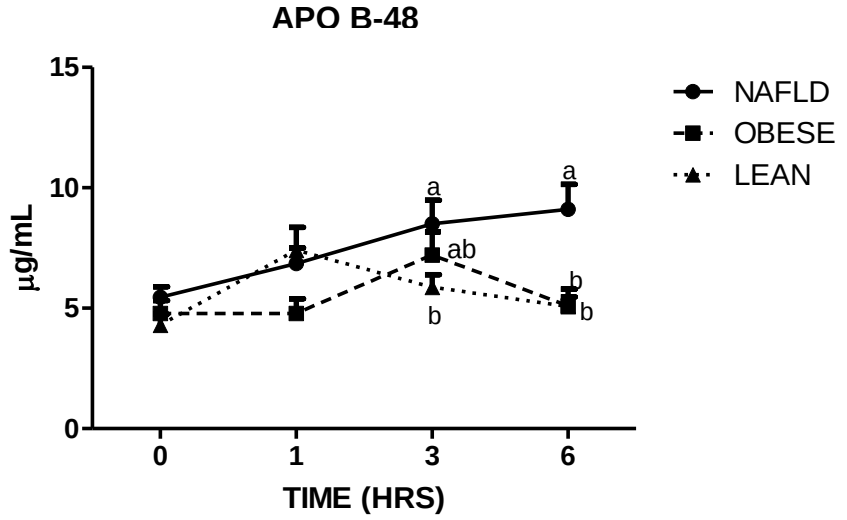


Figure 4.13. Apolipoprotein B-48 concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-age and gender matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons**.** Results are shown mean ± SEM.

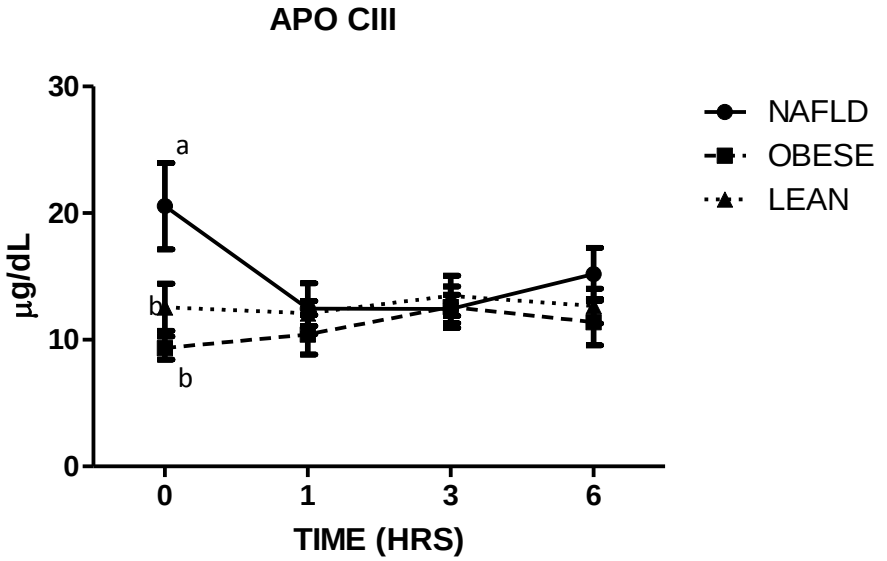


Figure 4.14. Apolipoprotein C-III concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-age and gender matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

4.3.9 Inflammatory mediators

4.3.9.1 *Adiponectin, TNF-α, IL-6 and IL-10*

Lean subjects exhibited the highest ($p>0.05$) fasting levels of adiponectin when compared to the children and adolescents with NAFLD and age-gender matched obese controls (**Figure 4.15)**. No significant differences between groups over the postprandial study period were observed when analyzing the adiponectin (overall fasted mean: 8.8$\pm$0.6 ng/mL) **(Appendix 2. Figure M**). TNF-α (absolute) fasting value ($p<0.001$) and AUC were significantly higher ($p<0.001$) in NAFLD compared to the lean and obese control subjects (**Figure 4.16, Appendix 2.**

Figure M). No significant interaction between group and time was observed ($p>0.05$). TNF-α iAUC was not significantly different between groups ($p>0.05$) (**Appendix 2. Figure M**). In contrast, IL-6 levels were not significantly different between groups at any time point, nor were the iAUC, AUC and interaction between group and time ($p>0.05$) (**Figure 4.17, Appendix 2. Figure M**). Fasting IL-10 values were significantly higher ($p<0.001$) in the lean subjects when compared to NAFLD and obese subjects (**Figure 4.18**). The former also exhibited higher AUC when compared to obese subjects ($p<0.01$) but not to NAFLD ($p>0.05$) (**Appendix 2. Figure M**). Nevertheless iAUC was not significantly different when comparing NAFLD vs. the other two groups ($p>0.05$) (**Appendix 2. Figure M**). No significant interaction between group and time was observed ($p>0.05$).

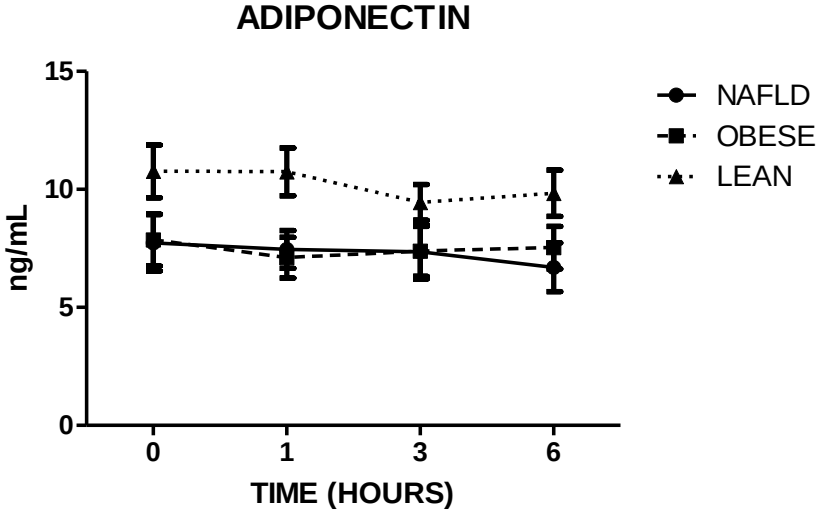


Figure 4.15. Adiponectin concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-age and gender matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

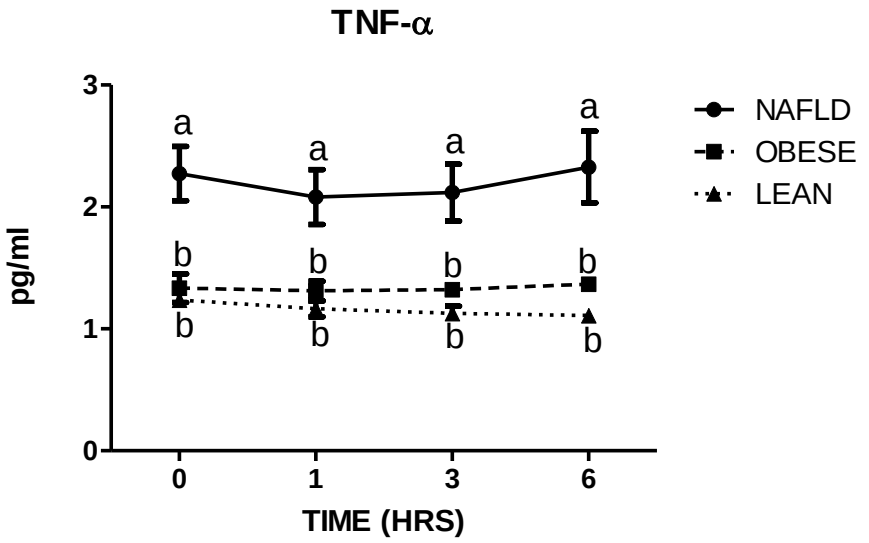


Figure 4.16. TNF-α concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-age, gender and BMI matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons**.** Results are shown mean ± SEM**.**

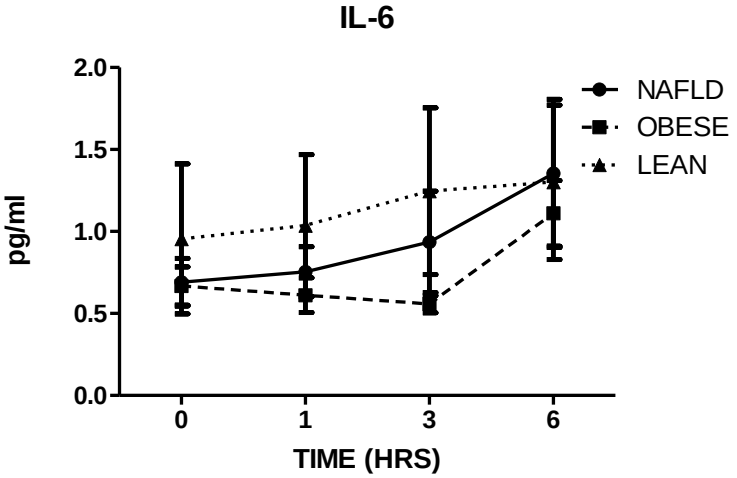


Figure 4.17. IL-6 concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-age, gender and BMI matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

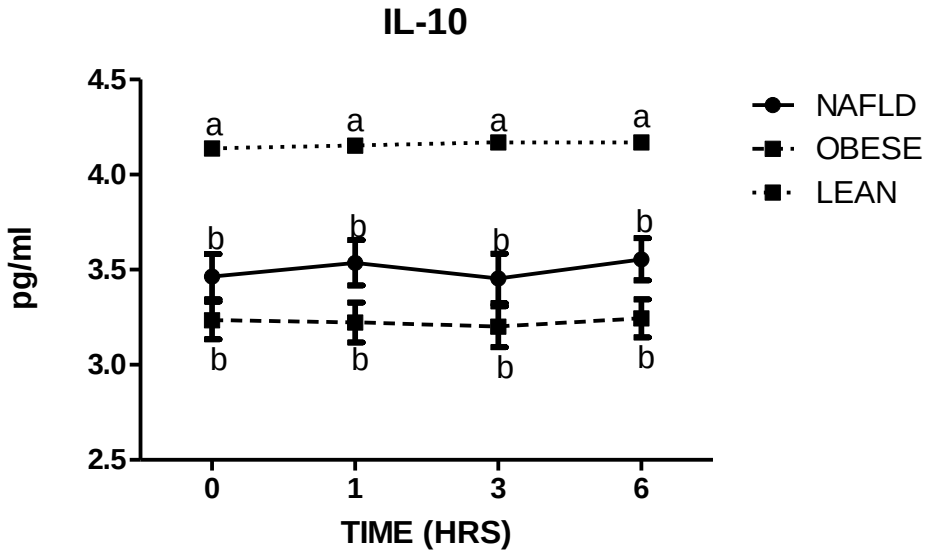


Figure 4.18. IL-10 concentrations prior (t=0) and following consumption of a high saturated/low polyunsaturated meal at 1, 3 and 6 hours in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-age, gender and BMI matched (n=9) and healthy lean-age, gender matched (n=11) controls. Values with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons**.** Results are shown mean $\pm$ SEM.

4.3.10 Habitual Food Intake

Data for habitual intake from three day food intake records is illustrated in **Table 4.6**. Macronutrient distribution in NAFLD subjects was within the Acceptable Macronutrient Distribution Range (AMDR), but absolute intake of carbohydrate and protein were higher than the Recommended Dietary Allowances (RDAs) for age and gender.

Fat percentage (% of TEI) was higher ($p>0.05$) in the NAFLD subjects but still within the AMDR. The amount of fat (g) was lower in the lean subjects when compared to the obese group (**see Table 4.6**). When analyzing the specific type of fat intake, MUFA and PUFA consumption (g) was significantly higher in the obese subjects when compared to NAFLD and lean subjects (**Table 4.6**). Additionally RDA/AI for omega-3 and omega-6 was not met in any of the groups. Nutrients at risk for insufficient intake (as defined by intakes less than the estimated average intake (EAR) or less than 50% of adequate intakes (AI) were vitamin A, biotin and vitamin E.

Chronic fructose intake has suggested having an effect in the postprandial response (Jin et al., 2012). Therefore, habitual intake was assessed (see section 4.2.5). All the groups had a fructose intake that was <7% TEI (leans: 4.3 $\pm$ 0.4; obese: 4.1 $\pm$ 0.5 and NAFLD: 3.6 $\pm$ 0.6; see **Table 4.6**). GL for NAFLD and lean subjects was >80 but <120 while the dietary intake of obese subjects had a GL >120 (See **Table 4.6** for the exact numbers). Values > 120 were considered high, <120 but >80 were considered medium and <80 low (Beulens et al., 2007).

Table 4.6. Habitual intake

	Lean (n=9)	Obese (n=9)	NAFLD (n=10)	p value
Energy (kcal)	1665±114.9[b]	2692±347.8[a]	2304±293.5[ab]	0.04
Carbohydrates (%)	57.8±2.1	53.2±1.5	50.2±2.5	0.06
Carbohydrates (g)[1]	241.6±20.0	359.6±49.6	288.9±38.6	0.1
Protein (%)	17.0±1.5	15.2±0.9	17.7±1.3	0.4
Protein (g)[2]	68.6±4.8	102.1±14.2	95.4±7.8	0.054
Fat (%)[3]	26.70±2.3	32.9±0.9	33.0±2.6	0.08
Fat (g)	49.9±6.3[b]	97.0±11.8[a]	87.6±14.9[a,b]	0.02
SFA (%)	10.9±1.2	12.1±0.9	11.8±0.8	0.6
SFA (g)	20.4±3.0	36.7±5.6	30.6±4.7	0.06
MUFA (%)	4.4±0.3[b]	8.6±1.0[a]	6.2±1.1[ab]	0.01
MUFA (g)	7.9±0.6[a]	25.8±4.4[b]	15.1±2.3[a]	0.001
PUFA (%)	2.2±0.3[b]	4.3±0.6[a]	2.7±0.5[ab]	0.04
PUFA (g)	4.3±0.9[a]	12.2±2.2[b]	6.4±1.2[a]	0.004
n6/n3	9.8±1.3	12.4±1.5	8.5±1.2	0.1
Fructose (g)*	17.6±1.9	28.4±5.3	27.3±4.8	0.1
Fructose (%TEI)[4]	4.3±0.4	4.1±0.5	3.6±0.6	0.7
Glycemic Load (GL)[5]	99.5±11.5	126.7±16.2	106.4±17.1	0.4

Values are mean ± SEM. Different superscripts indicate significant differences ($p<0.05$) between groups by repeated measures one way ANOVA followed by Bonferroni correction and pairwise comparisons. Percentages are based on the TEI.

RDA: [1]Carbohydrate: Children 4-8y, males 9−13 y, 14−18, females 9−13 y, 14−18: 130g/d.
[2]Protein: 4−8 y: 19g/d. Males 9−13 y: 34, 14-18y: 52g, females 9−13 y: 34 g/d, 14-18y: 46 g/d.
[3]Fat: 4−8 y: 25-35%AMDR, males 9−13 y, 14−18 y: 25-35% AMDR, females 9-13y, 14-18y 25-35%AMDR
[4]Fructose: low if <7% TEI (Bantle et al., 2000; Elliot et al., 2002; Hollenbeck, 1993; Reiser et al., 1989; Teff et al., 2002)
[5]GL: low if < 80 per day (Beulens et al., 2007)
*Fructose analysis was calculated using the USDA nutrient database, CNF and commercial food label information from the individual brand types.
Recommended Dietary Allowances (RDAs)
Acceptable Macronutrient Distribution Range (AMDR)

4.3.11 Interrelationships between insulin and other variables

Pearson correlation, linear and multiple regression were performed in order to determine the strength and direction of relationships between variables in the different groups. Variables analyzed were insulin, triglyceride, TNF-α, Apo B-48, B-100 and C-III, TG proportions in the fasted and postprandial stage as well as markers of liver function (ALT and AST; only fasting values).

4.3.12 Insulin and liver functions, apolipoprotein and triglycerides expression

Fasting HOMA-IR was positively correlated with ALT and AST (p=0.0001, 0.001; respectively, r^2=0.4, 0.3; respectively). When a subset group analysis (NAFLD, obese control and lean) was performed, fasting HOMA-IR was positively correlated in the NAFLD group with ALT and AST (p=0.01, r^2=0.5; p=0.03, r^2=0.4) (**see Appendix 2. Table 5**). Fasting insulin was significantly correlated with ALT and AST (p=0.001, 0.007; respectively, r^2=0.3, 0.2; respectively). When a subset group analysis (NAFLD, obese control and lean) was performed; fasting insulin was positively correlated with ALT (p=0.03, r^2=0.4) but not with AST (p=0.06, r^2=0.3) in the NAFLD group (**see Appendix 2. Table 5**). Data was categorized for ALT above and below 20 U/L and above and below 40 U/L; the strongest correlation between iAUC for insulin and iAUC for Apo C-III was observed in the subjects above 20 U/L for ALT (p=0.002, r^2=0.450).

Weak correlations were observed between fasting insulin, TG (fasted and postprandial) and fasting Apo B-100. Insulin iAUC correlated weakly with fasted and postprandial TG values and Apo B-48 at 6hrs post meal. When a subset group

analysis was performed no correlations between fasting and/or insulin iAUC and apolipoproteins (Apo B-48, B100 and C-III) were observed in any of the groups (**see Appendix 2. Table 5, 6 and 7**). Multiple regression was performed to assess important relationships; significant ($p<0.05$) associations were observed between iAUC for TG and iAUC Apo C-III, iAUC for Apo B-48 and iAUC Apo C-III, iAUC for TNF-α and iAUC Apo C-III, iAUC for TNF-α and iAUC for NEFA when insulin iAUC was the dependent variable.

In the NAFLD group significant correlations were found between iAUC for insulin and the C16:1n9 TG (proportions) at 1, 3 and 6 hrs post meal ($p=0.01$, 0.04, 0.009; respectively, $r^2=0.4$, 0.3, 0.5; respectively). Significant correlation were observed between fasting insulin and the C16:1 TG (proportions) at 1, 3 and 6 hrs post meal ($p=0.02$, 0.03, 0.005; respectively, $r^2=0.4$, 0.4, 0.5; respectively). When performing a multivariate regression analysis, variables that contributed significantly ($p<0.05$) to HOMA-IR explained by the model were ALT and fasted TG.

4.3.13 Non-insulin correlations

In the NAFLD group, TNF-α fasted and postprandial values showed a positive correlation with their respective C18:0 TG (proportions) fasted and postprandial (1, 3 and 6hrs post meal) values ($p=0.02$, 0.01, 0.01, 0.02; respectively, $r^2=0.4$, 0.5, 0.5, 0.4; respectively). AST was positively correlated with fasting TG ($p=0.003$; $r^2=0.2$). When a subset group analysis (NAFLD, obese control and lean) was performed NAFLD group showed a positive correlation between AST and fasting TG ($p=0.04$, $r^2=0.3$). Additionally, ALT and AST were

strongly correlated in the NAFLD, obese and lean group (p=0.0002, 0.01, 0.008; respectively, r^2=0.8, 0.5, 0.5; respectively) (**see Appendix 2. Table 5, 6 and 7).**

4.4. DISCUSSION

Obesity and high body fat percentage are major risk factors for developing NAFLD in adults, adolescents and children. NAFLD is a pathology that involves several metabolic abnormalities, such as hyperinsulinemia, IR, increased cytokine secretion and abnormalities in the postprandial state (Musso et al. 2005; 2003). Delayed postprandial lipid clearance has been observed in adults with NAFLD (Bravo et al., 2010; Musso et al. 2005; 2003); how this might contribute to disease pathology is not completely understood. Currently, there is limited data about the postprandial metabolic responses after consuming fast food meals (high saturated fat/low PUFA, especially LCPUFA) that are typically consumed by children and adolescents. The choices of food in the meal challenge allowed us to obtain a meal composition similar to a North American fast food breakfast (Fernández San Juan, 2000; Pachuki, 2011). To our knowledge this is the first study in children and adolescents with NAFLD that describes the postprandial responses following a high saturated/0% LCPUFA meal challenge. The current study demonstrated that children and adolescents with NAFLD exhibit different fasting and postprandial responses (e.g. insulin and lipid profile) to a standardized meal compared to obese and lean subjects. In our study children with NAFLD showed different body fat distribution when compared to obese controls as observed by the trend towards higher waist/hip ratio in spite of the lower BF% and WC.

4.4.1 *Insulin and glucose*

Children and adolescents with NAFLD exhibited significant fasting and postprandial hyperinsulinemia with normal glycemia compared with lean subjects suggesting that one of the major derangements (although not the only one) in our population is the hyperinsulinemia. Our results are similar to Manchanayake et al. (2011) in the sense that higher insulin values were observed across the postprandial spectrum in male adults with NAFLD when compared to non-NAFLD adults. High insulin secretion could explain the normal glucose values observed in the NAFLD population. Our results suggest that at this age fasting and postprandial hyperinsulinemia is still enough to maintain glucose values within normal ranges.

4.4.2 *Postprandial: TG, NEFA, LDL, HDL and cholesterol profile*

Postprandial lipid responses were similar between NAFLD and obese control subjects, but differed from lean subjects. Lean subjects cleared TG from plasma within 6 hours, but both obese children and adolescents and NAFLD children and adolescents had delayed clearance. Major differences between the obese children and adolescents and NAFLD children and adolescents were a) delayed clearance of Apo B-48 and b) suppression of NEFA.

This could potentially suggest that children and adolescents with NAFLD are probably more sensitive in terms of NEFA secretion to a high saturated fat meal in comparison to non NAFLD children and adolescents. Our results are somewhat different to what other researchers have observed in adults (Musso et al., 2009). Other researchers have observed consistently higher fasting and

postprandial NEFA values in adults with NAFLD when compared to non-NAFLD (Musso et al., 2009). Possible explanations of our study results could be: a) The different type of meal used (e.g. Musso et al. provided a carbohydrate free meal with higher energy content), b) the type of population (adults vs. children and adolescents), c) an underestimation of the NEFA flux to the liver as suggested by Diraison et al. (Diraison et al., 2003). It is very likely that children and adolescents with NAFLD have a higher NEFA storage in the liver and possibly the secretion of hepatic NEFA is decreased compared with non NAFLD subjects as observed by the reduced NEFA plasma levels (Caldwell et al., 2007; Cortez-Pinto, 2006). The high NEFA levels observed in the fasted state and at 1hr post meal suggests insensitivity to insulin or an inability to suppress NEFA in response to a meal. Higher NEFA values in NAFLD children and adolescents at 1 hr post meal would be expected since high insulin levels fail to suppress lipolysis (Caldwell et al., 2007; Cortez-Pinto, 2006; Vannia et al., 2010).

4.4.3 *Apolipoproteins*

Apolipoprotein B-48 (Apo B-48) is a protein found exclusively in the chylomicrons (CM) (Proctor et al., 2003). High Apo B-48 levels could mean either: a) defect in CM clearance, b) overproduction and assembly of CM or a combination of both (Proctor et al., 2003; Vine et al., 2008). Studies have suggested than Apo B-48 secretion and production are increased in insulin resistance and fasted Apo B-48 levels correlate with fasted TG values (Couillard et al., 2002; Valdivielso et al., 2010; Villodres et al., 2008). In our study, no significant differences in the Apo B-48 values were found between groups when

fasted, but were at 3 and 6 hours post meal in children and adolescents with NAFLD when compared to lean patients. Meal challenge composition (high SFA and low PUFA) may be influencing the postprandial response to some extent (Klob & Castro Cabezas, 2012; López-Miranda et al., 2007; Thomsen et al., 1999). Studies in animal models and in humans suggest that when a fat load rich in SFA is substituted by PUFA (especially omega-3) a reduced postprandial CM production has been observed (Chung et al., 2004; Harris & Muzio et al., 1993; Nestel et al., 1984; Tinker et al., 1999). Potential explanations to the different results obtained in our research could be a) a reflective of the type of population we studied (e.g. adults vs children and adolescents, adults with type 2 Diabetes and/or obese vs NAFLD), suggesting that children and adolescents with NAFLD exhibited a different Apo B-48 behavior (e.g. NAFLD population didn´t exhibit higher TG levels than the obese subjects), b) the type of meal (e.g. higher amount of kilocalories) (Villodres, 2008) or c) the methodology used (ELISA vs. SDS-PAGE) (Jackson & Williams, 2004). Advantages of the ELISA method include that it is specific for Apo B-48 on the contrary, when using the SDS-PAGE method an incomplete transfer of the proteins to membrane and antibody interactions may influence the Apo B-100 and B-48 reported levels (Jackson & Williams, 2004).

In our study we found significant higher values in fasting Apo B-100 in NAFLD subjects compared to lean subjects. Overall our data suggests that net uptake of fat into the liver is prolonged in the NAFLD subjects compared to obese controls which will promote accumulation of TG in the liver and consequently

simple steatosis. Apo B-100 is essential for hepatic assembly as well as secretion of TG rich lipoproteins and it is a major component of VLDL (Su et al., 2009). The observed higher Apo B-100 fasted levels could be due to a higher efflux from the liver, probably as a consequence of the high TG availability in the liver, which could be a consequence of the hyperinsulinemic state of this population. It is possible that our NAFLD population due to the hyperinsulinemic state exhibits an imbalance between influx and efflux to and from the liver; therefore the high availability of TG in the liver could promote an increased VLDL Apo B-100 secretion. Additionally, the meal challenge composition may be influencing the postprandial response. Evidence suggests that meals rich in SFA evoke higher Apo B-100 concentrations due to a possible altered secretion and degradation when compared to meals rich in PUFA and MUFA (Jackson et al., 2005). Meals higher in SFA will delay CM remnants clearance, promote fatty acid availability in the liver and consequently impair Apo B-100 secretion and therefore VLDL hepatic secretion (Carpentier et al., 2002; Charlton et al., 2002; Musso et al., 2003). It is possible that children and adolescents with NAFLD may be more susceptible to the adverse postprandial affects observed after a high SFA meal.

NAFLD subjects exhibited higher absolute levels of Apo C-III in the fasted state when compared to lean and obese control patients. Apo C-III levels have been correlated with IR and/or hyperinsulinemia (Kozlitina et al., 2011; Onat et al., 2003; Ooi et al., 2008). Insulin resistance and high Apo C-III could impair lipoprotein lipase (LPL) and hepatic lipase (HL) activity (Bobik, 2008; Jansen et al., 2001). LPL and HL play an important role in lipid metabolism in humans

(Jansen et al., 2001). In our study the high fasted Apo C-III as well as the fasting and postprandial hyperinsulinemia observed in the NAFLD subjects suggests impairment in LPL activity. This could lead to slow removal of chylomicrons and chylomicrons remnant from plasma. Chylomicrons are the preferred substrate of LPL (Bickerton et al., 2007; Frayn, 2001). Therefore, an increase uptake by the adipose tissue of the NEFA released by the LPL will be observed (Bickerton et al., 2007; Frayn, 2001, Paglialunga et al., 2009; Picard et al., 2002). Impairment of LPL may exacerbate postprandial hypertriglyceridemia through a delayed TG clearance as observed in the NAFLD group (Ferland et al., 2012; Fielding & Frayn, 1998; Frayn, 2001). Our results are slightly different than those reported by Mekki et al. (Mekki et al., 1999). In their study, normolipidemic subjects (women) exhibited higher Apo C-III values than hyperlipidemic patients in the fasting state and 2-4 hours after a meal. Differences between these observations and our own could be due to the different populations studied (e.g. female vs. NAFLD and/or adults vs. children and adolescents). Tilly et al. observed that factors influencing Apo C-III levels are age, BMI in adult men and contraceptive use in women (Tilly et al., 2003). In Mekki´s et al. study no mention about oral contraceptive use was mentioned being this another possible explanation of the different results observed in our study. It has been suggested that puberty affects the pathophysiology and severity of NAFLD (Suzuki et al., 2012). Potentially, puberty may be playing a role in the Apo C-III plasma expression. Nevertheless no major differences in postprandial Apo C-III expression were observed between children of different genders and no effects of age were observed.

4.4.4 *Fatty acid profile of plasma*

Fatty acid composition of plasma TG is reflective of short term dietary intake (Raatz et al., 2001). Since the major fatty acids observed in our meal challenge were C18:1n9> C16:0> C18:2n6> C14:0 and C18:0 changes of these in the plasma may be reflective of the way these FA are being handled to and from the liver following consumption of a meal. In our study, differences in proportions of SFA and MUFA TG were observed between obese subjects (NAFLD and non NAFLD obese controls) when compared to lean subjects. In general we found that the lean group exhibited postprandial increases in C14:0 and C16:0 FA, while the obese children and adolescents (obese controls and NAFLD) did not. Significantly lower SFA levels (percentages) were observed in NAFLD subjects when compared to lean subjects at 3 hrs post meal which may reflect how lean subjects clear dietary fatty acids in short term. It is worth to mention that at 3 and 6hrs post meal Apo B-48 were also higher in the NAFLD when compared to the lean groups. Saturated fat has shown to up regulate chylomicron production and therefore exacerbate postprandial lipemia (Klop & Castro Cabezas, 2012; López-Miranda et al., 2007; Thomsen et al., 1999). Our research suggests that the extent of up regulation is higher in subjects with NAFLD as observed by the significant higher values observed at 3 and 6 hours post meal.

In contrast, no major changes in either the individual MUFA (C16:1, C18:1n9) or the total sum of MUFA were observed in the obese children and adolescents (controls and NAFLD), although the lean children experienced postprandial declines in TG fractions. Gil-Campos et al. in a very similar

postprandial study from a meal composition perspective (similarities include: types of food used, butter was used as the source of fat and total amount of kilocalories) observed higher C16:1 acid level in obese children when compared to normal weight children (Gil-Campos et al., 2008). Additionally Okada et al. observed higher fasted C16:1 values in overweight children when compared to control (Okada et al., 2005). Our results are somewhat different from those reported by others; C16:1 acid, although not significant, was higher in the fasted and postprandial stage in the NAFLD subjects (but not in the obese control) in comparison to the lean subjects. C16:1 acid is not very predominant in the food, therefore it is a considered a product of endogenous lipogenesis (Okada et al., 2005). C16:1 acid may be reflective of stearoyl CoA desaturase (SCD) activity, therefore, reflective of the hepatic lipid pool and TG secretion (Okada et al., 2005; Palliard et al., 2008). A trend of higher C16:1 acid level in our NAFLD population could be suggestive of impairment in SCD activity which could be responsible in some degree for the accumulation of fat in the liver. A possible explanation for the difference between our study and that of Gil-Campos et al. (Gil-Campos et al., 2008) could be the different proportion of fat in the meals (32% vs 43% in our meal) and that our obese population had 2 subgroups (NAFLD and non NAFLD) (Gil-Campos et al., 2008; Okada et al., 20011; 2005). C18:2n6 is a substrate for synthesis of C20:4n6 (Elizondo et al., 2008), interestingly; C20:4n6 levels were similar between NAFLD and all other subjects. Controversy exists whether high intake of C18:2n6 will promote high levels of C20:4n6. Some researchers have suggested that C18:2n6 consumption may not be

associated with increased inflammation (Fritsche et al., 2008). In fact, some studies have observed that subjects consuming high C18:2n6 have lower inflammation status when compared to subjects who consume less C18:2n6 (Ferrucci et al., 2006; Fritsche et al., 2008; Kelley et al., 2008). Lower C20:4n6 levels in plasma may be reflective of the rapid conversion to pro inflammatory prostaglandins (Adam et al., 2008; Das, 2011; Mirza, 2011). A trend of higher n-6, lower n-3 and MUFA fasting values in NAFLD were observed which could potentially reflect chronic food intake in this population. More research is necessary in order to conclude that high n-6 levels as well as depletion of n-3 fatty acids may lead to up regulation of pro inflammatory mediators (e.g. TNF-α) promoting steatosis in the liver.

4.4.5 *Inflammatory mediators*

Our NAFLD patients exhibited significantly higher TNF-α concentrations in the fasting and postprandial state when compared to the lean and obese groups. The levels observed in our study were similar to what other researchers have reported (Gil Campos et al., 2009; Kallio et al., 2008; Poppit et al., 2008). IL-10 levels were significantly lower in the fasted and postprandial state in NAFLD and obese subjects when compared to lean subjects. High TNF-α and low IL-10 levels suggest that subjects with NAFLD exhibit a pro-inflammatory state. IL-6 was not different between groups nor did it change during the postprandial period. These results (TNF-α, IL-6 and IL-10 levels) suggest that a single meal high in SFA, low in PUFA may not be enough to evoke immediate changes in inflammation and that the overall inflammatory environment in NAFLD may be elicited more by

chronic intake, rather than by meal composition (e.g. high SFA, low PUFA, high fructose). Interestingly, a significant group and time effect was observed between TNF-α and NEFA, suggesting that even if there are no changes over time in TNF-α; a) a state of inflammation is observed in NAFLD patients, b) the higher TNF-α levels observed in the NAFLD population over time could influence NEFA release from adipose tissue. A pro-inflammatory state in addition to hyperinsulinemia will increase the NEFA supply to the liver (Tamura et al., 2005; Wree et al., 2011). The insulin resistant state fails to suppress the NEFA flux from the adipose tissue as a result of LPL impairment. Elevated NEFA could reduce hepatic insulin clearance as well as increased accumulation of TG in the liver which consequently leads to steatosis in the liver (Feldstein et al., 2004; Gan et al., 2011; Wree et al., 2011). Additionally, elevated NEFA have shown to promote insulin resistance in the muscle and to have toxic effects to pancreatic β-cells (Aguilera et al., 2008). Research in animal models has shown that TNF-α administration increases the NEFA release from the adipose tissue into the plasma (Kushibiki et al. 2002). In addition, TNF-α has shown to stimulate maturation of SREBP-1c (sterol regulatory element binding protein-1c) (Lawler et al., 1998) which will promote deposition of fat in the liver (Endo et al., 2007).

Overall, these results suggest that children and adolescents with NAFLD present a different metabolic environment when compared to the non-NAFLD subjects. Long term dietary intake may promote changes in the metabolic environment. Fructose intake was very similar in all the groups. Nevertheless values about the fructose content in the food are very limited in the nutrient data

base. This may lead to inaccuracies most likely underestimation in the chronic fructose intake calculation. More research is necessary in order to determine to how and to what extended higher chronic consumption of fructose affects the postprandial lipid clearance in children with NAFLD. Plasma phospholipid composition has been suggested to be directly affected by the predominant types of dietary fat (Raatz et al., 2001). Plasma phospholipid concentrations may reflect the intake over the last few days or meals (Arab, 2003; Fusconi et al., 2003; Patel et al., 2010; Raatz et al., 2001). In our study significant higher proportions of C18:0 acid were observed in the NAFLD population when compared to lean subjects in the fasted state and 1 hr post meal ($p<0.05$). C18:0 acid is a product of elongation of C16:0 acid and it is highly abundant in meat, poultry, fish, milk and cheese. The proportion of MUFA in phospholipid was not significantly different between groups. Nevertheless, children and adolescents with NAFLD consumed significantly less MUFA (g) when compared to obese subjects. It is possible that the high MUFA consumption might be playing some kind of protective role in the obese subjects as it has been suggested that MUFA have anti-inflammatory properties, decrease CRP and improve fasting glycemic levels (Ambring et al., 2006; Jenkins et al., 2003; Moussavi et al., 2008; Videla et al., 2004). Strong correlation between CRP and glucose levels has been observed, but the exact mechanism is not completely understood (Videla et al., 2004).

The appropriate balance between dietary MUFA and PUFA for NAFLD treatment is still unknown. In general the consensus about dietary MUFA/PUFA suggests that a 1:1 ratio may optimize the anti-atherogenic and anti-inflammatory

properties of PUFA and MUFA (Moussavi et al., 2008; Videla et al., 2004). Whether or not this represents the optimal ratio for dietary treatment of NAFLD remains unclear. Dietetic MUFA/PUFA ratio in children and adolescents with NAFLD may influence rates of lipogenesis and β-oxidation (Armoni et al., 2007; Moussavi et al., 2008; Svegliati-Baroni et al., 2006). The correction or improvement of these biochemical processes should help to decrease the amount of fat storage in the liver with subsequent improvements in the extent of steatosis, inflammation and fibrosis in NAFLD.

4.4.6 Strengths and limitations of study design

To our knowledge this is the first study examining postprandial responses to a mixed meal performed in children and adolescents with NAFLD that evaluates both insulin and lipid responses in the context of meal composition. Reasons of the limited data available include: a) difficulties to diagnose children and adolescents with NAFLD by the "gold standard" (liver biopsy) and b) postprandial studies are difficult to perform especially in children due to the number of hours that they need to fast after the meal challenge and the level of invasiveness. One of the strengths of our study is that the use of 3 groups (NAFLD, obese and lean controls) enabled us to study the effects of acute meal consumption over a spectrum of body composition subtypes and metabolic profiles. Additionally, our meal challenge had a very low fructose content (568 mg) as well a low GL (20) allowing us to observe metabolic responses to a meal challenge relatively high in fat for insulin and lipemia without confounding factors (e.g. fructose). The majority of the children and adolescents with NAFLD

were diagnosed using liver biopsy and an ultrasound (US) was used to rule out fat infiltration in our obese control subjects. This is a unique finding within the literature, as liver biopsy is not without risk for complications (e.g. bleeding) and therefore the use of liver biopsy to conclusively diagnose the presence of NAFLD in the clinical setting is not consistent. While the combined use of ultrasonography and plasma levels of liver biomarkers (ALT, AST) are more traditionally used within the clinical setting to diagnose NAFLD, liver biopsy is still considered the 'gold standard' to diagnose NAFLD in the clinical setting. Our study design has a conferred strength with the diagnosis of childhood NAFLD using this methodology and is among the few studies conducted using this modality.

One of the limitations of our study is the lack of ultrasound in some obese subjects and the small sample size. However it is unlikely that our obese children and adolescents had NAFLD as they underwent screening blood work to rule out risk for NAFLD. In addition, a post hoc statistical test was performed and the power (group) for most of the variables was above 0.9. Additionally, a statistical analysis for insulin, TG, LDL, HDL, Apo B-48, B-100 and C-III was performed comparing subjects with US vs. subjects without US (**Appendix2, Figures N and O**) and no statistical differences were found. Another limitation of the study was that the information regarding Tanner stage was not consistently available. An asset in our study is that subjects were age matched. We acknowledge that puberty may appear earlier in obese subjects. Nevertheless, obesity has shown to have a higher (by promoting earlier puberty) effect in girls when compared to

boys (Burt Solorzano & McCartney, 2010) therefore data was adjusted to explore gender differences. No interactions between gender, liver enzymes, iAUC for NEFA and iAUC for TG were observed.

4.4.7 Conclusions and Clinical Implications

In conclusion our study suggests that children and adolescents with NAFLD are characterized by a constant hyperinsulinemic (fasted and postprandial) and pro-inflammatory state (continuous) which is worsen by the presence of high body fat percentage. This conclusion might have been strengthening with the analysis of C-peptide. C-peptide has been suggested to be present in amounts equal to insulin but presents a longer half life (Binder et al., 1984; Marques et al, 2004). Therefore, C-peptide measurement might have enhanced the ability to understand how peripheral insulin secretion is influenced in NAFLD (Marques et al, 2004). Lipid abnormalities are also present but the abnormalities are very similar to the obese non-NAFLD subjects with the exception of Apo B-48 at 6hrs post meal and fasted Apo C-III levels which were remarkable different in the NAFLD population. It is not completely clear what happens first; the hyperinsulinemia or the excess of fat as both characteristics were present in our subjects. Our study suggests that hyperinsulinemia could be the NAFLD key feature. Hyperinsulinemia is probably triggering the lipid abnormalities such as altered lipolysis as observed by the postprandial NEFA behavior and delayed clearance as suggested by the higher 6hrs post meal Apo B-48 levels, but this hyperinsulinemic surge should be also considered as a potential

compensatory mechanism as observed by the high Apo C-III fasted values followed by normal post-meal values. Human beings are in a constant postprandial state (Klop et al., 2011; Schneeman et al., 1993) therefore it is possible that with every single meal there is a hyperinsulinemic assault which promotes and exacerbates the high pro-inflammatory environment (as observed by the constantly higher TNF-α values in the NAFLD subjects) in this population which worsens even more the hyperinsulinemic state creating a vicious cycle and promoting a metabolic environment for future disease complications such as NASH. It is worth to mention that certain individuals (e.g. hyperinsulinemic and/or obese subjects) may experience even worse postprandial responses depending on the type of meal consumed and therefore the intake of saturated fat should be avoided (Colak et al., 2012). Our study suggests that treatment in children and adolescents with NAFLD should definitively include a change in the eating patterns. Changes should focus not just in the amount of total kilocalories to allow weight loss but also special emphasis should be placed in decreasing the SFA intake from the meals in order to improve lipid clearance in children and adolescents with NAFLD. By decreasing the fat load in a single meal concurrent with an increase of the LCPUFA such as C20:5n3 and C22:6n3 content, an improved lipid clearance and lower postprandial insulin surges may be expected to be observed. As many fast food meals that children and adolescents consume also have varying amounts of LCPUFA (e.g. fish based fast food meal) and/or fructose (addition of sweetened beverages), it is also important to study the extent to which these additions to a meal may evoke metabolic disturbances that could

acutely or chronically exacerbate the risk for NAFLD and/or other metabolic complications in an obese child.

