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Diagnosis of non-alcoholic fatty liver disease in obese adolescents using non-invasive methods

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Thesis

DIAGNOSIS OF NON-ALCOHOLIC FATTY LIVER DISEASE IN OBESE ADOLESCENTS USING NON-INVASIVE METHODS

by

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I would like to dedicate this work to my parents, who have always encouraged me to pursue my dreams. Thank you for always believing in me and for supporting me in every decision I have made. All the experiences and education acquired during my life wouldn’t have been possible if it wasn’t for you.
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DIAGNOSIS OF NON-ALCOHOLIC FATTY LIVER DISEASE IN OBESE ADOLESCENTS USING NON-INVASIVE METHODS
LAURA LARA-CASTOR

ABSTRACT

Objective. To identify clinical, socio-demographic, dietary and biological markers to be used in a non-invasive and cost-effective clinical tool for screening for non-alcoholic fatty liver disease (NAFLD) in obese adolescents.

Methods. We conducted a cross-sectional analysis using baseline data from 77 obese adolescents enrolled in a drug trial for the Glaser Pediatric Research Network, between October 2003 and August 2007. NAFLD was defined as the presence of fatty liver infiltration assessed by computed tomography. Receiver operation characteristic (ROC) analyses were performed to identify variables with the highest area under the curve (AUC) for NAFLD. Serum biomarkers were dichotomized using sensitivity analyses to identify the best cutoff point for NAFLD. Multiple logistic regression models were created to predict prevalent NAFLD.

Results. Serum triglycerides was identified as the best biomarker for NAFLD (AUC 0.790; pseudo R² 0.235). Additional adjustment for sex, age and Tanner stage improved the AUC to 0.846 and the pseudo R² to 0.290. We then explored adding a simple biochemical marker for predicting NAFLD (HOMA-B, ALT or glutamate) and found that HOMA-B led to greater improvement in AUC, ALT to a greater improvement in sensitivity and glutamate to a greater improvement in the pseudo
R². Thus, all three factors individually improved overall model performance to some degree and inclusion of all three led to an AUC=0.907 and pseudo R²=0.433.

Our second objective was to develop a more complex exploratory model starting with the inclusion of important clinical predictors (triglycerides, sex, age, Tanner stage, SBP, BMI, waist circumference); this yielded an AUC of 0.871 and pseudo R² of 0.342. Further adjustment for HOMA-B, ALT and glutamate gave an AUC=0.913 and pseudo R²=0.497.

**Conclusion.** Simple clinical and biochemical factors may be used to screen for prevalent NAFLD. Our simplest clinically relevant model using triglycerides, age, sex and Tanner stage provided a reasonable screening tool for NAFLD in obese adolescents. A second more complex model that warrants further testing includes triglycerides, sex, age, Tanner stage, SPB, BMI, waist circumference, HOMA-B, ALT and glutamate. In this study, this model was more accurate for detecting undiagnosed cases of NAFLD in this pediatric population.
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LIST OF ABBREVIATIONS

ALT............................................................Alanine aminotransferase
AST .......................................................... Aspartate aminotransferase
AUC .......................................................... Area under the curve
BCAA ........................................................... Branched chained amino acids
BMC .......................................................... Boston Medical Center
BMI ............................................................ Body mass index
BU ............................................................. Boston University
BP-1 ............................................................ Binding protein-1
CDC .......................................................... Center of Disease Control and Prevention
ChREBP .................................................... Carbohydrate response element-binding protein
CPE .............................................................. C-peptide
CT .............................................................. Computed tomography
DNA .......................................................... Deoxyribonucleic acid
DHA .......................................................... Docosahexaenoic acid
EPA ............................................................ Eicosapentanoic acid
FIB-4 .......................................................... Fibrosis-4 score
FFA ............................................................ Free fatty acids
GI .............................................................. Glycemic index
GL ............................................................ Glycemic load
GPRN ......................................................... Glaser Pediatric Research Network
HbA1C ........................................................ Glycosylated hemoglobin
HDL ................................................................. High-density lipoprotein
HOMA-β .......................................................... Homeostatic model assessment for β-cell function
HOMA–IR ......................................................... Homeostatic model assessment for insulin resistance
HU .................................................................................. Hounsfield units
IGF-1 ........................................................................ Insulin-like growth factor-1
IRB .................................................................................. Institutional Review Board
LCPUFAs ............................................................... Long chain polyunsaturated fatty acids
LDL ........................................................................ Low-density lipoproteins
LFABP ........................................................................ Liver fatty acid binding protein
LPL .................................................................................. Lipoprotein Lipase
MRE ........................................................................ Magnetic resonance elastography
MRI ........................................................................ Magnetic resonance imaging
NAFLD ................................................................. Non-alcoholic fatty liver disease
NASH ........................................................................ Non-alcoholic steatohepatitis
n3 .................................................................................. Omega-3
n6 .................................................................................. Omega-6
OR .................................................................................. Odds ratio
PPR-α .......................................................... Peroxisome proliferator-activated receptor-alpha
PPR-γ .......................................................... Peroxisome proliferator-activated receptor-gamma
QUICKI .......................................................... Quantitative insulin sensitivity check index
ROC ................................................................. Receiver operation characteristics
ROS ........................................................................ Reactive oxygen species
R² ...........................................................R squared
SBP .............................................................. Systolic blood pressure
SREBP-1c ..................................................... Stearol regulatory element binding protein-
STATA ........................................................ Data analyst and statistical software
US ............................................................... United States of America
VLDL ............................................................ Very low-density lipoprotein
WC .............................................................. Waist circumference
YAQ ............................................................. Youth/Adolescent Questionnaire
INTRODUCTION

Public Health Importance

Obesity is of special concern in pediatrics as it is highly prevalent (17%)\textsuperscript{1}, tends to persist into adulthood and the longer the duration, the more severe the complications.\textsuperscript{2–5} Therefore, more efforts need to be directed toward prevention as well as screening and treatment of its comorbidities. One of the most serious complications of pediatric obesity and its concurrent metabolic changes is non-alcoholic fatty liver disease (NAFLD), which involves a spectrum of liver diseases, from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH).\textsuperscript{6–9} Simple hepatic steatosis consists of fat accumulation in >5% of the hepatocytes, whereas NASH goes along with hepatocyte ballooning, fibrosis and polymorphonuclear infiltration.\textsuperscript{10,11} Pediatric NAFLD remains underdiagnosed,\textsuperscript{12} even though it is the most common cause of liver disease in children.\textsuperscript{10,3} There is a great need to develop non-invasive tools to screen and diagnose NAFLD in at-risk populations before its progression to more severe stages and the development of cirrhosis, which are associated with a decreased quality of life, high medical costs and high mortality.\textsuperscript{7,12}

Prevalence

NAFLD affects 10–24% of the general population in the world.\textsuperscript{13–15} The real prevalence of pediatric NAFLD is unknown.\textsuperscript{12} However, some studies suggest that NAFLD affects between 0.7 to 17.3% of the general pediatric population, 38% of obese children\textsuperscript{16} and up to 83% of morbidly obese adolescents.\textsuperscript{17} NAFLD has been
found to be more common in children who are older, of Hispanic ethnicity, are male and have a family history of diabetes, obesity and hyperlipidemia.\textsuperscript{8,3,16,18,19}

**Pathogenesis**

NAFLD is considered as the liver expression of the metabolic derangement observed in obesity.\textsuperscript{19–22} Insulin resistance is believed the main driving factor and NAFLD is thus associated with obesity, hypertension and dyslipidemia.\textsuperscript{13,21,23} The most currently accepted theory for the development of NAFLD is a “multiple hit” process, where genetic, epigenetic and environmental factors play an important role in the appearance and progression of the disease.\textsuperscript{6,9,22,24,25} The first hit is recognized as the accumulation of triglycerides in the liver.\textsuperscript{13,26} The secondary hits come with the consecutive oxidative stress and inflammatory processes promoting the release of cytokines and reactive oxygen species (ROS), which lead to fibrosis, cirrhosis and, in some cases, cancer.\textsuperscript{7,10,21,23,26}