CHAPTER 5. COMPARISON OF TWO MEAL CHALLENGES (LCPU FA free vs 1.5% LCPUFA) IN POSTPRANDIAL LIPID AND LIPOPROTEIN METABOLISM IN CHILDREN AND ADOLESCENTS WITH NONALCOHOLIC FATTY LIVER DISEASE (NAFLD)

5.1 INTRODUCTION

The increase of the obesity rates has caused a higher incidence in the metabolic syndrome in both, adults and children (Adam & Angulo, 2006; Flores-Calderón et al., 2005; Papandreau et al., 2007; Schwimmer et al., 2003; Utzschneider et al., 2006). Postprandial lipemia and delayed lipid clearance has been shown to be a feature of the metabolic environment in adults with NAFLD; but little is known regarding whether this is a feature in childhood NAFLD. Postprandial features in children and adolescents with NAFLD in comparison to obese and lean controls were presented in chapter 4.

Long term consumption of fish oil (Long Chain Polyunsaturated Fatty Acids; LCPUFA) has shown to increase HDL cholesterol (Sanders et al., 2003), reduce fasting VLDL lipids and apolipoprotein B concentration (Nestel et al., 1984) as well as postprandial TG in both, lean and overweight healthy and subjects with moderate and severe hypertriglyceridemia (Harris et al., 1988, Harris & Muzio, 1993; Jackson et al., 2005; Weintraub et al., 1988) by reducing chylomicron production or secretion in the enterocyte (Adiels et al., 2012; Demacker et al., 1991; Harris et al., 1988; Harris & Muzio, 1993; Matikainen et al., 2007). According to Sanders a diet containing 3g of fish oil per day stimulates

lipoprotein lipase (LPL) expression leading to decreases in postprandial lipemia (Bickerton et al., 2007; Sanders, 2003). TG plasma levels following a fat challenge are dose dependent and characteristics of the TG rich lipoproteins will vary depending on the meal´s fatty acid composition (López-Miranda et al., 2007; Nestel et al., 1984; Sanders, 2003). Clearance of fat occurs in the following order PUFA>MUFA>SFA (Karupaiah et al., 2011). Within the SFA class; chain length lipidemic response in normal weight subjects has been observed in the following order C18:0>C16:0>C12:0+C14:0 (Karupaiah et al., 2011). With regards to the unsaturated fatty acids, smaller postprandial TG increases have been observed following meals containing LCPUFA specially omega-3 such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Jiménez-Gómez et al., 2010; López-Miranda et al., 2007; Shah et al., 2007).

With regard to the postprandial insulin response to acute meal challenges contradictory results have been observed. Meal challenges enriched with EPA and DHA (at least 20g of omega-3 PUFA in 100 g of fatty acids) performed in male and female adults with insulin resistance have shown lower insulin responses when compared to isocaloric meals rich in SFA (Jans et al., 2012; Robertson et al., 2002; Shah et al., 2007) while others have observed no differences in the postprandial insulin responses when SFA meals have been compared to meals high in MUFA (Rasmussen et al., 1996; Thomsen et al., 2003). Little is known about the insulin postprandial response in children and adolescents and whether or not feeding children and adolescents with doses of omega-3 that can be found in food are sufficient to evoke a favorable postprandial response.

The purpose of this study was to determine if changes in the **LCPUFA** content of a high saturated fat meal evoke a more favorable response in insulin, lipid and lipoprotein expression in children and adolescents with NAFLD. We hypothesized that a meal high in saturated fat but containing higher omega-3 LCPUFA (specifically EPA and DHA) content will ameliorate postprandial hyperinsulinemia, lipemia and altered lipoprotein expression and potentially contribute to changes in fat balance across the liver in children and adolescents with NAFLD.

5.2 SUBJECTS and METHODS

Subjects were recruited as part of larger prospective study examining the influence of dietary intake on hepatic fat metabolism in children and adolescents with NAFLD. A total of n=6 (n=3 leans; n=3 NAFLD) subjects participated in all three studies (larger prospective study, the first postprandial study (Chapter 4) and this current study (Chapter 5). A total of n=11 (n=7 leans; n=4 NAFLD) newly consented for this study (Chapter 5).

5.2.1 Subjects

We prospectively studied lean (n=21) and children and adolescents with NAFLD (n=18) from the NAFLD group: Non-alcoholic steatohepatitis [NASH] =3, Simple steatosis [SS] =15. Inclusion/exclusion criteria were described as previous in section 3.2.2 and 4.2.2. From the total of children and adolescents diagnosed with NAFLD, 10 had biopsy-proven disease in addition to ultrasonic evidence of fat in the liver and serum blood testing that included ALT, AST,

GGT, CRP, hepatitis B & C, immunoglobulins, testing of autoimmune hepatitis, serum copper and ceruloplasmin for testing of Wilson Disease. Criteria for liver biopsy was as previously described in Section 4.2.2. Written informed consent/assent was obtained from the responsible caregiver and the patient prior to study entry. The study was approved by the University of Alberta Health Research Ethics Board. (**Appendix 1. Forms A-I**). Operational Approval from Alberta Health Services (AHS) and Administrative Approval from the Northern Alberta Clinical Trials Centre, University of Alberta/AHS/Caritas was obtained prior to subject recruitment.

5.2.2 Anthropometric and Body Composition Assessment

5.2.3 Subjects

Subjects anthropometric and body composition assessment was as previously described in Section 4.2.3 and 4.2.4.

5.2.4 Habitual Food intake

Three day food records were used to determine subjects' habitual intake and to assess dietary fat intake prior to the study days (Food Processor SQL, version 10.4.0, ESHA Research, Salem, OR, 2008) as previously described in Section 4.2.5.

5.2.5 Composition of Test Meals (0% vs 1.5% LCPUFA)

Two meals reflective of a typical fast food breakfast consumed by children and adolescents in North America (Pachuki, 2011) were compared in this study (**Table 5.1 and 5.2**). Meal 1 (chapter 4) reflects macronutrient and fat composition observed in North America (Pachuki, 2011) while the second meal,

also included specific foods present in popular breakfast choices consumed in North America, similar to an egg muffin served by fast food restaurants. **Meal 1** was a **high saturated/LCPUFA free (0% LCPUFA** as per gas liquid chromatography analysis) and the data was presented previously in chapter 4. The meal consisted of mozzarella-cheddar cheese, butter, white bread and chocolate milk; the amounts were previously described in chapter 4. SFA/MUFA ratio was 1.14 and SFA/PUFA ratio was 2.28, n-3/n-6 ratio was 0.23 (. **Meal 2** consisted of a **high saturated/higher LCPUFA (1.5% LCPUFA** as per gas liquid chromatography analysis**)**. LCPUFA dose was reflective of the content present in fast food and snack food (Fernández San Juan, 2000). The meal consisted of mozzarella cheese (25g), egg (1 medium), soybean oil (4.5g), butter (11g), chocolate milk (125ml) and 2 capsules of omega-3 fish oil (**each capsule: 122 mg DHA and 30 mg EPA**). SFA/MUFA ratio was 1.28 and SFA/PUFA ratio was 2.49, n-3/n-6 ratio was 0.18 (g of fat as per GLC). Different foods had to be used in the two different meals to keep the SFA/MUFA and SFA/PUFA ratios as similar as possible. All meals were isocaloric, isonitrogenous and differed in their LCPUFA content. Subjects were divided into four groups. For **0% LCPUFA** meal; group 1: NAFLD (n=11), Group 2: Lean (n=11). For **1.5% LCPUFA** meal; group 3: NAFLD (n=7), Group 4: Lean (n=10). Meal **0% LCPUFA** and/or **1.5% LCPUFA** was given to all participants after taking the first blood work (0 hours or study baseline; see fasting and postprandial blood work section). Participants were given 10-15 minutes to finish their meal. Macronutrient distribution was analyzed based on the total amount of kilocalories present in the meal and was

analyzed using the Food Processor SQL (version 10.4.0, ESHA Research, Salem, OR, 2008) (**See table 5.1**). CNF, the USDA nutrient database and commercial food label information from the individual brand types were used to calculate the fructose content of the meal challenge. Mixed meal approach was used to calculate the GL (Collier et al., 1998; Ebbeling et al., 2004). Since dietary TG represent above the 90% of the total fat intake (Cohn et al., 2010) only the fatty acid composition of total TG was analyzed. Fatty acid composition of total TG of the meal was assessed using gas liquid chromatography (**See table 5.2**). The lipid components were extracted using the chloroform-methanol process (Folch et al., 1957). An internal standard was added (5mg of C15:0) to all samples. Samples were saponified (using KOH in methanol) and methylated using boron trifluoride (BF_3) and hexane (Murphy et al., 2011). The total amounts of individual FA were calculated from the proportion of FA of measured FA (by GC analysis) multiplied by the total fat (g) within the meal as determined by Food Processor.

Table 5.1. Macronutrient distribution

	Meal 1; 0% LCPUFA	Meal 2; 1.5% LCPUFA
Kilocalories	421.71	427.99
Protein (%)	17.35	17.67
Carbohydrates (%)	39.04	29.04
Fat (%)	43.34	53.21
Trans fat (g)	0.74	0.02
Cholesterol (mg)	52.18	215.02
Fructose (mg)[1]	568	568
Glycemic Index[2]	114	120
GL	20	17

[1]Fructose analysis was calculated using the USDA nutrient database, CNF and commercial food label information from the individual brand types. [2]Foster-Powell et al., 2002; Jenkins et al., 1981.

Table 5.2. Fatty acid composition of triglycerides in the test meal

	Meal 1; 0%LCPUFA	Meal 2; 1.5% LCPUFA
Fatty Acid as per GC analysis	Percentage (%)	Percentage (%)
Saturated		
C10:0	0.56	ND
C12:0	2.63	ND
C13:0	0.56	ND
C14:0	8.19	8.05
C16:0	25.22	31.49
C18:0	6.24	12.52
Monounsaturated		
C14:1	0.79	ND
C16:1	1.30	1.72
C18:1	35.51	31.42
Long chain polyunsaturated		
C18:2n6	15.23	11.59
C18:3n3	3.76	1.47
C20:2n6	ND	ND
C20:3n6	ND	ND
C20:4n6	ND	**0.38**
C20:5n3	ND	**0.27**
C22:6n3	ND	**0.83**

Average of SD between samples for 0% LCPUFA was 0.05, and for 1.5%LCPUFA was 0.12. Since dietary TG represent above the 90% of the total fat intake (Cohn et al., 2010) only the fatty acid composition of total TG was analyzed.

5.2.6 Baseline and Postprandial Blood work

Samples were collected as previously described in Section 4.2.7.

5.2.7 Fasting Blood work: Liver Biochemistries, C- reactive protein and leptin

Fasting blood (baseline or prior to meal consumption) was collected for measurement as previously described in Section 4.2.8.

5.2.8 Postprandial Blood work: Insulin and glucose, lipids, cholesterol and NEFA, Apolipoproteins B-48, B-100 and C-III.

Postprandial blood work was analyzed as previously described in Section 4.2.9.

5.2.9 Plasma Fatty Acid profile of Triglyceride (TG) and Phospholipid (PL) fractions

Postprandial blood work was analyzed ad previously described in Section 4.2.10.

5.2.10 Inflammatory mediators and adiponectin

Postprandial blood work was analyzed ad previously described in Section 4.2.11.

5.2.11 Statistical analysis

Results obtained followed the consumption of the **high SFA/1.5% LCPUFA** meal were compared to the **high SFA/0% LCPUFA** meal challenge. Data is expressed as mean $\pm$ SEM unless otherwise specified. Differences between groups were analyzed by repeated measures two way analysis of variance (ANOVA). Tests for deviations from Gaussian distribution were performed using the D'Agostino-Pearson omnibus and Shapiro-Wilk normality tests (Graph Pad

PRISM Software. version 5.0 La Joya California USA). Non-parametric tests (Kruskal Wallis test) were utilized for variables with skewed distributions. Repeated measures two-way analysis of variance (group and meal effects) were used to test the interaction between time and group (a total of 4 groups were analyzed: lean & NAFLD **0% LCPUFA** and lean & NAFLD **1.5% LCPUFA**). When a significant interaction was found between factors, differences across groups were analyzed by two way analysis of variance repeated measures followed by Bonferroni's correction (Graph Pad PRISM Software. version 5.0 La Joya California USA). Multiple regression was performed to examine the relationship between several independent (predictor) variables and a dependent variable (IBM SPSS statistics. version 19.0 Chicago IL USA). The incremental area under the curve (iAUC) of different metabolites during the meal test were calculated by the trapezoidal method (Graph Pad PRISM Software. version 5.0 La Joya California USA). Delta calculations between 6hrs post meal and fasted, 3hrs post meal and fasted and 1hr post meal and fasted (Δ6hrs-0hrs, Δ3hrs-0hrs, Δ1hr-0hrs) were performed in order to determine the postprandial clearance between the two meals. T-test followed by Welch´s correction was performed when comparing two groups (0% leans vs 1.5% leans & 0% NAFLD vs 1.5% NAFLD). Differences were considered statistically significant if $p < 0.05$.

5.3 RESULTS

5.3.1 Anthropometric and Demographic Variables

No significant differences were observed between the two lean groups or the two NAFLD groups. Both NAFLD groups were obese (BMI z-score 2.3$\pm$0.1

and 2.2±0.1, respectively) with waist circumference > 97th percentile (Cole et al., 2000). Intra group values (Lean 0% LCPUFA vs lean 1.5% LCPUFA and NAFLD lean 0% LCPUFA vs 1.5% LCPUFA) for body fat percentage, z-scores, waist circumference, waist to hip and height ratio were not significantly different. Baseline characteristics of the groups are described in **Table 5.3**.

Table 5.3. Anthropometric baseline characteristics

Anthropometric data	Lean 0% LCPUFA (n=11) 5F, 6M	Lean 1.5% LCPUFA (n=10) 6F, 4M	p value (0% lean vs 1.5% lean)	NAFLD 0% LCPUFA (n=11) 1F, 10M	NAFLD 1.5% LCPUFA (n=7) 1F, 6M	p value (0% NAFLD vs 1.5% NAFLD)	p value (ANOVA)
Age (y)	13.0 ± 0.8[a]	13.7 ± 0.9[a]	0.5	12.5 ± 0.9[a]	14.0 ± 0.7[a]	0.2	0.8
BMI z-scores	0.1 ± 0.3[a]	-0.01 ± 0.2[a]	0.6	2.3 ± 0.1[b]	2.2 ± 0.1[b]	0.6	< 0.0001
Weight z-scores	-0.05 ± 0.3[a]	0.1 ± 0.2[a]	0.7	2.5 ± 0.2[b]	2.6 ± 0.2[b]	0.7	<0.0001
Height z-scores	-0.06 ± 0.3	0.4 ± 0.2	0.2	0.8 ± 0.4	1.0 ± 0.3	0.6	0.12
Waist circumference(cm)	63.8 ± 2.6[a]	64.6 ± 1.6[a]	0.7	98.2 ± 5.0[b]	100.4 ± 4.9[b]	0.7	< 0.0001
w/hip ratio (cm)	0.7 ± 0.0[a]	0.7 ± 0.0[a]	0.1	0.9 ± 0.0[b]	0.9 ± 0.0[b]	0.8	< 0.0001
Waist to Height Ratio (WHR)	0.4 ± 0.0[a]	0.3 ± 0.0[a]	0.1	0.6 ± 0.0[b]	0.5 ± 0.0[b]	0.6	< 0.0001
Body fat (%); Bod Pod*	15.3 ± 1.7[a]	11.7 ± 2.3[a]	0.2	33.9 ± 3.8[b]	35.3 ± 4.4[b]	0.8	< 0.0001
Fat mass, kg (FM)	7.5 ± 1.3[a]	5.6 ± 1.0[a]	0.2	28.5 ± 1.5[b]	37.5 ± 9.9[b]	0.4	0.0001

Values are mean ± SEM. Different superscripts indicate significant differences (p<0.05) between groups by repeated measures one way anova after following Bonferroni correction pairwise comparison was performed.

*Normal body fat (%) according to the CDC study (Ogden et al., 2011): mean levels of body fat (%) at age 8 were 28% for boys and 31% for girls: mean levels and at age 19 were 23% for boys and 35% for girls.

5.3.2 Fasting Laboratory Parameters

Fasting laboratory values were similar at baseline between the two lean groups and between the two NAFLD groups. Comparing lean and NAFLD, NAFLD subjects had significant elevations in ALT and AST compared to the lean group ($p<0.0001$). CRP was significantly higher in the NAFLD subjects ($p=0.01$) when compared to leans. With regard to the lipid profile, no significant differences were observed between groups for fasting concentrations of LDL and total cholesterol (see **Table 5.4**). HDL-cholesterol was significantly lower in the NAFLD subjects when compared to healthy controls ($p<0.0001$). Fasting insulin and leptin were significantly higher ($p<0.0001$) in the NAFLD subjects when compared to the lean groups (**Table 5.4**).

Table 5.4. Fasted metabolic characteristics of the patients

	Lean 0% LCPUFA (n=11) 5F, 6M	Lean 1.5% PUFA (n=10) 6F, 4M	p value (0% lean vs 1.5% lean)	NAFLD 0% PUFA (n=11) 1F, 10M	NAFLD 1.5% PUFA (n=7) 1F, 6M	p value (0% NAFLD vs 1.5% NAFLD)	p value (ANOVA)
ALT (U/L)[1]	17.0 ± 1.5[a] (12-28)	16.0 ± 1.5[a] (10-25)	0.7	87.0 ± 16.1[b] (17-175)	76.0 ± 23.9[b] (16-200)	0.6	< 0.0001
AST(U/L)[2]	24.0 ± 1.9[a] (17-35)	22.0 ± 1.4[a] (15-30)	0.4	52.0 ± 9.9[b] (20-132)	43.5 ± 8.6[a,b] (18-86)	0.5	<0.0001
CRP (mg/L)[3]	0.6 ± 0.2[a] (0.2-3.5)	0.5 ± 0.1[a] (0.2-2.2)	0.7	4.1 ± 1.4[b] (0.5-15)	1.5 ± 0.3[a,b] (0.2-3.0)	0.1	0.01
GGT (U/L)[4]	7.0 ± 4.9 (5.0-21.0)	5.0 ± 0.0 (5.0-5.0)	0.2	47.1 ± 21.8 (10.0-256.0)	22.7 ± 4.0 (9.0-37.0)	0.3	0.06
Glucose (mmol/L)[5]	4.6 ± 0.1 (3.8-5.1)	4.5 ± 0.1 (3.6-5.1)	0.6	5.1 ± 0.2 (4.3-6.5)	5.0 ± 0.2 (4.1-5.8)	0.6	0.06
Insulin (mU/L)[6]	8.6 ± 1.4[a] (4.1-20.6)	10.7 ± 1.8[a] (2.2- 24.4)	0.3	30.8 ± 5.2[b] (11.8-56.1)	26.7 ± 5.5[b] (10.5-47.2)	0.6	< 0.0001

Values are mean ± SEM (except otherwise mentioned). Different superscripts indicate significant differences (p<0.05) between groups by repeated measures one way anova after following Bonferroni correction pairwise comparison was performed. Ranges (minimum-maximum) are expressed within the parentheses.

Normal values: [1] ALT <50(U/L), [2] AST<40 (U/L), [3] CRP <8.0 (mg/L), [4] GGT <70 (U/L), [5] glucose: 3.3-11.0 (mmol/L), [6] Insulin 5.0-20.0 (mU/L), [7] Triglycerides<1.50 (mmol/L), [8] Total cholesterol <4.40 (mmol/L), [9] HDL >1.00 (mmol/L), [10] LDL <2.80 (mmol/L), [11] Leptin: lean subjects with a body fat % of 16.2%: 2.74 mg/L in girls, 1.59 mg/L in boys (Blum et al., 1997), [12] HOMA-IR <3

Table 5.4 continues…

	Lean 0% LCPUFA (n=11) 5F, 6M	Lean 1.5% PUFA (n=10) 6F, 4M	p value (0% lean vs 1.5% lean)	NAFLD 0% PUFA (n=11) 1F, 10M	NAFLD 1.5% PUFA (n=7) 1F, 6M	p value (0% NAFLD vs 1.5% NAFLD)	p value (ANOVA)
Triglyceride (mmol/L)[7]	0.7 ± 0.0[a,b] (0.3-1.2)	0.6 ± 0.0[a] (0.3-0.9)	0.1	1.3 ± 0.2[b] (0.5-3.3)	1.5 ± 0.2[b] (0.8-2.4)	0.6	<0.0001
Total cholesterol (mmol/L)[8]	3.9 ± 0.2 (3.1-4.9)	3.6 ± 0.1 (3.2-4.3)	0.2	4.2 ± 0.4 (2.7-6.8)	3.7 ± 0.2 (3.0-4.8)	0.3	0.7
HDL cholesterol (mmol/L)[9]	1. 4 ± 0.0[a] (0.9-2.0)	1.2 ± 0.0[a] (0.9-1.5)	0.3	0.8 ± 0.0[b] (0.5.1.1)	0.9 ± 0.0[b] (0.7-1.1)	0.2	< 0.0001
LDL cholesterol (mmol/L)10	2.2 ± 0.1 (1.2-3.0)	2.1 ± 0.1 (1.7-2.7)	0.6	2.7 ± 0.3 (1.5-4.5)	2.1 ± 0.1 (1.6-2.7)	0.1	0.2
Leptin (ng/mL)[11]	0.7 ± 0.1[a] (0.02-1.7)	0.4 ± 0.1[a] (0.0-1.2)	0.2	2.2 ± 0.3[b] (0.6-4.3)	1.9 ± 0.3[b] (1.0-3.5)	0.5	< 0.0001
HOMA-IR[12]	1.8 ± 0.3[a] (4.3-0.8)	2.2 ± 0.3[a] (4.8-0.3)	0.4	7.3 ± 1.4[b] (15.7-2.7)	6.1 ± 1.4[b,c] (12.1-2.4)	0.5	0.0004

Values are mean ± SEM (except otherwise mentioned). Different superscripts indicate significant differences (p<0.05) between groups by repeated measures one way anova after following Bonferroni correction pairwise comparison was performed. Ranges (minimum-maximum) are expressed within the parentheses.

Normal values: [1] ALT <50(U/L), [2] AST<40 (U/L), [3] CRP <8.0 (mg/L), [4] GGT <70 (U/L), [5] glucose: 3.3-11.0 (mmol/L), [6] Insulin 5.0-20.0 (mU/L), [7] Triglycerides<1.50 (mmol/L), [8] Total cholesterol <4.40 (mmol/L), [9] HDL >1.00 (mmol/L), [10] LDL <2.80 (mmol/L), [11] Leptin: lean subjects with a body fat % of 16.2%: 2.74 mg/L in girls, 1.59 mg/L in boys (Blum et al., 1997), [12] HOMA-IR <3.

5.3.3 Postprandial meal responses for laboratory variables

Four groups are presented (Lean 0% LCPUFA, NAFLD 0% LCPUFA, Lean 1.5% LCPUFA and NAFLD 1.5% LCPUFA) as well as comparisons between treatments (NAFLD 0% LCPUFA vs NAFLD 1.5% LCPUFA and lean 0% LCPUFA vs lean 1.5% LCPUFA). Data is presented in time course series for postprandial meal response (at times 0, 1, 3 & 6 hrs) and as incremental area under the curve (iAUC) if significant differences were observed. For the entire data set no significant interactions between meal and group were observed.

5.3.4 Postprandial insulin and glucose response

Absolute and incremental postprandial changes in plasma insulin concentrations are shown in **Figure 5.1a and 5.1b.** Insulin levels in the four groups increased post meal, reaching the highest value at 1 hour. When comparing 0% LCPUFA NAFLD vs lean, NAFLD subjects exhibited significantly higher values at 1 and 3 hrs post meal ($p<0.001$, $p<0.05$; respectively) when compared to lean controls. The comparison between 1.5% LCPUFA NAFLD vs lean showed significant differences at 1 hr post meal ($p<0.001$) see **Figure 5.1a**. No significant differences were observed between meals within the NAFLD or lean. Incremental area under the curve (iAUC) was significantly higher in both NAFLD groups compared to the same meal in the lean group ($p=0.01$). No significant interactions between meal and group were observed ($p>0.05$). No intragroup statistical differences were observed ($p=0.2$ for leans and 0.4 for NAFLD) see **Figure 5.1b**.

Glucose absolute values were significantly higher in the NAFLD 0% LCPUFA population at one hour post meal when compared to the 0% LCPUFA lean controls ($p<0.001$) see **Figure 5.2**. No significant differences were observed between treatments. A trend towards lower iAUC ($p=0.1$) was observed in the NAFLD 1.5% LCPUFA when compared to the NAFLD 0% LCPUFA group (**Figure P. Appendix 2)**. No significant interaction between meal and group was observed ($p>0.05$).

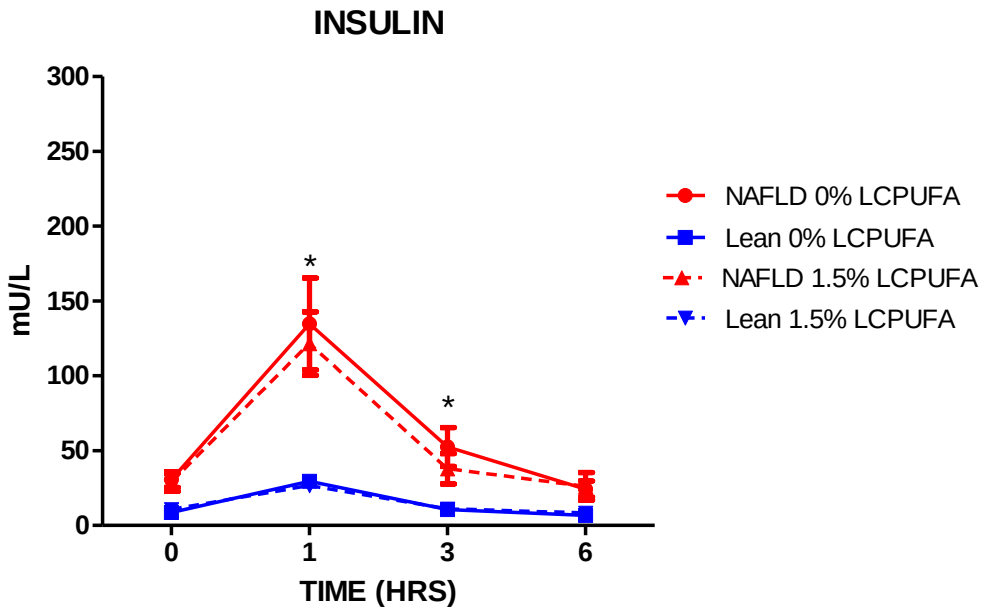


Figure 5.1a. Insulin concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal low in PUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=7) and healthy lean-age matched (n=10). NAFLD 0% LCPUFA vs lean 0% LCPUFA $p<0.05$ at 1 and 3 hrs post meal. NAFLD 1.5% LCPUFA vs lean 1.5% LCPUFA $p<0.05$ at 1 hr post meal. Results are shown mean $\pm$ SEM. * Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

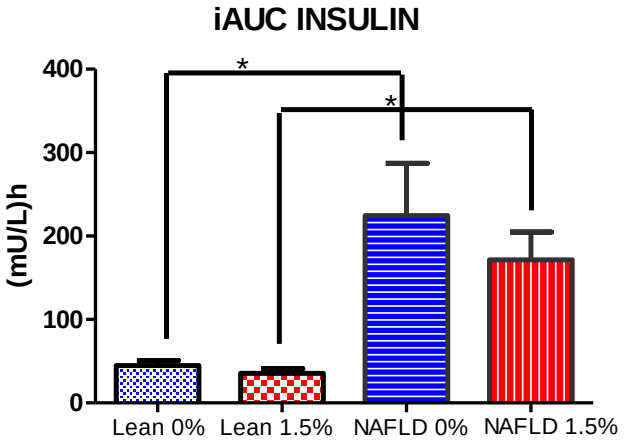


Figure 5.1b. Postprandial incremental area under the curve (iAUC) for insulin following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=7) and healthy lean-age matched (n=10). Values with different superscripts are significantly different by repeated measures two way ANOVA; following Bonferroni correction and pairwise comparisons. No significant interaction between meal and group was observed ($p>0.05$). Results are shown mean ± SEM. *Indicates significant difference when $p<0.05$.

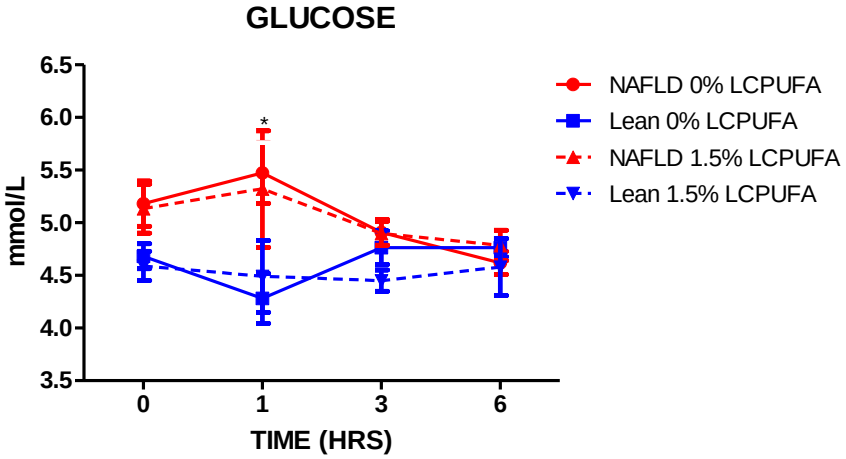


Figure 5.2. Glucose concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=7) and healthy lean-age matched (n=10). NAFLD 0% LCPUFA population vs lean 0% LCPUFA $p<0.05$ at 1 hr post meal Results are shown mean ± SEM. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

5.3.5 Postprandial TG, NEFA, LDL, HDL and cholesterol profile

5.3.5.1 *Triglycerides (TG)*

Peak plasma values for TG were observed at 3 hours post meal in all the groups. At 6hrs post meal NAFLD 0% LCPUFA subjects exhibited higher ($p<0.05$) TG values when compared to lean 0% LCPUFA subjects (**Figure 5.3**). On the other hand, NAFLD 1.5% LCPUFA subjects exhibited significant higher values at 0, 1, 3 and 6 hrs when compared to lean 1.5% LCPUFA subjects ($p<0.05$) see **Figure 5.3**. No significant differences between groups and treatments were observed when calculating iAUC (**Figure Q. Appendix 2)**. No significant interaction between meal and group was observed ($p>0.05$).

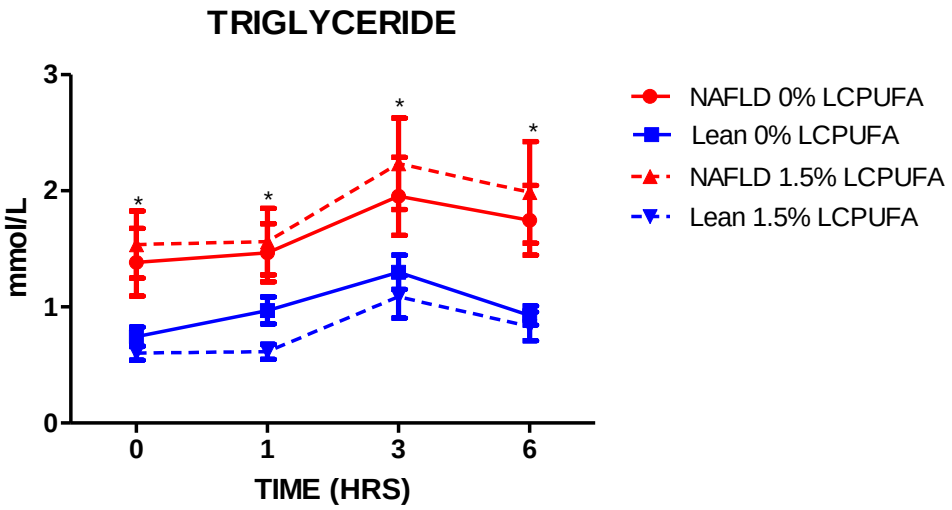


Figure 5.3. Triglyceride concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.48% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=7) and healthy lean-age matched (n=10). NAFLD 1.5% LCPUFA vs lean 1.5% LCPUFA significant at 0, 1, 3 and 6 hrs ($p<0.05$). Significant ($p<0.05$) higher values in NAFLD 0% LCPUFA vs lean 0% LCPUFA subjects at 6hrs. Results are shown mean $\pm$ SEM. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

5.3.5.2 *Non-esterified Fatty Acids (NEFA)*

Significantly higher NEFA values were observed in the NAFLD 0% LCPUFA subjects at 1 hour post meal ($p<0.05$) when compared to lean 0% LCPUFA controls (**Figure 5.4a**). No other significant differences between treatments were observed. Significant lower ($p=0.02$) iAUC was observed in the NAFLD 1.5% LCPUFA group when compared to the NAFLD 0% LCPUFA group. A positive trend between meal and group interaction was observed ($p=0.08$). No significant differences between the two lean groups were observed (**Figure 5.4b**).

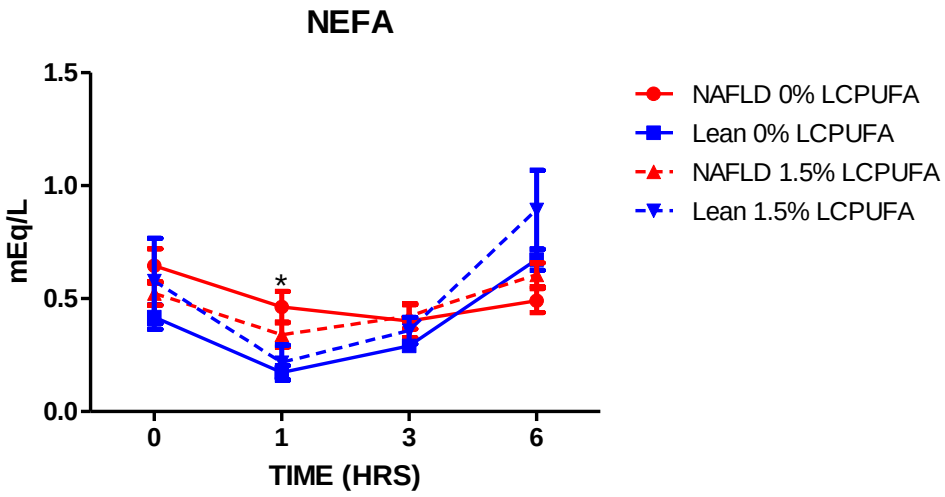


Figure 5.4a. Total non-esterified fatty acids (NEFA) concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). NAFLD 0% LCPUFA subjects vs lean control 0% LCPUFA $p<0.05$ at 1 hour post meal Results are shown mean $\pm$ SEM. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

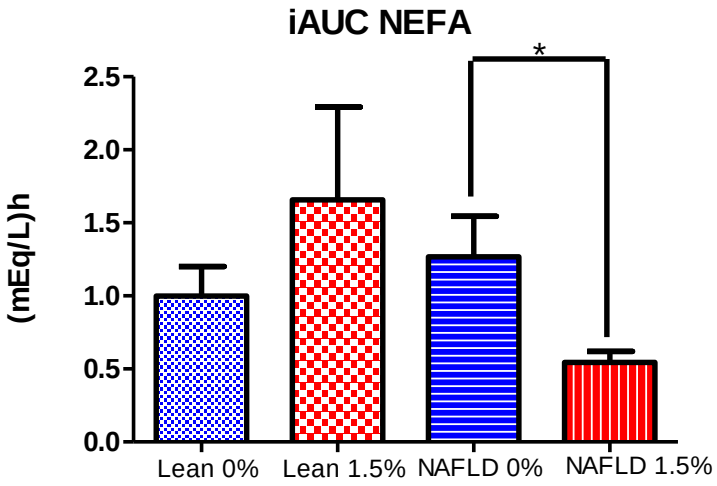


Figure 5.4b. Postprandial incremental area under the curve (iAUC) for non-esterified fatty acids (NEFA) following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). Values with different superscripts are significantly different by repeated measures two way ANOVA following Bonferroni correction and pairwise comparisons. No significant interaction between meal and group was observed ($p>0.05$). Results are shown mean $\pm$ SEM. *Indicates significant difference when $p<0.05$

5.3.5.3 *LDL, HDL and cholesterol profile*

No significant differences over time **(Figure 5.5)** or in the LDL-cholesterol iAUC were observed between the four groups **(Figure R. Appendix 2**). HDL levels were significantly lower at 0, 1, 3 and 6hrs post meal ($p<0.001$) in the NAFLD 0% LCPUFA subjects when compared to the lean 0% LCPUFA subjects. Additionally, significantly lower values ($p<0.05$) were observed in the NAFLD 1.5% LCPUFA group at 6hrs post meal when compared to lean 1.5% LCPUFA subjects. No significant differences were observed between treatments (**Figure 5.6**). No significant differences in iAUC for HDL were observed between groups and treatment (**Figure S. Appendix 2**). Total cholesterol showed no

significant differences between groups and treatments (**Figure 5.7a**). Significant lower iAUC for total cholesterol was observed in the 1.5% LCPUFA NAFLD group when compared to lean 1.5% LCPUFA controls (p=0.04). No significant postprandial differences between treatments were observed (**Figure 5.7b**).

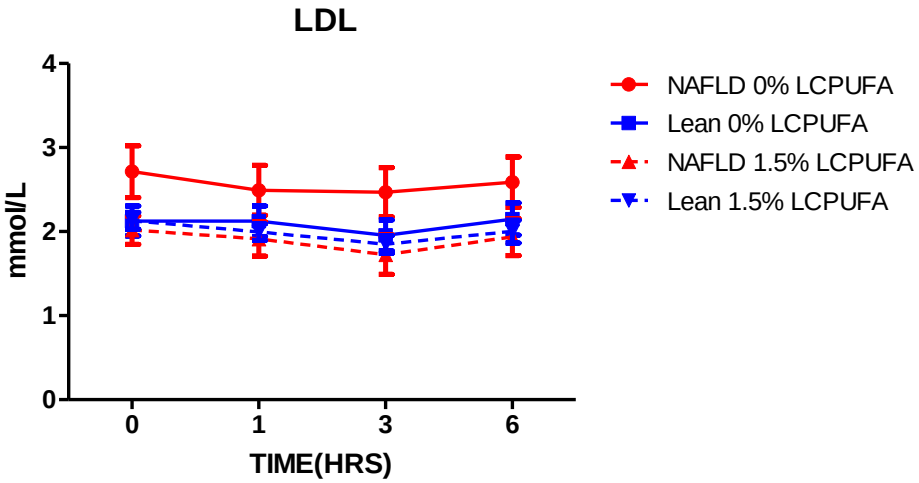


Figure 5.5. LDL concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=7) and healthy lean-age matched (n=10). Results are shown mean $\pm$ SEM. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

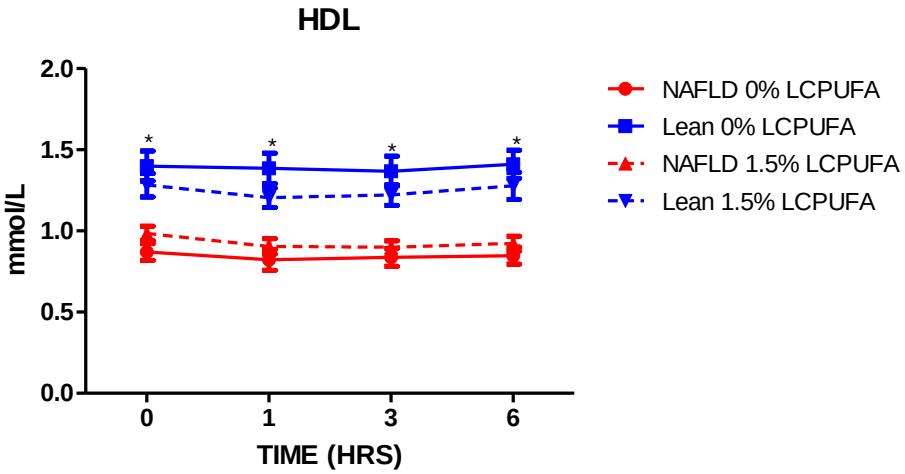


Figure 5.6. HDL concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=7) and healthy lean-age matched (n=10). Significant ($p<0.05$) NAFLD 0% LCPUFA vs lean 0% LCPUFA subjects at 0, 1, 3 and 6hrs. NAFLD 1.5% LCPUFA vs lean 1.5% LCPUFA significant ($p<0.05$) at 6hrs. Results are shown mean $\pm$ SEM. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

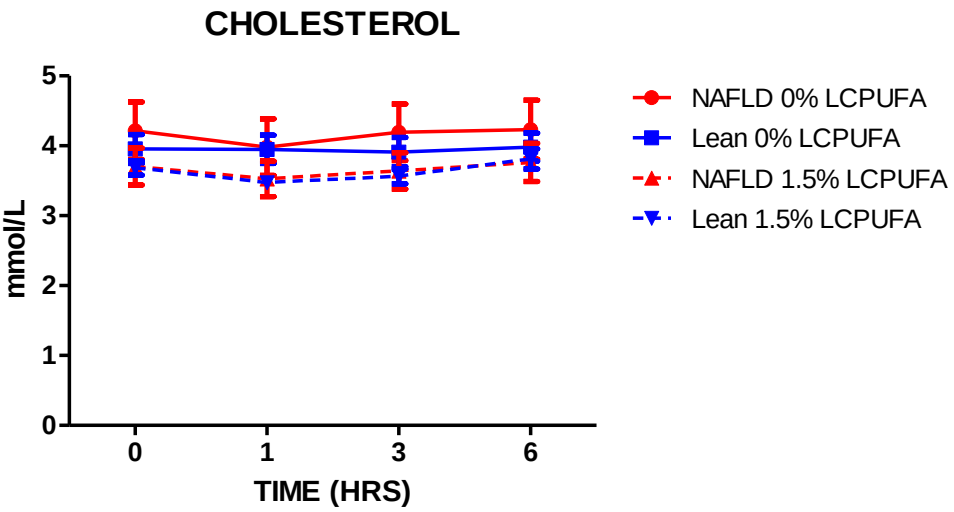


Figure 5.7a. Total cholesterol concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=7) and healthy lean-age matched (n=10). Results are shown mean ± SEM. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

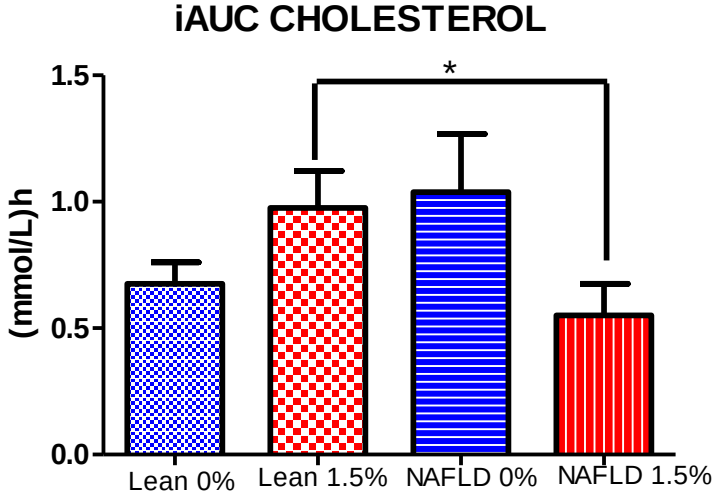


Figure 5.7b. Postprandial incremental area under the curve (iAUC) for Total cholesterol following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic

fatty liver disease (n=7) and healthy lean-age matched (n=10). Values with different superscripts are significantly different by repeated measures two way ANOVA; following Bonferroni correction and pairwise comparisons. No significant interaction between meal and group was observed ($p>0.05$). Results are shown mean ± SEM. * Indicates significant difference when $p<0.05$.

5.3.6 Fatty acid profile of plasma Triglyceride (TG) Fraction

The fatty acid (FA) profiles (in percentages) and concentrations (μg/ml) corresponding to high SFA/1.5% LCPUFA NAFLD and lean group are presented in **Table 5.5a and 5.5b**. Fatty acid (FA) profiles (in percentages) and concentrations (μg/ml) corresponding to high SFA/0% LCPUFA NAFLD and lean group are presented in **chapter 4 Table 4.4a and 4.4b**. Four groups' time course data [NAFLD 0% LCPUFA (meal 1), Lean 0% LCPUFA (meal 1), NAFLD 1.5% LCPUFA (meal 2) and Lean 1.5% LCPUFA (meal 2)] is presented for the fatty acids in which significant values between meal challenges were observed. Significant differences ($p<0.05$) between treatments are shown by an asterisk. For significant time intra group differences refer to the **Table 4.4a** and **4.4b, chapter 4** (high SFA/0% LCPUFA NAFLD vs lean) and **Table 5.5a** and **5.5b** below (high SFA/1.5% LCPUFA NAFLD vs lean).

Significant lower myristic acid (C14:0) fraction values ($p<0.05$) were observed in the lean 1.5% LCPUFA group when compared to the lean 0% LCPUFA group at 6hrs post meal (see **figure 5.8**). Significant higher C14:0 iAUC ($p<0.04$) was observed in the NAFLD 0% LCPUFA [44.10 ± 12.01 (μg/ml) h] when compared to the NAFLD 1.5% LCPUFA [15.82 ± 4.41 (μg/ml) h]. No significant differences in iAUC were observed when comparing the two lean

groups. Significant higher stearic acid (C18:0) fraction values (**see figure 5.9**) were observed on the lean 1.5% LCPUFA when compared to the lean 0% LCPUFA in the fasted state and at 1hr post meal ($p<0.01$, 0.05; respectively). No significant differences were observed in the C18:0 iAUC. Significantly higher linoleic acid (C18:2n6) fraction values (**see figure 5.10**) were observed in the lean 1.5% LCPUFA groups when compared to the lean 0% LCPUFA group at 6hrs post meal ($p<0.05$). No significant differences were observed in the C18:2n6 iAUC. Significant lower linolenic acid (C18:3n3) fraction values were observed in the lean 1.5% LCPUFA when fasted and at 1 hr post meal (see **figure 5.11**) when compared to the lean 0% LCPUFA ($p<0.001$, 0.01; respectively). No significant differences were observed in the C18:3n3 iAUC. Similar trend was observed when analyzing the sum of n-3 when fasted and 1 hr post meal ($p<0.001$, 0.01; respectively).

In order to calculate the postprandial clearance between meal challenges (intra group), Deltas between 1hr, 3hrs, 6hrs post meal and fasted (Δ1hr-fasted, Δ3hrs-fasted, Δ6hrs-fasted) were calculated (see **Table 5.6a and 5.6b**).

Table 5.5a. Plasma TG fatty acid composition (percentages) at postprandial time points for 1.5% LCPUFA (Meal 2)

Fatty Acid	TG (%) LEAN				TG (%) NAFLD			
	Fasted	1hr	3hr	6hr	Fasted	1hr	3hr	6hr
14:0	2.5±0.6	2.8±0.5	3.1±0.6	2.4±0.5	1.9±0.6	1.8±0.4	2.3±0.6	1.9±0.4
16:0	28.3±2.1	30.6±2.7	29.4±1.4	28.9±1.5	27.3±4.2	30.4±4.8	27.7±4.4	27.3±4.6
16:1	2.3±0.4	2.5±0.4	2.5±0.3	2.4±0.4	2.7±0.8	3.4±1.0	2.2±0.8	2.4±1.0
18:0	7.4±0.5[a]	7.6±0.5[a]	7.2±0.4[a]	7.4±0.7[a]	4.6±0.3[b]	4.9±0.7[b]	5.0±0.3[b]	4.5±0.4[b]
18:1	41.1±1.6	37.3±3.7	40.1±1.6	39.1±0.8	44.6±3.1	38.3±7.1	43.2±2.7	43.4±3.1
18:2n6	15.1±1.7	16.3±1.8	15.7±0.9	16.9±1.2	17.6±1.1	20.0±2.7	18.1±1.9	19.7±2.9
18:3n3	ND	ND	0.1±0.1	ND	ND	ND	0.1±0.1	ND
20:4n6	0.6±0.4	0.3±0.3	0.2±0.1	0.2±0.2	0.2±0.2	0.4±0.2	0.5±0.1	0.4±0.2
20:5n3	ND	ND	ND	ND	ND	ND	ND	ND
22:6n3	ND	ND	0.1±0.1	0.1±0.1	ND	ND	ND	ND
Σn-3	ND	ND	0.2±0.2	0.1±0.1	0.1±0.0	ND	0.1±0.1	ND
Σn-6	15.7±1.9	16.7±1.8	15.9±1.0	17.1±1.3	17.8±1.2	20.5±2.7	18.7±2.0	20.2±2.9
ΣMUFA	43.4±1.5	39.7±3.7	42.6±1.3	41.5±0.7	47.3±2.6	41.7±6.3	45.6±2.3	45.8±2.5
ΣSFA	38.2±2.7	41.0±3.4	39.7±1.8	38.7±1.6	33.8±4.2	37.1±4.9	35.0±4.0	33.8±4.5
Σμg/ml	608.5±337.3	535.9±194.3	1242.6±547.4	1052.9±502.0	628.5±253.8	542.3±133.1	603.2±150.9	581.2±158.4

Values are mean ± SEM (except otherwise mentioned). Variables with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Total fatty acids sum is expressed in μg/ml.

Table 5.5b. Plasma TG fatty acid composition (µg/ml) at postprandial time points for 1.5% LCPUFA (Meal 2)

Fatty Acid	TG (µg/ml) LEAN				TG (µg/ml) NAFLD			
	Fasted	1hr	3hr	6hr	Fasted	1hr	3hr	6hr
14:0	7.9±2.3	14.5±8.3	38.9±18.9	25.6± 13.4	7.2±2.1	7.2±2.3	11.1±3.0	10.0±2.8
16:0	167.7± 92.7	171.3± 85.7	381.8± 178.8	322.6± 161.1	186.4±74.4	168.6±44.6	189.5±54.0	185.0±54.0
16:1	18.4± 13.4	8.6± 2.5	36.9± 18.6	26.6± 13.4	14.5±3.2	16.8±5.1	15.0±6.0	13.9±5.1
18:0	37.6± 18.9	45.4± 19.4	83.6± 36.0	79.9± 38.5	32.4±16.4	22.3±4.6	27.4±5.5	24.4±4.7
18:1	278.1± 167.9	161.7± 61.7	487.9± 208.4	395.6± 180.4	275.7±116.6	230.0±60.3	254.0±67.5	241.0±76.8
18:2n6	85.9± 44.3	101.7± 45.5	199.1± 88.7	178.8± 91.2	106.7±43.4	92.5±21.7	99.7±22.4	102.0±23.0
18:3n3	ND	ND	0.8±0.8	ND	0.4±0.4	0.3±0.3	0.8±0.8	0.0±0.0
20:4n6	0.8±0.6	5.0±5.0	0.8±0.6	1.0±1.0	3.1±3.1	3.1±1.9	3.8±1.8	3.6±2.0
20:5n3	ND	ND	ND	ND	ND	ND	ND	ND
22:6n3	ND	ND	0.4±0.4	0.5±0.4	ND	ND	ND	ND
Σn-3	ND	ND	1.1±1.1	0.5±0.4	0.6±0.4	0.5±0.3	1.0±0.8	0.2±0.2
Σn-6	86.7± 44.3	108.4± 48.7	199.9± 88.6	179.8± 91.1	109.9±46.4	95.7±23.2	103.7±24.0	105.9±24.7
ΣMUFA	296.6± 181.1	170.4± 61.3	524.8± 226.3	422.2± 193.7	290.2±118.1	246.8±62.4	268.9±69.8	255.0±77.5
ΣSFA	213.2± 113.2	231.2± 112.2	504.4± 230.9	428.2± 212.8	226.0±90.3	198.2±49.4	228.1±60.1	219.5±59.9

Values are mean ± SEM (except otherwise mentioned). Variables with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

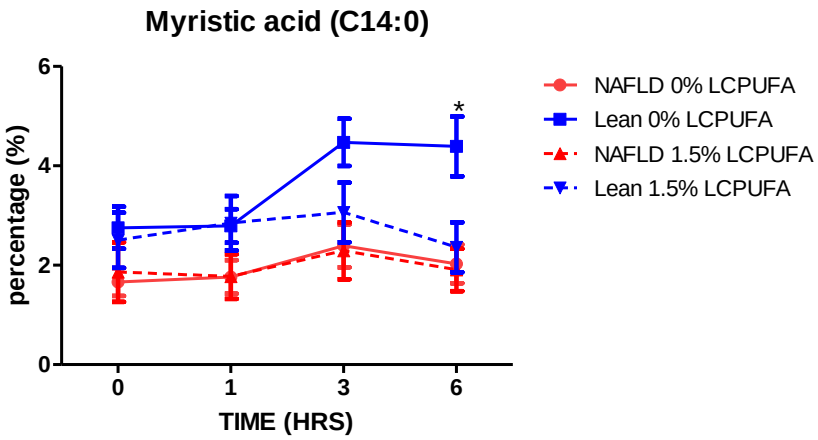


Figure 5.8. Plasma C14:0 concentrations in TG fractions prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). Only differences between meal challenges are shown in the graph. Lower C14:0 values ($p<0.05$) were observed in the lean 1.5% LCPUFA group when compared to the lean 0% LCPUFA group at 6hrs post meal. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

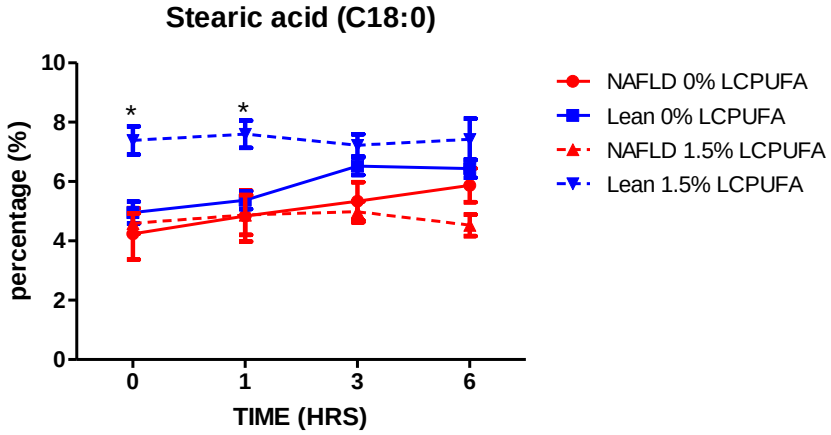


Figure 5.9. Plasma C18:0 concentrations in TG fractions prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). Only differences between meal challenges are shown in the graph. Higher C18:0 values ($p<0.05$) were observed on the lean 1.5% LCPUFA when compared to the lean

0% low LCPUFA on the fasted state and at 1hr post meal. *Indicates significant difference by repeated measures two way ANOVA when p<0.05 followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

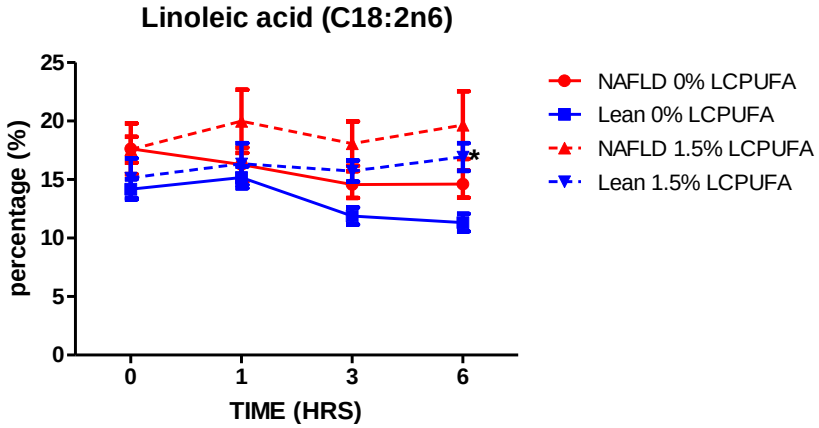


Figure 5.10. Plasma C18:2n6 concentrations in TG fractions prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). Only differences between meal challenges are shown in the graph. Higher C18:2n6 values (p<0.05) were observed in the lean 1.5% LCPUFA groups when compared to the lean 0% LCPUFA group at 6hrs post meal. *Indicates significant difference by repeated measures two way ANOVA when p<0.05 followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

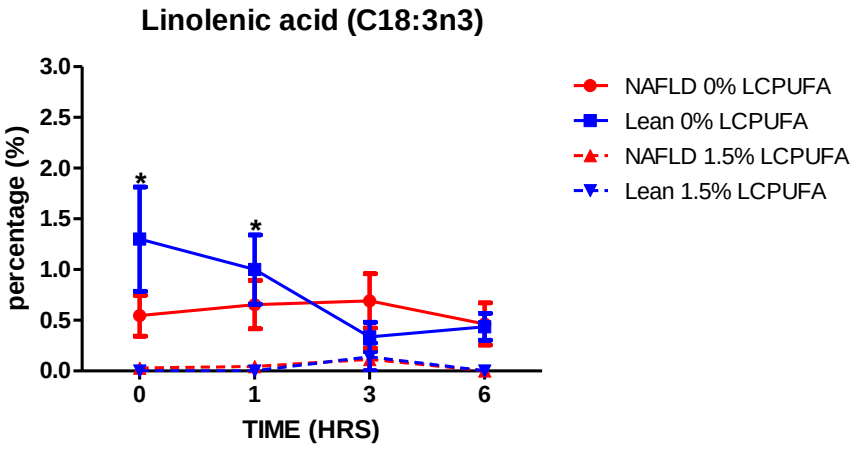


Figure 5.11. Plasma C18:3n3 concentrations in TG fractions prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease

(n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). Only differences between meal challenges are shown in the graph. Lower C18:3n3 values ($p<0.05$) were observed in the lean 1.5% LCPUFA when fasted and at 1 hr post meal when compared to the lean 0% LCPUFA. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

Table 5.6a. Comparison of postprandial clearance in lean subjects between meal challenges as expressed by delta (Δ1hrs-fasted, Δ3hrs-fasted, Δ6hr-fasted)

LEAN subjects									
	0% LCPUFA	1.5% LCPUFA		0% LCPUFA	1.5% LCPUFA		0% LCPUFA	1.5% LCPUFA	
Fatty acid (%)	Δ1-0hrs	Δ1-0hrs	p value 0% vs 1.5%	Δ3-0hrs	Δ3-0hrs	p value 0% vs 1.5%	Δ6-0hrs	Δ6-0hrs	p value 0% vs 1.5%
14:0	0.0±0.3	0.3±0.4	0.6	1.7±0.4	0.5±0.5	0.1	1.6±0.6	-0.4±0.3	0.01
16:0	0.7±0.9	2.2±2.1	0.5	5.9± 0.9	1.1±1.2	0.006	5.5±1.4	-1.1±1.2	0.002
16:1	0.1±0.3	-0.1±0.4	0.7	-0.4±0.4	-0.2±0.2	0.6	-0.3±0.3	0.1±0.3	0.3
18:0	0.4±0.2	0.2±0.5	0.7	1.5± 0.4	-0.1±0.3	0.01	1.5±0.3	0.1±0.5	0.05
18:1	0.1±1.0	-3.8±4.4	0.4	-3.4±1.4	-1.0±1.1	0.2	-4.4±1.4	-0.9±1.4	0.09
18:2n6	0.9±0.6	1.2±1.2	0.8	-2.2±0.6	0.5±1.1	0.04	-2.8±0.6	1.9±1.2	0.004
18:3n3	-0.2±0.2	0.0±0.0	--	-0.9±0.5	0.1±0.1	0.07	-0.8±0.4	0.0±0.0	--
20:4n6	-0.5±0.2	-0.3±0.1	0.4	-0.8±0.1	-0.4±0.3	0.4	-0.6±0.2	-0.1±0.1	0.1
20:5n3	0.0±0.0	0.0±0.0	--	0.0±0.0	0.0±0.0	--	0.0±0.0	0.0±0.0	--
22:6n3	0.0±0.0	0.0±0.0	--	0.0±0.0	0.0±0.0	--	0.0±0.0	0.1±0.1	--
Σn-3	-0.1±0.3	0.0±0.0	--	-0.9±0.5	0.2±0.2	0.09	-0.9±0.5	0.0±0.0	0.07
Σn-6	-1.1±0.8	0.9±1.4	0.2	-4.7±0.6	0.1±1.2	0.004	-5.2±0.8	1.8±1.1	0.0001
ΣSFA	1.1±1.2	2.8±3.0	0.6	9.1±1.2	1.5±1.5	0.001	8.6±1.9	-1.4±1.4	0.0007
ΣMUFA	0.0±0.8	-3.6±4.7	0.4	-3.0±1.1	-0.8±1.1	0.1	-4.1±1.4	-1.0±1.4	0.1
Σμg/ml	194.8±113.9	-72.5±199.4	0.2	17.1± 220.3	634.2±500.9	0.2	-138.4±199.5	819.7±635.9	0.1

Values are mean ± SEM (except otherwise mentioned). Values are significant when $p<0.05$ by T-test when comparing 0%LCPUFA vs 1.5%LCPUFA.

Table 5.6b. Comparison of postprandial clearance in NAFLD subjects between meal challenges as expressed by delta (Δ1hrs-fasted, Δ3hrs-fasted, Δ6hr-fasted)

	NAFLD subjects								
	0% LCPUFA	1.5% LCPUFA		0% LCPUFA	1.5% LCPUFA		0% LCPUFA	1.5% LCPUFA	
Fatty acid (%)	Δ1-0hrs	Δ1-0hrs	p value 0% vs 1.5%	Δ3-0hrs	Δ3-0hrs	p value 0% vs 1.5%	Δ6-0hrs	Δ6-0hrs	p value 0% vs 1.5%
14:0	0.1±0.1	-0.0±0.2	0.5	0.7±0.2	0.4±0.3	0.4	0.4±0.2	0.0±0.3	0.3
16:0	-0.1±0.9	3.0±3.1	0.3	0.2±0.9	0.4±0.6	0.8	0.3±1.5	-0.0±0.7	0.8
16:1	0.3±0.3	-0.7±0.6	0.1	0.3±0.3	0.5±0.9	0.8	0.2±0.4	0.3±1.0	0.9
18:0	0.5±0.2	0.2±0.7	0.7	1.1±0.3	0.3±0.5	0.3	1.7±0.7	-0.1±0.6	0.08
18:1	2.4±2.0	-6.3±6.6	0.2	1.6±2.0	-1.2±0.9	0.2	2.4±2.1	-1.3±1.3	0.1
18:2n6	-1.3±1.9	2.4±1.9	0.1	-3.0±1.6	0.5±0.9	0.07	-3.0±1.8	2.1±2.0	0.07
18:3n3	0.1±0.0	0.0±0.0	--	0.1±0.1	0.0±0.1	0.7	-0.1±0.1	-0.0±0.0	0.7
20:4n6	0.0±0.1	0.2±0.1	0.2	-0.0±0.0	0.3±0.1	0.07	0.1±0.2	0.2±0.1	0.5
20:5n3	0.0±0.0	0.0±0.0	--	0.0±0.0	0.0±0.0	--	0.0±0.0	0.0±0.0	--
22:6n3	0.0±0.0	0.0±0.0	--	0.0±0.0	0.0±0.0	--	0.0±0.1	0.0±0.0	--
Σn-3	0.1±0.1	0.0±0.0	--	0.1±0.1	0.0±0.1	0.6	-0.1±0.2	-0.1±0.0	0.8
Σn-6	-1.2±1.9	2.6±1.9	0.1	-3.1±1.6	0.8±1.0	0.055	-3.0±1.8	2.4±1.9	0.06
ΣSFA	0.5±1.0	3.2±3.8	0.5	2.0±1.0	1.2±0.5	0.4	2.3±1.5	-0.0±0.7	0.1
ΣMUFA	2.1±2.3	-5.6±6.4	0.3	1.3±2.3	-1.7±1.0	0.2	2.3±2.1	-1.5±1.51	0.1
Σμg/ml	97.7±87.4	-86.1±162.8	0.3	254.4±135.7	-25.2±130.6	0.1	303.5±225.2	-47.2±130.2	0.1

Values are mean ± SEM (except otherwise mentioned). Values are significant when $p<0.05$ by T-test when comparing 0%LCPUFA vs 1.5%LCPUFA.

5.3.7 Fatty acid profile of plasma phospholipid (PL) Fraction

The Fatty acid (FA) profiles (in percentages) and concentrations (μg/ml) corresponding to high SFA/1.5% LCPUFA NAFLD and lean group are presented in **Table 5.7a and 5.7b.** No significant changes across the time were observed. Fatty acid (FA) profiles (in percentages) and concentrations (μg/ml) corresponding to high SFA/0% LCPUFA NAFLD and lean group are presented in **chapter 4 Table 4.5a and 4.5b**. Four groups' time course data [NAFLD 0% LCPUFA (meal 1), Lean 0% LCPUFA (meal 1), NAFLD 1.5% LCPUFA (meal 2) and Lean 1.5% LCPUFA (meal 2)] is presented for the fatty acids in which significant values between meal challenges were observed. Significant differences ($p<0.05$) between treatments are shown by an asterisk. For significant time intra group differences refer to the **Table 4.5a** and **4.5b, chapter 4** (high SFA/0% LCPUFA NAFLD vs lean) and **Table 5.7a** and **5.7b** below (high SFA/1.5% LCPUFA NAFLD vs lean).

Significant higher C14:0 and lower C18:2n6 fractions were observed in the NAFLD 1.5% LCPUFA at 6hrs post meal when compared to NAFLD 0% LCPUFA ($p<0.001$, <0.05; respectively); see **Figures 5.12 and 5.13**. iAUC for both, C14:0 and C18:2n6 were not significantly different in both groups when comparing meals. Lower 22:6n3 values were observed at all-time points in lean 1.5% LCPUFA subjects when compared to the lean 0% LCPUFA group ($p<0.01$). Similar behavior was observed in the NAFLD 1.5% LCPUFA group at 1, 3 and 6hrs post meal ($p<0.05$) **Figure 5.14.** The total sum of omega-3 was significantly lower in the NAFLD 1.5% LCPUFA when compared to the NAFLD 0%

LCPUFA group at 0, 1 and 3 hrs post meal ($p<0.05$); see **figure 5.15**. No other significant differences were observed.

Table 5.7a. Postprandial Plasma PL fatty acid composition (percentages) at postprandial time points 1.5% LCPUFA (Meal 2)

Fatty Acid	TPL (%) LEAN				TPL (%) NAFLD			
	Fasted	1hr	3hr	6hr	Fasted	1hr	3hr	6hr
14:0	0.5±0.1	0.6±0.2	0.2±0.1	0.3±0.1	0.6±0.2	0.4±0.1	0.5±0.1	1.0±0.3
16:0	31.6±2.5	33.2±0.7	32.9±0.7	33.2±0.9	36.3±2.1	34.4±1.2	34.9±1.0	32.9±0.6
16:1	0.0±0.0	0.0±0.0	0.1±0.1	0.1±0.1	0.6±0.3	0.4±0.3	0.7±0.3	0.8±0.3
18:0	17.6±0.7	17.2±0.7	18.5±0.7	17.9±0.5	17.8±1.3	18.7±1.0	17.9±1.1	17.4±1.5
18:1	12.2±0.5^{ab}	12.8±0.7^{ab}	12.5±0.5^{a}	12.0±0.4^{ab}	9.2±1.4^{ab}	10.6±0.5^{ab}	9.2±1.4^{b}	11.7±1.2^{ab}
18:2n6	22.0±0.6^{ab}	21.5±0.9^{ab}	22.3±0.7^{ab}	23.4±0.7^{a}	22.4±1.5^{ab}	21.9±1.2^{ab}	21.2±0.6^{ab}	18.7±1.7^{b}
18:3 n3	ND	ND	ND	ND	ND	0.1±0.1	0.1±0.1	0.1±0.1
20:4n6	7.9±0.6^{ab}	8.1±0.5^{ab}	7.8±0.4^{ab}	8.0±0.4^{a}	7.2±0.2^{ab}	7.7±0.4^{ab}	7.6±0.9^{ab}	6.0±0.4^{b}
20:5n3	0.3±0.1	0.0±0.0	0.2±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.2±0.1
22:6n3	1.1±0.5	0.9±0.3	1.0±0.4	0.7±0.3	0.4±0.3	0.3±0.2	0.2±0.2	0.0±0.0
Σn-3	1.5±0.3	0.9±0.3	1.2±0.4	0.9±0.4	0.5±0.4	0.5±0.3	0.8±0.4	1.0±0.6
Σn-6	27.7±2.0^{ab}	32.8±1.2^{ab}	32.3±0.8^{ab}	32.7±0.5^{a}	33.0±1.5^{ab}	33.1±1.1^{ab}	31.9±0.9^{ab}	27.7±2.0^{b}
ΣMUFA	12.4±0.5	12.9±0.7	12.6±0.5	12.1±0.4	9.8±1.2	11.0±0.3	10.0±1.6	12.5±1.4
ΣSFA	49.7±2.2^{a}	51.3±0.8^{ab}	51.8±0.8^{ab}	51.5±1.0^{ab}	55.4±1.1^{b}	53.8±0.8^{ab}	53.7±1.3^{ab}	51.5±1.6^{ab}
Σμg/ml	605.0±56.4^{ab}	618.4±58.0^{a}	557.0±50.6^{ab}	549.8±22.6^{a}	2107.8±711.3^{ab}	2323.9±818.1^{b}	1984.7±702.5^{ab}	2501.6±886.1^{b}

Values are mean ± SEM (except otherwise mentioned). Variables with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Total fatty acids sum is expressed in μg/ml.

Table 5.7b. Postprandial Plasma PL fatty acid composition (µg/ml) at postprandial time points 1.5% LCPUFA (Meal 2)

Fatty Acid	TPL (µg/ml) LEAN				TPL (µg/ml) NAFLD			
	Fasted	1hr	3hr	6hr	Fasted	1hr	3hr	6hr
14:0	3.7±1.2^{ab}	5.0±2.1^{ab}	1.8±0.9^{ab}	1.9±1.1^{a}	6.7±3.9^{ab}	9.3±4.8^{ab}	8.2±3.6^{ab}	16.9±7.9^{b}
16:0	209.3±20.3^{ab}	210.2±21.2^{a}	188.4±19.4^{ab}	184.6±10.8^{a}	750.2±256.4^{ab}	802.8±284.7^{b}	688.5±238.7^{ab}	864.8±305.4^{b}
16:1	0.0±0.0	0.5±0.5	0.6±0.6	0.6±0.3	14.7±10.2	12.4±10.9	16.5±9.1	20.1±12.5
18:0	104.2±8.3^{ab}	103.8±8.1^{a}	99.9±8.7^{ab}	98.7±4.2^{a}	405.5±142.3^{ab}	464.8±169.5^{b}	389.2±143.7^{ab}	512.1±193.8^{b}
18:1	72.7±5.8^{ab}	81.3±10.0^{ab}	68.2±5.7^{ab}	66.3±2.6^{a}	179.0±76.3^{ab}	229.9±84.0ab	189.1±86.7^{ab}	306.6±113.3^{b}
18:2n6	135.3±15.1^{ab}	133.1±15.0^{a}	126.2±10.3^{ab}	129.5±5.9^{a}	465.0±150.6^{ab}	498.2±170.5^{b}	417.5±142.3^{ab}	496.1±171.2^{b}
18:3n3	ND	ND	ND	ND	ND	0.5±0.5	0.5±0.5	0.5±0.5
20:4n6	45.9±5.9	52.6±7.1	43.4±4.7	44.6±3.6	166.5±59.7	190.7±70.7	169.3±62.9	187.5±74.2
20:5n3	1.8±1.0	0.3±0.3	1.4±1.0	0.8±0.6	1.2±1.2	0.6±0.6	0.5±0.5	0.5±0.5
22:6n3	4.4±1.6	5.7±1.6	6.7±2.2	4.1±1.9	11.3±10.4	14.5±8.8	2.9±2.9	0.0±0.0
Σn-3	6.5±2.3	6.0±1.7	8.1±2.6	5.0±2.3	12.4±10.3	15.7±8.6	5.8±3.0	4.6±3.5
Σn-6	197.2±22.1^{ab}	200.3±21.6^{a}	184.5±15.4^{ab}	188.8±8.5^{a}	708.7±236.5^{ab}	779.0±274.0^{b}	651.9±228.8^{ab}	767.7±270.8^{b}
ΣMUFA	74.6±6.8^{ab}	81.7±10.1^{ab}	69.7±6.5^{ab}	67.0±2.7^{a}	193.7±77.5^{ab}	242.2±86.0^{ab}	205.5±95.6^{ab}	326.7±123.6^{b}
ΣSFA	317.6±29.0^{ab}	319.9±30.0^{a}	291.1±28.6^{ab}	285.5±14.3^{a}	1170.4±398.9^{ab}	1278.2±456.6^{b}	1088.7±384.9^{ab}	1394.2±503.6^{b}

Values are mean ± SEM (except otherwise mentioned). Variables with different superscripts are significantly different by repeated measures two way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons.

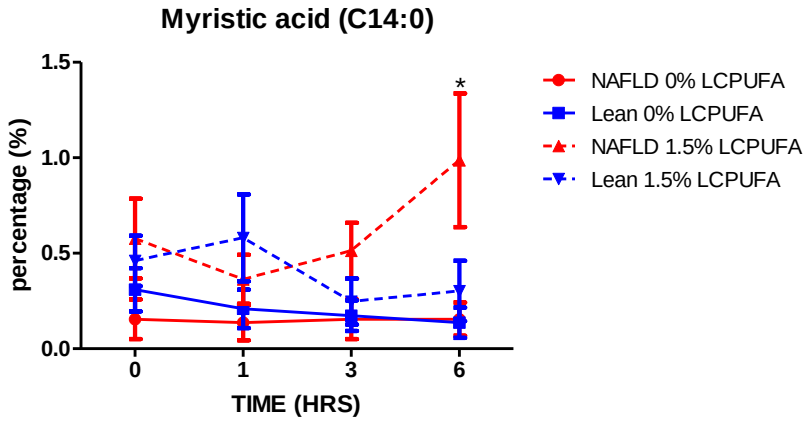


Figure 5.12. Plasma C14:0 concentrations in TPL fractions prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). Only differences between meal challenges are shown in the graph. Significant higher C14:0 ($p<0.05$) in the NAFLD 1.5% LCPUFA at 6hrs post meal when compared to NAFLD 0% LCPUFA. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

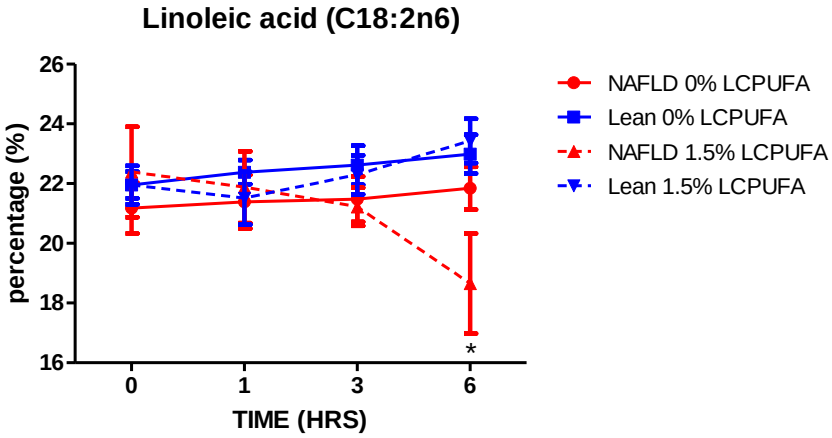


Figure 5.13. Plasma C18:2n6 concentrations in TPL fractions prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). Only differences between meal challenges are shown in the graph. Significant lower C18:2n6 ($p<0.05$) in the NAFLD 1.5% LCPUFA at 6hrs post meal when

compared to NAFLD 0% LCPUFA. *Indicates significant difference by repeated measures two way ANOVA when p<0.05 followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

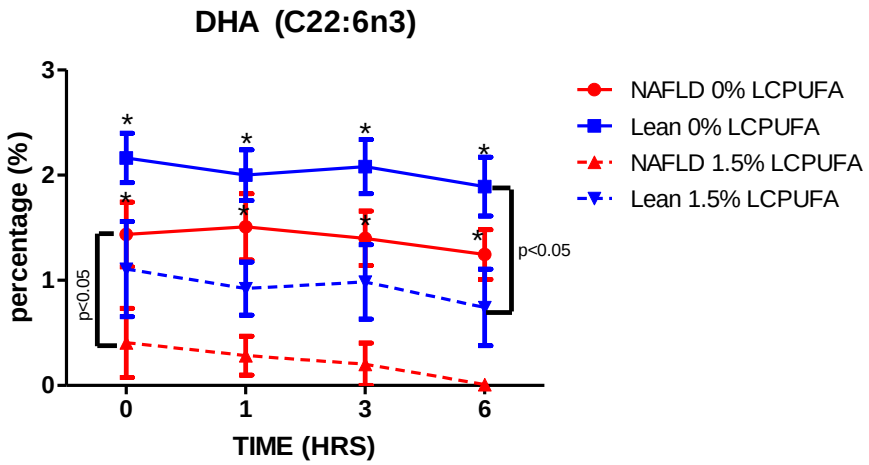


Figure 5.14. Plasma C22:6n3 concentrations in TPL fractions prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). Only differences between meal challenges are shown in the graph. Lower (p<0.05) C22:6n3 at 0, 1, 3 and 6hrs in lean 1.5% LCPUFA subjects when compared to the lean 0% LCPUFA group. Lower (p<0.05) C22:6n3 at 1, 3 and 6hrs in NAFLD 1.5% LCPUFA subjects when compared to the NAFLD 0% LCPUFA group. *Indicates significant difference by repeated measures two way ANOVA when p<0.05 followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

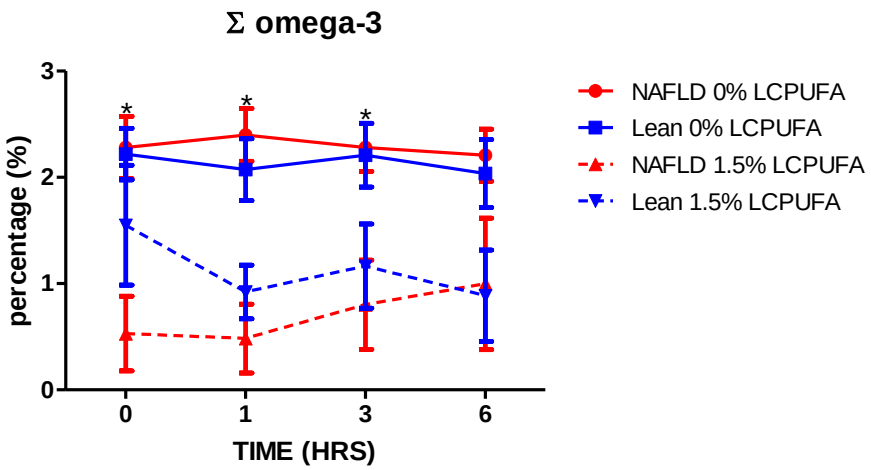


Figure 5.15. Plasma sum of omega-3 concentrations in TPL fractions prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). Only differences between meal challenges are shown in the graph. Lower ($p<0.05$) values in the NAFLD 1.5% LCPUFA when compared to the NAFLD 0% LCPUFA group at 0, 1 and 3 hrs post meal. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

5.3.8 Apolipoproteins

5.3.8.1 *Apolipoprotein B-48*

NAFLD children and adolescents had significantly higher apolipoprotein B-48 levels when compared to lean subjects in response to the 0% LCPUFA at 3 and 6 hours ($p<0.05$, 0.01; respectively) post meal consumption (**Figure 5.16**). No other significant differences were observed (**Figure 5.16**). With regard to the iAUC no significant differences between groups and treatments were observed. No significant interaction between meal and group was observed (**Figure T. Appendix 2**).

5.3.8.2 *Apolipoprotein B-100.*

Significant differences were observed between NAFLD and lean at all-time points ($p<0.05$) in response to the 0% LCPUFA (**Figure 5.17)**. No significant differences between treatments were observed. With regard to the iAUC no significant differences between groups and treatments were observed. No significant interaction between meal and group was observed (**Figure U. Appendix 2**).

5.3.8.3 *Apolipoprotein C-III*

Fasting plasma values of apolipoprotein C-III were significantly higher ($p<0.05$) in the NAFLD group when compared to the lean group following the 0% LCPUFA challenge (**Figure 5.18**). Significant higher values where observed at 0, 1, 3 hrs post meal in the NAFLD group when compared to the lean group ($p<0.05$, 0.001, 0.01) after the 1.5% LCPUFA meal (**Figure 5.18**). In addition, significant differences between meals in the NAFLD group were observed at 1 and 3 hrs post

meal ($p<0.05$). No significant differences in the iAUC between treatments and groups were observed. No significant interaction between meal and group was observed (**Figure V. Appendix 2**).