It is not clear whether liver triglyceride accumulation is the cause or the consequence of insulin resistance.\textsuperscript{25} Liver triglyceride accumulation may occur because of an insulin resistance state, which may be associated with prolonged exposure to high insulin serum levels\textsuperscript{27} associated with hyper caloric diets, as well as genetic susceptibility and sedentary lifestyles.\textsuperscript{22,25} Insulin resistance causes compensatory hyperinsulinemia and a consequent disruption in lipid metabolism.\textsuperscript{28,29} The tissue uptake of fatty acids from the apolipoproteins is decreased\textsuperscript{23} as the insulin sensitive enzyme lipoprotein lipase (LPL) is not being properly activated to breakdown the fatty acids embedded in the chylomicrons and VLDL (very low-
density lipoproteins.\textsuperscript{30} The release of free fatty acids (FFA) from the adipocytes is increased due to the lack of inhibition of the hormone sensitive lipase by insulin and to meet the energetic demands of the body.\textsuperscript{25,28–31} As a result serum FFA increase and these is subsequently delivered to the liver.\textsuperscript{25,28} As opposed to the other tissues, the liver shows an increase in the uptake and synthesis of triglycerides and a decrease in lipid beta-oxidation and synthesis of apolipoproteins for the export of triglycerides to the blood.\textsuperscript{9,3,23,24,28} These molecular mechanisms are regulated by the transcription factors sterol regulatory element-binding protein-1c (SREBP-1c), carbohydrate response element-binding protein (ChREBP), and peroxisome proliferator-activated receptor-gamma (PPAR-\textgamma).\textsuperscript{3,24,28} An imbalance between the synthesis/uptake of triglycerides versus the export/ beta oxidation in liver leads to liver steatosis.\textsuperscript{28} The secondary hits come as a consequence of a lipotoxic effect of the FFAs and lipid intermediates causing oxidative stress, mitochondrial dysfunction and pro-inflammatory cytokines contributing to the progression to NASH which is characterized by increased fibrosis and consequent cirrhosis.\textsuperscript{7,9,3,24,28} Some of the adipocytokines, adipose tissue substances, that has been found to be involved are adiponectin, leptin, resistin and tumor necrosis factor-alpha.\textsuperscript{12,28} This lipotoxic effect aggravates the impaired insulin signaling, which further decreases export of triglycerides to the blood.\textsuperscript{24} Finally, imbalances in gut permeability or in gut microbiota might also contribute to the liver damage via gut-derived bacterial
products, which have been found to result in the activation of the innate immune response and increased fibrogenesis and inflammation.\textsuperscript{32}

**Diagnosis**

The diagnosis of pediatric NAFLD in early stages is difficult due to the need for invasive\textsuperscript{33,34} and costly\textsuperscript{35} diagnostic procedures, lack of consensus in the screening methods and absence of symptoms.\textsuperscript{22,36–39}

Liver biopsy is currently considered as the gold standard to diagnose NAFLD because it allows for the identification of the histopathological stage of the hepatocyte, which allows to differentiate simple steatosis from NASH.\textsuperscript{12,40,41} However, this technique is highly invasive and costly and is associated with risk of bleeding and may also miss the diagnosis due to sampling error given the small portion of parenchyma liver examined.\textsuperscript{33,34,41–43}

Imaging techniques such as ultrasonography, computed tomography (CT), magnetic resonance imaging (MRI) and magnetic resonance elastography (MRE) have also been used for diagnostic purpose.\textsuperscript{44,45} CT scan is easy, fast and available, and one-slice CT scan, which conveys a low radiation exposure, has been identified as a good predictor of NAFLD.\textsuperscript{46–48} CT scan has the disadvantage of not being sensitive enough to differentiate early from more advanced stages such as NASH or advanced fibrosis, but it has shown to be good at detecting fatty infiltration in the liver and has a better sensitivity than ultrasonography.\textsuperscript{12,45}

Serum biomarkers and equations derived from these biomarkers are also used in practice to screen for NAFLD. Potential biomarkers of NAFLD may markers of
energy, lipids and protein metabolism such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), AST:ALT ratio, fasting glucose, fasting insulin, C-peptide (CPE), the quantitative insulin sensitivity check index (QUICKI), homeostatic model assessment for insulin resistance (HOMA-IR), low-density lipoproteins (LDL), high-density lipoproteins (HDL), triglycerides. However, these serum biomarkers alone have usually poor sensitivity for detecting NAFLD The serum biomarker most commonly used clinically to screen for NAFLD is ALT. ALT not only has found to have low accuracy because other conditions can be associated with elevation of ALT, but there is also a lack of consensus regarding the threshold for defining abnormal serum levels of ALT, especially in pediatric populations. Using liver biopsy as gold standard, the sensitivity of ALT has been shown to be 57%, well below the conventionally accepted 80% sensitivity of a biomarker. Given ALT’s low sensitivity and lack of other clinically practical alternatives to screen for NAFLD, ALT is used as a screening tool but definitely not as diagnostic tool of NAFLD. Biomarkers such as markers of cellular injury or apoptosis (caspase generated cytokeratin 18, Fas ligand and procollagen III), inflammation (adipokines and ferritin), oxidative stress (glutathione peroxidase dismutase, superoxide dismutase and oxidized low density lipoprotein) and lipotoxicity are also associated with NAFLD. However, these biomarkers are not readily available in clinical practice, thus its utility for screening for NAFLD is low.
Recently, the serum long chain polyunsaturated fatty acids (LCPUFAs) omega-6 (n6) and omega-3 (n3) were identified as potential biomarkers for predicting NAFLD. In fact, NAFLD has been associated with high serum and liver n6:n3 ratio. An increase in the n6:n3 ratio leads to a decrease in fatty acid oxidation, which is caused by the down regulation of the transcription factor peroxisome proliferator-activated receptor α (PPAR-α), which in turns blunts the transcription of fatty acid oxidation genes, thus decreasing the mitochondrial and peroxisomal β-oxidation. The increase in the n6:n3 ratio also decreases the export of triglycerides due to the down regulation of PPAR-α also inhibits the transcription of the cytosolic protein liver fatty acid binding protein (LFABP) which influences the assembly of very low-density lipoproteins (VLDL). Finally, the increased n6:n3 ratio increases fatty acid synthesis by an increase in the expression and/or processing of the transcription factor SREBP-1. All these metabolic changes promote the fatty acid deposition in the liver. LCPUFA serum biomarkers are also not readily available in clinical practice. Of note, previous studies validated the relationship of dietary n3 and n6 with their correspondent serum levels, and dietary, not serum, LCPUFA data has been recommended for use as a proxy of serum levels in community studies.

NAFLD has also been associated with other clinical and socio-demographic characteristics such as sex, age, Tanner stage, ethnicity, body mass index (BMI), systolic blood pressure (SBP), hypertension, Tanner stage and waist circumference.
Formerly evaluated, some clinical predictive models for NAFLD have accounted for clinical and socio-demographic characteristics as well as serum biomarkers of energy metabolism and inflammation. These clinical predictive models are easy, affordable, available and accurate\textsuperscript{45}. The most recent models include the D fibrosis score, BARD score, fibrosis-4 score (FIB-4), the Fatty Liver Index, the Framingham Steatosis Index and the Pediatric NAFLD Fibrosis Index.\textsuperscript{69–73} The D fibrosis score includes hyperglycemia, albumin, age, BMI, platelet count and AST:ALT ratio. The FIB-4 score utilizes AST, ALT, platelets and age. The Fatty Liver Index uses BMI, diagnosis of diabetes, age, sex and AST:ALT. The Framingham Steatosis Index includes age, sex, BMI, triglycerides, hypertension, diabetes and the ratio AST:ALT and finally, the Pediatric NAFLD Fibrosis Index uses age, waist circumference and triglycerides. However, these models, are either only been validated in adult population or screen for fibrosis and not for NAFLD from the early to late stages.