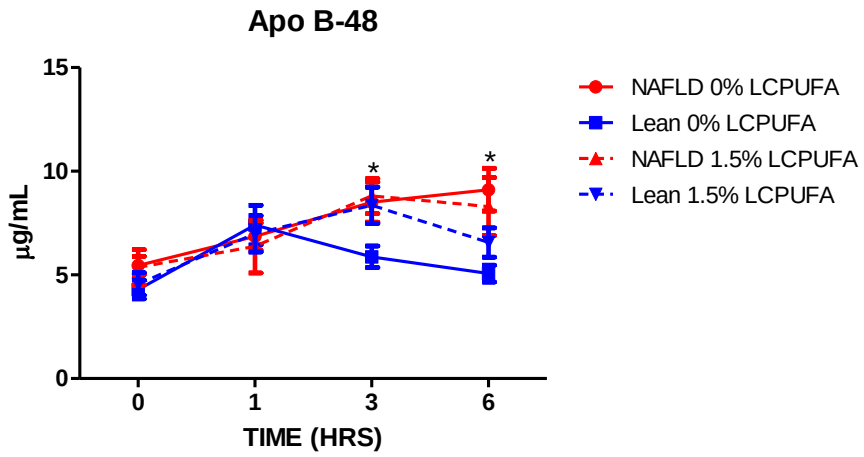


Figure 5.16. Total Apolipoprotein B-48 concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=5) and healthy lean-age matched (n=10). NAFLD 0% LCPUFA vs lean 0% LCPUFA $p<0.05$ at 3 and 6 hours. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

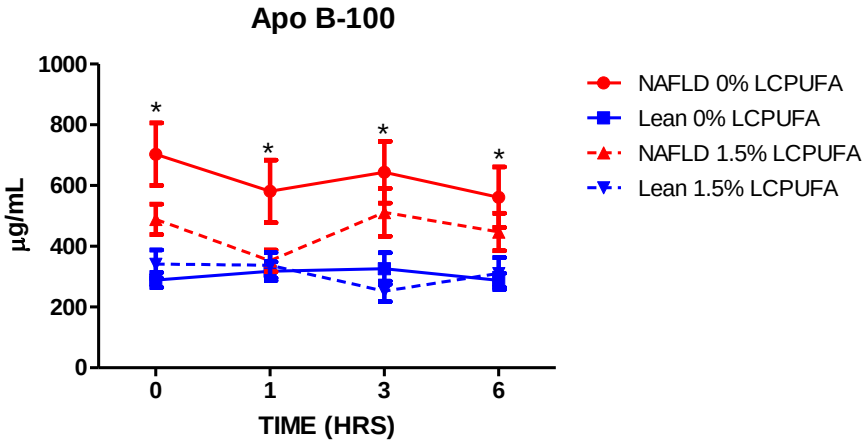


Figure 5.17. Total Apolipoprotein B-100 concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic

fatty liver disease (n=6) and healthy lean-age matched (n=10). NAFLD 0% LCPUFA vs lean 0% LCPUFA significant ($p<0.05$) at all-time points No significant differences between treatments were observed. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

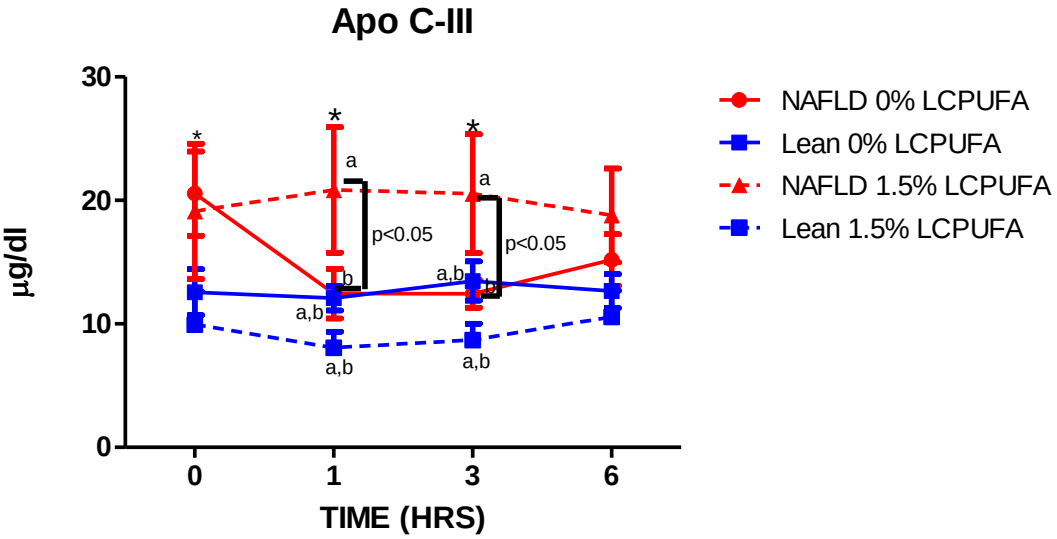


Figure 5.18. Total Apolipoprotein C-III concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=7) and healthy lean-age matched (n=10). NAFLD 0% LCPUFA vs lean 0% LCPUFA $p<0.05$ at 0hrs. NAFLD 1.5% LCPUFA vs Lean 1.5% LCPUFA $p<0.05$ at 0, 1, 3 hrs. NAFLD 0% LCPUFA vs NAFLD 1.5% LCPUFA significant differences ($p<0.05$) at 1 and 3hrs post meal. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

5.3.9 Inflammatory mediators and adiponectin

5.3.10 *Adiponectin, TNF-α, IL-6 and IL-10*

No significant differences in adiponectin levels were observed between the NAFLD and lean subjects following the 0% LCPUFA meal. On the contrary, significant higher values were observed in the lean subjects at 0, 1, 3, 6 hrs when compared to the NAFLD group ($p<0.05$, 0.05, 0.01, 0.05; respectively) in response to the 1.5% LCPUFA meal challenge; see **Figure 5.19**. No significant differences in the iAUC between treatments and groups were observed (**Figure W. Appendix 2**).

Significant higher TNF-α values were observed at 0, 1, 3 and 6hrs in the NAFLD group when compared to the lean group ($p<0.001$) following the 0% LCPUFA meal; see **Figure 5.20a**. No significant differences at any time point were observed when comparing the NAFLD vs the lean group in response to the 1.5% LCPUFA meal challenge. Significant differences were observed on the fasted state, 1, 3 and 6 hrs post meal when comparing the NAFLD groups on the 0% and 1.5% LCPUFA meals ($p<0.01$, 0.01, 0.05, 0.05; respectively); see **Figure 5.20a**. When comparing the iAUC significant higher ($p=0.02$) values where observed in the NAFLD 0% LCPUFA group when compared to the lean 0% LCPUFA group (**Figure 5.20b**). No other significant differences were observed.

IL-6 levels were not significantly different between groups and treatments at any time point (**Figure 5.21a**). Nevertheless, a trend of lower iAUC ($p=0.06$) was observed when comparing NAFLD vs lean following the 1.5% LCPUFA meal challenge (**Figure 5.21b**).

IL-10 fasting, 1, 3 and 6hrs post meal were significantly lower in the NAFLD group when compared to the lean subjects ($p<0.001$) following the 0% LCPUFA meal; see **Figure 5.22**. Significant lower values were observed at 1hr post meal in the NAFLD group when compared to the lean controls ($p<0.05$) on the 1.5% LCPUFA challenge. No significant differences in the iAUC between treatments and groups were observed (**Figure X. Appendix 2**).

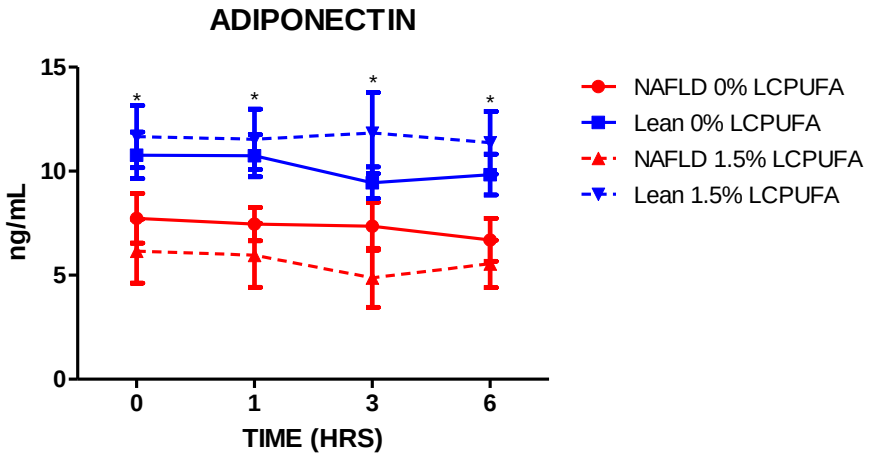


Figure 5.19. Total adiponectin concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=5) and healthy lean-age matched (n=10). Higher values ($p<0.05$) were observed in the lean 1.5% LCPUFA subjects at 0, 1, 3, 6 hrs when compared to the NAFLD 1.5% LCPUFA group. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

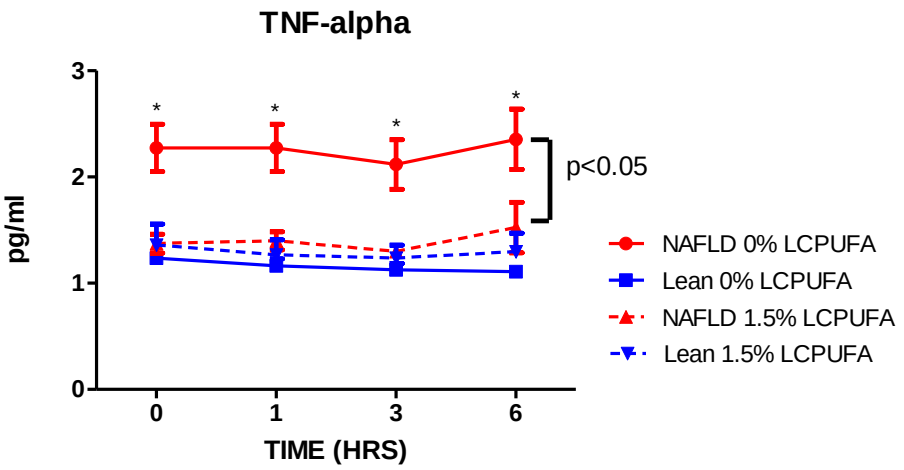


Figure 5.20a. Total TNF-α concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). Higher ($p<0.05$) TNF-α values were observed at 0, 1, 3 and 6hrs in the 0% LCPUFA NAFLD groups when compared to the 0% LCPUFA lean group. Significant ($p<0.05$) differences were observed on the fasted state, 1, 3 and 6 hrs post meal when comparing the 0% LCPUFA NAFLD vs 1.5% LCPUFA NAFLD groups. *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

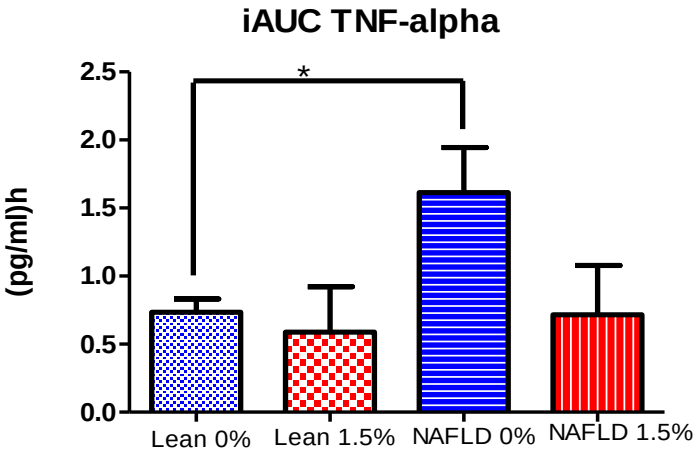


Figure 5.20b. Postprandial incremental area under the curve (iAUC) for TNF-α following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). No significant interaction between meal and group was observed ($p>0.05$). *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$; following Bonferroni correction and pairwise comparison. Results are shown mean ± SEM.

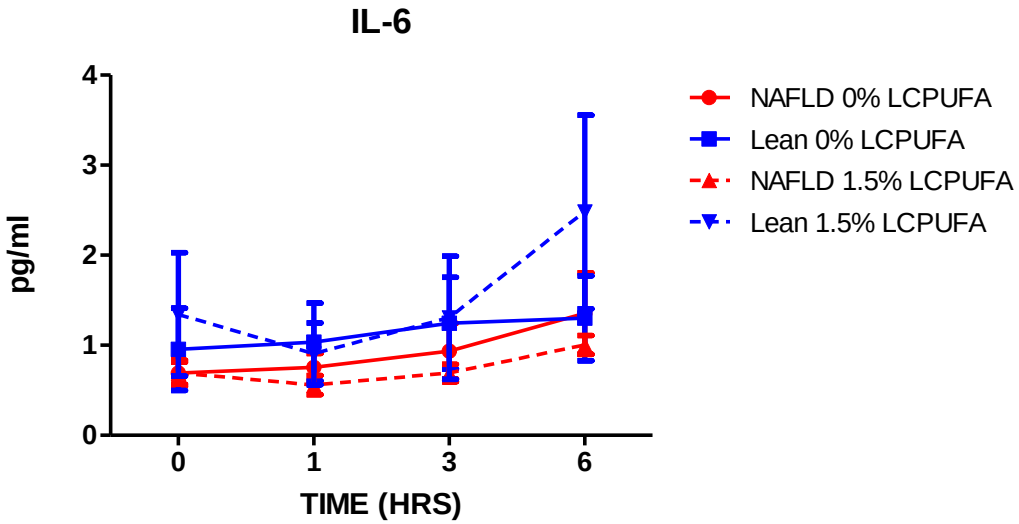


Figure 5.21a. Total IL-6 concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=5) and healthy lean-age matched (n=10). *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

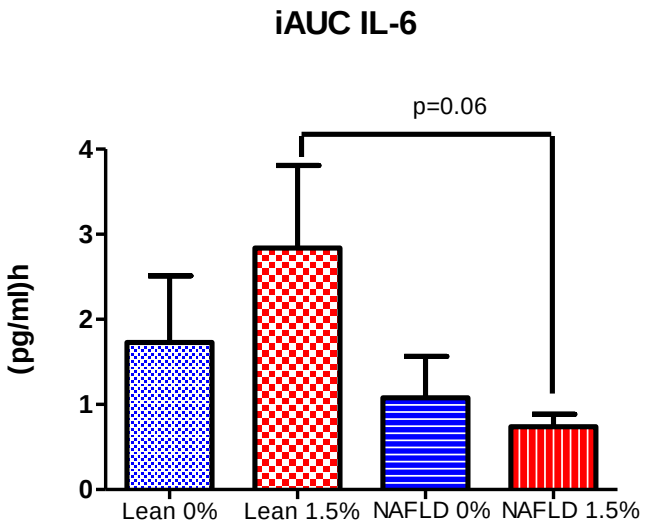


Figure 5.21b. Postprandial incremental area under the curve (iAUC) for IL-6 following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=5) and healthy lean-age matched (n=10). No significant interaction between meal and group was observed ($p>0.05$). Values with different superscripts are significantly different by repeated measures two way ANOVA when $p<0.05$; following Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

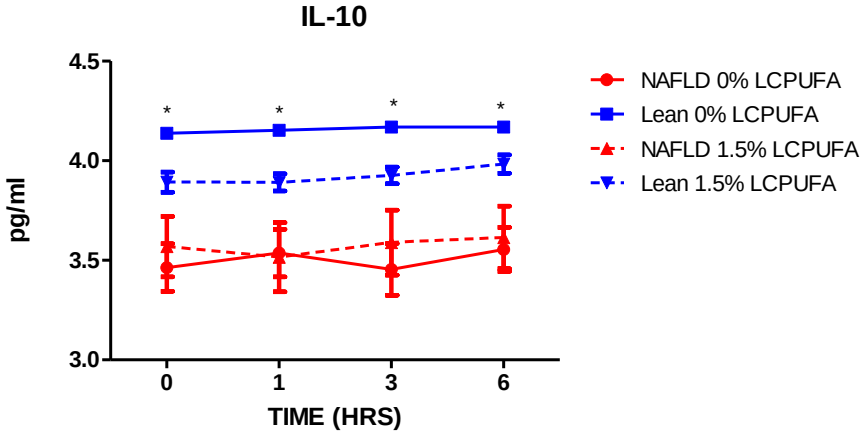


Figure 5.22. Total IL-10 concentrations prior (t=0) and following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA at 1, 3 and 6 hours. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=5) and healthy lean-age matched (n=10). Fasting, 1, 3 and 6hrs post meal were

significantly ($p<0.05$) lower in the 0% LCPUFA NAFLD group when compared to the 0% LCPUFA lean subjects. Significant lower ($p<0.05$) values were observed at 1hr post meal in the 1.5% LCPUFA NAFLD group when compared to the 1.5% LCPUFA lean controls ($p<0.05$). *Indicates significant difference by repeated measures two way ANOVA when $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

Table 5.8a. Comparison of postprandial clearance for different metabolic variables in lean subjects between meal challenges as expressed by Delta (Δ1hr-fasted, Δ3hrs-fasted, Δ6hrs-fasted)

	LEAN subjects								
	0% LCPUFA	**1.5% LCPUFA**		**0% LCPUFA**	**1.5% LCPUFA**		**0% LCPUFA**	**1.5% LCPUFA**	
Variable	**Δ1-0hrs**	**Δ1-0hrs**	**p value 0% vs 1.5%**	**Δ3-0hrs**	**Δ3-0hrs**	**p value 0% vs 1.5%**	**Δ6-0hrs**	**Δ1-0hrs**	**p value 0% vs 1.5%**
Insulin (mU/L)	20.8±3.4	16.0±2.7	0.2	2.0±1.8	0.2±1.9	0.5	-2.4±1.2	-2.6±2.6	0.9
Glucose (mmol/L)	-0.4±0.2	-0.1±0.3	0.5	0.0±0.1	-0.1±0.0	0.2	0.1±0.1	0.0±0.2	0.7
Triglycerides (mmol/L)	0.2±0.0	0.0±0.0	0.01	0.5±0.0	0.4±0.1	0.6	0.2±0.1	0.2±0.1	0.8
Total Cholesterol (mmol/L)	-0.0±0.0	-0.2±0.0	0.005	-0.0±0.0	-0.1±0.0	0.1	0.0±0.1	0.1±0.1	0.6
HDL (mmol/L)	No change	No change	--	No change	No change	--	No change	No change	--
LDL (mmol/L)	-0.1±0.0	-0.1+0.0	0.7	-0.2±0.0	-0.2±0.0	0.8	-0.1±0.0	-0.1±0.0	0.6
NEFA (mEq/L)	-0.2±0.0	-0.3±0.1	0.4	-0.1±0.0	-0.2±0.1	0.5	0.2±0.0	0.3±0.1	0.8
Apo B-48 (μg/mL)	3.1±0.9	2.4±0.8	0.6	1.5±0.5	3.7±0.8	0.04	0.7±0.3	1.7±0.5	0.1
Apo B-100 (μg/mL)	45.1±36.5	-3.9±24.1	0.2	37.4±48.9	-89.0±35.5	0.051	33.6±40.2	-34.0±22.2	0.1
Apo C-III (μg/dl)	-0.5±1.2	-1.8±1.9	0.5	0.8±1.0	-1.2±2.3	0.4	0.1±0.9	0.0±3.2	0.9
IL-6 (pg/ml)	-0.2±0.0	-0.3±0.1	0.2	0.2±0.2	-0.0±0.1	0.3	0.3±0.1	1.2±0.4	0.09
IL-10 (pg/ml)	No change	No change	--	No change	No change	--	No change	No change	--
TNF-α (pg/ml)	No change	No change	--	-0.1±0.0	-0.1±0.0	0.7	-0.1±0.0	-0.0±0.0	0.6
Adiponectin (ng/ml)	-0.0±0.8	-0.1±0.7	0.9	-1.3±0.5	0.1±0.9	0.1	-0.9±0.4	-0.7±0.8	0.8

Values are mean ± SEM (except otherwise mentioned). Values are significant when $p<0.05$ by T-test when comparing 0%LCPUFA vs 1.5%LCPUFA.

Table 5.8b. Comparison of postprandial clearance for different metabolic variables in NAFLD subjects between meal challenges as expressed by Delta (Δ1hr-fasted, Δ3hrs-fasted, Δ6hrs-fasted)

	NAFLD subjects								
	0% LCPUFA	1.5% LCPUFA		0% LCPUFA	1.5% LCPUFA		0% LCPUFA	1.5% LCPUFA	
Variable	**Δ1-0hrs**	**Δ1-0hrs**	**p value 0% vs 1.5%**	**Δ3-0hrs**	**Δ3-0hrs**	**p value 0% vs 1.5%**	**Δ6-0hrs**	**Δ6-0hrs**	**p value 0% vs 1.5%**
Insulin (mU/L)	104.0±28.0	92.6±17.6	0.7	21.6±10.6	8.9±4.7	0.2	-6.4±3.2	-2.8±4.5	0.5
Glucose (mmol/L)	0.2±0.2	0.2±0.3	0.9	-0.2±0.2	-0.2±0.1	0.8	-0.6±0.2	-0.3±0.1	0.4
Triglycerides (mmol/L)	No change	No change	--	0.5±0.0	0.6±0.21	0.6	0.4±0.1	0.4±0.2	0.7
Total Cholesterol (mmol/L)	-0.2±0.1	-0.1±0.0	0.7	No change	No change	--	0.0±0.1	0.0±0.0	0.6
HDL (mmol/L)	No change	No change	--	No change	0.0±0.1	0.5	No change	No change	--
LDL (mmol/L)	-0.2±0.0	-0.1±0.0	0.3	-0.2±0.0	-0.2±0.1	0.7	-0.1±0.0	-0.0±0.1	0.7
NEFA (mEq/L)	-0.1±0.0	-0.1±0.0	0.8	-0.2±0.0	-0.1±0.0	0.1	-0.1±0.0	0.1±0.0	0.004
Apo B-48 (μg/mL)	1.4±0.6	1.0±0.8	0.7	3.0±1.0	3.4±0.9	0.7	3.6±1.1	2.9±0.5	0.5
Apo B-100 (μg/mL)	-122.2±67.0	-137.2±51.1	0.8	-59.3±22.3	22.4±82.1	0.3	-141.7±70.6	-42.0±34.3	0.2
Apo C-III (μg/dl)	-8.1±2.4	2.6±6.0	0.1	-8.1±3.3	1.8±6.2	0.2	-5.3±2.2	-2.7±6.1	0.7
IL-6 (pg/ml)	0.0±0.0	-0.1±0.0	0.1	0.2±0.1	0.0±0.0	0.2	0.6±0.3	0.3±0.0	0.3
IL-10 (pg/ml)	No change	No change	--	No change	No change	--	0.1±0.0	0.0±0.0	0.3
TNF-α (pg/ml)	-0.2±0.0	0.0±0.0	0.02	-0.1±0.1	0.0±0.0	0.4	0.1±0.1	0.1±0.2	0.8
Adiponectin (ng/ml)	-0.3±0.6	-0.2±0.5	0.9	-0.4±0.8	-1.2±0.6	0.4	-1.0±0.6	-0.6±0.4	0.5

Values are mean ± SEM (except otherwise mentioned). Values are significant when $p<0.05$ by T-test when comparing 0%LCPUFA vs 1.5%LCPUFA.

5.4 DISCUSSION

Delayed postprandial insulin and lipid clearance has been observed in adults with NAFLD (Bravo et al., 2010; Musso et al., 2005; 2003). Our previous study allowed us to conclude that delayed postprandial hyperinsulinemia and altered lipoprotein expression are metabolic derangements present in children and adolescents with NAFLD following the consumption of a high saturated fat meal (Mager et al., 2010; Rodríguez Dimitrescu & Mager, 2008; Rodríguez Dimitrescu et al., 2010). There is controversy around whether the addition of LCPUFA to a high saturated meal challenge may improve clearance in normal and healthy overweight/obese subjects. The main objective of this study was to compare two meal challenges reflective of a westernized fast food breakfast, both high in saturated fat but with varying LCPUFA content. A small amount of LCPUFA was included in the meal as usually very small amounts of these are present in a fast food meal (Fernández San Juan, 2000). To our knowledge this is the first study that explores the acute postprandial metabolic response to two meal fat challenges essentially equivalent for GI and GL, similar saturated fat content and fructose and varied in the LCPUFA content. These two meals simulated the nutritional composition (saturated fat content) of fast food meals in children and adolescents with biopsy proven NAFLD.

No significant differences were observed in the postprandial insulin levels between meals; albeit a modest reduction in the iAUC for both NAFLD and lean groups was found following consumption of the 1.5% LCPUFA meal. The

improvement of iAUC for insulin is similar to what Shah et al. observed (Shah et al., 2007). In their study, significantly lower insulin and iAUC postprandial insulin values were observed in adult male subjects with type 2 diabetes after a postprandial meal rich in DHA and EPA (salmon oil) when compared to other test oils (salmon vs palm vs olive vs safflower). Differences between our study and Shah et al. include the studied population, (NAFLD vs type 2 diabetic; as NAFLD subjects are usually characterized by hyperinsulinemia and normal glucose values) age (children and adolescents vs adults) and higher amount of calories (1000 kcal) within the meal challenge used in their study. Higher fat content (50g) and a higher PUFA dose (PUFA omega-3 (n-3): 38.6% and omega-6 (n-6): 2.3% of the total meal challenge kilocalories) were present in Shah et al. meal challenge when compared to the meal challenge within our study. The higher amount of PUFA present in Shah et al. meal challenge could have probably been more effective at promoting reductions in postprandial lipemia than our dose. Similarly to Shah et al. we did not observed significant differences in postprandial glucose responses between meal challenges (Shah et al., 2007). In our study glucose postprandial response may be independent of the type of meal fed and more dependent on phenotype as observed by Gil-Campos et al. where significantly higher fasting and postprandial glucose values were observed in overweight children when compared to leans regardless of the diet fed. Glucose values did not change over the postprandial period which is similar to our observations in our study in each of the four groups (Gil-Campos et al., 2011).

Triglyceride clearance was very similar between treatments and similar trends and values were observed when comparing the 0% LCPUFA meal vs the 1.5% LCPUFA meal in both NAFLD subjects and lean controls (intra group comparison) with regard to TG clearance. Our results are similar to what Shah et al. observed in their type 2 diabetic subjects. Lack of significant differences in TG clearance between the two meal challenges in our study could be partially be explained by the moderate power between groups of our sample size and by the lower dose of LCPUFA added to the meal challenge. Significantly improved postprandial lipid clearance has been observed by others in subjects following chronic consumption of providing a total of 1000 mg of EPA and 700 mg of DHA (Slivkoff-Clark et al., 2012); rather than at low doses given within our study. Nevertheless, the literature is still controversial about the effects of prior total fat intake as well as chronic intakes of MUFA, LCPUFA and saturated prior to an acute meal challenge and the extent to which acute changes in LCPUFA intake may influence postprandial TG clearance. While differences in prior fat intake (type and amount) may influence acute metabolic responses, this is unlikely to have been a major factor in our study outcomes as no absolute differences in total fat intake, saturated fat, MUFA and or PUFA intakes were noted in either of the two groups studied.

Significantly lower iAUC for NEFA was observed in the NAFLD in response to the 1.5% LCPUFA meal when compared to 0% LCPUFA meal. This result is similar to what Jackson et al. observed when comparing omega-6 PUFA to SFA and MUFA meals in middle aged men. It is possible that a meal richer in

LCPUFA may be metabolized more rapidly by LPL leading to a lower release of NEFA into the circulation (Jackson et al., 2005). Nevertheless, the minimum necessary dose in order to observe these changes has not been determined. Additionally, the small (not significant) improvement in insulin response may be another mechanism that could explain the observed NEFA response potentially by lipolysis improvement. The NAFLD 1.5% LCPUFA group showed in general, a lower insulin postprandial response, lower iAUC and lower values for the early phase response as observed by Δ1hr-0hrs values. The small ($p>0.05$) improvement in insulin sensitivity at 1hr post meal could explain the lower values observed in the NEFA at 1hr. The potential improvement of the LPL activity following the consumption of a 1.5% LCPUFA meal, possibly allows improved fatty acid uptake and therefore lower release into the venous plasma. It is also possible that a meal higher in LCPUFA by maintaining constant levels of Apo C-III could evoke a constant inhibition of HL which could potentially decrease the uptake of TG rich lipoproteins (e.g. CMr and VLDL) by the liver (Ooi et al., 2008; Perret et al., 2002). Additionally, the significant differences observed when comparing the Δ6hrs-1hr suggest an improvement in the "trapping" (Frayn et al, 1998) of fatty acids into the adipose tissue. Efficient trapping has been observed in lean subjects and suggested to be anti-atherogenic (Fielding, 2011; Frayn, 1998).

Lower C14:0 and higher C18:2n6 in the TG were observed in the lean subjects (but not in the NAFLD subjects) consuming the 1.5% LCPUFA meal when compared to the lean 0% LCPUFA group. In addition, C18:0 plasma TG

content was higher in the lean 1.5% LCPUFA group when compared to the lean 0% LCPUFA group and this was not observed in the NAFLD subjects. Analysis of the three day food record showed almost identical consumption of SFA and MUFA in both lean groups. Nevertheless higher total values in the plasma phospholipids were observed in 1.5% LCPUFA NAFLD group. Saturated fat intake has been strongly correlated with higher CRP levels (Araya et al., 2006) and CRP levels in both lean groups were very similar. C14:0 and C18:2n6 contents (g of fat) were similar in both meals and C18:0 was higher in the 1.5% LCPUFA meal. Our plasma results suggest that TG postprandial fatty composition is partially affected, but not solely, by the composition of a meal challenge; at least over the first six hours following consumption of a meal. TG postprandial fatty composition is probably a combination of chronic and acute meal intake; with the influences of meal composition expressed after 6-8 hrs (Parks, 2001). These results are similar to those reported by Weintraub et al. and Harris et al. where the major determinant of the postprandial response was determined to be chronic intake (Harris et al., 1988; Weintraub et al., 1988). Additionally, it is possible that in order to see significant postprandial differences a higher fat load (total fat) as well as higher LCPUFA content may be necessary (Lairon et al., 2008).

Significant differences were observed in the apolipoprotein B-48 and B-100 between the NAFLD and the lean group in the 0% LCPUFA meal challenge but not in the 1.5% LCPUFA meal challenge. Nevertheless, low Apo B-100 Δ6-0hrs values in both, NAFLD and lean 1.5% LCPUFA in addition to a trend

towards lower Apo B-48 iAUC in the NAFLD 1.5% LCPUFA when compared to the NAFLD 0% LCPUFA group suggest that probably a higher dose of LCPUFA would evoke a significant effect. It is possible that higher LCPUFA doses evoke a better postprandial triglyceride response due to a higher activation of LPL leading to faster clearance rates of CM, which would be observed by lower Apo B-48 levels (Harris & Muzio et al., 1993; Weintraub et al., 1998). Changes in postprandial lipoprotein values have been observed by others when the amount of LCPUFA omega-3 oil was at least 30% of the total meal challenge kilocalories (Shah et al., 2007; Weintraub et al., 1998; Zampelas et al., 1994). Similar Apo B-48 results were observed by Roche et al., when comparing the effects of SFA vs MUFA meals (Roche et al., 1998). According to Weintraub et al. a meal challenge rich in omega-3, especially LCPUFA, will evoke a higher chylomicron lipolysis susceptibility which will evoke a faster postprandial lipoprotein catabolic rate; but if the chronic diet is high in SFA a slow lipoprotein catabolic rate will be observed (Weintraub et al., 1988). Therefore, it is very likely that in order to observe improved clearance chronic intake should also be modified, since it has been suggested that chronic intake in normal and hypertriglyceridemic subjects plays the major role when it comes to a postprandial response (Harris et al., 1988).

Apo C-III inhibits the lipoprotein lipase and it is down regulated by insulin concentrations (Olivieri et al., 2005). Δ6-0hrs changes in Apo C-III were lower in the 0% LCPUFA NAFLD subjects when compared to the 1.5% LCPUFA NAFLD group. Additionally, negative values were observed during the early phase response (Δ1-0hrs) in the NAFLD group in the 0% LCPUFA meal (meal 1)

while positive delta was observed in the NAFLD subjects consuming the 1.5% LCPUFA meal (meal 2). It is possible that the lower insulin rise observed at 1hr post meal (in the 1.5% LCPUFA NAFLD group) as well as a lower iAUC (both, not significant) may be one of the reasons why Apo C-III levels remained almost the same after the higher LCPUFA intervention. A second hypothesis is the one proposed by Olivieri et al., who suggested that there are individuals who will respond to the omega-3 PUFA supplementation diet by decreasing Apo C-III levels and there are others that will not respond (Olivieri et al., 2005; Ye & Kwiterovich, 2000). It is possible that most of our NAFLD subjects in the 1.5% LCPUFA group are non-diet responders or the amount of LCPUFA was relatively low. Nevertheless, PUFA effect in the Apo C-III has been suggested to occur after chronic consumption (Olivieri et al., 2005) whether or not a single meal with higher LCPUFA content evokes changes in the Apo C-III expression in NAFLD children and adolescents requires further investigation.

No significant cytokine changes in the Δ6-0hrs between treatments were observed. TNF-α group but not time effect were observed between the two NAFLD groups. Higher fasted and postprandial values were observed in the NAFLD group that consumed the 0% LCPUFA meal when compared to the NAFLD group the consumed 1.5% LCPUFA meal. Interestingly, the anti-inflammatory cytokine IL-10 levels were not significantly different between the two NAFLD groups; suggesting that the NAFLD 0% LCPUFA group had a higher pro-inflammatory environment when compared to the 1.5% LCPUFA NAFLD group. It is very likely, as observed in our study that cytokine levels are

more reflective of a long term effect and as suggested in our previous postprandial study a single meal may not necessarily evoke or change the pro or anti-inflammatory environment present in the body; particularly within the time frame of study examined within this study.

5.4.1 Strengths and Limitations

Overall, our study suggests that small changes in the amount of LCPUFA in a high saturated fat meal challenge may not be enough to alter postprandial insulin and lipid clearance in children and adolescents with and without NAFLD in the first six hours after meal consumption.

Strengths of our study include the extensive exploration of metabolic variables (e.g. insulin, lipid profile, Apo B-48, B-100, C-III) thought to be related to disease pathogenesis in both healthy lean and children and adolescents with biopsy proven disease NAFLD. Additionally, our study was characterized by homogeneous ethnicity; which allowed us to determine whether the presence or absence of changes were due to the meal or group phenotype. A major strength of our study is that two isocaloric, similar in fructose and glycemic load meal challenges (composition was similar to fast food breakfasts typically consumed by children and adolescents) with varying LCPUFA content were compared. The use of “real food” (instead of fat liquid emulsions) in both meal challenges is an additional strength because it enables the ability to study the postprandial metabolic changes elicited from typical meal patterns consumed by children and adolescents. However, at the same time designing two meals with very similar

nutritional composition from a macronutrient and fatty acid composition can be very challenging; resulting in some small non-significant differences in meal composition. Most of these differences were related to total fat intake (5 g difference), rather than the type of fat (saturated vs MUFA vs PUFA). Significant differences in the postprandial TG rise were observed by others when comparing meals with different type of fat content, but the amounts of fat content differed by at least 15g of fat rather than by the small amount that was present between our two meal challenges (Dallongeville et al., 2002; Kim et al., 2006; Schneeman et al., 2003). In addition, we did not observe major differences in postprandial rises in insulin and TG between the meals within groups. Therefore, the possibility that this difference (5g) in fat influenced study outcomes is relatively minor. Additionally, the cholesterol (mg) and carbohydrate (%) content in the meal were slightly but not significantly different. Despite the higher cholesterol content in the 1.5% LCPUFA no significant postprandial cholesterol differences were observed between the two groups. Minimal cholesterol changes across the postprandial period have also been observed by others in lean and obese adults (Svenson et al., 2011). On the other hand, the carbohydrate content between meals varied by 10% (equivalent to 10g) of the total kilocalorie content of the meal, nevertheless the GL, GI and fructose were almost identical. Since no significant changes in the glucose and insulin between meals in both lean and NAFLD groups were observed we think this 10% difference is probably not a confounding factor of the results observed. Different glucose and insulin changes across the postprandial period have been observed by others when comparing to meals in

lean and obese adults when the carbohydrate content was different by at least 100 g (Schindhelm et al., 2008).

It is possible that if the postprandial study period was extended (e.g. 8 hours) patterns of delayed clearance especially for TG, NEFA, Apo B-100 and B-48 may have been more evident particularly in the children and adolescents with NAFLD. Some research in adults with NAFLD suggests that delayed lipemia may last for up to six to eight hours after consumption of a high fat meal (Jackson et al., 2005; Jiménez-Gómez et al., 2009; Montegard et al., 2010). However, other studies in obese children and adolescents have not shown this type of pattern of response (Bickerton et al., 2007; Iovine et al., 2004); with meal responses indicating postprandial lipid clearance within the first 3-4 hours. An option to extend the study period to up to 8 hours was considered within the study design. This however, was not feasible in a population with poor vascular access and because both study groups found it difficult to remain NPO (nothing by mouth) for more than six hours. Although a bigger sample size in the NAFLD group may have potentially also conferred additional strength to study findings, it is unlikely to be a major factor influencing study findings as a post-hoc power test in the primary outcome variables of interest displayed a power that was greater than 0.8.

Another potential limitation within the study design includes the fact that subjects within the second meal challenge were not gender matched with the lean subjects (as in the first postprandial study in Chapter 4). This was due to the challenges associated with subject recruitment of healthy lean subjects which

precluded a precise ability to gender match controls with NAFLD children and adolescents. While some gender interactions between iAUC for HOMA-IR and Apo C-III were observed in this study, we did not see any other gender-outcome variable interactions in the other metabolic variables studies (i.e. no interactions between gender, liver enzymes, iAUC for NEFA and iAUC for TG were observed). Our conclusions would have been strengthened if Tanner stage would have been consistently available. An asset in our study is that subjects were age matched. Puberty may appear earlier in obese subjects, but obesity has shown to have a higher (by promoting earlier puberty) effect in girls than in boys (Burt Solorzano & McCartney, 2010) therefore data was adjusted to explore gender differences as previously mentioned. We also acknowledge that ideally in order to compare two meal effects the same subjects should have participated in both postprandial studies. This was not possible due to several subjects not consenting to the second postprandial study. The few subjects that participated in both studies showed similar insulin, NEFA and TG postprandial meal responses to what the other subjects did. A potential limitation in our study is that the amount of fat content in the meals differed by 5g (equivalent to a teaspoon). The postprandial TG rise was not significantly different between meals. Significant differences in the postprandial TG rise were observed by others when comparing meals with different type of fat content, but the amounts of fat content differed by at least 15g of fat (Dallongeville et al., 2002; Kim et al., 2006; Schneeman et al., 2003). Therefore, the possibility that this difference (5g) in fat may be masking our results is small.

5.4.2 Conclusions and Clinical Implications

Postprandial metabolic response may be mostly affected by chronic intake and general lifestyle; rather than acute changes in food intake. While intake of meal challenges high in SFA did evoke some postprandial changes in metabolic parameters regular intake of meals high in SFA and low in PUFA, especially LCPUFA (characteristic of western fast food) could promote simple steatosis in children and adolescents with NAFLD by increased NEFA spillover into the circulation and enhanced uptake into the liver, lower rates of CM clearance, increased VLDL production and increased Apo B-100 levels (Lambert & Parks, 2012; Parks, 2001; Tinker et al., 1999). This delayed lipid clearance in addition to the hyperinsulinemic state present in children and adolescents with NAFLD may promote a pro-inflammatory environment characterized by constantly higher TNF-α levels which would worsen the hyperinsulinemic state leading to a vicious cycle characterized by hyperinsulinemia-delayed clearance and a pro-inflammatory environment. How this may be ameliorated following the addition of a small amount of LCPUFA in a meal remains unclear as we did not see any major changes in postprandial lipid and lipoprotein expression with this small addition of the LCPUFA. The amount of LCPUFA that was added to the high saturated meal was based upon typical amounts that might be present in a fast food meal (e.g. in a fish based fast food meal) and therefore suggests that higher doses would be needed to evoke more favorable changes to the postprandial metabolic environment in children and adolescents with NAFLD. Nevertheless, a small increase in the amount of LCPUFA in the meal did evoke very modest

postprandial changes in plasma concentrations of the NEFA; a major substrate that is thought to contribute to the intrahepatic fatty acid pool within the liver in NAFLD. While the addition of LCPUFA to high fast food meal, may promote subtle changes in the acute postprandial period, they clearly do not overcome the adverse metabolic effects of consumption of a high saturated fast food meal. Therefore, this study reinforces that treatment in children and adolescents with NAFLD should promote changes in eating patterns. As mentioned in the previous chapter (chapter 4) attention should be placed not just in the quantity of fat but also in the quality. SFA intake should be discouraged especially in children and adolescents in order to improve lipid clearance. Further research is necessary to determine how LCPUFA intake (acute and chronic) may modulate the postprandial metabolic environment in children and adolescents with NAFLD.

CHAPTER 6. CLINICAL FINDINGS AND FINAL CONCLUSIONS

Postprandial lipemia or delayed lipid clearance is currently considered a risk factor for nonalcoholic fatty liver disease (NAFLD) in adults (Umpaichitra et al., 2004). Delayed lipid clearance has been associated with development and progression of cardiovascular disease (CVD) (Couch et al., 2000; Umpaichitra et al., 2004). The postprandial response in adults with NAFLD is characterized by delayed lipid clearance and insulin resistance (Flores-Calderón et al., 2005; Musso et al., 2003; Papandreau et al., 2007), but the postprandial responses in children and adolescents with NAFLD to certain types of meal challenges have not been described. The overall major objective of our study was to determine how varying the amount of LCPUFA in a high saturated fat meal reflective of typical meals consumed by children and adolescents, influences postprandial insulin, lipid and lipoprotein expression in children and adolescents with NAFLD and to compare these changes to the responses that occur in both healthy obese and lean children and adolescents. We also studied the body composition (skinfold fat distribution) to examine what simple body composition measures need to be used to screen for risk of NAFLD.

The first objective of our study was to describe the somatotype and body composition in children and adolescents with NAFLD as well as to study the potential interrelationships between body somatotype and biomarkers of liver disease risk and inflammation. It was hypothesized that the somatotype and body composition of children and adolescents with NAFLD will be different from

children and adolescents without NAFLD and would be characterized by higher body fat percentage and different areas of body fat distribution. Additionally, it was expected that measures of visceral and subcutaneous fat would be associated with fasting hyperinsulinemia, insulin resistance and increased ALT and AST. Our results showed that children and adolescents with NAFLD have similar body fat percentage in comparison to obese subjects but the body fat distribution is different. Major differences were that children and adolescents with NAFLD accumulate most of their subcutaneous fat in the trunk area within the abdominal skinfold. These results are similar to what Jun et al. observed in adults with NAFLD who exhibited higher subcutaneous fat when compared to subjects without NAFLD (Jun et al., 2008). In our study we determined that fat distribution is even more important than total body fat percentage as observed by the strong correlations of the subscapular and abdominal skinfolds with ALT and insulin values. Association between laboratory variables (e.g. ALT, insulin) and subcutaneous adipose tissue in children and adolescents with NAFLD have been previously observed (Fishbein et al., 2006) but the uniqueness of our study was that we were able to determine the subcutaneous areas (subscapular and abdominal) that contribute the most to abnormal laboratory variables in children and adolescents with NAFLD such as insulin and liver enzymes.

Despite the differences in the fat distribution; obese control and NAFLD subjects showed similar somatotype as they were all, except one subject, placed in the same category (mesomorphic endomorph). One possible explanation is that the category observed in these populations could be considered as the “risky

NAFLD zone" and maybe it is just a matter of time in which the obese non NAFLD will develop NAFLD. Development of NAFLD could depend on genetics, duration of metabolic abnormalities such as obesity, hyperinsulinemia and altered lipid profile. A second possible explanation could be that somatotype may not be considered as a diagnostic tool since it did not distinguis between NAFLD and obese controls and therefore more emphasis should be placed on body fat distribution.