Given the increasing prevalence of pediatric NAFLD linked to the obesity epidemic, there is a need to identify new cost-effective and non-invasive strategies to diagnose NAFLD in its earlier stages.\textsuperscript{12,39} Most studies target adult population, but there is a need to focus on the pediatric population as duration of obesity is critical.\textsuperscript{3,35} Although, biomarkers of NAFLD have been evaluated in a few studies of pediatric NAFLD, the accuracy of those indicators using receiver operating characteristics (ROC) analysis has not been performed. Therefore, potential clinical models for the screening of NAFLD in pediatric populations have not been
tested, even though such a method could provide a critically-needed, a cost effective and non-invasive method for identifying subjects with NAFLD. 59,60

**Principal Objective**

We used baseline data available from adolescents enrolled in a multicenter study of metformin and lifestyle from the Glaser Pediatric Research Network74 to identify biological, clinical, dietary and socio-demographic characteristics strongly associated with NAFLD to develop a tool for its screening in a pediatric population.
METHODS

General Design
We conducted a cross-sectional analysis using baseline data (from October 2003 to August 2007) from 77 obese adolescents enrolled in a clinical trial of metformin for the Glaser Pediatric Research Network (GPRN) to identify characteristics most strongly correlated with NAFLD among obese adolescents. The study is described in more details elsewhere and in the Appendix.74,75

Inclusion and exclusion criteria of the subjects
The inclusion criteria for the subjects entered in the study of metformin were as follows: ages 13.0 – 17.9 years, BMI ≥95th percentile, weight ≤136 kg. Subjects were excluded if they had a diagnosis of diabetes (symptoms of diabetes plus casual plasma glucose concentration ≥200 mg/dl (11.1 mmol/l); fasting plasma glucose ≥126mg/dl (7.0 mmol/l) or 2 hour plasma glucose ≥200 mg/dl (11.1 mmol/l) during an oral glucose tolerance test), prior drug therapy to treat diabetes or insulin sensitivity (i.e. insulin, insulin analogs, acarbose, acetihexamide, chloropropamide, glimepiride, glipizide, glyburide, metformin, pioglitazone, repaglinide, rosiglitazone, tolazamide, tolbutamide or troglitazone), previous medication to aid in weight loss (i.e. Benzphetamine Hcl, Diethylpropion Hcl, Fenfluramine Hcl, Phendimetrazine Tartrate, Phentermine Hcl or Orlistat, Sibutramine Hcl Monohydrate), current medication that might increase metformin levels (i.e. Cimetidine, amiloride, digoxin, furosemide, morphine, nifedipine, procainamide, ranitidine, triamterene, trimethoprim, vancomycin and quinidine),
recent glucocorticoid therapy, history of any syndrome or medical disorder associated with significant obesity (i.e. Prader Willi Syndrome, Bardet-Biedl Syndrome, Cohen Syndrome or Cushing syndrome or disease), prior surgical therapy for obesity, recent history of involvement in a formal weight loss program, alcohol use (if in the past 6 months had more than 3 alcohol containing beverages in a 24hr period and/or consumed alcohol more than twice per week), elevated creatinine (>1.2 mg/dl), untreated disorders of thyroid function, elevated liver enzymes ALT or AST >80 (approximately 2 times upper limit of normal), mobility impairment, other serious medical condition that the principal investigator or the lead site investigator determined may put the patient at undue risk if enrolled in the study, unable to comply with the protocol in opinion of the principal investigator or the lead site investigator, subjects with child-bearing potential who were unwilling to remain abstinent or use and effective method for birth control (see Appendix).

**Primary outcome for the current cross-sectional analysis**

The primary outcome of this cross-sectional study was the absence of NAFLD defined by a cutoff point of 5HU (Hounsfield Units) in fatty liver infiltration assessed by CT scan. CT uses X-rays that are collimated to provide a fan-shaped beam that is passed through the body, to produce a cross-sectional image of the body region scanned. One-slice CT scan implies minimal radiation and has proved to provide a good estimate of body composition. Therefore it doesn’t imply major risk unless indicated by the principal or lead investigator (See Appendix).
For assessment of fatty liver infiltration, the liver and spleen were visualized using a single CT slice at the level of T11-T12 disk space. A 5-10-mm slice collimation was acquired through the liver and spleen. Attenuation values of the liver and spleen were measured on these images using an operator-defined circular or elliptical region-of-interest cursor. Lower attenuation means lower density and thus higher fat content. Acquisition of a single CT image is recognized as a practical and reliable method for routine measurement of liver fat in research and clinical settings. A cut of point of 5 Hounsfield Units (HU) for the difference (L-S) in attenuation between liver (L) and spleen (S) was used to define presence vs. absence of fat on liver CT-scan as this value has been shown to be highly specific.

Secondary measures

Other variables used in this analysis included demographic (sex and race), anthropometric (weight, height, waist circumference), clinical (SBP and Tanner stage), biochemical (fasting glucose, fasting insulin, glycosylated hemoglobin (HbA1C), CPE, glucagon, leptin, ALT, AST, triglycerides, HDL, glutamate, glutamine, leucine, branched chained amino acids (BCAA), binding protein-1 (BP-1) and insulin-like growth factor-1 (IGF-1)) and dietary variables (kilocalories, protein, carbohydrate, fiber, total fat, polyunsaturated fat, n3, n6, eicosapentanoic acid (EPA), docosahexaenoic acid (DHA), glycemic index (GI) and glycemic load (GL)).

Demographics
Race was determined by self-identification. Subjects were asked to choose one or more of the following categories: American Indian or Alaska Native, Asian, Native Hawaiian or other Pacific Islander, black or African American, white or other. Ethnicity was also determined by self-identification by asking whether the subjects consider themselves to be Hispanic or Latino. Given the small numbers of subjects in some of these categories, we reclassified the race/ethnicity as non-Hispanic white, non-Hispanic black, Hispanic/Latino and others.

**Height, weight and Body Mass Index (BMI)**

Height and weight were measured twice using a calibrated wall-mounted stadiometer to the nearest 0.1 cm for height and an electronic scale to the nearest 0.1 kg for weight. The patient weight was measured in light clothing and without shoes. A third reading was taken if the difference between the first two readings was >0.5 cm for height or 0.3 kg for weight. The means of these measurements were calculated for the statistical analysis.

**Waist circumference**

Waist circumference was measured by the umbilical method and each measure was done twice and the mean was used in the analyses. Subjects were measured around the smallest area below the rib cage and above the umbilicus.

**Puberty assessment**

The Tanner staging of pubic hair by direct inspection of a trained clinician was used to determine the stage of puberty of adolescents from both sexes. Tanner stage
was treated as dichotomous joining stages 2 and 3 because it has been shown that the greatest hormonal changes occur in these 2 stages. 68,74

**Youth/Adolescent Questionnaire (YAQ)**

Dietary and supplement intake were estimated for the past year by using the validated Youth Adolescent food frequency questionnaire. 79 The questionnaires were sent and scored at Channing Laboratory in Boston, MA.