The usefulness and application of study findings to the clinical setting is that the determination of body composition and somatotype by well-trained clinicians is a relatively easy and low cost technique that could provide valuable information to assess the risk of NAFLD in obese children and adolescents as well as to monitor efficacy of interventions on body composition. Biopsy is the standard of care and ALT and US are the tools often used by health care practitioners to screen for disease. The use of skinfold measures could be used as an additional tool to decide whether or not a more invasive procedure (e.g. liver biopsy) is necessary. Future research studies could include both the determination of the somatotype by skinfolds as well as magnetic resonance spectroscopy (MRS) which would provide useful information about the visceral fat including the intrahepatic triglyceride content (Deivanayagam et al., 2008; Kamba et al., 2001). The potential relationship between the somatotype and intrahepatic fat content in children and adolescents with NAFLD has not been explored. Studying how changes in body fat distribution using simple, and easy to use anthropometric measures (such as multiple skinfold measures) in children and adolescents with

NAFLD following a lifestyle intervention in combination with MRS, would afford a comprehensive and non-invasive evaluation of the effects of a lifestyle intervention on body fat distribution (including hepatic fat deposition) and markers of disease risk in this population.

An important clinical finding in this study was observed between the obese healthy controls and the obese children and adolescents with NAFLD. This finding was that children and adolescents with NAFLD have persistent fasting hyperinsulinemia typically in the presence of normoglycemia. Some, but not all children have elevations in liver function transaminases; but this is not a persistent finding. In our study the majority of the NAFLD children and adolescents exhibited elevated ALT while all the obese had normal values. This suggests that overweight and obese children and adolescents should have fasting measurements of insulin and glucose performed; with a consideration of a fasting HOMA-IR calculation performed to assess insulin sensitivity. This is particularly important in children who exhibit relative central adiposity in relation to their peripheral extremities. One limitation to this approach would be the consideration of puberty where transient insulin resistance might be expected to influence overall clinical assessment.

Overweight and obese children and adolescents may have delayed diagnosis of NAFLD especially if the fasting glucose and liver enzymes show normal values. Consideration of lowering the threshold for liver aminotransferases (below 20 U/L) in the assessment for disease risk for NAFLD

may be an important feature in this overall assessment, as we found that changes in body composition associated with NAFLD (central adiposity: both visceral and subcutaneous) were associated with ALT levels above 20 U/L (high normal values). Using simple and easy to measure anthropometric tools in the clinical setting in addition to consideration of lowering the threshold of specific laboratory parameters, might confer an added benefit to earlier detection of childhood NAFLD. This warrants further investigation.

The second objective of our study was to define the impact of acute intake of a high saturated fat meal/long chain polyunsaturated fatty acids free (LCPUFA) content on postprandial lipid, insulin, lipoproteins, apolipoproteins (apolipoprotein B-48, B100 and C-III) and cytokines (IL-10, IL-6 and TNF-α) in children and adolescents who are lean, obese or overweight controls and those with NAFLD. It was hypothesized that prolonged postprandial hyperinsulinemia induced by acute intakes of a high saturated fat/LCPUFA free meal will result in altered and delayed lipid, apolipoprotein (specifically: apolipoprotein B-48, B-100 and C-III) and cytokine expression (specifically: IL-10, IL-6 and TNF-α) in children and adolescents with NAFLD (see **Figure 6.1**). Similat to the results in study 1, children with NAFLD showed different body fat distribution when compared to obese controls as observed by the trend towards higher waist/hip ratio in spite of the lower BF% and WC. In this study, hyperinsulinemia was present in both the fasting and postprandial state in NAFLD children and adolescents (**Figure 6.1**). At this age lipid abnormalities such as high TG, LDL, total cholesterol and low HDL were similar between obese children and

adolescents and those without NAFLD. One of the interesting findings in our study was the impaired apolipoprotein behavior (higher postprandial Apo B-48, B-100 and fasted C-III) observed solely in children and adolescents with NAFLD suggesting an imbalance between influx and efflux of TG to and from the liver (**Figure 6.1**). This is one of the key differences observed in children and adolescents with NAFLD when compared to non NAFLD subjects. Our conclusion is that hyperinsulinemia is pivotal in the apolipoprotein derangements (Apo B-48, B-100 and C-III). We observed that excess of body fat by itself may not be enough to develop NAFLD; hyperinsulinemia was present in all the NAFLD children and adolescents suggesting that hyperinsulinemia may be the NAFLD epicenter, but clearly not the only metabolic derangement observed.

Hyperinsulinemia may evoke further changes in metabolism that contribute to childhood NAFLD. Whether or not an impaired hepatic and intestinal clearance of dietary fat delays or prolongs postprandial metabolic events or if intrahepatic differences in fat synthesis/catabolism contribute to an increased risk for steatosis, remains unclear from our data set. However, it is clear that disturbances in fat balance across the liver do exist in children and adolescents with NAFLD, even those with very mild disease (simple steatosis) as observed in all of our NAFLD patients with the exception of 3 subjects.

A unique finding in our study was the Apo C-III behaviour observed in the NAFLD population. High fasting levels followed by a dramatic postprandial decrease were observed following the consumption of a high saturated/LCPUFA-

free meal. It is possible that the surge of insulin observed in the NAFLD children and adolescents at 1 hour is helping to regulate and decrease the Apo C-III levels which activate LPL on peripheral tissues to enable a greater clearance after a meal rich in saturated fat and LCPUFA free (**Figure 6.1**). LPL impairment in both adipose and muscle tissue in children and adolescents with NAFLD could potentially lead to delayed removal of chylomicrons and chylomicron remnants (Frayn, 2001; Paglialunga et al., 2009; Picard et al., 2002; Preiss-Landl et al., 2002). The higher Apo B-48 values observed at 3 and 6hrs post meal as well as the higher Apo B-100 at baseline for children and adolescents with NAFLD may be evidence of LPL impairment probably as a consequence of the hyperinsulinemic state of the NAFLD population. These observations appear to be exacerbated by a meal high in SFA and very low in PUFA/LCPUFA (Ferland et al., 2012, Fielding & Frayn, 1998; Frayn, 2001). Hyperinsulinemia may be causing CM overproduction leading to high TG availability in the liver causing increased Apo B-100 VLDL production (Couillard et al., 2002; Valdivielso et al., 2010; Villodres et al., 2008) (**Figure 6.1**). Derangements of apolipoproteins could potentially worsen NAFLD by increasing the accumulation of fat in the liver and increase the risk for future cardiovascular disease events (CVD) (Alipour et al., 2008; Karpe et al., 1999).

Differences in postprandial fatty acid composition of the TG fractions were seen in the NAFLD group. As shown in chapter 4, the proportions of saturated and monounsaturated fatty acids within the TG changed across the postprandial period in the leans but no changes were observed in the NAFLD and

obese controls. These differences are reinforcing the idea that children and adolescents with NAFLD present impaired LPL, B-48, B-100 and C-III activities and indirectly infer that intrahepatic processes within the liver might result in the retention of saturated fat in particular. This is particularly evident as the healthy lean children and adolescents appear to clear saturated fatty acids 4-6 hours post meal consumption and the obese children and adolescents (with and without NAFLD) do not appear to do this. Another important conclusion obtained in our study was that consumption of a meal rich in SFA is not augmenting the already existing pro-inflammatory state of children and adolescents with NAFLD. Nevertheless, it is possible that no inflammatory changes were observed because most of our NAFLD subjects were diagnosed with simple steatosis and not NASH which is characterized by a higher proinflammatory state. The pro inflammatory state as observed by significantly elevated TNF-α values in our NAFLD population were similar to the ones observed by Manco et al. (Manco et al., 2007). Similar to what others have observed, C-reactive protein was also elevated in our NAFLD population (Chen et al., 2012; Kogiso et al., 2009; Neuman et al., 2010; Zimmermann et al., 2011). This pro-inflammatory state (**Figure 6.1**) may be mostly a consequence of hyperinsulinemia and excessive body fat and could promote deposition of fat in the liver by stimulating SREBP-1c (sterol regulatory element binding protein-1c) maturation (Endo et al., 2007; Lawler et al., 1998).

Briefly, this study suggests that children and adolescents with NAFLD should decrease the consumption of SFA in order to avoid exacerbating the delayed lipid clearance observed in this population as a consequence of the

hyperinsulinemia/insulin resistance. Although not studied in this research, it is possible that the delayed lipid clearance observed in children and adolescents with NAFLD may be worse if the meal has a high fructose, high GL content.

The third objective of our study was to determine if changing the long chain polyunsaturated fatty acids (LCPUFA) content of a high saturated meal evokes changes in insulin, lipid, apolipoprotein and cytokines expression in children and adolescents with NAFLD compared to meal tested in objective 2. It was hypothesized that incorporating LCPUFA into the meal would alter metabolic responses (glucose, insulin, changes in lipoproteins, apolipoproteins and cytokines). Additionally, higher LCPUFA amounts in a high saturated fat meal would ameliorate postprandial hyperinsulinemia, lipemia and altered lipoprotein and apolipoprotein expression in children and adolescents with NAFLD. We have concluded that a meal rich in SFA and higher in LCPUFA does not evoke significant postprandial changes. An exception to this was the decrease of NEFA and Apo C-III following the LCPUFA meal. We suspect that one of the major reasons why we observed only a few postprandial changes is due to the low amount of LCPUFA in the meal challenges. Nevertheless, the LCPUFA provided in the meal is similar to expected amount in a fast food meal (e.g. fish hamburger) (Fernández San Juan, 2000). An improvement in the insulin sensitivity (postprandial) was observed but it did not reach significance. Lower levels of NEFA in plasma were observed at the early postprandial phase respond (1 hr post meal) and a lower iAUC for NEFA were observed in the NAFLD children and adolescents consuming 1.5% LCPUFA versus the NAFLD group consuming 0%

LCPUFA. Our results suggest that a meal with more LCPUFA may improve lipoprotein lipase (LPL) activity allowing slower release into the plasma (Fielding, 2011; Frayn, 1998; Jackson et al., 2005). The Apo C-III levels are suggestive of a LPL and probably hepatic lipase (HL) improvement possibly due to reduced insulin levels. Improvement in HL and peripheral LPL would potentially decrease the uptake and/or delivery of TG rich lipoproteins to the liver (Ooi et al., 2008; Perret et al., 2002).

How to improve the delayed clearance observed in children and adolescents with NAFLD is currently a challenge. In our study we were not able to show that addition of LCPUFA to a high saturated fat meal challenge improves insulin and TG clearance in children and adolescents with NAFLD. Therefore, a very important clinical message is that SFA intake should be discouraged especially in children and adolescents with NAFLD in order to improve lipid clearance. Future studies could be improved by increasing the amount of LCPUFA to 1-2.5g of EPA and DHA as given in other supplementation trials (Robertson et al., 2002; Shah et al., 2007; Shapiro et al., 2011). Hyperglycemic and hyperinsulinemic clamp techniques could be used to study whether the insulin secretion capacity as well as insulin secretion is improved by the addition of LCPUFA to a high saturated fat meal challenge. Additionally, stable isotopes could be used to trace apolipoproteins and NEFA kinetics of two different meals (Fabbrini et al., 2009; Patterson & Veillon, 2001).

Future postprandial studies could be improved by including a higher amount of fat in the meal (30-50g) and omega-3 LCPUFA (> 1.5g) as well as extending the length of the postprandial period to 8 hours (Jans et al., 2012; Lairon, 2008; Parks, 2001; Robertson et al., 2002; Shah et al., 2007; Shapiro et al., 2011). By providing a larger a more robust response may be noted. Children and adolescents may also be more likely to comply to finish an eight hours postprandial study. Additionally two identical meal challenges could be compared by only adding the LCPUFA to one of the meals. In our study much of the suggested postprandial kinetic responses are speculative. Deuterated palmitate tracer infusion could be used to understand and determine with more accuracy the metabolism of chylomicron, chylomicron remnants, Apo B-100 VLDL, Apo B-100 LDL and NEFA kinetics (Fabbrini et al., 2009).

Why some children and adolescents with very similar body weight, body composition and somatotype develop NAFLD and why some others don´t remains still unclear. Genetic polymorphisms such as the Apo C-III variant alleles observed by Petersen et al., in an Asian population have been associated with NAFLD and insulin resistance (Duvnjak et al., 2009; Petersen et al., 2010). Genetic polymorphisms are beyond the scope of this research but it could be speculated that since our population of study was fairly homogeneous in terms if ethnicity it is possible that some of the differences observed in the Apo C-III are due to polymorphisms. Higher prevalence of NAFLD has been observed in Asian and Hispanic population (Duvnjak et al., 2009), but it must be emphasized that genetic predisposition is only one factor contributing to disease expression and

several factors that are able to be controlled such as diet, are definitively playing a role in the phenotype expression and NAFLD development. Such factors include physical activity during the first years of age (Power & Parsons, 2000) current type and intensity physical activity, nutrition in early life stages such as breastfeeding vs formula (Nobili et al., 2009) and quality of diet (macro and micronutrient distribution) among others.

To our knowledge this is the first study in children and adolescents with NAFLD to include a wide variety of fasting and postprandial metabolic markers such as insulin, lipid profile including the fatty acid composition of TG and PL fractions, apolipoproteins and inflammatory markers. Another strength of our study is the in depth and first time description of the body composition and somatotype in NAFLD children and adolescents in comparison to obese control and healthy leans. Additionally, all of our NAFLD subjects except the ones that did not meet the criteria for biopsy as per the hospital protocol were diagnosed according to the "gold standard" which is liver biopsy (Pietro et al., 2012). Liver biopsy proven disease can be hard to achieve in clinical studies with children. The use of biopsy proven disease also allowed us to identify that metabolic perturbations occur even in the presence of milder disease (simple steatosis); a finding that is unique with the literature. Our inclusion and exclusion criteria allowed us to explore an ample range of body types and health status. Another unique feature of our study was the comparison of two isocaloric meals which were reflective of the fast food composition that children and adolescents in North

America actually eat, similar in the amount of fat, fructose and GL but with different LCPUFA amount.

Some of the study weaknesses are that ideally all the NAFLD subjects should have had biopsy and all the obese control should have had an US. However, given that liver biopsy has substantial health risks associated, it was only performed when clinically indicated. Nevertheless a statistical analysis was performed with all the obese controls as well as only those with US and no statistical differences were observed in any of the other outcomes. Another weakness for our study is that the lean subjects were age but not gender matched but as mentioned in the chapter 3, both genders were equally distributed in the lean group. In the chapter 3 we were able to show that gender did not affect the interrelationships between subcutaneous and visceral adiposity markers with markers of liver function, inflammation and altered lipemia. Although our subjects were age matched, puberty may appear earlier in obese subjects, but the effect of obesity to early puberty has shown to have a higher effect in girls (Burt Solorzano & McCartney, 2010). Research suggests that during puberty, higher lipid profile and temporary insulin resistance may be experienced (Moran et al., 2002; Spinneker et al., 2007). Nevertheless, studies have also shown that the most determinant factors for these metabolic abnormalities are BMI and body fat percentage (Moran et al., 2002; Spinneker et al., 2007). We also acknowledge that in order to establish a "pure" meal effect the same subjects should have participated in both postprandial studies.

6.1 Clinical implications and overall conclusion

This research suggests that elevated subscapular skinfold, fasting ALT and Apo C-III in addition to high 1hr post meal insulin values are anthropometric and laboratory markers that distinguish children and adolescents with NAFLD from the non-NAFLD subjects. The measurement of these variables in the clinical setting may be helpful to detect more promptly who is at higher risk to develop NAFLD. Additionally, in our study we observed that Brozek formula showed the closest body fat percentages in both lean and obese subjects when compared to Bod Pod values measured by ADP. Therefore, Brozek formula could be used in the clinical setting when other more expensive techniques are not available. Clinical implications of the postprandial findings in terms of treatment include that the adverse effects of a meal rich in SFA but low in fructose, GI and GL are not diminished by a small amount of LCPUFA. Both of the postprandial meal challenges were rich in C16:0. Main sources of this fatty acid include milk, cheese and butter. It should be emphasized that none of these foods should be prohibited since there are good sources of other nutrients (e.g. calcium). Special attention should be placed in the amount these foods are being consumed. Based on our observations SFA promote delayed lipid clearance. Therefore, SFA intake should be discouraged in children and adolescents.

Overall, our study suggests that children and adolescents with NAFLD are characterized by accumulation of fat in the trunk area, fasting and postprandial hyperinsulinemia as well as altered apolipoprotein (Apo B-48, B-100 and C-III)

expression. NAFLD subjects exhibited lower rates of postprandial appears of plasma saturate fatty acids after consuming a meal in comparison to the lean group, but obese control subjects behaved similar to NAFLD. Interestingly postprandial behaviors did not change in the NAFLD and leans with the 1.5% LCPUFA meal. Taken all together this data suggests that lipid clearance was not impacted specifically by the addition of a small amount of LCPUFA. Further investigation is necessary to determine to what the postprandial response can be modulated by modifications of a meal challenge or if the postprandial response is mostly determined by the physique such as body composition and regular dietary intake.

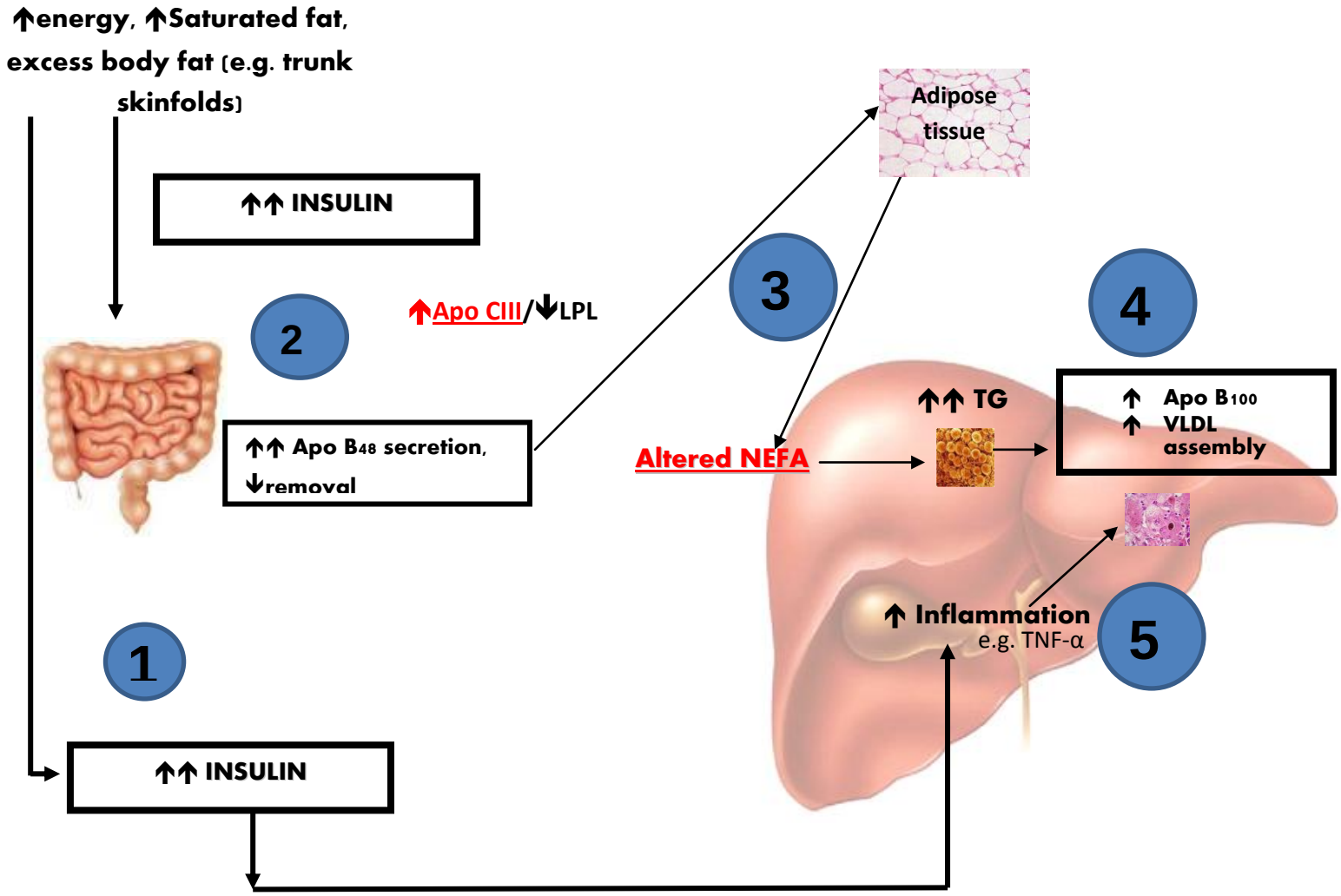


Figure 6.1. NAFLD metabolic derangements. Abbreviations: CM: Chylomicron; NASH: nonalcoholic steatohepatitis; NEFA: non esterified fatty acids; SS: simple steatosis; TG: triglycerides; VLDL: very low density lipoproteins. Red color: Improved by a higher consumption of LCPUFA

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Appendix 1. Form A

UNIVERSITY OF ALBERTA

Assent Form (Healthy children and adolescents)

Title of Project: Post-prandial lipemia in children with nonalcoholic steatohepatitis (NASH)

Principal Investigator: Diana Mager, PhD RD Telephone: 780-492-7687

Co-Investigator: Dr. Susan M. Gilmour, MD Telephone: 780-407-3339
Dr. Jason Yap, MD Telephone: 780-407-3698

You have a fatty liver. We would like you to participate in a research study that will help us understand how the foods that you eat might affect your liver. We think that the type of foods you eat (fatty and sugary foods) might affect the way your liver handles fat, especially after you eat a meal. We would like to study how the food you eat affects the way your liver stores fat.

Change to the study. We would like to ask you to come back to the Human Nutrition Research Unit (HNRU) to repeat the study again. This time we would like you to eat a slightly different breakfast. This breakfast will consist of most of the same foods as on the first study day, except there will be more vegetables in it and some different types of fat. We wish to see how the different types of fat in food affects the way your liver stores fat. We are doing this part of the study now as we just got some more funding to do the study.

What will you have to do?
If you and your parents agree that it is okay to take part in this study, we will ask you to come to the Human Nutrition Research Unit (HNRU) at the University of Alberta.

1) We will measure your weight, height, waist and some arm measurements. We will measure your waist by putting a tape measure around your waist and take a measurement of your skin from the back of your arm with a caliper. Calipers look like tongs. It will look like you will be getting a little pinch but it will not feel this way. This does not hurt.

2) We will also measure the amount of muscle in your body with a special machine called the Bod Pod. This test takes about 5 minutes. We will ask you to wear a swimsuit and a swim cap when you do this test. You will not get wet. This test is very safe.

3) We will ask you to eat a meal (breakfast) and to let us take a blood sample just before you eat the meal, and 1 hour and 3 and 6 hours after you eat the meal. We will offer you some choices in the type of foods that we want you to eat on the day that we do these tests to make sure you are eating breakfast foods that you like. The blood tests are extra blood tests. We want to take approximately ½ teaspoon of blood each time so we can measure how your body metabolizes

fat in your liver after a meal. You will be asked not to eat anything after your supper the night before you come to the Human Nutrition Research Unit at the University of Alberta.

3) We will ask you to write down what you eat on three different days (2 weekdays and 1 weekend day). It will take about 10 minutes to fill out the food record on each of the three days. Your parents can help you write down what you eat. We will provide you and your parents/caregiver with a self-addressed stamped envelope so you can mail this back to the research team.

4) We would also like to look at your medical chart to see the results from other tests you have had.

Will it help? We don't know if finding out about the way your liver metabolizes fat will help you. We think that understanding the way your liver does this will help us know how what you should eat.

Will it hurt? All of the additional tests are harmless. Giving the extra blood might be a little uncomfortable. It will feel the same as when you give blood at your clinic visits with the doctor. You may not also like the taste of the breakfast that we provide to you. However, we will give you a list of the foods that you like before the study day so you can let us know which foods you like. We will offer you only the foods that you chose from this list.

Can you quit?: You don't have to take part in the study, and you can quit at any time. No one will be mad at you if you decide you don't want to do this, or if you decide to stop part way through. You should tell the doctor or dietitian or the research team that you want to quit.

Who will know?: No one except your parents and the doctor will know you're taking part in the study unless you want to tell them. Your name and your chart won't be seen by anyone except the doctors and nurses and dietitians and research team.

Your signature: We would like you to sign this form to show that you agree to take part. Your mom or dad will be asked to sign another form agreeing for you to take part in the study.

Do you have more questions? You can ask your mom or dad about anything you don't understand. You can also talk to Dr. Gilmour or Dr. Jason Yap or Diana Mager (researcher). Dr. Gilmour's telephone number is 780-407-3339 and Dr. Mager's telephone number is 780-492-7687. Dr. Yap's telephone number is 780-407-3698.

I agree to take part in the study.

Signature of research participant:_____Date: _____

Signature of witness:_____Date: _____

Signature of investigator:_____Date:_____

Appendix 1. Form B

UNIVERSITY OF ALBERTA

Assent Form (Healthy Children and Adolescents)

Title of Project: Post-prandial lipemia in children with nonalcoholic steatohepatitis (NASH).

Principal Investigator: Diana Mager, PhD RD Telephone: 780-492-7687

Co-Investigator: Dr. Susan M. Gilmour, MD Telephone: 780-407-3339
Dr. Jason Yap MD Telephone: 780-407-3698

Some children get fatty livers. We are not sure why. We think that the type of foods you eat (fatty and sugary foods) might affect the way your liver handles fat, especially after a fatty meal. We would like you to participate in a research study that will help us understand how foods might affect your liver.

What will you have to do?

If you and your parents agree that it is okay to take part in this study, we will ask you to come to the Human Nutrition Research Unit (HNRU) on two different days, University of Alberta:

On each study day:

1) We will measure your weight, height, waist and some arm measurements. We will measure your waist by putting a tape measure around your waist and take a measurement of your skin from the back of your arm with a caliper. Calipers look like tongs. It will look like you will be getting a little pinch but it will not feel this way. This does not hurt.

2) We will also measure the amount of muscle in your body with a special machine called the Bod Pod. This test takes about 5 minutes. We will ask you to wear a swimsuit and a swim cap when you do this test. You will not get wet. This test is very safe.

3) We will ask you to eat a meal (breakfast) and to let us take a blood sample just before you eat the meal, and 1 hour and 3 and 6 hours after you eat the meal. We will offer you some choices in the type of foods that we want you to eat on the day that we do these tests to make sure you are eating breakfast foods that you like. On each of the different days the meals will be a little different. One day will have some extra vegetables with the meals and slightly different fats in the meal. The blood tests are extra blood tests. We want to take approximately ½ teaspoon of blood each time so we can measure how your body metabolizes fat in your liver after a meal. You will be asked not to eat anything after your supper the night before you come to the Human Nutrition Research Unit at the University of Alberta.

4) We will ask you to write down what you eat on three different days (2 weekdays

and 1 weekend day). It will take about 10 minutes to fill out the food record on each of the three days. Your parents can help you write down what you eat. We will provide you and your parents/caregiver with a self-addressed stamped envelope so you can mail this back to the research team.

4) We would also like to look at your medical chart to see the results from other tests you have had.

5) If you agree that you will participate in this study, we will ask you to have an ultrasound of your liver. An ultrasound is a test that takes pictures of your liver to see if your liver is healthy. It will not hurt and is completely safe. This test takes about 15-20 minutes.

Will it help? We don't know if finding out about the way your liver metabolizes fat will help you. We think that understanding the way your liver does this will help us know how what you should eat.

Will it hurt? All of the additional tests are harmless. Giving the extra blood might be a little uncomfortable. It will feel the same as when you give blood at your clinic visits with the doctor. You may not also like the taste of the breakfast that we provide to you. However, we will give you a list of the foods that you like before the study day so you can let us know which foods you like. We will offer you only the foods that you chose from this list.

Can you quit?: You don't have to take part in the study, and you can quit at any time. No one will be mad at you if you decide you don't want to do this, or if you decide to stop part way through. You should tell the doctor or dietitian or the research team that you want to quit.

Who will know?: No one except your parents and the doctor and research team will know you're taking part in the study unless you want to tell them. Your name and your chart won't be seen by anyone except the doctors and nurses and dietitians and research team.

Your signature: We would like you to sign this form to show that you agree to take part. Your mom or dad will be asked to sign another form agreeing for you to take part in the study.

Do you have more questions? You can ask your mom or dad about anything you don't understand. You can also talk to Dr. Gilmour or Diana Mager (researcher) or Dr. Yap. Dr. Gilmour's telephone number is 780-407-3339 and Dr. Mager's telephone number is 780-492-7687. Dr. Yap's telephone number is 780-492-3698.

I agree to take part in the study.

Signature of research participant:_____Date: _____

Signature of witness:_____Date: _____

Signature of
vestigator:_____Date:_____

Appendix 1. Form C

UNIVERSITY OF ALBERTA

Assent Form (Healthy children and adolescents with NASH)

Title of Project: Post-prandial lipemia in children with nonalcoholic steatohepatitis (NASH)

Principal Investigator: Diana Mager, PhD RD Telephone: 780-492-7687

Co-Investigator: Dr. Susan M. Gilmour, MD Telephone: 780-407-3339
Dr. Jason Yap, MD Telephone: 780-407-3698

You have a fatty liver. We would like you to participate in a research study that will help us understand how the foods that you eat might affect your liver. We think that the type of foods you eat (fatty and sugary foods) might affect the way your liver handles fat, especially after you eat a meal. We would like to study how the food you eat affects the way your liver stores fat.

Change to the study. We would like to ask you to come back to the Human Nutrition Research Unit (HNRU) to repeat the study again. This time we would like you to eat a slightly different breakfast. This breakfast will consist of most of the same foods as on the first study day, except there will be more vegetables in it and some different types of fat. We wish to see how the different types of fat in food affects the way your liver stores fat. We are doing this part of the study now as we just got some more funding to do the study.

What will you have to do?
If you and your parents agree that it is okay to take part in this study, we will ask you to come to the Human Nutrition Research Unit (HNRU) at the University of Alberta.

1) We will measure your weight, height, waist and some arm measurements. We will measure your waist by putting a tape measure around your waist and take a measurement of your skin from the back of your arm with a caliper. Calipers look like tongs. It will look like you will be getting a little pinch but it will not feel this way. This does not hurt.

2) We will also measure the amount of muscle in your body with a special machine called the Bod Pod. This test takes about 5 minutes. We will ask you to wear a swimsuit and a swim cap when you do this test. You will not get wet. This test is very safe.

3) We will ask you to eat a meal (breakfast) and to let us take a blood sample just before you eat the meal, and 1 hour and 3 hours and 6 hours after you eat the meal. We will offer you some choices in the type of foods that we want you to eat on the day that we do these tests to make sure you are eating breakfast foods that you like. The blood tests are extra blood tests. We want to take

approximately ½ teaspoon of blood each time so we can measure how your body metabolizes fat in your liver after a meal. You will be asked not to eat anything after your supper the night before you come to the Human Nutrition Research Unit at the University of Alberta.

3) We will ask you to write down what you eat on three different days (2 weekdays and 1 weekend day). It will take about 10 minutes to fill out the food record on each of the three days. Your parents can help you write down what you eat. We will provide you and your parents/caregiver with a self-addressed stamped envelope so you can mail this back to the research team.

4) We would also like to look at your medical chart to see the results from other tests you have had.

Will it help? We don't know if finding out about the way your liver metabolizes fat will help you. We think that understanding the way your liver does this will help us know how what you should eat.

Will it hurt? All of the additional tests are harmless. Giving the extra blood might be a little uncomfortable. It will feel the same as when you give blood at your clinic visits with the doctor. You may not also like the taste of the breakfast that we provide to you. However, we will give you a list of the foods that you like before the study day so you can let us know which foods you like. We will offer you only the foods that you chose from this list.

Can you quit?: You don't have to take part in the study, and you can quit at any time. No one will be mad at you if you decide you don't want to do this, or if you decide to stop part way through. You should tell the doctor or dietitian or the research team that you want to quit.

Who will know?: No one except your parents and the doctor will know you're taking part in the study unless you want to tell them. Your name and your chart won't be seen by anyone except the doctors and nurses and dietitians and research team.

Your signature: We would like you to sign this form to show that you agree to take part. Your mom or dad will be asked to sign another form agreeing for you to take part in the study.

Do you have more questions? You can ask your mom or dad about anything you don't understand. You can also talk to Dr. Gilmour or Dr. Jason Yap or Diana Mager (researcher). Dr. Gilmour's telephone number is 780-407-3339 and Dr. Mager's telephone number is 780-492-7687. Dr. Yap's telephone number is 780-407-3698.

I agree to take part in the study.

Signature of research participant:_____Date: _____

Signature of witness:_____Date: _____

Signature of investigator:_____Date:_____

Appendix 1. Form D

UNIVERSITY OF ALBERTA

Assent Form (Healthy Children and Adolescents with NASH)

Title of Project: Post-prandial lipemia in children with nonalcoholic steatohepatitis (NASH).

Principal Investigator: Diana Mager, PhD RD Telephone: 780-492-7687

Co-Investigator: Dr. Susan M. Gilmour, MD Telephone: 780-407-3339
Dr. Jason Yap MD Telephone: 780-407-3698

Some children get fatty livers. We are not sure why. We think that the type of foods you eat (fatty and sugary foods) might affect the way your liver handles fat, especially after a fatty meal. We would like you to participate in a research study that will help us understand how foods might affect your liver.

What will you have to do?
If you and your parents agree that it is okay to take part in this study, we will ask you to come to the Human Nutrition Research Unit (HNRU) on two different days, University of Alberta:
On each study day:

1) We will measure your weight, height, waist and some arm measurements. We will measure your waist by putting a tape measure around your waist and take a measurement of your skin from the back of your arm with a caliper. Calipers look like tongs. It will look like you will be getting a little pinch but it will not feel this way. This does not hurt.

5) We will also measure the amount of muscle in your body with a special machine called the Bod Pod. This test takes about 5 minutes. We will ask you to wear a swimsuit and a swim cap when you do this test. You will not get wet. This test is very safe.

6) We will ask you to eat a meal (breakfast) and to let us take a blood sample just before you eat the meal, and 1 hour and 3 and 6 hours after you eat the meal. We will offer you some choices in the type of foods that we want you to eat on the day that we do these tests to make sure you are eating breakfast foods that you like. On each of the different days the meals will be a little different. One day will have some extra vegetables with the meals and slightly different fats in the meal. The blood tests are extra blood tests. We want to take approximately ½ teaspoon of blood each time so we can measure how your body metabolizes fat in your liver after a meal. You will be asked not to eat anything after your supper the night before you come to the Human Nutrition Research Unit at the University of Alberta.

4) We will ask you to write down what you eat on three different days (2 weekdays

and 1 weekend day). It will take about 10 minutes to fill out the food record on each of the three days. Your parents can help you write down what you eat. We will provide you and your parents/caregiver with a self-addressed stamped envelope so you can mail this back to the research team.

7) We would also like to look at your medical chart to see the results from other tests you have had.

5) If you agree that you will participate in this study, we will ask you to have an ultrasound of your liver. An ultrasound is a test that takes pictures of your liver to see if your liver is healthy. It will not hurt and is completely safe. This test takes about 15-20 minutes.

Will it help? We don't know if finding out about the way your liver metabolizes fat will help you. We think that understanding the way your liver does this will help us know how what you should eat.

Will it hurt? All of the additional tests are harmless. Giving the extra blood might be a little uncomfortable. It will feel the same as when you give blood at your clinic visits with the doctor. You may not also like the taste of the breakfast that we provide to you. However, we will give you a list of the foods that you like before the study day so you can let us know which foods you like. We will offer you only the foods that you chose from this list.

Can you quit?: You don't have to take part in the study, and you can quit at any time. No one will be mad at you if you decide you don't want to do this, or if you decide to stop part way through. You should tell the doctor or dietitian or the research team that you want to quit.

Who will know?: No one except your parents and the doctor and research team will know you're taking part in the study unless you want to tell them. Your name and your chart won't be seen by anyone except the doctors and nurses and dietitians and research team.

Your signature: We would like you to sign this form to show that you agree to take part. Your mom or dad will be asked to sign another form agreeing for you to take part in the study.

Do you have more questions? You can ask your mom or dad about anything you don't understand. You can also talk to Dr. Gilmour or Diana Mager (researcher) or Dr. Yap. Dr. Gilmour's telephone number is 780-407-3339 and Dr. Mager's telephone number is 780-492-7687. Dr. Yap's telephone number is 780-492-3698.

I agree to take part in the study.

Signature of research participant:_____Date: _____

Signature of witness:_____Date: _____

Signature of Investigator:_____Date:_____

Appendix 1. Form E

UNIVERSITY OF ALBERTA

INFORMATION LETTER
(Children and Adolescents with NASH)

Title of Project:	Post-prandial lipemia in children with nonalcoholic steatohepatitis (NASH).	
Principal Investigator:	Diana Mager, PhD RD	Telephone: 780-492-7687
Co-Investigator:	Dr. Susan M. Gilmour, MD	Telephone: 780-407-3339
	Dr. Jason Yap MD	Telephone: 780-407-3698

This information letter is intended for the study subject's parents/caregiver. If you are signing on behalf of your child, the words 'you' and 'your' should be read as your 'your child'.

Purpose of this study

We would like you to participate in a research study that will help us understand why children get fatty livers. There is some evidence that fat build-up in the liver may be due to the type of foods (fatty and sugary foods) that children eat and the way the body processes these foods after you eat them. Our plan is to study how your diet affects the way fat is metabolized in the liver after you eat a meal. We want to study if the fat from your food affects your liver.

Addendum: Since, you already consented to our study, we would like to ask you to come to the HNRU to eat another small meal (breakfast) and to perform the 'exact' same tests as you did when you participated in our study the first time. We want to see if differences in the type of fat also affect the way the liver metabolizes fat after you eat. The meal that we are going to provide to you is a little different from the last meal. It will consist of fats that are thought to be healthy in the diet (long chain polyunsaturated fats). We will serve you a slightly different meal than the meal that we gave you before which was more typical of the North American diet. We are now able to study this as we have been given some extra funding to study this that we did not have at the time that we asked you to be in the study the first time.

Procedure(s) of the study

1. Anthropometric Measurements

We will measure your weight, height and waist. We will measure your waist circumference by putting a tape measure around your waist. We will also measure how big your muscles are by taking a measurement of your skin from the back of your arm, with a caliper. Your wrist and elbow diameter will be measured will a small bone caliper. Calipers look like tongs. It will look like a little pinch but will not feel like one. The research nurse will also take your blood pressure. This will happen during your regular clinic appointments in the Pediatric Centre for Weight and Health.

We will also measure the amount of muscle in your body with a special machine called the Bod Pod machine. The Bod Pod is a machine that measures the amount of muscle in a person's body by measuring body weight and by detecting the difference in volume of the air before and after a person sits in the machine. This allows the machine to calculate how much of your body is muscle and how much is fat. The Bod Pod consists of two chambers: the test chamber, where you will be sitting and the reference chamber which is mainly the seat. The Bod Pod test takes about 5 minutes, and you will be asked to wear a swimsuit and a swim cap. You need to wear these instead of regular clothes so the machine can measure your muscles correctly. You will not get wet and the test is not uncomfortable at all and it is completely safe. The Bod Pod test will be done at the Human Nutrition Research Unit, HNRU, University of Alberta, at time of entry into our study and 6 months later during your regularly scheduled clinic appointment. Because this test is not happening at the same time as your clinic appointment we will pay for parking costs at the university.

2. Oral Fat Meal

When you come to the HNRU, we will also ask you to eat a small meal (breakfast). The meal that we will provide to you will consist of foods that are typically eaten at breakfast (bread/breakfast cereal, butter, jam, juice, eggs, cheese and/or milk).We will offer you a choice of meals to choose from prior to coming to the HNRU on the study day to make sure that you only eat food that you like. We will also review with you about any history of food allergies you may have to make sure we do not offer any foods that you are allergic to. Our kitchen is not a peanut-free zone, so if you have a peanut allergy you should not participate in this study. On the day that you are coming to the HNRU you will be asked not to eat anything after your supper the night before you come. For this test we will also need to collect four blood samples from you (approximately ½ teaspoon each time). We need to do this before you eat the small meal, and then again 1 hour after, 3 and 6 hours after. This is in *addition t*o the routine blood work that you would do at the Stollery Children's Hospital after a clinic visit. To avoid any extra unnecessary pokes, the nurse will put in an indwelling catheter in your arm so we can get the three blood samples without having to poke you each time. The reason we want to collect this blood from you is to study how the fats in the meal affects the amount of fat and some proteins in your blood and how the levels change in your blood after you eat a meal. This information will help us understand the way your liver metabolizes fat after a meal and provide us with more information about why some children deposit fat in their livers and why others do not. While you are waiting in the HNRU you can watch movies or listen to music.

New Meal: We will serve you a slightly different meal than the meal that we gave you before which was more typical of the North American diet. The foods in this meal will include many of the same foods (eggs, milk), but will have some new choices as well (vegetables, differences in cooking oils).

3. Food Intake

We will ask you to fill out a three day food record based on what you eat. For the three day food records we will ask you to write down what you have eaten for the three days (2 weekdays and 1 weekend day). It will take about 10 minutes to fill out the food record on each of the three days. We will provide you with a self-addressed stamped envelope so you can mail this back to the research team.

4. Medical Records

We would also like to look at your medical records to find out about medications, relevant lab work (for example at the amount of fat and sugar that is present in the blood) and results of other medical tests that were used to find out about your liver. We would also like to review your medical records to look at your liver biopsy results. This will help us

understand everything about your liver.

5. Abdominal Ultrasound

If you agree that you will participate in this study, we will ask you to have an ultrasound to rule out the possibility that you have any extra fat in your liver. An ultrasound is a test that takes pictures of your liver to see if your liver is healthy. It will not hurt and is completely safe. This test takes about 15-20 minutes. We will make sure that you know the results of this test. If there are any concerns about the results, we will refer you to a liver doctor.

.**Possible Risks**

All of the ***additional*** tests used in this study have little potential for harm. Our kitchen is not a peanut-free zone, so if you have a peanut allergy you should not participate in this study. Another risk for this study is the potential discomfort with providing the blood samples. This may include some minor discomfort or bruising.

Possible Benefits

There are no direct benefits to you in this study. We are not sure of the best way to prevent and treat a fatty liver in children. Your participation in this study will help us to understand how some children get fat in their livers. This information will help us understand what is the best way to prevent getting a fatty liver.

Confidentiality: We will not share any information in your personal health record with anyone. Any research data collected about you during this study will not identify you by name, only by your initials and a coded number. Your name will not be shared with anyone outside the research clinic and your name will not be in any reports published from this research.

For this study, the doctor or other members of the research team (dietitian, graduate student) may need to access your personal health records for health information. He/she may also need to contact your family doctor and your other health care providers to obtain additional medical information. The health information collected as part of this study will be kept confidential unless release is required by law, and will be used only for the purpose of the research study. By signing the consent form you give permission to the study staff to access any personally identifiable health information which is under the custody of other health care professionals. This will only be done if it is thought to be necessary to carry out this research project.

The personal health information collected in this study may need to be checked by the Health Research Ethics Board (HREB) at the University of Alberta. This may be necessary so the HREB can make sure that the data collected in the study is accurate. .

By signing the consent form you give permission for the collection, use and sharing of information from your medical records for purpose of this research. In the University of Alberta, study information is required to be kept for 7 years. Even if you withdraw from the study, the medical information which is obtained from you the research will not be destroyed. You have a right to check your health records and request changes if your personal information is incorrect.

Voluntary Participation: You are free to stop participating in the study at any time. This will not affect the quality of medical care that you are provided with by your doctor or dietitian. If there is any information that is gained from the study that may affect your decision to continue with this study, we will let you know right away.

Reimbursement of Expenses: You will be provided with parking vouchers to cover the cost of your parking expenses.

Compensation for Injury*:* If you become ill or injured as a result of participating in this study, necessary medical treatment will be available at no additional cost to you. By signing this consent form you are not releasing the investigator(s)or institution(s) from their legal and professional responsibilities.

Do you have more questions?

You can ask your dietitian about anything you don't understand. You can also talk to Diana Mager or Susan M. Gilmour or Dr. Yap. Diana Mager's phone number is 492-7687. Susan Gilmour's telephone number is 407-3339. Jason Yap's phone number is 780-407-3698. If you have any problems or concerns about any part of this study please call the Patient Relations Office of Capital Health at (780)-482-8080.This office has no connection with the study researchers.

Principal Investigator:	Diana Mager, PhD RD	Telephone: 780-492-7687
Co-Investigator:	Dr. Susan M. Gilmour, MD	Telephone: 780-407-3339
	Dr. Jason Yap MD	Telephone: 780-407-3698

Appendix 1. Form F

UNIVERSITY OF ALBERTA

INFORMATION LETTER
(Healthy Children and Adolescents)

Title of Project:	Post-prandial lipemia in children with nonalcoholic steatohepatitis (NASH).	
Principal Investigator:	Diana Mager, PhD RD	Telephone: 780-492-7687
Co-Investigator:	Dr. Susan M. Gilmour, MD	Telephone: 780-407-3339
	Dr. Jason Yap MD	Telephone: 780-407-3698

This information letter is intended for the study subject's parents/caregiver. If you are signing on behalf of your child, the words 'you' and 'your' should be read as your 'your child'.

Purpose of this study

We would like you to participate in a research study that will help us understand why children get fatty livers. There is some evidence that fat build-up in the liver may be due to the type of foods (fatty and sugary foods) that children eat and the way the body processes these foods after you eat them. Our plan is to study how your diet affects the way fat is metabolized in the liver after you eat a meal. We want to study if the fat from your food affects your liver.

Addendum: Since, you already consented to our study, we would like to ask you to come to the HNRU to eat another small meal (breakfast) and to perform the 'exact' same tests as you did when you participated in our study the first time. We want to see if differences in the type of fat also affect the way the liver metabolizes fat after you eat. The meal that we are going to provide to you is a little different from the last meal. It will consist of fats that are thought to be healthy in the diet (long chain polyunsaturated fats). We will serve you a slightly different meal than the meal that we gave you before which was more typical of the North American diet. We are now able to study this as we have been given some extra funding to study this that we did not have at the time that we asked you to be in the study the first time.

Procedure(s) of the study

1. Anthropometric Measurements

We will measure your weight, height and waist. We will measure your waist circumference by putting a tape measure around your waist. We will also measure how big your muscles are by taking a measurement of your skin from the back of your arm, with a caliper. Your wrist and elbow diameter will be measured will a small bone caliper. Calipers look like tongs. It will look like a little pinch but will not feel like one. The research nurse will also take your blood pressure. This will happen during your regular clinic appointments in the Pediatric Centre for Weight and Health.

We will also measure the amount of muscle in your body with a special machine called the Bod Pod machine. The Bod Pod is a machine that measures the amount of muscle in a person's body by measuring body weight and by detecting the difference in volume of the air before and after a person sits in the machine. This allows the machine to calculate how much of your body is muscle and how much is fat. The Bod Pod consists of two chambers: the test chamber, where you will be sitting and the reference chamber which is mainly the seat. The Bod Pod test takes about 5 minutes, and you will be asked to wear a swimsuit and a swim cap. You need to wear these instead of regular clothes so the machine can measure your muscles correctly. You will not get wet and the test is not uncomfortable at all and it is completely safe. The Bod Pod test will be done at the Human Nutrition Research Unit, HNRU, University of Alberta, at time of entry into our study and 6 months later during your regularly scheduled clinic appointment. Because this test is not happening at the same time as your clinic appointment we will pay for parking costs at the university.

2. Oral Fat Meal

When you come to the HNRU, we will also ask you to eat a small meal (breakfast). The meal that we will provide to you will consist of foods that are typically eaten at breakfast (bread/breakfast cereal, butter, jam, juice, eggs, cheese and/or milk).We will offer you a choice of meals to choose from prior to coming to the HNRU on the study day to make sure that you only eat food that you like. We will also review with you about any history of food allergies you may have to make sure we do not offer any foods that you are allergic to. Our kitchen is not a peanut-free zone, so if you have a peanut allergy you should not participate in this study. On the day that you are coming to the HNRU you will be asked not to eat anything after your supper the night before you come. For this test we will also need to collect three blood samples from you (approximately ½ teaspoon each time). We need to do this before you eat the small meal, and then again 1 hour after and 3 hours. This is in *addition t*o the routine blood work that you would do at the Stollery Children's Hospital after a clinic visit. To avoid any extra unnecessary pokes, the nurse will put in an indwelling catheter in your arm so we can get the three blood samples without having to poke you each time. The reason we want to collect this blood from you is to study how the fats in the meal affects the amount of fat and some proteins in your blood and how the levels change in your blood after you eat a meal. This information will help us understand the way your liver metabolizes fat after a meal and provide us with more information about why some children deposit fat in their livers and why others do not. While you are waiting in the HNRU you can watch movies or listen to music.

New Meal: We will serve you a slightly different meal than the meal that we gave you before which was more typical of the North American diet. The foods in this meal will include many of the same foods (eggs, milk), but will have some new choices as well (vegetables, differences in cooking oils).

3. Food Intake

We will ask you to fill out a three day food record based on what you eat. For the three day food records we will ask you to write down what you have eaten for the three days (2 weekdays and 1 weekend day). It will take about 10 minutes to fill out the food record on each of the three days. We will provide you with a self-addressed stamped envelope so you can mail this back to the research team.

4. Medical Records

We would also like to look at your medical records to find out about medications, relevant lab work (for example at the amount of fat and sugar that is present in the blood) and results of other medical tests that were used to find out about your liver. We would also like to review your medical records to look at your liver biopsy results. This will help us

understand everything about your liver.

3. Abdominal Ultrasound

If you agree that you will participate in this study, we will ask you to have an ultrasound to rule out the possibility that you have any extra fat in your liver. An ultrasound is a test that takes pictures of your liver to see if your liver is healthy. It will not hurt and is completely safe. This test takes about 15-20 minutes. We will make sure that you know the results of this test. If there are any concerns about the results, we will refer you to a liver doctor.

.**Possible Risks**

All of the ***additional*** tests used in this study have little potential for harm. Our kitchen is not a peanut-free zone, so if you have a peanut allergy you should not participate in this study. Another risk for this study is the potential discomfort with providing the blood samples. This may include some minor discomfort or bruising.

Possible Benefits

There are no direct benefits to you in this study. We are not sure of the best way to prevent and treat a fatty liver in children. Your participation in this study will help us to understand how some children get fat in their livers. This information will help us understand what is the best way to prevent getting a fatty liver.

Confidentiality: We will not share any information in your personal health record with anyone. Any research data collected about you during this study will not identify you by name, only by your initials and a coded number. Your name will not be shared with anyone outside the research clinic and your name will not be in any reports published from this research.

For this study, the doctor or other members of the research team (dietitian, graduate student) may need to access your personal health records for health information. He/she may also need to contact your family doctor and your other health care providers to obtain additional medical information. The health information collected as part of this study will be kept confidential unless release is required by law, and will be used only for the purpose of the research study. By signing the consent form you give permission to the study staff to access any personally identifiable health information which is under the custody of other health care professionals. This will only be done if it is thought to be necessary to carry out this research project.

The personal health information collected in this study may need to be checked by the Health Research Ethics Board (HREB) at the University of Alberta. This may be necessary so the HREB can make sure that the data collected in the study is accurate. .

By signing the consent form you give permission for the collection, use and sharing of information from your medical records for purpose of this research. In the University of Alberta, study information is required to be kept for 7 years. Even if you withdraw from the study, the medical information which is obtained from you the research will not be destroyed. You have a right to check your health records and request changes if your personal information is incorrect.

Voluntary Participation: You are free to stop participating in the study at any time. This will not affect the quality of medical care that you are provided with by your doctor or dietitian. If there is any information that is gained from the study that may affect your decision to continue with this study, we will let you know right away.

Reimbursement of Expenses: You will be provided with parking vouchers to cover the cost of your parking expenses.

Compensation for Injury*:* If you become ill or injured as a result of participating in this study, necessary medical treatment will be available at no additional cost to you. By signing this consent form you are not releasing the investigator(s)or institution(s) from their legal and professional responsibilities.

Do you have more questions?

You can ask your dietitian about anything you don't understand. You can also talk to Diana Mager or Susan M. Gilmour or Dr. Yap. Diana Mager's phone number is 492-7687. Susan Gilmour's telephone number is 407-3339. Jason Yap's phone number is 780-407-3698. If you have any problems or concerns about any part of this study please call the Patient Relations Office of Capital Health at (780)-482-8080.This office has no connection with the study researchers.

Principal Investigator:	Diana Mager, PhD RD	Telephone: 780-492-7687
Co-Investigator:	Dr. Susan M. Gilmour, MD	Telephone: 780-407-3339
	Dr. Jason Yap MD	Telephone: 780-407-3698

Appendix 1. Form G

UNIVERSITY OF ALBERTA

INFORMATION LETTER
(Healthy Children and Adolescents)

Title of Project:	Post-prandial lipemia in children with nonalcoholic steatohepatitis (NASH).	
Principal Investigator:	Diana Mager, PhD RD	Telephone: 780-492-7687
Co-Investigator:	Dr. Susan M. Gilmour, MD	Telephone: 780-407-3339
	Dr. Jason Yap MD	Telephone: 780-407-3698

This information letter is intended for the study subject's parents/caregiver. If you are signing on behalf of your child, the words 'you' and 'your' should be read as your 'your child'.

Purpose of this study

We would like you to participate in a research study that will help us understand why children get fatty livers. There is some evidence that fat build-up in the liver may be due to the type of foods (fatty and sugary foods) that children eat and the way the body processes these foods after you eat them. Our plan is to study how your diet affects the way fat is metabolized in the liver after you eat a meal. We want to study if the fat from your food affects your liver.

Addendum: Since, you already consented to our study, we would like to ask you to come to the HNRU to eat another small meal (breakfast) and to perform the 'exact' same tests as you did when you participated in our study the first time. We want to see if differences in the type of fat also affect the way the liver metabolizes fat after you eat. The meal that we are going to provide to you is a little different from the last meal. It will consist of fats that are thought to be healthy in the diet (long chain polyunsaturated fats). We will serve you a slightly different meal than the meal that we gave you before which was more typical of the North American diet. We are now able to study this as we have been given some extra funding to study this that we did not have at the time that we asked you to be in the study the first time.

Procedure(s) of the study

1. Anthropometric Measurements

We will measure your weight, height and waist. We will measure your waist circumference by putting a tape measure around your waist. We will also measure how big your muscles are by taking a measurement of your skin from the back of your arm, with a caliper. Your wrist and elbow diameter will be measured will a small bone caliper. Calipers look like tongs. It will look like a little pinch but will not feel like one. The research nurse will also take your blood pressure. This will happen during your regular clinic appointments in the Pediatric Centre for Weight and Health.

We will also measure the amount of muscle in your body with a special machine called the Bod Pod machine. The Bod Pod is a machine that measures the amount of muscle in a person's body by measuring body weight and by detecting the difference in volume of the air before and after a person sits in the machine. This allows the machine to calculate how much of your body is muscle and how much is fat. The Bod Pod consists of two chambers: the test chamber, where you will be sitting and the reference chamber which is mainly the seat. The Bod Pod test takes about 5 minutes, and you will be asked to wear a swimsuit and a swim cap. You need to wear these instead of regular clothes so the machine can measure your muscles correctly. You will not get wet and the test is not uncomfortable at all and it is completely safe. The Bod Pod test will be done at the Human Nutrition Research Unit, HNRU, University of Alberta, at time of entry into our study and 6 months later during your regularly scheduled clinic appointment. Because this test is not happening at the same time as your clinic appointment we will pay for parking costs at the university.

2. Oral Fat Meal

When you come to the HNRU, we will also ask you to eat a small meal (breakfast). The meal that we will provide to you will consist of foods that are typically eaten at breakfast (bread/breakfast cereal, butter, jam, juice, eggs, cheese and/or milk).We will offer you a choice of meals to choose from prior to coming to the HNRU on the study day to make sure that you only eat food that you like. We will also review with you about any history of food allergies you may have to make sure we do not offer any foods that you are allergic to. Our kitchen is not a peanut-free zone, so if you have a peanut allergy you should not participate in this study. On the day that you are coming to the HNRU you will be asked not to eat anything after your supper the night before you come. For this test we will also need to collect three blood samples from you (approximately ½ teaspoon each time). We need to do this before you eat the small meal, and then again 1 hour after and 3 hours. This is in *addition t*o the routine blood work that you would do at the Stollery Children's Hospital after a clinic visit. To avoid any extra unnecessary pokes, the nurse will put in an indwelling catheter in your arm so we can get the three blood samples without having to poke you each time. The reason we want to collect this blood from you is to study how the fats in the meal affects the amount of fat and some proteins in your blood and how the levels change in your blood after you eat a meal. This information will help us understand the way your liver metabolizes fat after a meal and provide us with more information about why some children deposit fat in their livers and why others do not. While you are waiting in the HNRU you can watch movies or listen to music.

New Meal: We will serve you a slightly different meal than the meal that we gave you before which was more typical of the North American diet. The foods in this meal will include many of the same foods (eggs, milk), but will have some new choices as well (vegetables, differences in cooking oils).

3. Food Intake

We will ask you to fill out a three day food record based on what you eat. For the three day food records we will ask you to write down what you have eaten for the three days (2 weekdays and 1 weekend day). It will take about 10 minutes to fill out the food record on each of the three days. We will provide you with a self-addressed stamped envelope so you can mail this back to the research team.

4. Medical Records

We would also like to look at your medical records to find out about medications, relevant lab work (for example at the amount of fat and sugar that is present in the blood) and results of other medical tests that were used to find out about your liver. We would also like to review your medical records to look at your liver biopsy results. This will help us

understand everything about your liver.

6. Abdominal Ultrasound

If you agree that you will participate in this study, we will ask you to have an ultrasound to rule out the possibility that you have any extra fat in your liver. An ultrasound is a test that takes pictures of your liver to see if your liver is healthy. It will not hurt and is completely safe. This test takes about 15-20 minutes. We will make sure that you know the results of this test. If there are any concerns about the results, we will refer you to a liver doctor.

.**Possible Risks**

All of the ***additional*** tests used in this study have little potential for harm. Our kitchen is not a peanut-free zone, so if you have a peanut allergy you should not participate in this study. Another risk for this study is the potential discomfort with providing the blood samples. This may include some minor discomfort or bruising.

Possible Benefits

There are no direct benefits to you in this study. We are not sure of the best way to prevent and treat a fatty liver in children. Your participation in this study will help us to understand how some children get fat in their livers. This information will help us understand what is the best way to prevent getting a fatty liver.

Confidentiality: We will not share any information in your personal health record with anyone. Any research data collected about you during this study will not identify you by name, only by your initials and a coded number. Your name will not be shared with anyone outside the research clinic and your name will not be in any reports published from this research.

For this study, the doctor or other members of the research team (dietitian, graduate student) may need to access your personal health records for health information. He/she may also need to contact your family doctor and your other health care providers to obtain additional medical information. The health information collected as part of this study will be kept confidential unless release is required by law, and will be used only for the purpose of the research study. By signing the consent form you give permission to the study staff to access any personally identifiable health information which is under the custody of other health care professionals. This will only be done if it is thought to be necessary to carry out this research project.

The personal health information collected in this study may need to be checked by the Health Research Ethics Board (HREB) at the University of Alberta. This may be necessary so the HREB can make sure that the data collected in the study is accurate. .

By signing the consent form you give permission for the collection, use and sharing of information from your medical records for purpose of this research. In the University of Alberta, study information is required to be kept for 7 years. Even if you withdraw from the study, the medical information which is obtained from you the research will not be destroyed. You have a right to check your health records and request changes if your personal information is incorrect.

Voluntary Participation: You are free to stop participating in the study at any time. This will not affect the quality of medical care that you are provided with by your doctor or dietitian. If there is any information that is gained from the study that may affect your decision to continue with this study, we will let you know right away.

Reimbursement of Expenses: You will be provided with parking vouchers to cover the cost of your parking expenses.

Compensation for Injury*:* If you become ill or injured as a result of participating in this study, necessary medical treatment will be available at no additional cost to you. By signing this consent form you are not releasing the investigator(s)or institution(s) from their legal and professional responsibilities.

Do you have more questions?

You can ask your dietitian about anything you don't understand. You can also talk to Diana Mager or Susan M. Gilmour or Dr. Yap. Diana Mager's phone number is 492-7687. Susan Gilmour's telephone number is 407-3339. Jason Yap's phone number is 780-407-3698. If you have any problems or concerns about any part of this study please call the Patient Relations Office of Capital Health at (780)-482-8080.This office has no connection with the study researchers.

Principal Investigator:	Diana Mager, PhD RD	Telephone: 780-492-7687
Co-Investigator:	Dr. Susan M. Gilmour, MD	Telephone: 780-407-3339
	Dr. Jason Yap MD	Telephone: 780-407-3698

Appendix 1. Form H

UNIVERSITY OF ALBERTA

INFORMATION LETTER
(Healthy Children and Adolescents)

Title of Project:	Post-prandial lipemia in children with nonalcoholic steatohepatitis (NASH).	
Principal Investigator:	Diana Mager, PhD RD	Telephone: 780-492-7687
Co-Investigator:	Dr. Susan M. Gilmour, MD	Telephone: 780-407-3339
	Dr. Jason Yap MD	Telephone: 780-407-3698

This information letter is intended for the study subject's parents/caregiver. If you are signing on behalf of your child, the words 'you' and 'your' should be read as your 'your child'.

Purpose of this study

We would like you to participate in a research study that will help us understand why children get fatty livers. There is some evidence that fat build-up in the liver may be due to the type of foods (fatty and sugary foods) that children eat and the way the body processes these foods after you eat them. Our plan is to study how your diet affects the way fat is metabolized in the liver after you eat a meal. We want to study if the fat from your food affects your liver. We will ask you to come to the University of Alberta on two separate test days.

Procedure(s) of the study on each Study Day

1. Anthropometric Measurements

We will measure your weight, height and waist. We will measure your waist circumference by putting a tape measure around your waist. We will also measure how big your muscles are by taking a measurement of your skin from the back of your arm, with a caliper. Your wrist and elbow diameter will be measured will a small bone caliper. Calipers look like tongs. It will look like a little pinch but will not feel like one. The research nurse will also take your blood pressure. This will happen during your regular clinic appointments in the Pediatric Centre for Weight and Health.

We will also measure the amount of muscle in your body with a special machine called the Bod Pod machine. The Bod Pod is a machine that measures the amount of muscle in a person's body by measuring body weight and by detecting the difference in volume of the air before and after a person sits in the machine. This allows the machine to calculate how much of your body is muscle and how much is fat. The Bod Pod consists of two chambers: the test chamber, where you will be sitting and the reference chamber which is mainly the seat. The Bod Pod test takes about 5 minutes, and you will be asked to wear a swimsuit and a swim cap. You need to wear these instead of regular clothes so the machine can measure your muscles correctly. You will not get wet and the test is not uncomfortable at all and it is completely safe. The Bod Pod test will be done at the Human Nutrition Research Unit, HNRU, University of Alberta, at time of entry into our study and 6 months

later during your regularly scheduled clinic appointment. Because this test is not happening at the same time as your clinic appointment we will pay for parking costs at the university.

2. Fat Meal

When you come to the HNRU on two different days, we will also ask you to eat a small meal (breakfast). The meal on one of the days that we will provide to you will consist of foods that are typically eaten at breakfast (bread/breakfast cereal, butter, jam, juice, eggs, cheese and/or milk).We will offer you a choice of meals to choose from prior to coming to the HNRU on the study day to make sure that you only eat food that you like. On the other test day, we will give you a meal that is slightly different that the one we offer you on the other test day. This meal will consist of many of the same foods (eggs, milk), but will also have some new choices (vegetables and grains) and will be cooked in some different cooking oils that are thought to be very healthy for you. We will offer you a choice in the types of foods we are asking you to eat.

We will also review with you about any history of food allergies you may have to make sure we do not offer any foods that you are allergic to. Our kitchen is not a peanut-free zone, so if you have a peanut allergy you should not participate in this study. On the day that you are coming to the HNRU you will be asked not to eat anything after your supper the night before you come. For this test we will also need to collect four blood samples from you (approximately ½ teaspoon each time). We need to do this before you eat the small meal, and then again 1 hour after and 3 and 6 hours. To avoid any extra unnecessary pokes, the nurse will put in an indwelling catheter in your arm so we can get the three blood samples without having to poke you each time. The reason we want to collect this blood from you is to study how the fats in the meal affects the amount of fat and some proteins in your blood and how the levels change in your blood after you eat a meal. This information will help us understand the way your liver metabolizes fat after a meal and provide us with more information about why some children deposit fat in their livers and why others do not. We will make sure that you know the results of this test. The liver doctor on our research team will review the results of your blood test and will let you know about any concerns (if any). If there are any concerns you're your blood work, the liver doctor on our team will send the results to your family doctor and refer you to any clinics (e.g. liver clinics), if needed. While you are waiting in the HNRU you can watch movies or listen to music.

3. Food Intake

We will ask you to fill out a three day food record based on what you eat. For the three day food records we will ask you to write down what you have eaten for the three days (2 weekdays and 1 weekend day). It will take about 10 minutes to fill out the food record on each of the three days. We will provide you with a self-addressed stamped envelope so you can mail this back to the research team.

Possible Risks

All of the tests used in this study have little potential for harm. Our kitchen is not a peanut-free zone, so if you have a peanut allergy you should not participate in this study. Another risk for this study is the potential discomfort with providing the blood samples. This may include some minor discomfort or bruising.

Possible Benefits

There are no direct benefits to you in this study. We are not sure of the best way to prevent and treat a fatty liver in children. Your participation in this study will help us to understand how some children get fat in their livers. This information will help us understand what is the best way to prevent getting a fatty liver.

Confidentiality: We will not share any information in your personal health record with anyone. Any research data collected about you during this study will not identify you by name, only by your initials and a coded number. Your name will not be shared with anyone outside the research clinic and your name will not be in any reports published from this research.

For this study, the doctor or other members of the research team (dietitian, graduate student) may need to access your personal health records for health information. He/she may also need to contact your family doctor to share the results of blood tests. This will only be done if it is thought to be necessary in order to carry out this research project properly or if results that are outside the normal range that need to be reported. The health information collected as part of this study will be kept confidential unless release is required by law, and will be used only for the purpose of the research study. By signing the consent form you give permission to the study staff to access any personally identifiable health information which is under the custody of other health care professionals.

The personal health information collected in this study may need to be checked by the Health Research Ethics Board (HREB) at the University of Alberta. This may be necessary so the HREB can make sure that the data collected in the study is accurate.

By signing the consent form you give permission for the collection, use and sharing of information from your medical records for purpose of this research. In the University of Alberta, study information is required to be kept for 7 years. Even if you withdraw from the study, the medical information which is obtained from you the research will not be destroyed. You have a right to check your health records and request changes if your personal information is incorrect.

Voluntary Participation: You are free to stop participating in the study at any time. This will not affect the quality of medical care that you are provided with by your doctor or dietitian. If there is any information that is gained from the study that may affect your decision to continue with this study, we will let you know right away.

Reimbursement of Expenses: You will be provided with parking vouchers to cover the cost of your parking expenses.

Compensation for Injury*:* If you become ill or injured as a result of participating in this study, necessary medical treatment will be available at no additional cost to you. By signing this consent form you are not releasing the investigator(s)or institution(s) from their legal and professional responsibilities.

Do you have more questions?

You can ask your dietitian about anything you don't understand. You can also talk to Diana Mager or Susan M. Gilmour or Dr. Yap. Diana Mager's phone number is 492-7687. Susan Gilmour's telephone number is 407-3339. Jason Yap's phone number is 780-407-3698. If you have any problems or concerns about any part of this study please call the University of Alberta Research Ethics Office at 780-492-2615.This office has no connection with the study researchers.

Principal Investigator:	Diana Mager, PhD RD	Telephone: 780-492-7687
Co-Investigator:	Dr. Susan M. Gilmour, MD	Telephone: 780-407-3339
	Dr. Jason Yap MD	Telephone: 780-407-3698

Appendix 1. Form I

UNIVERSITY OF ALBERTA

INFORMATION LETTER
(Children and Adolescents with NASH)

Title of Project:	Post-prandial lipemia in children with nonalcoholic steatohepatitis (NASH).	
Principal Investigator:	Diana Mager, PhD RD	Telephone: 780-492-7687
Co-Investigator:	Dr. Susan M. Gilmour, MD	Telephone: 780-407-3339
	Dr. Jason Yap MD	Telephone: 780-407-3698

This information letter is intended for the study subject's parents/caregiver. If you are signing on behalf of your child, the words 'you' and 'your' should be read as your 'your child'.

Purpose of this study

We would like you to participate in a research study that will help us understand why children get fatty livers. There is some evidence that fat build-up in the liver may be due to the type of foods (fatty and sugary foods) that children eat and the way the body processes these foods after you eat them. Our plan is to study how your diet affects the way fat is metabolized in the liver after you eat a meal. We want to study if the fat from your food affects your liver. We will ask you to come to the University of Alberta on two separate test days.

Procedure(s) of the study on each Study Day

1. **Anthropometric Measurements**

We will measure your weight, height and waist. We will measure your waist circumference by putting a tape measure around your waist. We will also measure how big your muscles are by taking a measurement of your skin from the back of your arm, with a caliper. Your wrist and elbow diameter will be measured will a small bone caliper. Calipers look like tongs. It will look like a little pinch but will not feel like one. The research nurse will also take your blood pressure. This will happen during your regular clinic appointments in the Pediatric Centre for Weight and Health.

We will also measure the amount of muscle in your body with a special machine called the Bod Pod machine. The Bod Pod is a machine that measures the amount of muscle in a person's body by measuring body weight and by detecting the difference in volume of the air before and after a person sits in the machine. This allows the machine to calculate how much of your body is muscle and how much is fat. The Bod Pod consists of two chambers: the test chamber, where you will be sitting and the reference chamber which is mainly the seat. The Bod Pod test takes about 5 minutes, and you will be asked to wear a swimsuit and a swim cap. You need to wear these instead of regular clothes so the machine can measure your muscles correctly. You will not get wet and the test is not uncomfortable at all and it is completely safe. The Bod Pod test will be done at the Human Nutrition Research Unit, HNRU, University of Alberta, at time of entry into our study and 6 months later during your regularly scheduled clinic appointment. Because this test is not happening at the same time as your clinic appointment we will pay for parking costs at the university.

2. Oral Fat Meal

When you come to the HNRU on two different days, we will also ask you to eat a small meal (breakfast). The meal on one of the days that we will provide to you will consist of foods that are typically eaten at breakfast (bread/breakfast cereal, butter, jam, juice, eggs, cheese and/or milk).We will offer you a choice of meals to choose from prior to coming to the HNRU on the study day to make sure that you only eat food that you like. On the other test day, we will give you a meal that is slightly different that the one we offer you on the other test day. This meal will consist of many of the same foods (eggs, milk), but will also have some new choices (vegetables and grains) and will be cooked in some different cooking oils that are thought to be very healthy for you. We will offer you a choice in the types of foods we are asking you to eat.

We will also review with you about any history of food allergies you may have to make sure we do not offer any foods that you are allergic to. Our kitchen is not a peanut-free zone, so if you have a peanut allergy you should not participate in this study. On the day that you are coming to the HNRU you will be asked not to eat anything after your supper the night before you come. For this test we will also need to collect four blood samples from you (approximately ½ teaspoon each time). We need to do this before you eat the small meal, and then again 1 hour after and 3 and 6 hours. This is in *addition t*o the routine blood work that you would do at the Stollery Children's Hospital after a clinic visit. To avoid any extra unnecessary pokes, the nurse will put in an indwelling catheter in your arm so we can get the three blood samples without having to poke you each time. The reason we want to collect this blood from you is to study how the fats in the meal affects the amount of fat and some proteins in your blood and how the levels change in your blood after you eat a meal. This information will help us understand the way your liver metabolizes fat after a meal and provide us with more information about why some children deposit fat in their livers and why others do not. While you are waiting in the HNRU you can watch movies or listen to music.

3. Food Intake

We will ask you to fill out a three day food record based on what you eat. For the three day food records we will ask you to write down what you have eaten for the three days (2 weekdays and 1 weekend day). It will take about 10 minutes to fill out the food record on each of the three days. We will provide you with a self-addressed stamped envelope so you can mail this back to the research team.

4. Medical Records

We would also like to look at your medical records to find out about medications, relevant lab work (for example at the amount of fat and sugar that is present in the blood) and results of other medical tests that were used to find out about your liver. We would also like to review your medical records to look at your liver biopsy results. This will help us understand everything about your liver.

7. Abdominal Ultrasound

If you agree that you will participate in this study, we will ask you to have an ultrasound to rule out the possibility that you have any extra fat in your liver. An ultrasound is a test that takes pictures of your liver to see if your liver is healthy. It will not hurt and is completely safe. This test takes about 15-20 minutes. We will make sure that you know the results of this test. If there are any concerns about the results, we will refer you to a liver doctor.

Possible Risks

All of the ***additional*** tests used in this study have little potential for harm. Our kitchen is not a peanut-free zone, so if you have a peanut allergy you should not participate in this study. Another risk for this study is the potential discomfort with providing the blood

samples. This may include some minor discomfort or bruising.

Possible Benefits
There are no direct benefits to you in this study. We are not sure of the best way to prevent and treat a fatty liver in children. Your participation in this study will help us to understand how some children get fat in their livers. This information will help us understand what is the best way to prevent getting a fatty liver.

Confidentiality: We will not share any information in your personal health record with anyone. Any research data collected about you during this study will not identify you by name, only by your initials and a coded number. Your name will not be shared with anyone outside the research clinic and your name will not be in any reports published from this research.

For this study, the doctor or other members of the research team (dietitian, graduate student) may need to access your personal health records for health information. He/she may also need to contact your family doctor and your other health care providers to obtain additional medical information. The health information collected as part of this study will be kept confidential unless release is required by law, and will be used only for the purpose of the research study. By signing the consent form you give permission to the study staff to access any personally identifiable health information which is under the custody of other health care professionals. This will only be done if it is thought to be necessary to carry out this research project.

The personal health information collected in this study may need to be checked by the Health Research Ethics Board (HREB) at the University of Alberta. This may be necessary so the HREB can make sure that the data collected in the study is accurate. .

By signing the consent form you give permission for the collection, use and sharing of information from your medical records for purpose of this research. In the University of Alberta, study information is required to be kept for 7 years. Even if you withdraw from the study, the medical information which is obtained from you the research will not be destroyed. You have a right to check your health records and request changes if your personal information is incorrect.

Voluntary Participation: You are free to stop participating in the study at any time. This will not affect the quality of medical care that you are provided with by your doctor or dietitian. If there is any information that is gained from the study that may affect your decision to continue with this study, we will let you know right away.

Reimbursement of Expenses: You will be provided with parking vouchers to cover the cost of your parking expenses.

Compensation for Injury*:* If you become ill or injured as a result of participating in this study, necessary medical treatment will be available at no additional cost to you. By signing this consent form you are not releasing the investigator(s)or institution(s) from their legal and professional responsibilities.

Do you have more questions?
You can ask your dietitian about anything you don't understand. You can also talk to Diana Mager or Susan M. Gilmour or Dr. Yap. Diana Mager's phone number is 492-7687. Susan Gilmour's telephone number is 407-3339. Jason Yap's phone number is 780-407-3698. If you have any problems or concerns about any part of this study please call the

Patient Relations Office of Alberta Health Services at (780)-482-8080.This office has no connection with the study researchers.

Principal Investigator:	Diana Mager, PhD RD	Telephone: 780-492-7687
Co-Investigator:	Dr. Susan M. Gilmour, MD	Telephone: 780-407-3339
	Dr. Jason Yap MD	Telephone: 780-407-3698

Appendix 2.

Table 1. Body fat predictive equations

Predictive equations used:

Air Displacement Plethysmography (Bod Pod): Siri equation used: %BF = (495 / Body Density) -450.

Slaughter: %BF: 1.021*(triceps + subscapular)-0.008*(triceps + subscapular) ^{2}-1.7

If the sum of the triceps + subscapular >35 the following equation should be used:

BF%: 0.783*(triceps + subscapular) + 1.6.

Brozek: %BF: = [(4.57/BD)-4.142]*100. BD = [1.0982-(ΣSF*0.000815)] + [(ΣSF) ^{2} * 0.00000084]

ΣSF= Triceps + subscapular + abdomen skinfold measurements.

Deurenberg for children: %BF: (1.5*BMI) - (0.7*age) – (3.6*gender) + 1.4. Gender=0 girls and 1 for boys.

Durnin and Womersley: %BF: (503.3/D-459.2). D= 1.1599-0.0717*(log triceps + subscapular + biceps + suprailiac).

Table 1. Continues

Study	Type of population	Formula	Status
Janz et al., 1992.	122 lean subjects pre to post pubertal subjects (ages 8-17)	Slaughter et al.	Validation of the Slaughter equation by comparing the values with Siri age-adjusted body density equation. Results: Slaughter and Siri evoke similar results.
Rodríguez et al., 2005.	238 lean Caucasian adolescents (167 females and 113 males; ages 13-17.9)	Slaughter et al. and Brook (females)	Slaughter et al and Brook (females) equations were compared to DXA. Results: Non-significant differences when compared to DXA.
Roemmich et al., 1997.	Lean males and females (47 boys and girls all pre pubertal)	Slaughter et al.	Cross validation with the 3 compartment water density (3C-H2O) DXA, Slaughter et al. skinfold equations and BIA. Results: Similar Technical error when compared to BIA and DXA.
Dezenberg et al., 1999.	Pre and post pubertal, lean and overweight Heterogeneous groups. Caucasian (n=133) and African American (n=69).	Slaughter et al.	Slaughter et al. cross validated against DXA. Results: New anthropometric formula necessary for African americans.
Goran et al., 1996.	Pre pubertal lean subjects: boys and girls 98 (49 and 49)	Slaughter et al.	Slaughter et al. equation cross validated against DXA. Results: New formulas are necessary as they may be over or under estimating the body fat percentage.
Reilly et al., 1995.	98 lean and overweight pre pubertal children (64 boys, 34 girls).	Slaughter et al. and Deurenberg et al.	Hidrodensitometry vs Slaughter and Deurenberg. Results: magnitude of the error will depend on the body fatness of the children being studied.
Deurenberg et al., 1990.	Pre pubertal (n=114), pubertal (n=35) and post pubertal (n=21). Lean subjects	Deurenberg et al.	Determine the relationship between skinfold thicknesses and body density. Results: Skinfolds are acceptable ways to predict body density and body fat.

Table 1. Continues

Deurenberg et al., 1991.	1229 lean, overweight and obese subjects (521 males and 708 females. BMI; 13.9–40.9 kg/m^2, age range of 7–83 years	Deurenberg et al.	Body composition was determined by densitometry and anthropometry using Deurenberg et al. equation. Results: in obese subjects the formula slightly overestimated the BF%.
Wang & Deurenberg, 1996.	Chinese lean to overweight females, aged 18-67 years.	Deurenberg et al.	Deurenberg vs Bioelectrical bio impedance. Results: In very lean subjects the predictive methods overestimate body fat compared with values obtained from body densitometry.
Garcia et al., 2005.	117 lean German subjects (46 men and 71 women) 26 to 67 years of age	Durnin and Womersley	Durnin and Womersley vs DXA. Results: No significant differences between methods.
Andrade et al., 2002.	29 malnourished, HIV-positive women.	Durnin and Womersley	Results: The Durnin-Womersley formula can be used to predict change in SAT. Error to determine SAT by MRI is approximately twice as great as Durnin-Womersley measures in control subjects.
Brandon, 1998.	78 lean women (39 African American and 39 white women)	Durbin and Wimberley, Sloan et al, Jackson et al.	Comparison of existing skinfold equations for estimating body fat in African American and white women vs. UWW. Results: Skinfolds equations designed for white women are less accurate in estimating body fat in African American women. Durnin and Womersley formula showed similar values of body fat when compared to UWW.
Deurenberg et al., 2003.	101 lean and overweight adolescents (49 girls 52 boys) 16-18 y.	Durnin and Womersley, Slaughter et al., Deurenberg et al.	Durnin and Womersley, Slaughter et al., Deurenberg et al. formulas were compared. Results: Slaughter et al., and Deurenberg et al., showed very similar results. Durnin and Womersley slightly overestimated BF% when compared to the other 2.

Table 1. Continues

Martín Moreno et al., 2001.	149 lean and overweight subjects (n=83 males and 66 women).	Brozek and Deurenberg equations	BIA vs Brozek and Deurenberg equations. Results: Similar values obtained with Brozek. Deurenberg overestimated the values when compared to BIA.
Arroyo et al., 2004.	University lean students (n=653).	Brozek and Deurenberg	BIA vs Brozek and Deurenberg formulas. Results: Brozek showed the highest agreement followed by Deurenberg.
Kagawa et al., 2008.	234 athletic boys and 292 non athletic and 180 athletic girls (all less than 18 years old). Lean and overweight subjects	Brozek et al. and Tobe et al.	Brozek et al and Tobe et al. vs UWW. Results: For athletic Japanese children Tobe et al. may be the preferred choice of formula.
España Romero et al., 2009.	Elite sports climbers (lean). 19 subjects (Young adults <30 years old).	17 Formulas (Durnin and Womersley, Brozek)	17 Formulas vs DXA. Results: Durnin equation was the most accurate. Slightly higher inter method difference observed with Brozek´s formula.
Valizadeh et al., 2007.	15-17 years old lean subjects (boys)	Brozek, Slaughter and Jackson -Pollock	BIA vs Brozek, Slaughter and Jackson-Pollock. Results: Brozek´s formula is a reliable method. Jackson-Pollock overestimates %BF.

Table 2. Pearson correlations (univariate) between BMI, WC, WHtR, WHR with insulin, liver enzymes, cytokines and lipid profile

	GGT	Glucose	Insulin	HOMA	ALT	AST	Cholesterol	TG	HDL	CRP	Adiponectin
BMI	**0.54***	**0.11***	**0.55***	**0.54***	**0.30***	**0.12***	0.07	**0.35***	**0.44***	**0.28***	**0.14***
waist	**0.59***	**0.12***	**0.54***	**0.52***	**0.40***	**0.25***	0.09	**0.38***	**0.47***	**0.36***	**0.17***
w/hip	**0.20***	**0.18***	**0.36***	**0.38***	**0.45***	**0.24***	0.04	**0.12***	**0.21***	**0.14***	**0.12***
w/height	**0.59***	**0.16***	**0.55***	**0.54***	**0.47***	**0.31***	0.11	**0.46***	**0.44***	**0.39***	**0.12***

*p<0.05, values are expressed in r^2. HOMA-IR, ALT, AST, GGT, Insulin, TG natural logarithm (base e) was performed for the entire cohort.

	IL6	IL10	LEPTIN	TNF-α	Adiponectin > mean
BMI	0.02	**0.42***	0.04	0.04	**0.22***
waist	0.03	**0.44***	0.06	**0.14***	**0.29***
w/hip	0.05	**0.23***	0.01	**0.28***	0.18
w/height	0.03	**0.38***	0.05	**0.22***	0.19

*p<0.05, values are expressed in r^2. HOMA-IR, ALT, AST, GGT, Insulin, TG natural logarithm (base e) was performed for the entire cohort.