**Clinical Laboratory assessments**

Fasting glucose, fasting insulin, HbA1C, CPE, triglycerides, HDL, ALT, AST, glucagon, leptin, fasting amino acids, BP-1 and IGF-1 in serum were obtained from the subjects after following a 3-day normal carbohydrate diet (at least 150 g/day) and a 10 h fast.74,75 Amino acids levels were available in this study but have not previously been evaluated in NAFLD and thus are not reimbursed for this condition by third party payer. More details on the measurements can be found in the Appendix and elsewhere74.

**Calculated variables**

**BMI**

BMI was calculated as the mean weight in kilograms divided by the mean height in meters squared and converted to sex- and age specific z-score based on data from the Centers for Disease Control and Prevention (CDC).74,80

**Characterization of insulin dynamics and insulin sensitivity**

For this study, fasting insulin and glucose measurements were obtained for calculation of both the HOMA and QUICKI indices. The homeostasis model
assessment-insulin resistance (HOMA-IR) was calculated as \([\text{Fasting Glucose (mmol/L)} \times \text{Fasting Insulin (μIU/ml)}] / 22.5\). The model assessment of beta-cell function (HOMA-β) was calculated as \([20 \times \text{Fasting Insulin (μIU/ml)}] / \text{Fasting Glucose (mmol/L)} - 3.5\).\(^{74,81–84}\)

**Institutional approval and informed consent**

Institutional Review Board (IRB) approval were obtained from the five GPRN sites and from the Boston Medical Center (BMC). Before the study began, signed informed consent and age appropriate assent were obtained from a parent or other legal guardian of each subject. In addition, an exempt approval was obtained for this NAFLD analysis.\(^{74,75}\)

**Statistical Analysis**

We conducted a cross-sectional analysis in 77 adolescent obese subjects to identify clinical, socio-demographic, dietary and biological characteristics to develop a non-invasive and cost-effective screening tool.

**Exploratory analysis**

Exploratory analyses with histograms and boxplots showed that most of the characteristics were not normally distributed, which was confirmed with Shapiro-Wilk tests for normality.

**Hypothesis testing**

Since several of the biomarkers were not normally distributed and the sample size was small, we decided to conduct non-parametric analysis. Wilcoxon tests were
used for continuous variables and Fisher’s exact tests for categorical variables. Statistical significance was determined as p≤0.05.

**Receiver Operation Characteristics (ROC) analyses**

ROC analyses were performed to obtain the C-statistics (area under the curve, AUC) of the different variables associated with NAFLD. The best cutoff point of continuous variables were determined using the `cutpt` command in STATA and to generate a binary variable that would allow for the estimation of the variables’ sensitivity, specificity, and percent of correctly classified subjects. Dichotomized biomarkers at a set cutoff point is an alternative method to treat non-normally distributed biomarkers when log transformation is not desired.85

**Correlation analysis**

Given that many of the variables of interest that were compared were continuous vs. continuous but also continuous vs. dichotomous, we used Pearson correlation and the point biserial correlation coefficients (mathematically equivalent to Pearson correlation) to examine the association variables. Variables with correlation coefficient ≥0.7 were not included in the same model to rule out interaction effect.

**Logistic regression analyses**

Simple logistic regression analyses were performed to estimate the odds ratio (OR) for NAFLD, as well as AUC, pseudo R², sensitivity, specificity and percent of subjects correctly classified for each variable.
After identifying the best predictor using ROC and simple logistic regression analyses we build potential models for the screening of NAFLD. Our goal was to identify the best model as well as a practical, clinically relevant model using the best main predictor adjusted for a few variables. In the attempt of avoiding adjustment given the small sample size, we initially adjusted for a few relevant variables. We built a core model with the three clinically relevant variables (age, sex and Tanner stage (stages 2 and 3). We further adjusted the model for other variables wherever possible.

**Reclassification and log transformation of variables**

Given that many biomarkers were not normally distributed and the limited sample size, we tested strategies such as reclassification and log transformation. We examined continuous variables before and after log-transformation or classification in tertiles. We also created three types of dichotomous variables for those continuous variables to identifying NAFLD: 1) joining the 2 less extreme tertiles, 2) using the median as cutoff point for NAFLD and 3) the best dichotomous cutoff point determined by ROC analysis. Given the stronger associations with NAFLD and its easier interpretation of the results, we retained the dichotomous variables defined as its best cutoff point in the final analyses.

**Handling of missing data**

From the original 77 subjects, two had missing baseline CT scan data for assessing liver infiltration. They were included in the study by substituting with outcome data from their second CT scan (week 52 of the metformin trial). We
tested possible bias from including these subjects by determining to which group of the intervention each of these subjects was assigned and by assessing their basal HOMA-B, triglycerides and ALT levels. Both subjects were females. One of the subjects had positive CT scan for liver infiltration which was consistent with its baseline HOMA-B (634 (Uu/mL) / (mmol/L)), triglycerides (152 mg/dl) and ALT (34 IU/L) serum levels. This subject was enrolled in the group treated with metformin, but still had NAFLD after the 52 weeks. The other subject had negative CT scan for liver infiltration, which also was consistent with its baseline HOMA-B (169 (Uu/mL) / (mmol/L)), triglycerides (66 mg/dl) and ALT (27 IU/L) serum levels. This subject was enrolled in the control group of the metformin study.

For other variables with missing values, the group mean from all non-missing values was substituted for the particular variable. Fasting insulin, HOMA-IR, HOMA-B, CPE, QUICKI and HbA1c had 2 missing values. SBP, ALT, AST, glucagon, glutamate, glutamine, leucine, BCAA, kcal, protein, carbohydrates, fiber, total fat, polyunsaturated fat, n6, n3, EPA, DHA, glycemic index and glycemic load had 1 missing value for each.

All the statistical analysis were performed using STATA 13.

RESULTS

The participants median (Q1 [25th percentile] - Q3 [75th percentile]) age of the study sample (n = 77) was 14.7(13.7 - 16.0) years old, 26(34%) were male and
15(20%) had a Tanner stage of 2 or 3 (Table 1). The study participants were 49(64%) non-Hispanic white, 16(21%) non-Hispanic black, 7(9%) Asian, 3(4%) Hispanic/Latino, 1(1%) other (Hawaiian Pacific/white) and 1(1%) was missing value (Table 1). The participants median (Q1 – Q3) BMI was 35.3 (32.3 - 39.3), they were all obese and 38(49%) had a BMI ≥99th percentile (Table 1). Using one slice CT-scan, the prevalence of NAFLD was 20.8%. The subjects with NAFLD were more likely to be male, to be at Tanner stage 2 or 3, to have higher SBP, higher levels of fasting insulin, HOMA-B, ALT, AST and triglycerides but lower levels of HDL and AST:ALT compared to those without NAFLD (Table 1).

Table 1. General characteristics of the subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Median (Q1 - Q3) or n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All subjects n=77</td>
</tr>
<tr>
<td>Demographics</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>14.7 (13.7 - 16.0)</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>26 (34%)</td>
</tr>
<tr>
<td>Tanner stage (2 or 3)</td>
<td>15 (20%)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
</tr>
<tr>
<td>Non Hispanic white</td>
<td>49 (64%)</td>
</tr>
<tr>
<td>Non Hispanic black</td>
<td>16 (21%)</td>
</tr>
<tr>
<td>Asian</td>
<td>7 (9%)</td>
</tr>
<tr>
<td>Hispanic/Latino</td>
<td>3 (4%)</td>
</tr>
<tr>
<td>Other</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Missings</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Antropometry and clinic</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>98.6 (88.4 - 113.1)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165.0 (160.0 - 172.2)</td>
</tr>
<tr>
<td>Description</td>
<td>Mean Value</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>35.3</td>
</tr>
<tr>
<td>BMI z score</td>
<td>2.3</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>103.9</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>122.0</td>
</tr>
</tbody>
</table>

**Serum biomarkers**

<table>
<thead>
<tr>
<th>Description</th>
<th>Mean Value</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting glucose (mg/dL)</td>
<td>92</td>
<td>85.5 - 97.0</td>
<td>97.0</td>
</tr>
<tr>
<td>Fasting insulin (μU/mL)</td>
<td>15.0</td>
<td>10.0 - 24.0</td>
<td>24.0</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.4</td>
<td>5.2 - 5.6</td>
<td>5.6</td>
</tr>
<tr>
<td>HOMA-IR (mmol/L * uU/mL)</td>
<td>3.5</td>
<td>2.2 - 5.7</td>
<td>5.7</td>
</tr>
<tr>
<td>Glucagon (mmol/L)</td>
<td>100.0</td>
<td>87.0 - 116.0</td>
<td>116.0</td>
</tr>
<tr>
<td>Leptin</td>
<td>33.0</td>
<td>23.0 - 47.5</td>
<td>47.5</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>19.0</td>
<td>15.0 - 27.0</td>
<td>27.0</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>23.0</td>
<td>19.0 - 27.5</td>
<td>27.5</td>
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<tr>
<td>Glutamine: Glutamate</td>
<td>8.2</td>
<td>5.7 - 13.3</td>
<td>13.3</td>
</tr>
<tr>
<td>Leucine (μmol/L)</td>
<td>118.0</td>
<td>109.5 - 135.0</td>
<td>135.0</td>
</tr>
<tr>
<td>BCAA (μmol/L)</td>
<td>413.0</td>
<td>368.5 - 458.5</td>
<td>458.5</td>
</tr>
<tr>
<td>IGF1 (ng/ml)</td>
<td>302.0</td>
<td>229.0 - 386.0</td>
<td>386.0</td>
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</tbody>
</table>

**Diet**

<table>
<thead>
<tr>
<th>Description</th>
<th>Mean Value</th>
<th>Lower Bound</th>
<th>Upper Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcal</td>
<td>1772</td>
<td>1211 - 2197</td>
<td>2197</td>
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<tr>
<td>Protein (g)</td>
<td>75.3</td>
<td>50.6 - 94.8</td>
<td>94.8</td>
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<tr>
<td>Carbohydrates (g)</td>
<td>223.3</td>
<td>150.0 - 278.2</td>
<td>278.2</td>
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<tr>
<td>Fiber (g)</td>
<td>14.1</td>
<td>9.7 - 17.7</td>
<td>17.7</td>
</tr>
<tr>
<td>Total fat (g)</td>
<td>61.8</td>
<td>45.3 - 81.7</td>
<td>81.7</td>
</tr>
<tr>
<td>Polyunsaturated fat (g)</td>
<td>12.0</td>
<td>9.1 - 15.5</td>
<td>15.5</td>
</tr>
<tr>
<td>Omega 6, n6 (g)</td>
<td>11.8</td>
<td>8.7 - 15.4</td>
<td>15.4</td>
</tr>
<tr>
<td>Omega 3, n3 (mg)</td>
<td>60.0</td>
<td>20.0 - 120.0</td>
<td>120.0</td>
</tr>
<tr>
<td>n6/n3</td>
<td>243.2</td>
<td>98.4 - 497.4</td>
<td>497.4</td>
</tr>
<tr>
<td>EPA (mg)</td>
<td>10.0</td>
<td>0.0 - 35.0</td>
<td>35.0</td>
</tr>
<tr>
<td>DHA (mg)</td>
<td>40.0</td>
<td>20.0 - 85.0</td>
<td>85.0</td>
</tr>
<tr>
<td>Glycemic index</td>
<td>52.4</td>
<td>50.3 - 55.1</td>
<td>55.1</td>
</tr>
</tbody>
</table>
Glycemic load  116.7 (77.6 – 149.7)  109.5 (64.2 – 158.1)  116.7 (79.3 – 148.4)

Data are median (Q1 [25th percentile] – Q3 [75th percentile]) or counts and percent (n(%)), as appropriate.

*p≤0.05 from Fisher and Wilcoxon test for binary test and continuous variables respectively.

Table 2 provides data on the performance of a number of biomarkers and other subject characteristics for diagnosing NAFLD. The biomarkers with the best sensitivity (88%) of NAFLD included triglycerides, while those with the highest specificity estimate (≥80%) included Tanner stage, ALT and AST (Table 2). None of the biomarkers had both a sensitivity and specificity greater than 80% and none had an AUC above 80%. The biomarkers with the best AUC were triglycerides (0.79), HOMA-B (0.74) and ALT (0.76) (Table 2). Simple logistic regression analyses were done to estimate the relative odds of having NAFLD associated with individual characteristics or biomarkers and to help to identify important factors for inclusion in subsequent models. (Table 2).

Since ALT is the most commonly biomarker used in practice for detecting NAFLD, we tested the C-statistics for cutoff points conventionally utilized in hospitals. A cutoff point of 40 UI/L decreased the sensitivity to 31.3% (95% CI, 11.0 to 58.7) and the AUC to 0.62 (0.50 - 0.74). A cutoff point of 50 UI/L decreased the sensitivity to 12.5% (95% CI, 1.6 – 38.3) and the AUC to 0.54 (95% CI, 0.45 – 0.63).

Using unadjusted predictors of NAFLD and ROC analysis, triglycerides, HOMA-B, ALT and glutamate had the odds ratio and best AUC (Table 3) (Figure 1). Since ALT is known to be a better marker of NAFLD while AST is a better marker of hepatitis from other conditions and alterations in other organs (i.e. heart, muscle
and kidney), we used ALT rather than AST in the models. We selected triglycerides as the main predictor of NAFLD because of the magnitude of its association in the logistic regression analysis (OR: 16.7, 95% CI, [3.4 to 81.2]) and higher ROC analysis (AUC 0.79, 95% CI, [0.69 to 0.89]).

A clinically practical model (Core A) was first constructed; triglycerides, age, sex and Tanner stage were retained in this model. Next, we explored the addition of several other important clinical variables to the Core A model, including BMI, waist circumference, SBP, HOMA-B, ALT and glutamate.

After adjusting for sex, age and Tanner stage (Core A model), improved the AUC from 0.79 to 0.85, above the cutoff (>0.80) for identifying a good diagnostic tool (Table 3)(Figure 2, graph A). Further adjustment for one biochemical variable at a time showed improvement in the AUC estimates (Table 3). The best simple models were those models that included Core A variables of triglycerides, age, sex and Tanner stage plus a single additional biomarker for NAFLD (Core A + 1 in Table 3). We selected the best clinically relevant model as the one with Core A + BMI (AUC 0.85) given that BMI is a more readily available measurement than waist circumference, SBP, HOMA-B, ALT and glutamate (Table 3) (Figure 2, graph C).