Table 3. Pearson correlations (univariate) between total body fat (BF) and fat free mass (FFM) with insulin and lipid profile.

	GGT	Glucose	Insulin	HOMA	ALT	AST	Cholesterol	TG	HDL	Adiponectin
%FFM	**0.41***	0.08	**0.45***	**0.42***	**0.25***	0.10	0.03	**0.30***	**0.44***	0.10
FFM (kg)	**0.38***	0.08	**0.32***	**0.30***	**0.19***	0.07	0.04	**0.14***	**0.34***	**0.28***
Fat Mass (kg)	**0.45***	0.06	**0.48***	**0.45***	**0.21***	0.08	0.05	**0.27***	**0.40***	**0.12***
%Fat ADP	**0.41***	0.08	**0.45***	**0.42***	**0.25***	0.10	0.03	**0.30***	**0.44***	0.10

*p<0.05, values are expressed in r^2. HOMA-IR, ALT, AST, GGT, Insulin, TG natural logarithm (base e) was performed for the entire cohort.

	IL6	IL10	Leptin	TNF-α
%FFM	0.01	**0.50***	0.05	0.04
FFM (kg)	0.03	**0.25***	0.04	0.01
Fat Mass (kg)	0.01	**0.48***	0.03	0.01
%Fat ADP	0.01	**0.50***	0.05	0.04

*p<0.05, values are expressed in r^2. HOMA-IR, ALT, AST, GGT, Insulin, TG natural logarithm (base e) was performed for the entire cohort.

Table 4. Pearson correlations (univariate) between skinfold measures, trunk-extremity ratio and somatotype with insulin, cytokines and lipid profile.

	GGT	Glucose	Insulin	HOMA	ALT	AST	Cholesterol	TG	HDL	CRP	Adiponectin
Endomorphic	**0.54***	0.09	**0.52***	**0.49***	**0.40***	**0.19***	0.04	**0.45***	**0.54***	**0.28***	0.11
Mesomorphic	**0.33***	0.06	**0.38***	**0.36***	**0.32***	0.12*	0.06	**0.26***	**0.41***	**0.18***	0.11
Ectomorphic	**0.39***	0.09	**0.38***	**0.36***	**0.34***	**0.15***	0.01	**0.37***	**0.45***	**0.15***	0.08
Triceps	**0.47***	0.03	**0.50***	**0.45***	**0.27***	**0.14***	0.08	**0.38***	**0.44***	**0.29***	0.05
Biceps	**0.32***	0.07	**0.40***	**0.38***	**0.21***	0.08	0.02	**0.26***	**0.46***	**0.24***	0.05
Subscapular	**0.59***	0.07	**0.56***	**0.52***	**0.47***	**0.26***	0.03	**0.39***	**0.56***	**0.40***	0.15
Supraspinale	**0.58***	0.08	**0.49***	**0.46***	**0.41***	**0.23***	0.08	**0.46***	**0.57***	**0.31***	**0.13***
Abdominal	0.53*	**0.15***	**0.49***	**0.49***	**0.50***	**0.29***	0.02	**0.41***	**0.53***	**0.26***	**0.12***
Ileac crest	0.49*	0.11	**0.42***	**0.42***	**0.34***	**0.16***	0.03	**0.45***	**0.51***	**0.21***	0.09
TER trunk/ext	0.16*	0.05	0.06	0.06	**0.23***	0.09	0.00	**0.14***	0.18	0.06	**0.15***
Trunk	0.63*	0.08	**0.51***	**0.49***	**0.44***	**0.23***	0.02	**0.43***	**0.58***	**0.29***	**0.12***

*$p<0.05$, values are expressed in r^2. HOMA-IR, ALT, AST, GGT, Insulin, TG natural logarithm (base e) was performed for the entire cohort.

Table 4. Continues…

	IL6	IL10	Leptin	TNF-α
Endomorphic	0.05	**0.53***	0.06	**0.20***
Mesomorphic	0.05	**0.46***	0.04	**0.28***
Ectomorphic	0.08	**0.41***	0.09	**0.18***
Triceps	0.03	**0.49***	0.06	**0.15***
Biceps	0.07	**0.68***	0.09	0.10
Subscapular	0.04	**0.54***	0.03	**0.23***
Supraspinale	0.06	**0.52***	0.05	**0.19***
Abdominal	0.05	**0.48***	0.05	**0.22***
Ileac crest	0.07	**0.63***	0.08	**0.22***
TER trunk/ext	0.03	0.05	0.00	**0.24***
Trunk	0.06	**0.65***	0.06	**0.24***

*$p<0.05$, values are expressed in r^2. HOMA-IR, ALT, AST, GGT, Insulin, TG natural logarithm (base e) was performed for the entire cohort.

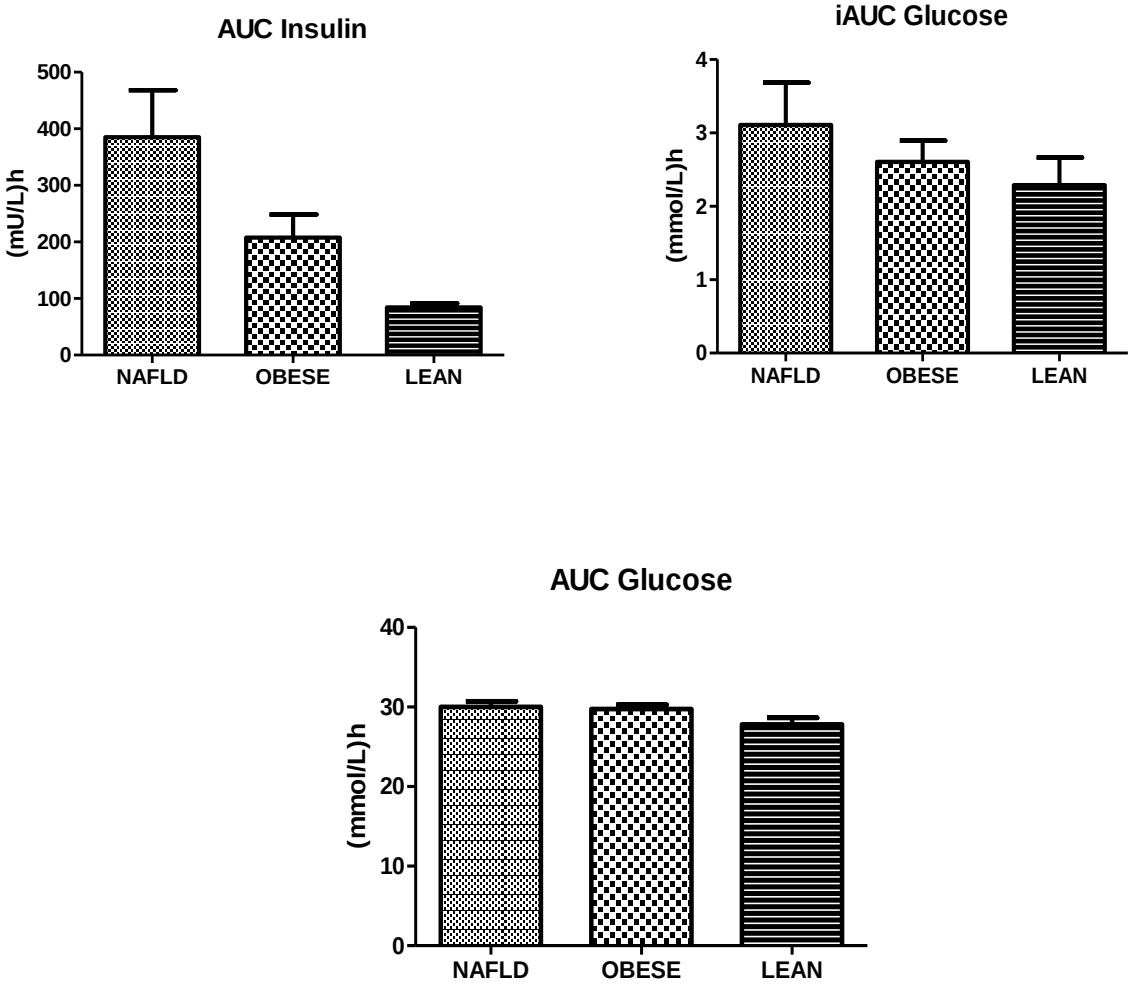

Figure A. Postprandial area under the curve (AUC) for insulin and glucose, postprandial incremental area under the curve (iAUC) for glucose in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender- and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

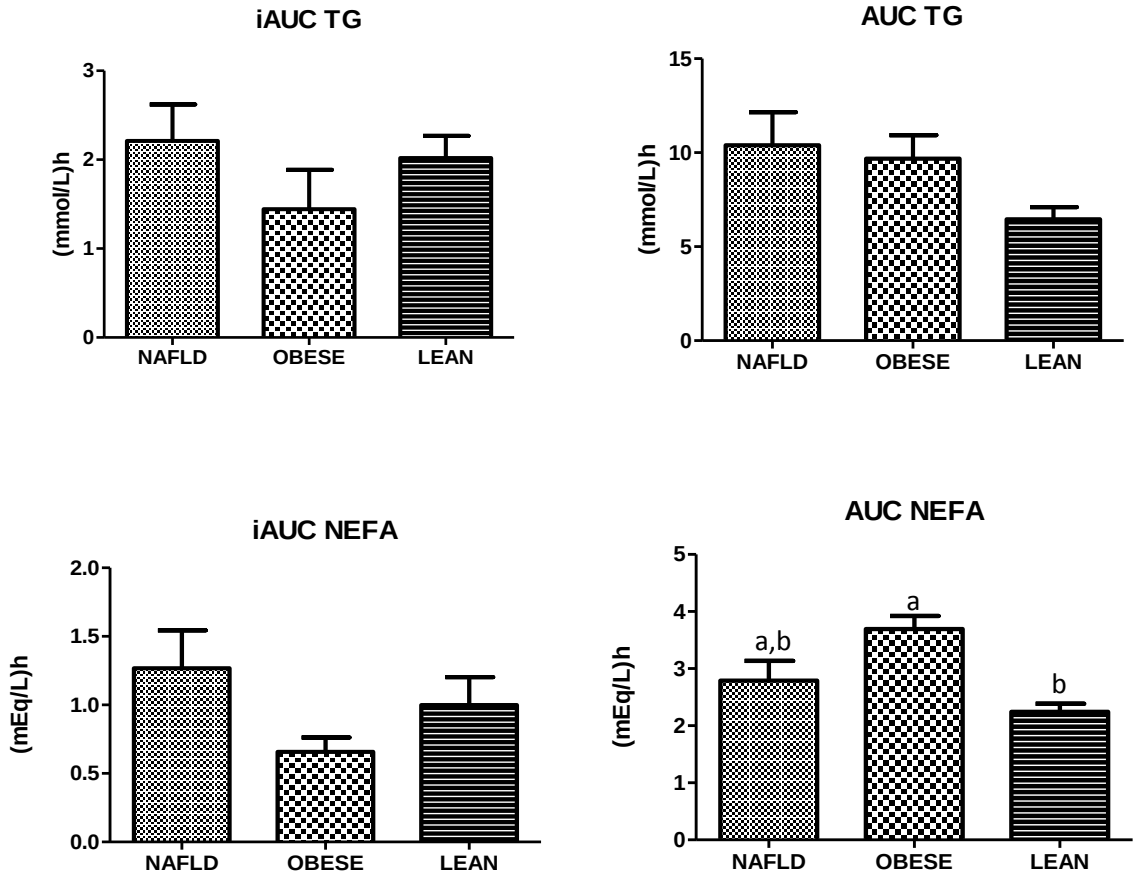

Figure B. Postprandial area under the curve (AUC) and incremental area under the curve (iAUC) for triglycerides and non-esterified fatty acids in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender- and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

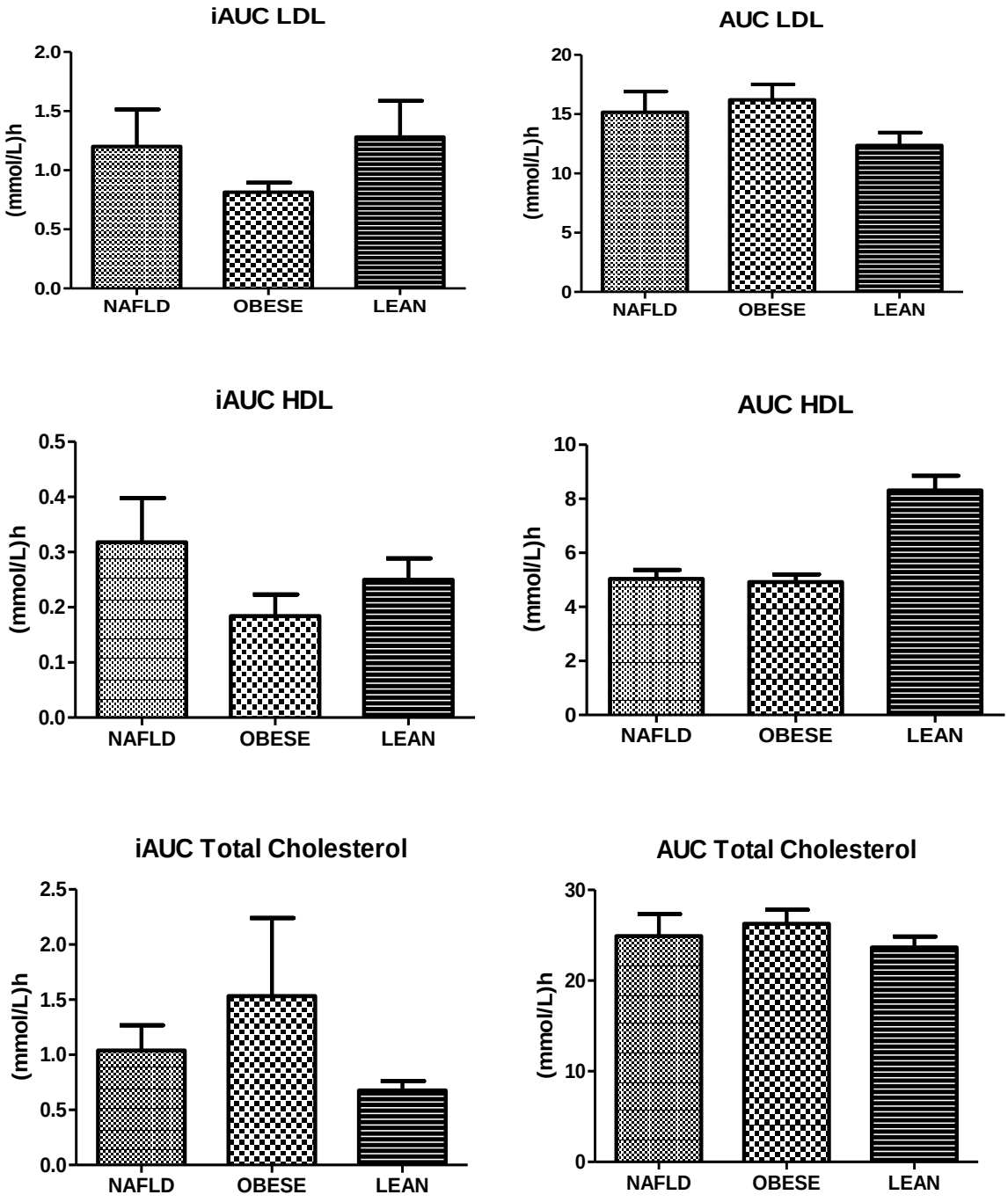

Figure C. Postprandial area under the curve (AUC) and incremental area under the curve (iAUC) for LDL, HDL and total cholesterol in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

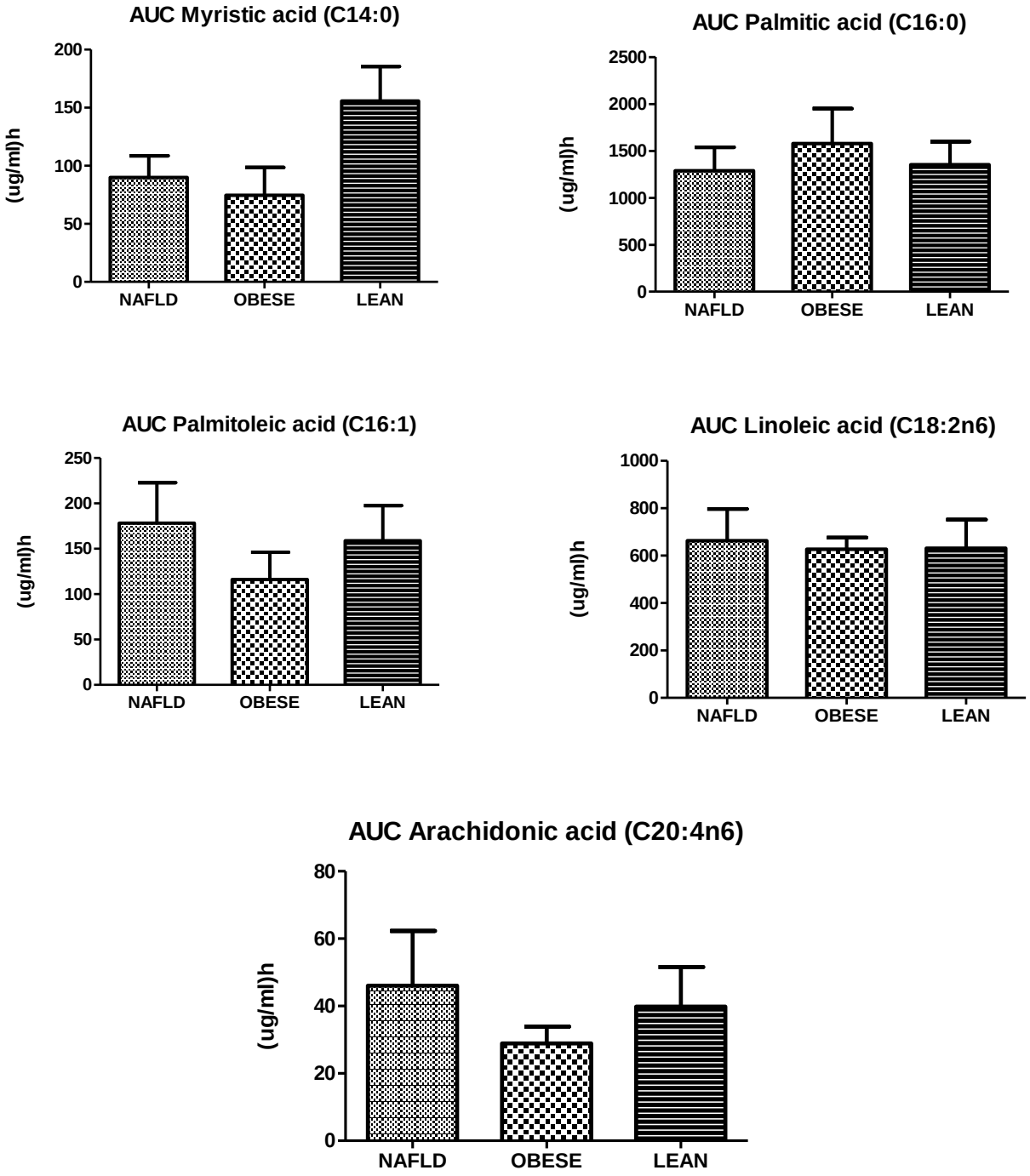

Figure D. Postprandial area under the curve (AUC) TG fractions for C14:0, C16:0, C16:1, C18:2n6 and C20:4n6 fatty acids in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

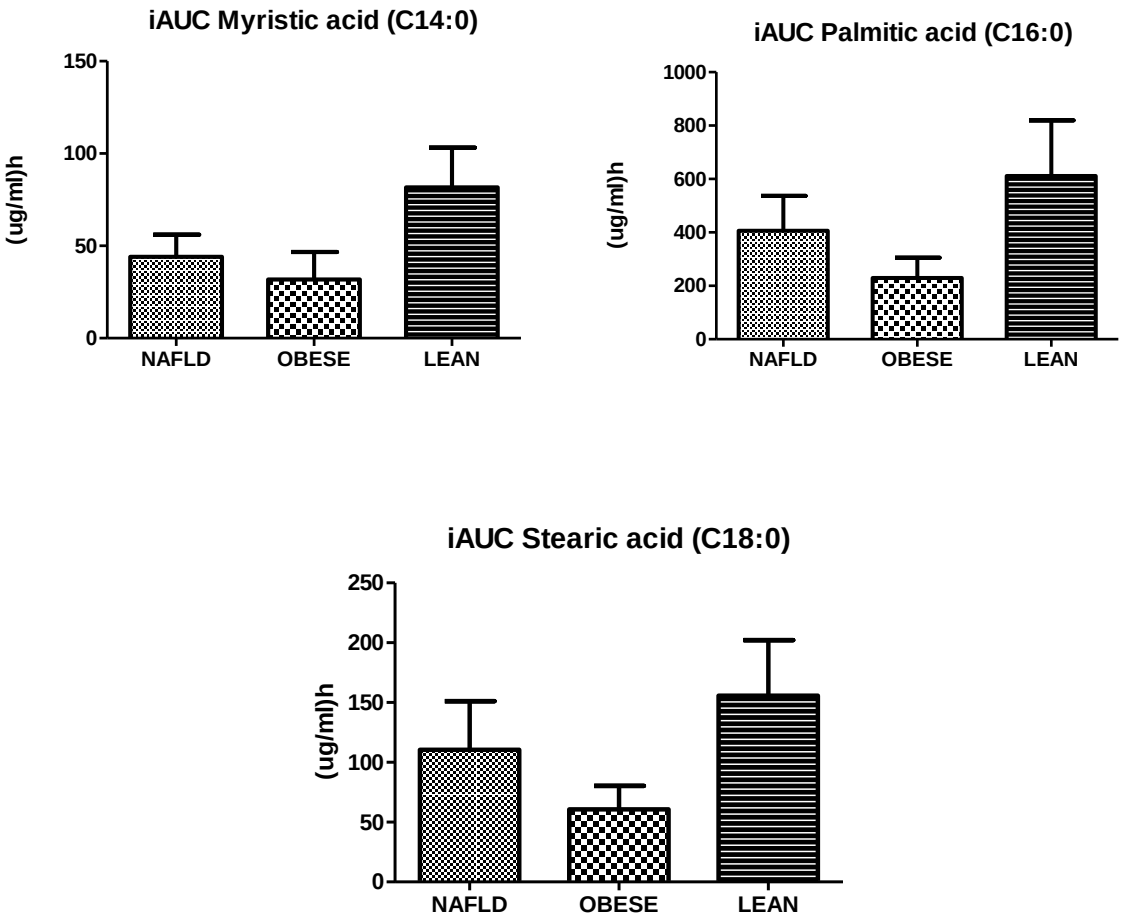

Figure E. Postprandial incremental area under the curve (iAUC) for TG fractions for C14:0, C16:0 and C18: 0 fatty acids in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

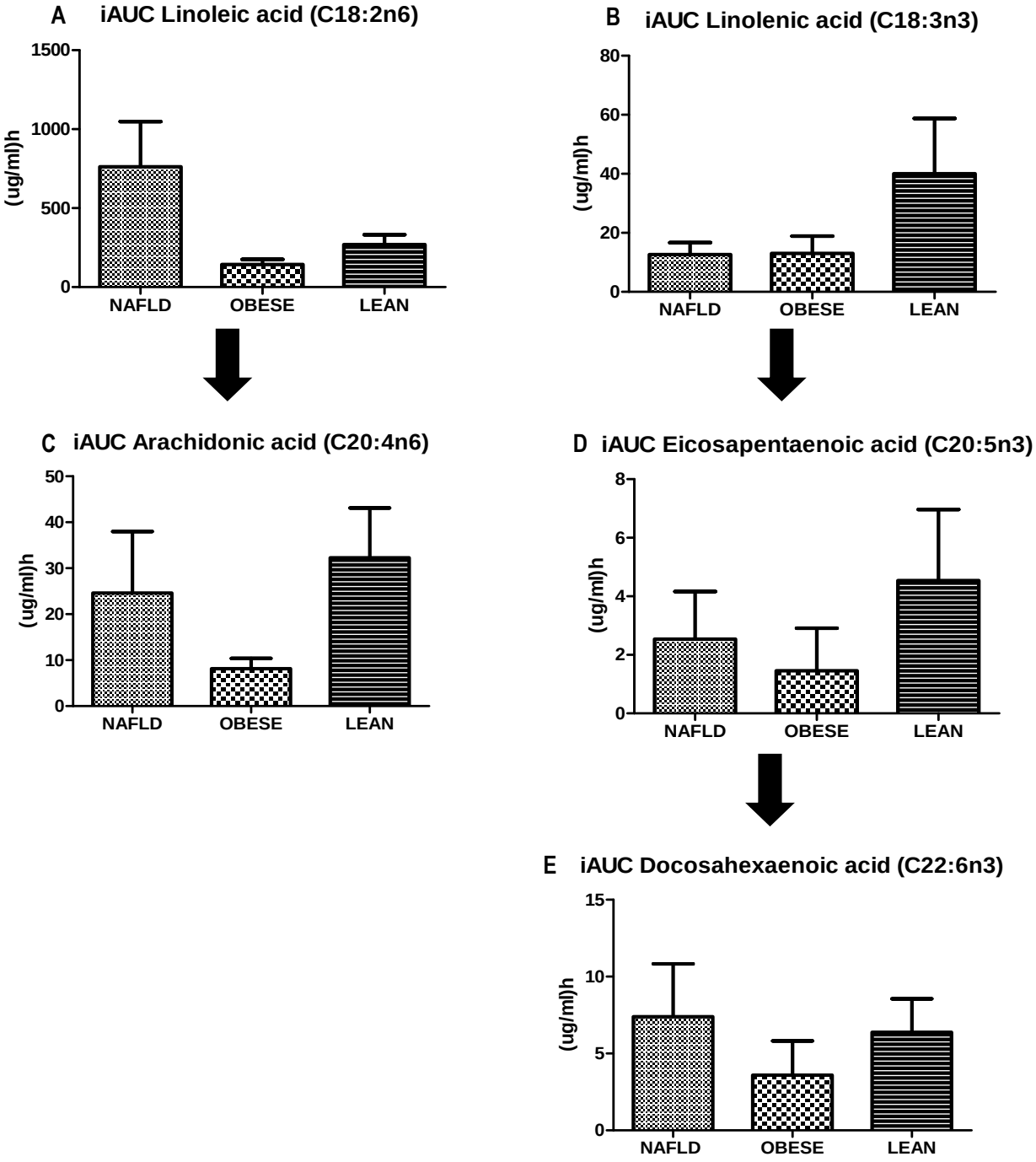

Figure F. Postprandial incremental area under the curve (iAUC) TG fractions in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. **A**: C18:2n6, **B**: C18:3n3, **C**: C20:4n6, **D**: C20:5n3, **E**: C22:6n3. Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

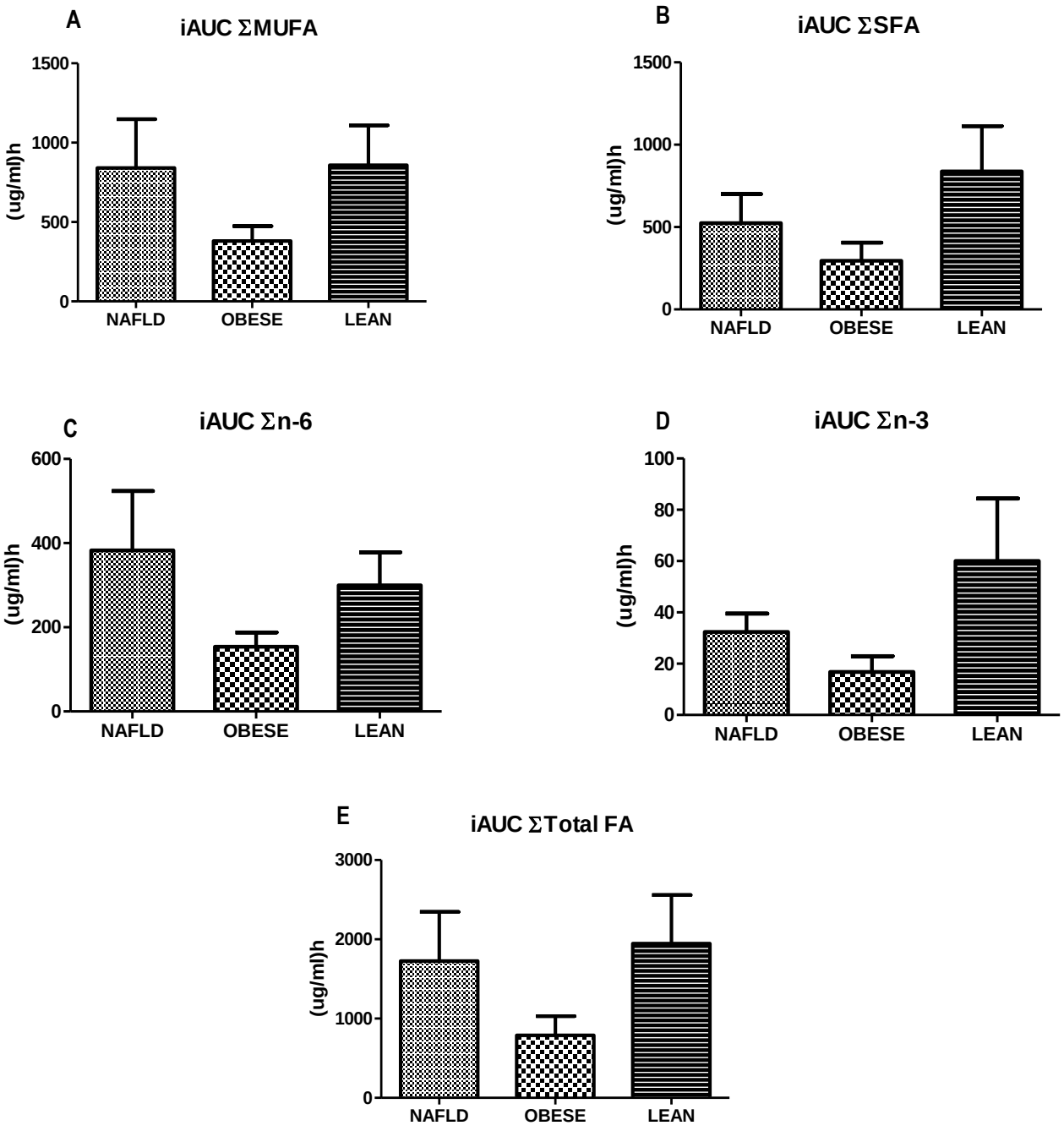

Figure G. Postprandial incremental area under the curve (iAUC) for TG fractions in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. **A**: total MUFA, **B**: total SFA, **C**: total omega-6, **D**: total omega 3, **E**: Total fatty acids (sum) in the plasma TG fractions. Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

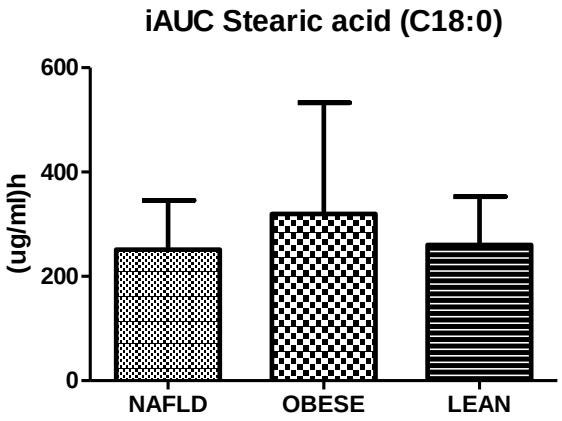

Figure H. Postprandial incremental area under the curve (iAUC) for PL fractions for C18:0 fatty acid in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

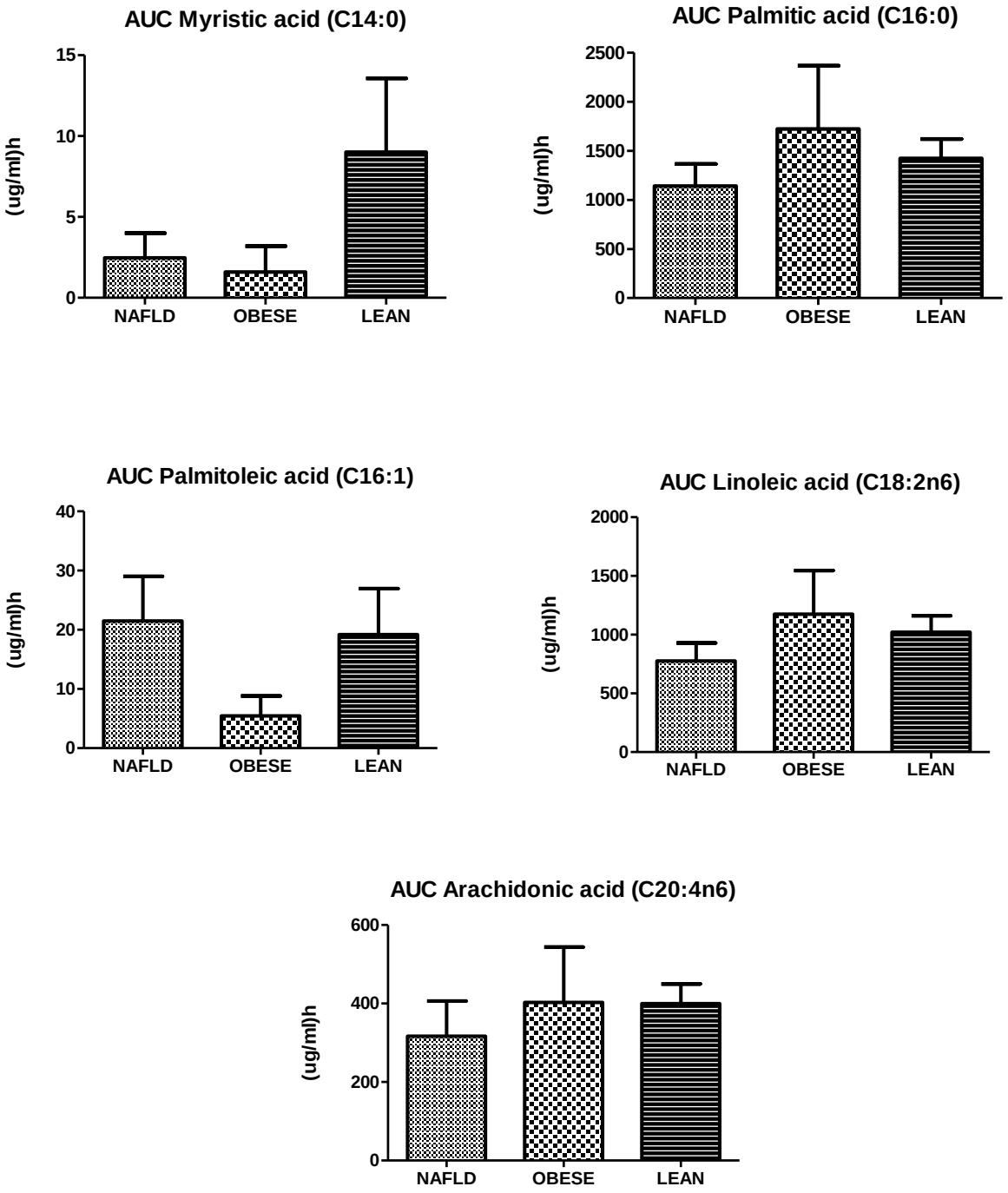

Figure I. Postprandial area under the curve (AUC) for PL fractions for C14:0, C16:0, C16:1, C18:2n6 and C20:4n6 fatty acids in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

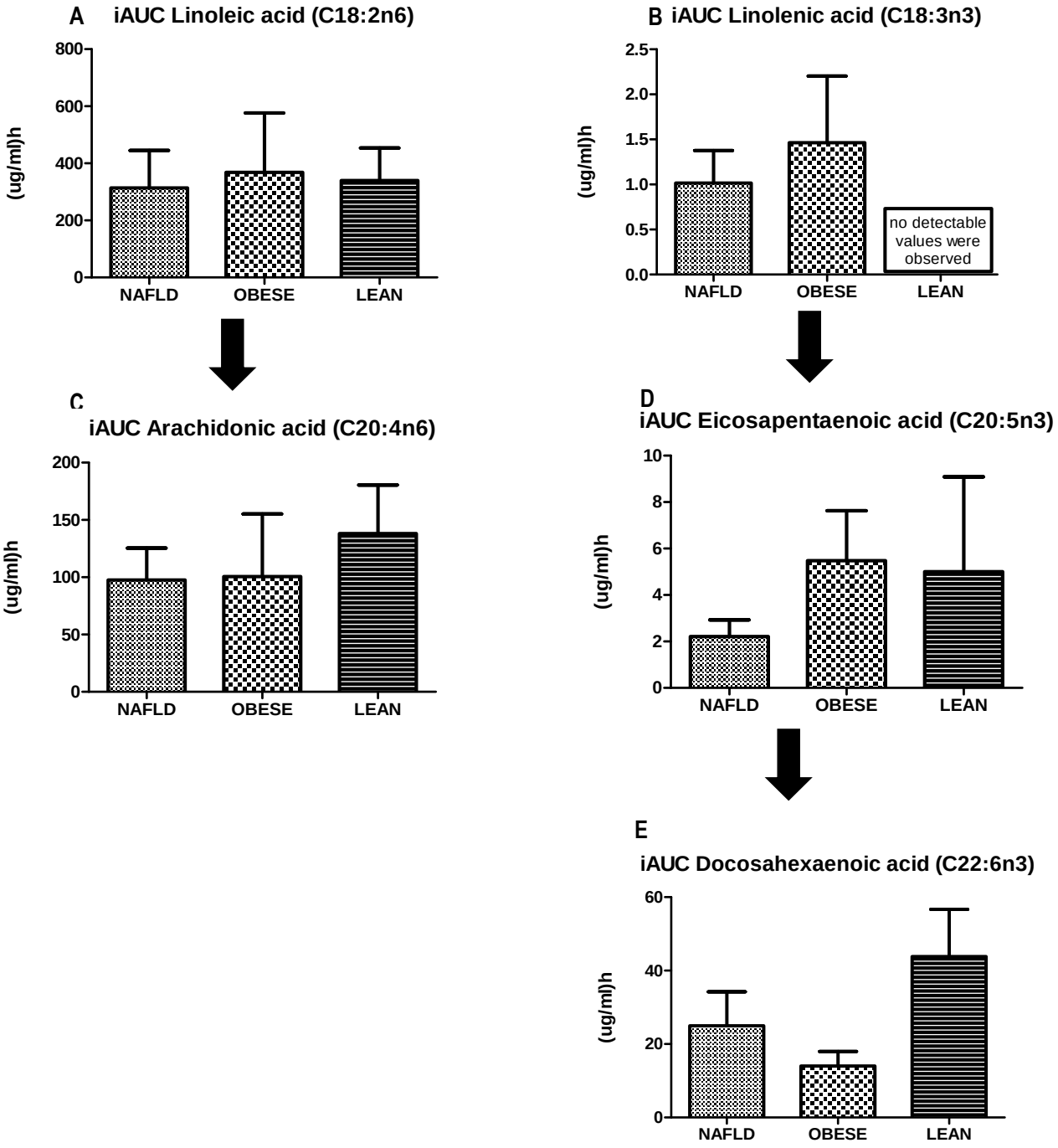

Figure J. Postprandial incremental area under the curve (iAUC) for PL fractions in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. **A**: C18:2n6, **B**: C18:3n3, **C**: C20:4n6, **D**: C20:5n3, **E**: C22:6n3. Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

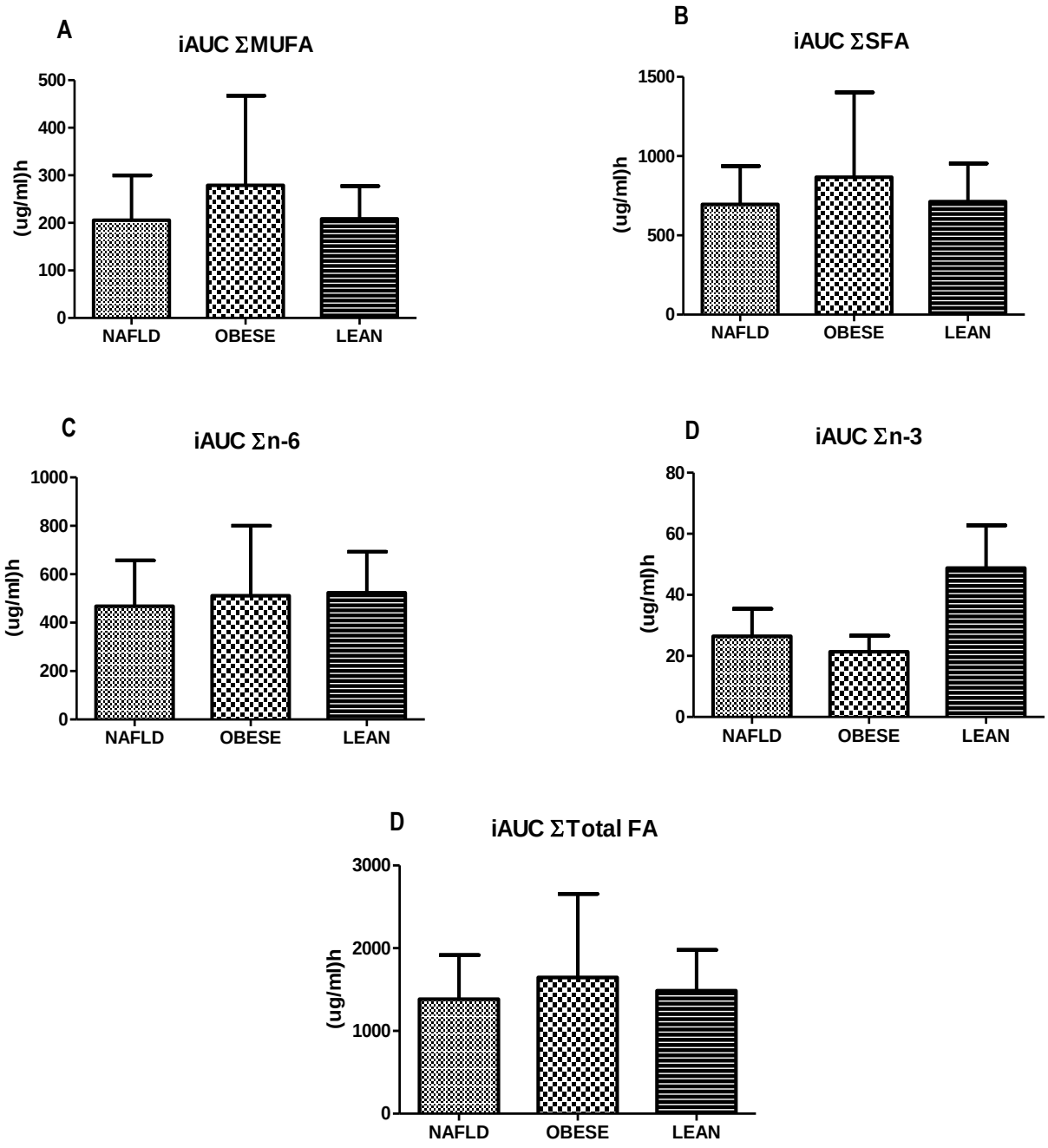

Figure K. Postprandial incremental area under the curve (iAUC) for PL fractions in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. **A**: MUFA, **B**: SFA, **C**: omega-6, **D**: omega 3, **E:** Total fatty acids (sum). Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM**.**

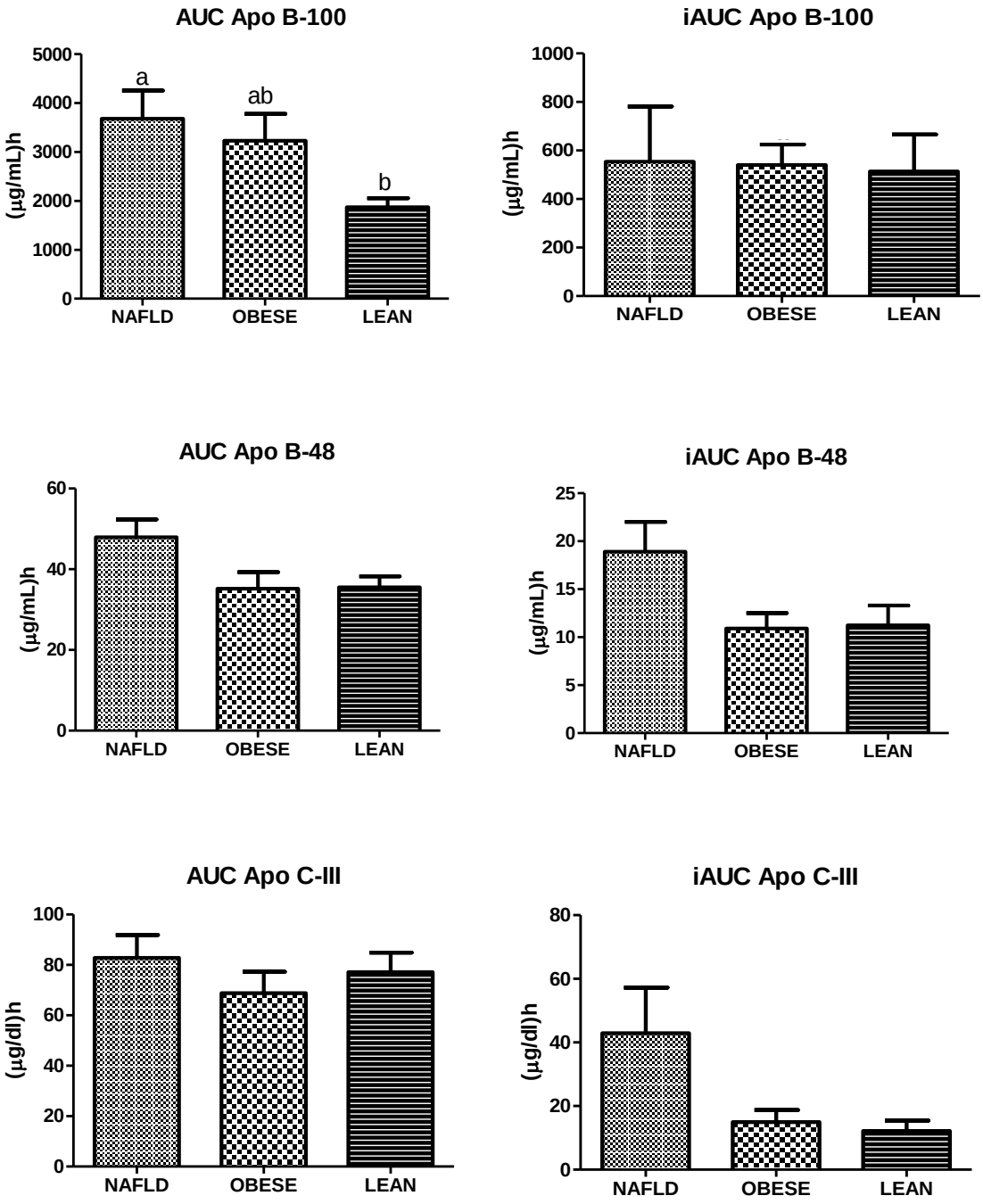

Figure L. Postprandial area under the curve (AUC) and incremental area under the curve (iAUC) for Apo B-100, B-48 and C-III in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender-and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. Variables with different superscripts are significantly different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons Results are shown mean $\pm$ SEM.