In these analyses we found that HOMA-B led to greater improvement in AUC, ALT to a greater improvement in sensitivity and glutamate to a greater improvement in the pseudo R². Thus, all three factors individually improved overall model performance to some degree and inclusion of all three led to an AUC=0.91 and pseudo R²=0.43.
We later generated a second core model (Core B) to adjust for more clinical relevant variables including not only sex, age and Tanner stage, but also SBP, BMI and waist circumference. This model had a higher AUC and pseudo $R^2$ than Core A (Table 3) (Figure 2, graph B). Adjustment for additional variable using the Core B compared to Core A, showed improvement in the AUC and the pseudo $R^2$. The best of these models was Core B adjusted for HOMA-B, ALT and glutamate with AUC of 0.91 and pseudo $R^2$ of 0.50 (Table 3) (Figure 2, graph D). However, the adjusted odds ratio estimates for triglycerides in this final model was less precise. The sensitivity, specificity and percent of correctly classified subjects varied based on the co-variates added to models (Table 3). The sample size in this study was small and therefore we were aware that we risked over-specifying the predictive model when too many variables were added. We tested the impact of adding other explanatory variables to the model in terms of the model's overall performance and the precision of the triglyceride estimate.
Table 2. ROC and simple logistic regression analyses

<table>
<thead>
<tr>
<th></th>
<th>Cutoff point</th>
<th>Below cutoff point, n(%)</th>
<th>Correctly classified, n(%)</th>
<th>Sensitivity, Estimate (95% CI), %</th>
<th>Specificity, Estimate (95% CI), %</th>
<th>Area Under the Curve, Estimate (95% CI)</th>
<th>Odds Ratio for triglycerides, Estimate (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Continuous predictors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>--</td>
<td>26 (34%)</td>
<td>53 (69%)</td>
<td>56% (30 - 80)</td>
<td>72% (59 - 83)</td>
<td>0.53 (0.37 - 0.69)</td>
<td>1.1 (0.7 - 1.6)</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mmHg)</td>
<td>--</td>
<td>15 (19%)</td>
<td>58 (73%)</td>
<td>38% (15 - 65)</td>
<td>85% (74 - 93)</td>
<td>0.64 (0.48 - 0.81)</td>
<td>1.0 (1.0 - 1.1)</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>--</td>
<td>34 (56%)</td>
<td>45 (58%)</td>
<td>69% (41 - 89)</td>
<td>56% (42 - 69)</td>
<td>0.49 (0.33 - 0.65)</td>
<td>1.0 (0.9 - 1.1)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>--</td>
<td>39 (51%)</td>
<td>45 (58%)</td>
<td>69% (41 - 89)</td>
<td>56% (42 - 69)</td>
<td>0.54 (0.39 - 0.70)</td>
<td>1.0 (1.0 - 1.1)</td>
</tr>
<tr>
<td><strong>Binary variables</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (male)</td>
<td>--</td>
<td>26 (34%)</td>
<td>53 (69%)</td>
<td>56% (30 - 80)</td>
<td>72% (59 - 83)</td>
<td>0.64 (0.50 - 0.78)</td>
<td>3.3 (1.1 - 10.4)</td>
</tr>
<tr>
<td>Tanner stage (2 and 3)</td>
<td>--</td>
<td>15 (19%)</td>
<td>58 (73%)</td>
<td>38% (15 - 65)</td>
<td>85% (74 - 93)</td>
<td>0.61 (0.48 - 0.74)</td>
<td>3.5 (1.1 - 11.6)</td>
</tr>
<tr>
<td>Fasting insulin (μU/mL)</td>
<td>15.5</td>
<td>34 (56%)</td>
<td>45 (58%)</td>
<td>69% (41 - 89)</td>
<td>56% (42 - 69)</td>
<td>0.62 (0.49 - 0.76)</td>
<td>2.8 (0.9 - 8.6)</td>
</tr>
<tr>
<td>HOMA-B (Uu/mL) / (mmol/L)</td>
<td>247.9</td>
<td>49 (64%)</td>
<td>57 (74%)</td>
<td>75% (48 - 93)</td>
<td>74% (61 - 84)</td>
<td><strong>0.74 (0.62 - 0.87)</strong></td>
<td>8.4 (2.5 - 28.5)</td>
</tr>
<tr>
<td>HOMA-IR (Uu/mL) / (mmol/L)</td>
<td>3.7</td>
<td>39 (51%)</td>
<td>45 (58%)</td>
<td>69% (41 - 89)</td>
<td>56% (42 - 69)</td>
<td>0.62 (0.49 - 0.76)</td>
<td>2.8 (0.9 - 8.6)</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>109.5</td>
<td>45 (58%)</td>
<td>57 (74%)</td>
<td>88% (62 - 98)</td>
<td>71% (57 - 82)</td>
<td><strong>0.79 (0.69 - 0.89)</strong></td>
<td>16.7 (3.4 - 81.2)</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>37.5</td>
<td>30 (39%)</td>
<td>28 (36%)</td>
<td>44% (20 - 70)</td>
<td>34% (23 - 48)</td>
<td>0.39 (0.25 - 0.53)</td>
<td>0.4 (0.1 - 1.2)</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>25</td>
<td>56 (73%)</td>
<td>62 (81%)</td>
<td>69% (41 - 89)</td>
<td>84% (72 - 92)</td>
<td><strong>0.76 (0.64 - 0.89)</strong></td>
<td>11.2 (3.3 - 38.1)</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>26.5</td>
<td>55 (71%)</td>
<td>59 (77%)</td>
<td>63% (35 - 85)</td>
<td>80% (68 - 89)</td>
<td><strong>0.71 (0.58 - 0.85)</strong></td>
<td>6.8 (2.1 - 21.8)</td>
</tr>
<tr>
<td>AST:ALT</td>
<td>1.1</td>
<td>31 (40%)</td>
<td>31 (40%)</td>
<td>50% (25 - 75)</td>
<td>38% (26 - 51)</td>
<td>0.44 (0.30 - 0.58)</td>
<td>0.61 (0.21 - 1.78)</td>
</tr>
<tr>
<td>Glutamate (μmol/L)</td>
<td>63.5</td>
<td>42 (55%)</td>
<td>60 (65%)</td>
<td>75% (48 - 93)</td>
<td>62% (49 - 74)</td>
<td><strong>0.69 (0.56 - 0.81)</strong></td>
<td>5.0 (1.5 - 16.3)</td>
</tr>
</tbody>
</table>

Data is presented as counts and percentage (n(%)) or estimate and 95% confidence interval (95%, CI) as appropriate. Below cutoff point applies only for the dichotomous biomarker variables fasting insulin, HOMA-B, HOMA-IR, triglycerides, HDL, ALT, AST, AST:ALT and glutamate. For sex it indicates the n(%) of males and for Tanner stage the n(%) of the combined stages 2 and 3.
### Table 3. Multiple logistic regression models for NAFLD

<table>
<thead>
<tr>
<th>Model Description</th>
<th>Sensitivity, Estimate (%)</th>
<th>Specificity, Estimate (%)</th>
<th>Correctly classified, %</th>
<th>AUC, Estimate</th>
<th>Pseudo R²</th>
<th>Adjusted Pseudo R²</th>
<th>Odds Ratio for triglycerides, Estimate (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted triglycerides</td>
<td>88.0</td>
<td>70.1</td>
<td>79.2</td>
<td>0.790</td>
<td>0.235</td>
<td>0.184</td>
<td>16.7 (3.4 - 81.2)</td>
</tr>
<tr>
<td>CORE A (triglycerides*, age, sex, Tanner stage)</td>
<td>62.5</td>
<td>91.8</td>
<td>85.7</td>
<td>0.846</td>
<td>0.290</td>
<td>0.163</td>
<td>18.6 (3.3 - 103.9)</td>
</tr>
<tr>
<td>CORE A + 1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core A + BMI</td>
<td>56.3</td>
<td>91.8</td>
<td>84.4</td>
<td>0.853</td>
<td>0.295</td>
<td>0.142</td>
<td>17.4 (3.2 - 96.0)</td>
</tr>
<tr>
<td>Core A + WC</td>
<td>62.5</td>
<td>91.8</td>
<td>85.7</td>
<td>0.848</td>
<td>0.291</td>
<td>0.138</td>
<td>18.2 (3.2 - 102.7)</td>
</tr>
<tr>
<td>Core A + SPB</td>
<td>56.3</td>
<td>93.4</td>
<td>85.7</td>
<td>0.870</td>
<td>0.340</td>
<td>0.187</td>
<td>17.2 (3.1 - 95.8)</td>
</tr>
<tr>
<td>Core A + HOMA-B*</td>
<td>56.3</td>
<td>95.1</td>
<td>87.0</td>
<td>0.887</td>
<td>0.353</td>
<td>0.201</td>
<td>9.2 (1.5 - 55.7)</td>
</tr>
<tr>
<td>Core A + ALT*</td>
<td>68.8</td>
<td>93.4</td>
<td>88.3</td>
<td>0.867</td>
<td>0.357</td>
<td>0.205</td>
<td>11.3 (2.0 - 65.6)</td>
</tr>
<tr>
<td>Core A + glutamate*</td>
<td>50</td>
<td>95.1</td>
<td>85.7</td>
<td>0.882</td>
<td>0.360</td>
<td>0.207</td>
<td>15.0 (2.7 - 85.6)</td>
</tr>
<tr>
<td>CORE A + 2</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core A + HOMA-B* + ALT*</td>
<td>68.8</td>
<td>95.1</td>
<td>89.6</td>
<td>0.893</td>
<td>0.396</td>
<td>0.218</td>
<td>7.0 (1.1 - 44.3)</td>
</tr>
<tr>
<td>Core A + HOMA-B* + glutamate*</td>
<td>56.3</td>
<td>96.7</td>
<td>88.3</td>
<td>0.893</td>
<td>0.406</td>
<td>0.228</td>
<td>9.6 (1.6 - 57.5)</td>
</tr>
<tr>
<td>Core A + ALT* + glutamate*</td>
<td>62.5</td>
<td>95.1</td>
<td>88.3</td>
<td>0.900</td>
<td>0.400</td>
<td>0.223</td>
<td>11.3 (1.9 - 65.9)</td>
</tr>
<tr>
<td>CORE A + 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core A + HOMA-B* + ALT* + glutamate*</td>
<td>62.5</td>
<td>96.7</td>
<td>89.6</td>
<td>0.907</td>
<td>0.433</td>
<td>0.229</td>
<td>7.8 (1.2 - 49.6)</td>
</tr>
<tr>
<td>CORE A + 4</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Core A + HOMA-B* + ALT* + glutamate* + SBP</td>
<td>68.8</td>
<td>95.1</td>
<td>89.6</td>
<td>0.911</td>
<td>0.468</td>
<td>0.239</td>
<td>7.8 (1.3 - 48.2)</td>
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<tr>
<td>CORE B (CORE A + BMI + WC + SBP)</td>
<td>56.3</td>
<td>93.4</td>
<td>85.7</td>
<td>0.871</td>
<td>0.342</td>
<td>0.139</td>
<td>17.7 (3.1 - 98.9)</td>
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<td><strong>CORE B + 1</strong></td>
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</tr>
<tr>
<td>Core B + HOMA-B*</td>
<td>62.5</td>
<td>95.1</td>
<td>88.3</td>
<td>0.893</td>
<td>0.385</td>
<td>0.157</td>
<td>9.7 (1.6 - 60.1)</td>
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<td>Core B + ALT*</td>
<td>68.8</td>
<td>98.4</td>
<td>92.2</td>
<td>0.865</td>
<td>0.391</td>
<td>0.162</td>
<td>11.5 (1.9 - 69.9)</td>
</tr>
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<td>Core B + glutamate*</td>
<td>75</td>
<td>96.7</td>
<td>92.2</td>
<td>0.904</td>
<td>0.446</td>
<td>0.271</td>
<td>18.3 (2.7 - 122.8)</td>
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<tr>
<td>Core B + HOMA-B* + ALT*</td>
<td>62.5</td>
<td>95.1</td>
<td>88.3</td>
<td>0.891</td>
<td>0.423</td>
<td>0.168</td>
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<td>Core B + HOMA-B* + glutamate*</td>
<td>68.8</td>
<td>96.7</td>
<td>90.9</td>
<td>0.920</td>
<td>0.469</td>
<td>0.215</td>
<td>13.0 (1.8 - 92.2)</td>
</tr>
<tr>
<td>Core B + ALT* + glutamate*</td>
<td>68.8</td>
<td>95.1</td>
<td>89.6</td>
<td>0.900</td>
<td>0.479</td>
<td>0.225</td>
<td>15.0 (2.1 - 108.5)</td>
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<tr>
<td>Core B + HOMA-B* + ALT* + glutamate*</td>
<td>75</td>
<td>98.4</td>
<td>93.5</td>
<td>0.913</td>
<td>0.497</td>
<td>0.218</td>
<td>11.7 (1.5 - 91.3)</td>
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*Biomarker defined by ROC analysis as its best cut off point for NAFLD.
Pseudo $R^2$ is McFadden's formula.
Figure 1 shows the ROC curves for the unadjusted biomarkers triglycerides, ALT, HOMA-B and glutamate. Figure 2 illustrates the ROC curves for the multiple logistic regression models Core A (AUC 0.84), Core B (AUC 0.87), most clinically relevant (AUC 0.85) and best overall model (AUC 0.91).

**FIGURE 1.** ROC curves for unadjusted markers of NAFLD
FIGURE 2. ROC for Core A, Core B, clinically relevant and best overall multiple logistic regression models for NAFLD
DISCUSSION

Blood triglycerides were the best biomarker for the screening of NAFLD in this cross-sectional analysis of obese adolescents. The blood biomarkers ALT, HOMA-B and glutamate, were also good individual predictors for NAFLD. Adjustment for age, sex and Tanner stage improved the AUC of the model. Further adjustment for BMI, SBP, waist circumference, HOMA-B, ALT and glutamate, provided even better estimates and AUC. As shown elsewhere, age, sex, ethnicity, BMI, hypertension, waist circumference, fasting glucose, triglycerides, ALT and AST are known characteristics associated with NAFLD in adult and pediatric populations.\textsuperscript{8–10,12,19,20,23,39,45,49,50,52} In addition, we have observed that protein metabolism markers such as glutamate are also predictors of NAFLD in obese adolescents.

While ALT is the most commonly used biomarker to screen for NAFLD, the lack of consensus the optimal cutoff value for predicting NAFLD in children and adolescents\textsuperscript{53} highlights the importance of finding other predictors to screen for this disease. What is more, ALT is frequently found to be at the normal range in children with NAFLD.\textsuperscript{12} Therefore, we aimed to develop a clinical tool that could screen for NAFLD that had a better AUC than ALT or another individual marker. Even though similar models have been developed for adult populations\textsuperscript{50,73}, research in pediatric population is scarce.\textsuperscript{12} Except for the diagnosis of NASH\textsuperscript{50}, we are not aware of the availability of non-invasive comprehensive models of NAFLD in clinical practice. Given the importance of detecting the disease before
it advances to more severe stages, the development of such a screening tool is of great importance. Currently, the most accepted theory for the development of NAFLD is a multifactorial process, including insulin resistance, liver triglycerides accumulation, high BMI, male gender, Hispanic ethnicity, genetics, low physical activity and a poor diet among others. Therefore, it is important to develop a predictive model that accounts for most of these covariates. Confirming findings of Schwimmer et al. and Manco et al., we found that the best cutoff point for ALT for detecting NAFLD in this sample was lower than what is commonly used in clinic for screening for NAFLD (50 UI/L [30 to 90] for boys and 40 UI/L [29 to 65] for girls. When a cutoff point is set too high, it might lead an underdiagnoses of the disease. Therefore, these higher cutoff values for NAFLD screening will lead to substantial underdiagnoses of the disease, a finding that has critically important implications since the longer the duration of NAFLD, the more severe the complications will be prior to diagnosis, which means greater medical costs, lower quality of life and possibly premature death. The high association of triglycerides with NAFLD in this analysis is consistent with other studies. The multiple logistic regression models had lower sensitivity but higher specificity than the unadjusted triglycerides. The models also showed higher AUC and they explained more of the variance (greater pseudo R²), as well as higher percent of subjects were classified correctly compared to the simple logistic regression analyses (Table 3). In this analysis we found that
the blood biomarkers ALT, HOMA-B and glutamate, could be used as a surrogate marker in screening for NAFLD given the small differences in the AUC with triglycerides.