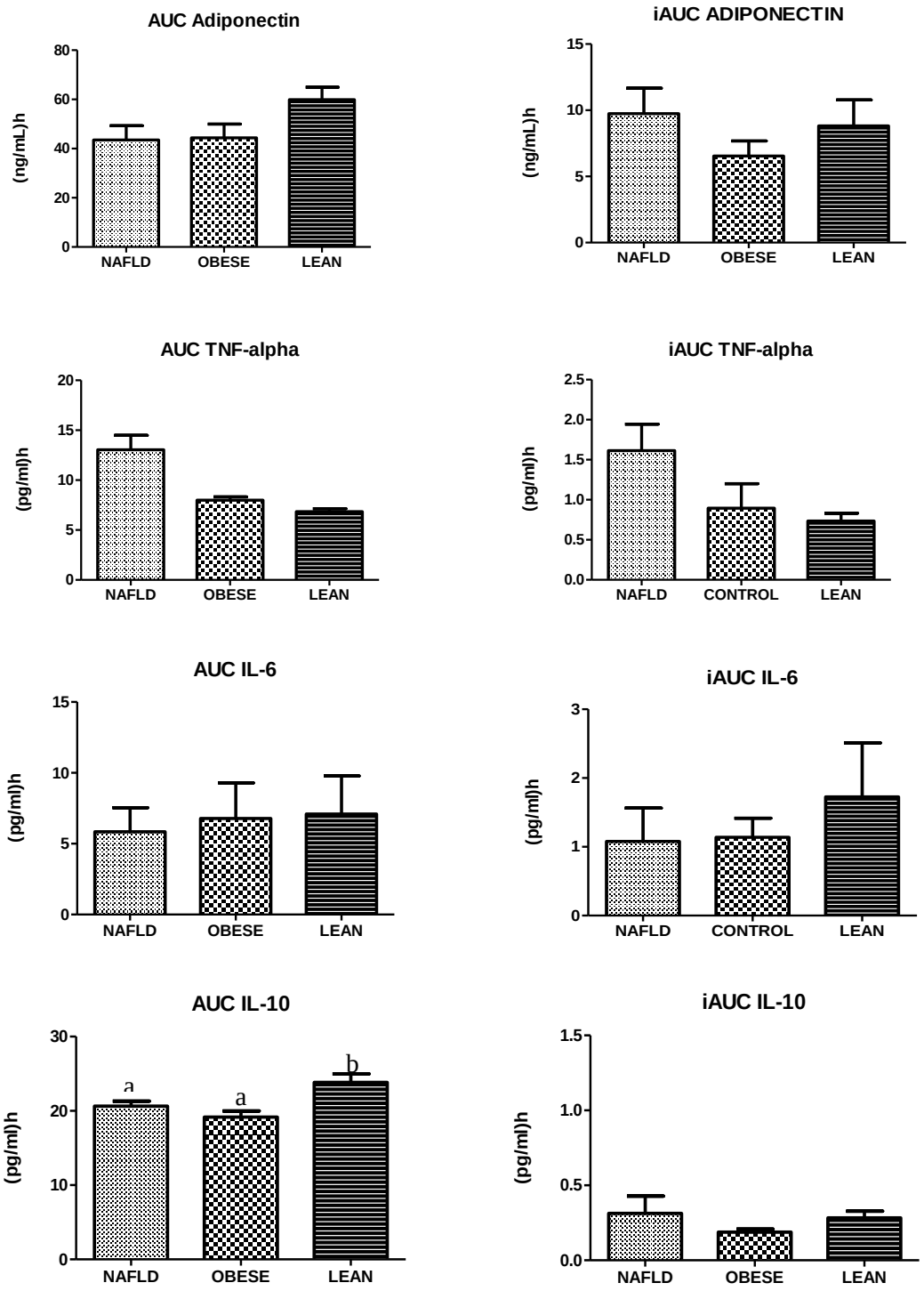

Figure M. Postprandial area under the curve (AUC) and incremental area under (iAUC) the curve for adiponectin, TNF-α, IL-6 and IL-10 in children and adolescents with nonalcoholic fatty liver disease (n=11), healthy obese-gender- and aged matched (n=9) and lean healthy controls (n=11) following consumption of 0% LCPUFA meal. Variables with different superscripts are significantly

different by repeated measures one way ANOVA $p<0.05$ followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

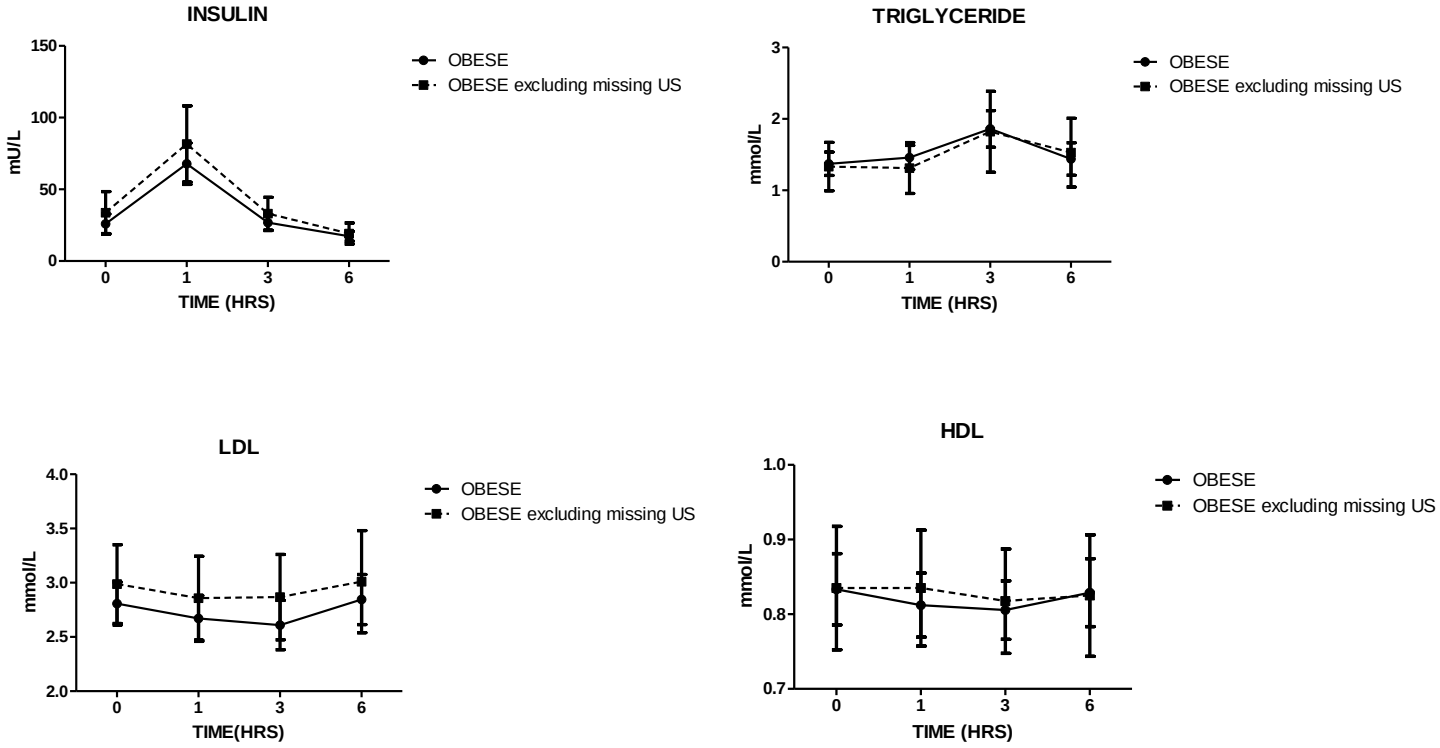

Figure N. Insulin, triglyceride, LDL and HDL **comparison between the obese (n=9) with and without US vs. obese with US (n=4)** concentrations prior (t=0) and following consumption of 0% LCPUFA meal at 1, 3 and 6 hours. Values with different superscripts are significantly different at $p<0.05$ by repeated measures two way ANOVA for time and group effect followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

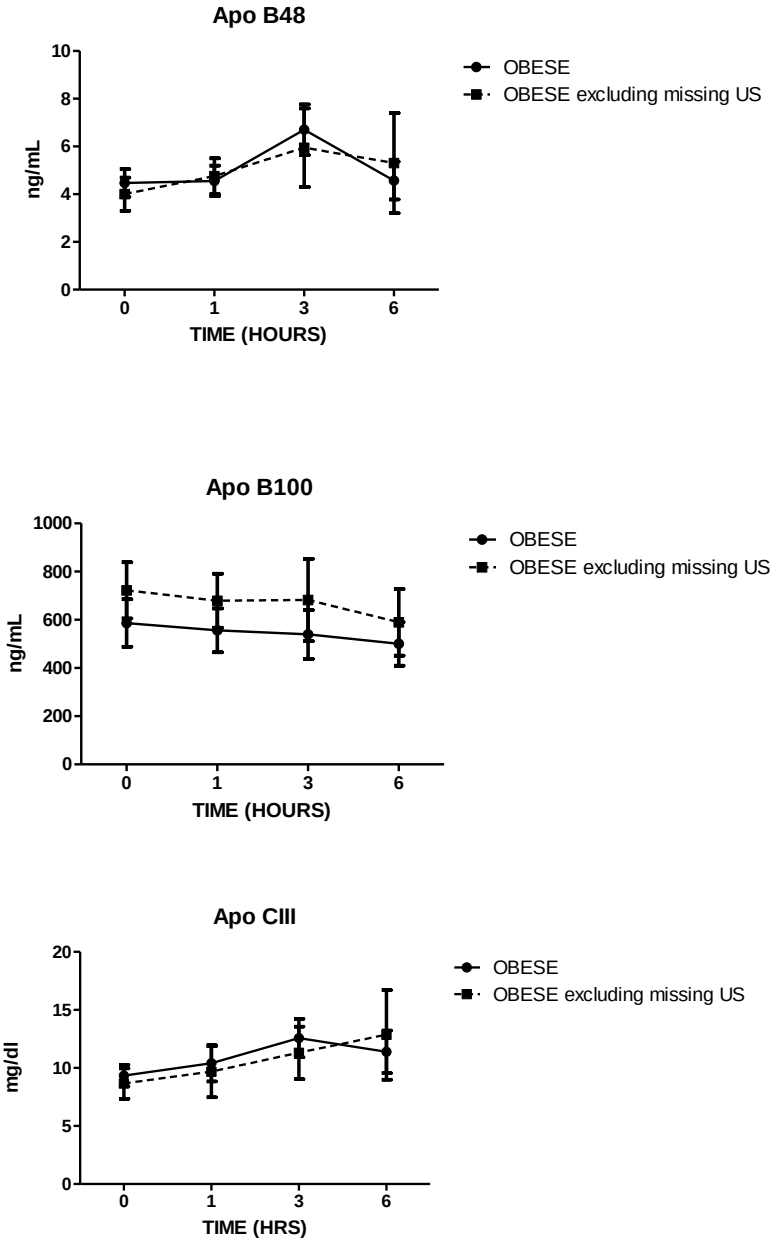

Figure O. Apo B-48, B-100 and C-III **comparison between the obese (n=9) with and without US vs. obese with US (n=4)** concentrations prior (t=0) and following consumption of 0% LCPUFA meal at 1, 3 and 6 hours Values with different superscripts are significantly different at $p<0.05$ by repeated measures two way ANOVA for time and group effect followed by Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

Table 5. Pearson (univariate) correlations NAFLD (group analysis for 0% LCPUFA meal).

	Age	ALT	AST	Insulin fasted	Insulin 1hr	Insulin 3hr	Insulin 6hr	iauc insulin	HOMA IR fasted
ALT	0.00		0.81*	0.40*	0.22	0.01	0.09	0.06	0.51*
AST	0.00	0.81*		0.32	0.16	0.01	0.06	0.04	0.41*
Insulin fasted	0.02	0.40*	0.32		0.33	0.37*	0.65*	0.20	0.93*
Insulin 1hr	0.27	0.22	0.16	0.33		0.64*	0.61*	0.92*	0.18
Insulin 3hr	0.23	0.01	0.01	0.37*	0.64*		0.69	0.80*	0.17
Insulin 6hr	0.23	0.09	0.06	0.65*	0.61*	0.69*		0.55*	0.43*
iauc insulin	0.32	0.06	0.04	0.20	0.92*	0.80*	0.55*		0.07
HOMA IR fasted	0.00	0.51*	0.41*	0.93*	0.18	0.17	0.43*	0.07	
TG fasted	0.03	0.17	0.37*	0.16	0.27	0.25	0.12	0.28	0.11
TG 1hr	0.02	0.16	0.38*	0.15	0.19	0.20	0.11	0.20	0.10
TG 3hr	0.03	0.07	0.22	0.09	0.22	0.30	0.10	0.29	0.04
TG 6hr	0.16	0.10	0.19	0.29	0.41*	0.52*	0.42*	0.47	0.17
iAUC TG	0.13	0.09	0.11	0.01	0.05	0.29	0.21	0.14	0.00
TNF-α fasted	0.01	0.06	0.16	0.00	0.04	0.02	0.01	0.00	0.01
TNF-α 1hr	0.03	0.02	0.08	0.00	0.07	0.00	0.02	0.04	0.00
TNF-α 3hr	0.00	0.03	0.11	0.00	0.09	0.00	0.01	0.05	0.00
TNF-α 6hr	0.00	0.02	0.11	0.01	0.05	0.00	0.04	0.02	0.01
Apo B-48 fasted	0.06	0.05	0.10	0.20	0.00	0.03	0.01	0.00	0.22
B-48 1hr	0.08	0.06	0.00	0.11	0.13	0.09	0.08	0.10	0.12
B-48 3hr	0.02	0.00	0.03	0.05	0.00	0.04	0.12	0.01	0.03
B-48 6hr	0.04	0.10	0.08	0.00	0.00	0.07	0.11	0.01	0.01
Apo B-100 fasted	0.10	0.00	0.04	0.07	0.00	0.00	0.00	0.00	0.10
B-100 1hr	0.00	0.01	0.00	0.19	0.03	0.06	0.14	0.02	0.18
B-100 3hr	0.11	0.00	0.04	0.06	0.00	0.00	0.00	0.00	0.09
B-100 6hr	0.00	0.00	0.00	0.19	0.02	0.03	0.12	0.01	0.20
Apo CIII fasted	0.00	0.11	0.08	0.07	0.07	0.12	0.26	0.09	0.02
C-III 1hr	0.03	0.05	0.01	0.00	0.03	0.06	0.00	0.05	0.00
C-III 3hr	0.72*	0.04	0.02	0.10	0.24	0.26	0.26	0.25	0.06
C-III 6hr	0.11	0.02	0.01	0.03	0.00	0.00	0.05	0.00	0.02

*values are expressed in r^2; * when $p<0.05$

Table 6. Pearson (univariate) correlations Obese control group (group analysis for 0% LCPUFA meal).

	Age	ALT	AST	Insulin fasted	Insulin 1hr	Insulin 3hr	Insulin 6hr	iauc insulin	HOMA IR fasted
ALT	0.30		0.59*	0.03	0.04	0.00	0.03	0.08	0.01
AST	0.26	0.59*		0.02	0.04	0.01	0.04	0.09	0.01
Insulin fasted	0.01	0.03	0.02		0.53*	0.88*	0.77*	0.36	0.98*
Insulin 1hr	0.00	0.04	0.04	0.53*		0.48*	0.83*	0.95*	0.55*
Insulin 3hr	0.05	0.00	0.01	0.88*	0.48*		0.79*	0.36	0.88*
Insulin 6hr	0.01	0.03	0.04	0.77*	0.83*	0.79*		0.71*	0.77*
iauc insulin	0.00	0.08	0.09	0.36	0.95*	0.36	0.71*		0.38
HOMA IR fasted	0.04	0.01	0.01	0.98*	0.55*	0.88*	0.77*	0.38	
TG fasted	0.10	0.01	0.00	0.33	0.07	0.30	0.22	0.06	0.31
TG 1hr	0.03	0.00	0.01	0.29	0.12	0.29	0.29	0.12	0.27
TG 3hr	0.18	0.00	0.01	0.38	0.09	0.34	0.25	0.06	0.39
TG 6hr	0.12	0.07	0.03	0.48*	0.36	0.53*	0.49*	0.35	0.54*
iAUC TG	0.01	0.14	0.07	0.42	0.54*	0.46*	0.57*	0.52*	0.48*
TNF-α fasted	0.16	0.26	0.19	0.00	0.00	0.03	0.00	0.00	0.00
TNF-α 1hr	0.42	0.11	0.07	0.01	0.04	0.05	0.00	0.06	0.04
TNF-α 3hr	0.35	0.06	0.01	0.01	0.07	0.00	0.05	0.09	0.00
TNF-α 6hr	0.50	0.05	0.01	0.02	0.13	0.00	0.08	0.13	0.01
Apo B-48 fasted	0.16	0.38	0.61	0.07	0.00	0.03	0.01	0.00	0.04
B-48 1hr	0.21	0.10	0.17	0.02	0.00	0.02	0.04	0.02	0.02
B-48 3hr	0.01	0.03	0.44*	0.00	0.01	0.00	0.02	0.01	0.01
B-48 6hr	0.01	0.00	0.12	0.23	0.29	0.21	0.19	0.27	0.26
Apo B-100 fasted	0.00	0.12	0.01	0.28	0.35	0.16	0.22	0.33	0.25
B-100 1hr	0.00	0.19	0.02	0.47*	0.42	0.32	0.36	0.34	0.42
B-100 3hr	0.05	0.07	0.03	0.23	0.21	0.23	0.19	0.23	0.21
B-100 6hr	0.01	0.14	0.00	0.47*	0.45*	0.33	0.41	0.38	0.42
Apo C-III fasted	0.00	0.02	0.00	0.05	0.17	0.00	0.10	0.20	0.05
C-III 1hr	0.10	0.10	0.02	0.08	0.00	0.12	0.04	0.01	0.06
C-III 3hr	0.03	0.15	0.02	0.00	0.10	0.00	0.03	0.16	0.01
C-III 6hr	0.17	0.10	0.05	0.54*	0.55*	0.52*	0.60*	0.53*	0.61*

*values are expressed in r2; * when $p<0.05$

Table 7. Pearson (univariate) correlations Lean control group (group analysis for 0% LCPUFA meal).

	Age	ALT	AST	Insulin fasted	Insulin 1hr	Insulin 3hr	Insulin 6hr	iauc insulin	HOMA IR fasted
ALT	0.01		0.55*	0.00	0.18	0.00	0.14	0.20	0.00
AST	0.17	0.55*		0.13	0.19	0.00	0.36	0.10	0.12
Insulin fasted	0.03	0.00	0.13		0.38	0.00	0.13	0.18	0.99*
Insulin 1hr	0.00	0.18	0.19	0.38		0.08	0.02	0.81*	0.32
Insulin 3hr	0.07	0.00	0.00	0.00	0.08		0.02	0.00	0.00
Insulin 6hr	0.12	0.14	0.36	0.13	0.02	0.02		0.01	0.16
iauc insulin	0.00	0.20	0.10	0.18	0.81*	0.00	0.01		0.15
HOMA IR fasted	0.03	0.00	0.12	0.99	0.32	0.00	0.16	0.15	
TG fasted	0.01	0.00	0.02	0.09	0.18	0.01	0.00	0.18	0.05
TG 1hr	0.09	0.00	0.03	0.17	0.23	0.02	0.03	0.18	0.12
TG 3hr	0.00	0.02	0.06	0.07	0.12	0.04	0.00	0.14	0.04
TG 6hr	0.22	0.01	0.00	0.00	0.00	0.01	0.03	0.00	0.00
iAUC TG	0.27	0.03	0.02	0.01	0.01	0.00	0.05	0.00	0.01
TNF-α fasted	0.00	0.01	0.00	0.13	0.21	0.00	0.00	0.12	0.11
TNF-α 1hr	0.02	0.06	0.16	0.06	0.19	0.04	0.01	0.15	0.04
TNF-α 3hr	0.01	0.00	0.03	0.18	0.33	0.01	0.05	0.21	0.14
TNF-α 6hr	0.00	0.01	0.00	0.01	0.39	0.12	0.00	0.33	0.00
Apo B-48 fasted	0.06	0.01	0.04	0.25	0.03	0.00	0.03	0.01	0.32
B-48 1hr	0.08	0.03	0.02	0.04	0.27	0.15	0.06	0.25	0.02
B-48 3hr	0.01	0.02	0.09	0.26	0.09	0.11	0.17	0.00	0.28
B-48 6hr	0.09	0.24	0.00	0.38	0.00	0.00	0.03	0.03	0.44
Apo B-100 fasted	0.36	0.06	0.14	0.11	0.07	0.13	0.06	0.05	0.10
B-100 1hr	0.01	0.25	0.11	0.18	0.07	0.00	0.06	0.10	0.17
B-100 3hr	0.00	0.11	0.01	0.41	0.05	0.00	0.12	0.02	0.45
B-100 6hr	0.35	0.00	0.03	0.03	0.00	0.01	0.02	0.00	0.06
Apo C-III fasted	0.02	0.00	0.00	0.03	0.01	0.16	0.03	0.00	0.06
C-III 1hr	0.02	0.04	0.00	0.00	0.02	0.36	0.04	0.00	0.00
C-III 3hr	0.01	0.06	0.11	0.04	0.07	0.14	0.00	0.16	0.02
C-III 6hr	0.02	0.04	0.02	0.01	0.03	0.18	0.00	0.12	0.00

*values are expressed in r^2; * when $p<0.05$

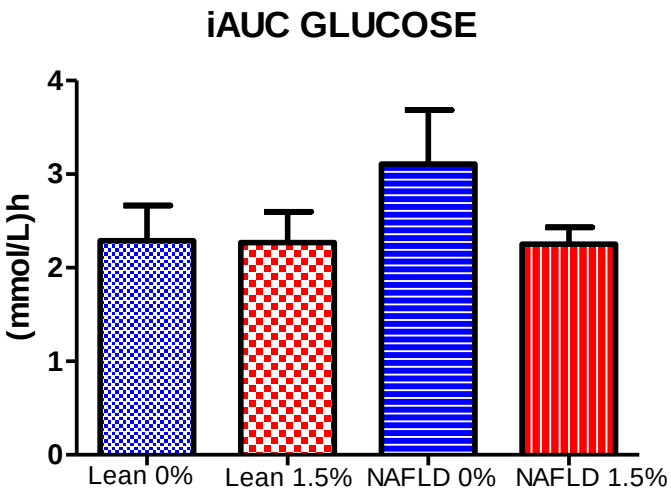

Figure P. Postprandial incremental area under the curve (iAUC) for glucose following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). Values with different superscripts are significantly different by repeated measures two way ANOVA when $p<0.05$ following Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

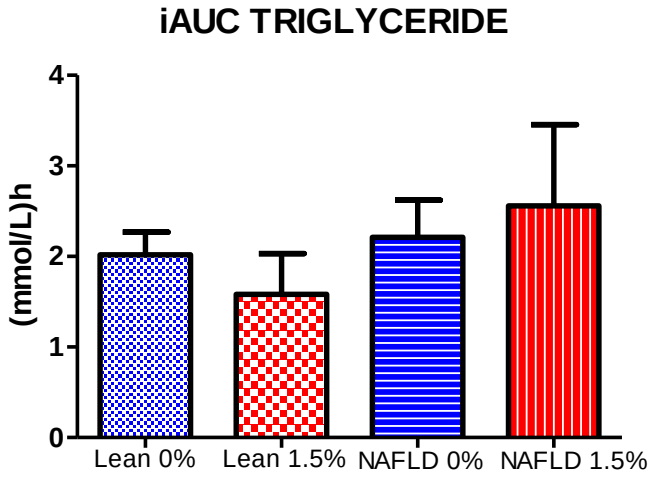

Figure Q. Postprandial incremental area under the curve (iAUC) for triglyceride following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. 1.5% LCPUFA: nonalcoholic fatty liver disease (n=7) and healthy lean-age matched (n=10). Values with different superscripts are significantly different by repeated measures two way ANOVA when $p<0.05$ following Bonferroni correction and pairwise comparisons Results are shown mean $\pm$ SEM.

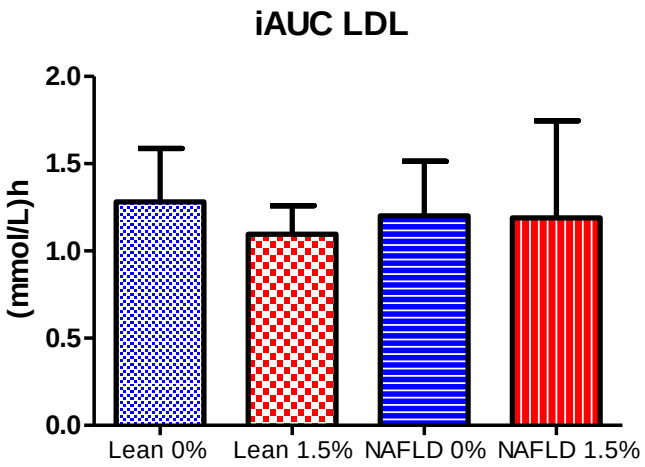

Figure R. Postprandial incremental area under the curve (iAUC) for LDL following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=7) and healthy lean-age matched (n=10). Values with different superscripts are significantly different by repeated measures two way ANOVA when $p<0.05$ following Bonferroni correction and pairwise comparisons Results are shown mean $\pm$ SEM.

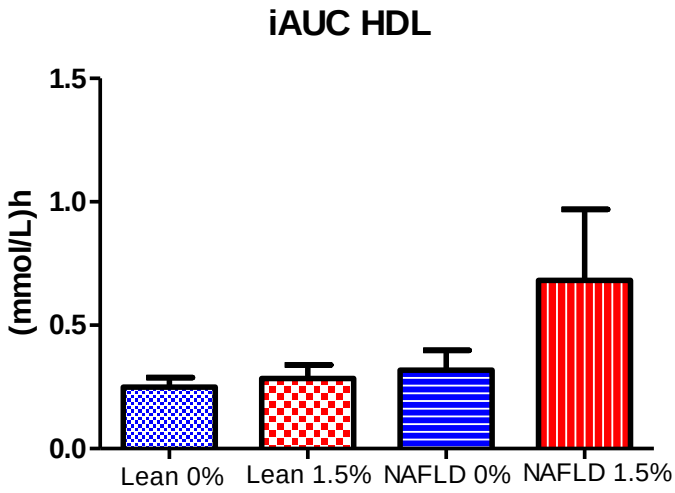

Figure S. Postprandial incremental area under the curve (iAUC) for HDL following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=7) and healthy lean-age matched (n=10). Values with different superscripts are significantly different by repeated measures two way ANOVA when $p<0.05$ following Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

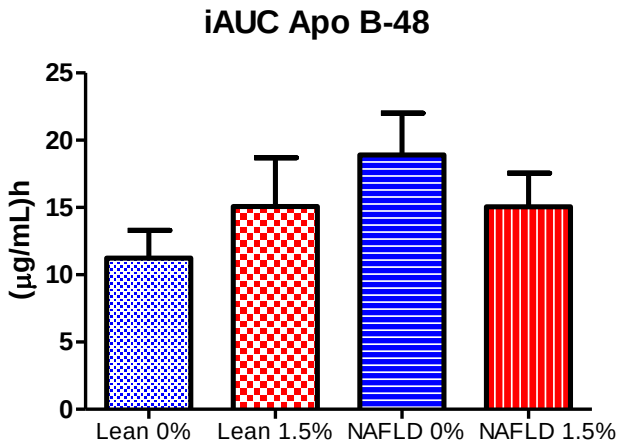

Figure T. Postprandial incremental area under the curve (iAUC) for Apolipoprotein B-48 following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=5) and healthy lean-age matched (n=10). Values with different superscripts are significantly different by repeated measures two way ANOVA when $p<0.05$ following Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

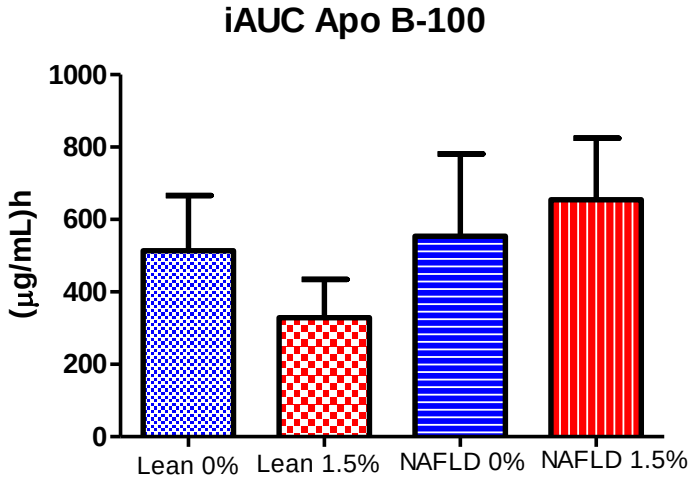

Figure U. Postprandial incremental area under the curve (iAUC) for Apolipoprotein B-100 following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=6) and healthy lean-age matched (n=10). Values with different superscripts are significantly different by repeated measures two way ANOVA when $p<0.05$ following Bonferroni correction and pairwise comparisons. Results are shown mean ± SEM.

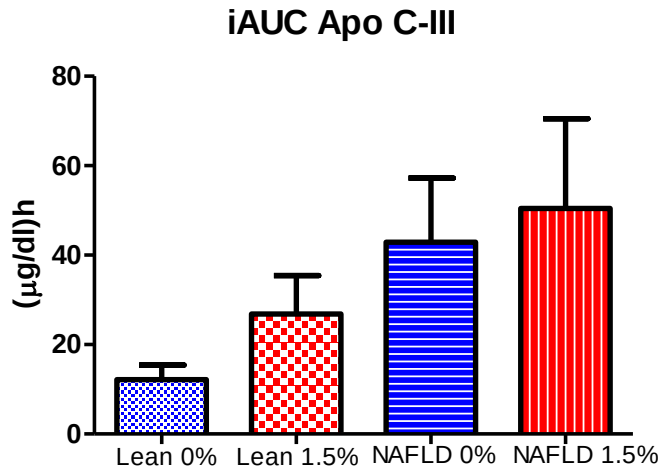

Figure V. Postprandial incremental area under the curve (iAUC) for Apolipoprotein C-III following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=7) and healthy lean-age matched (n=10). Values with different superscripts are significantly different by repeated measures two way ANOVA when $p<0.05$ following Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

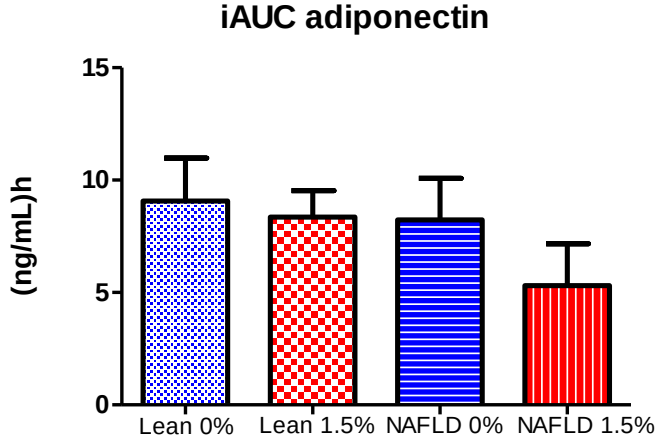

Figure W. Postprandial incremental area under the curve (iAUC) for adiponectin following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=5) and healthy lean-age matched (n=10). Values with different superscripts are significantly different by repeated measures two way ANOVA when $p<0.05$ following Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

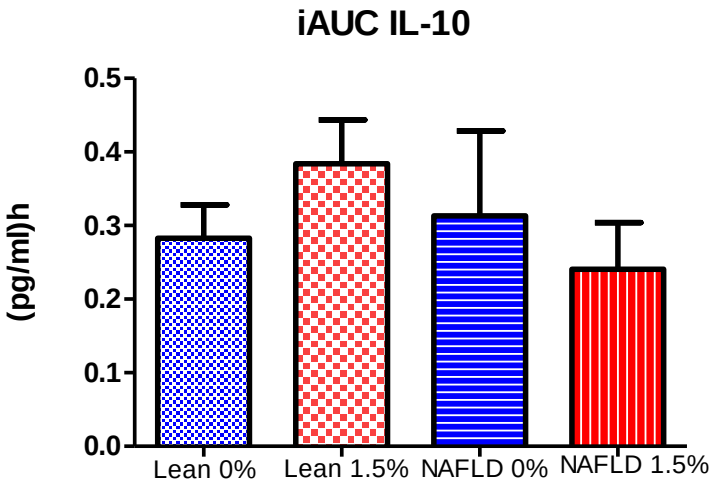

Figure X. Postprandial incremental area under the curve (iAUC) for IL-10 following consumption of two different meals: 1) 0% LCPUFA and 2) 1.5% LCPUFA. Meal 0% LCPUFA: nonalcoholic fatty liver disease (n=11) and healthy lean-age matched (n=11) controls. Meal 1.5% LCPUFA: nonalcoholic fatty liver disease (n=5) and healthy lean-age matched (n=10). Values with different superscripts are significantly different by Values with different superscripts are significantly different by repeated measures two way ANOVA when $p<0.05$ following Bonferroni correction and pairwise comparisons. Results are shown mean $\pm$ SEM.

Table 8. Additional statistical analysis for the entire cohort

Model	Predictive variables	Dependent variables	p value	r^2 value
Partial correlation 0% LCPUFA meal	iAUC for TG and iAUC for Apo C-III	Insulin iAUC	p=0.2	0.047
Partial correlation 1.5% LCPUFA meal	iAUC for TG and iAUC for Apo C-III	Insulin iAUC	p=0.5	0.023
Partial correlation 0% LCPUFA meal	iAUC for TG and iAUC for NEFA	Insulin iAUC	p=0.3	0.034
Partial correlation 1.5% LCPUFA meal	iAUC for TG and iAUC for NEFA	Insulin iAUC	p=0.5	0.093
Partial correlation 0% LCPUFA meal	iAUC for insulin and iAUC for Apo C-III	TG iAUC	p<0.0001	0.376
Partial correlation **1.5% LCPUFA meal**	iAUC for insulin and iAUC for Apo C-III	TG iAUC	p=0.4	0.136
Partial correlation 0% LCPUFA meal	iAUC for insulin and iAUC for Apo C-III	Apo B-48 iAUC	p<0.0001	0.421
Partial correlation 1.5% LCPUFA meal	iAUC for insulin and iAUC for Apo C-III	Apo B-48 iAUC	p=0.2	0.249
Multiple regression 0% LCPUFA meal	iAUC for TG and iAUC Apo C-III	Insulin iAUC	p<0.0001,	0.458
Multiple regression 1.5% LCPUFA meal	iAUC for TG and iAUC Apo C-III	Insulin iAUC	p=0.4	0.110
Multiple regression	iAUC for Apo B-48 and iAUC Apo C-	Insulin iAUC	p<0.0001,	0.447

0% LCPUFA meal	III			
Multiple regression 1.5% LCPUFA meal	iAUC for Apo B-48 and iAUC Apo C-III	Insulin iAUC	p<0.4	0.108
Multiple regression 0% LCPUFA meal	iAUC for TNF-α and iAUC Apo C-III	Insulin iAUC	p<0.0001,	0.480
Multiple regression 1.5% LCPUFA meal	iAUC for TNF-α and iAUC Apo C-III	Insulin iAUC	p<0.5	0.085
Multiple regression 0% LCPUFA meal	iAUC for TNF-α and iAUC for NEFA	Insulin iAUC	p=0.04	0.203
Multiple regression 1.5% LCPUFA meal	iAUC for TNF-α and iAUC for NEFA	Insulin iAUC	p=0.7	0.034
Multiple regression 0% LCPUFA meal	iAUC for Apo CIII and ALT>20 U/L	Insulin iAUC	p=0.002	0.450
Multiple regression 1.5% LCPUFA meal	iAUC for Apo CIII and ALT>20 U/L	Insulin iAUC	p=0.3	0.133