The high accuracy of our multiple logistic regression models labeled as Core A and Core B, suggests that they may be good non-invasive and cost effective tools to implement in clinical practice for the screening of NAFLD after further validation in larger samples. We selected the Core A model adjusted for BMI as the best clinically relevant model since BMI is a readily available measurement and it showed to improve the C-statistics of the model.

We also found that further adjustment for an additional biomarker improved the AUC and pseudo $R^2$. We selected the Core B model adjusted for HOMA-B, ALT, and glutamate as the best overall model given its best combination of sensitivity, specificity, AUC, pseudo $R^2$ and percent of correctly classified subjects. Since this is a pilot study, we cannot confirm the inclusion of such variables for a screening tool, but only recommend to test this models in a larger sample before application in clinical practice.

As opposed to other studies, some biomarkers were not as strongly associated with NAFLD (i.e., fasting glucose, fasting insulin, CPE, AST/ALT, HDL and leptin).\textsuperscript{20,49,50,91} Leptin is an adipocytokine that has been found to be protective against the development of NAFLD in adolescents given its influence on increasing energy expenditure and decreasing food intake.\textsuperscript{91} However, in our sample the difference in leptin levels between groups was not statistically significant, and did
not have a strong AUC for predicting NAFLD. This lack of association in our sample may be the best explained by the exclusion in this sample of patients with weight >136 kg.

As opposed to other adult and pediatric studies, none of the dietary variables in our study had a strong association with NAFLD. The lack of association between dietary variables and NAFLD in our study may be explained by the small sample size, high levels of random error form the food frequency methodology and potential for bias in the dietary intake estimates.

The main limitation of this pilot study is its sample size, which limits the creation of a definitive tool for the screening of NAFLD. This may explain why the odds ratio were large and their confidence intervals spread. The study sample size and recruitment procedures may explain the prevalence of NAFLD, which has been reported to be between 30% and 83% in other obese and morbidly obese pediatric population. In other studies, Hispanics have been found to be more likely than non-Hispanics blacks or whites, and Asians to have NAFLD. Given the limited sample size, we were not able to examine the relationship of race and ethnicity with NAFLD. Finally, age and BMI were identified as confounders of the relationship between triglyceride and NAFLD but not as predictors of NAFLD. A study with a broader range of age and BMI or with a larger sample size would be needed to revise the predictive value of these factors in the screening instrument. However, our study has specific strengths. In first place, we did a careful statistical analysis, selecting non-parametric over parametric methods given that the
distribution of several variables was not normal in our sample. We also corroborated that the variables entered in the models were not correlated for more than 0.7, to avoid over inflation of the results. We tested the inclusion of study variables to eliminate errors derived by the non-normal distribution.
CONCLUSION

We successfully identified substantial biomarkers for the screening of NAFLD that could be used in clinical practice. Blood triglycerides were the best marker for the screening of NAFLD. A clinically relevant model using triglycerides, age, sex and Tanner stage showed to be a good screening tool for NAFLD in obese adolescents. We believe that a multivariate screening tool that includes triglycerides, sex, age, Tanner stage, SPB, BMI, waist circumference, HOMA-B, ALT and glutamate will provide much greater accuracy for detecting undiagnosed cases of NAFLD in pediatric populations. This pilot study provided the data to define the sample needed to conduct larger validation studies to provide a clinical tool to screen for NAFLD and thus help manage NAFLD among obese adolescents in earlier stages.
APPENDIX

Insights about the clinical trial of metformin and lifestyle

The metformin study was a multicenter, randomized, placebo controlled, double blinded trial, designed to test the hypothesis that 48 weeks of treatment with metformin would decrease the BMI of obese adolescents. The study had a duration of 124 weeks in total, going from October 2003 to August 2007. There were 2 Enrollment phases, one anticipated at week 0 and another one at week 24. Each Enrollment phase had a length of 24 weeks.\textsuperscript{74,75}

Study enrollment

Recruitment

The hospitals forming part of the GPRN were Packard Children’s Hospital, Stanford University School of Medicine; Texas Children’s Hospital, Baylor College of Medicine; Children’s Medical Center, University of California San Francisco; Mattel Children’s Hospital, University of California, Los Angeles and Children’s Hospital Boston and Harvard Medical School.\textsuperscript{75} Each of the 5 participant sites screened approximately 25-30 subjects. Enrollment and screening took approximately 24 weeks.

Recruitment from clinic and hospital databases

Prospective subjects who met the appropriate age and approximate BMI criteria were identified during review of the hospital database or referred by their personal physicians in the pediatric clinics at the five participating institutions, as well as
from local private and community practices. Methods of recruitment included invitations by the patient’s physician during a clinic visit, or a letter of invitation from the patient’s physician to the prospective subjects.

**Advertisements**

All advertisements were approved by each institution’s Institutional Review Board.

**Minority recruitment**

One of the goals of the study is to determine the impact race/ethnicity has on the outcome measured. Investigators and support staff at all five participating institutions were strongly encourage to recruit minority subjects into the study.

**Initial contact and pre-screening encounter**

If a prospective subject expressed interest in the study, the Nurse Coordinator, under the direction of the Lead Site Investigator, met the subject and his/her parent or legal guardian in clinic, or contacted the subject by phone to review the study and complete a pre-screening encounter.

**Pre-screening encounter**

The subject (and parent or legal guardian) was questioned regarding inclusion and exclusion criteria. In addition, potential subjects were asked about how they heard about the study in order to focus future recruitment efforts. If they were not planning to participate in the study, reasons for not doing so were assessed and recorded. Subjects who met the non-laboratory eligibility criteria and who were interested in participating were consented and schedule for a Screening visit. If the subject was
contacted by phone for pre-screening, consent was obtained at the beginning of the Screening visit.

**Study registry**

Each of the five participating institutions maintained a cumulative record of all potential subjects that were pre-screened at that site. This registry included data regarding how the potential subjects were identified, whether they agreed to be part of the study, and if not, why not. By comparing non-enrolled subjects to enrolled subjects, recruitment can be optimized over time.

**Informed Consent process**

Informed consent was obtained prior to screening procedures, either during the initial pre-screening contact with subjects in clinic, or immediately prior to initiation the Screening visit. The subject (and parent or legal guardian) were given the consent form (and assent form) to read, and the Lead Site Investigator was available to review the study, clarify any areas that were confusing and answer any questions. The purpose, study duration, procedures, potential risk and benefits and the subject’s rights were explained. The subjects were instructed that participation was voluntary and that s/he was free to withdraw at any time. It was also noted that the subject may be withdrawn by the Principal or Lead Site Investigator at any time.

By asking the subject to explain the study in his/her own words, it was assessed the subject’s understanding and autonomy. Each subject was informed that access to his/her medical records and trial related source documents will be granted for
research related monitoring, IRB review, and regulatory inspection. Subjects were also informed that if abnormal test results that could affect the care they receive from their primary care physician were obtained during the screening process, their tests results were going to be provided to their physician.

**Overview of visit assessments and procedures of the study of metformin**

**Screening visit**
The screening visit consisted in obtaining the following: informed content (if subject and parent had not yet completed the consent form), contact information, measurement of subject’s height and weight (in order to calculate BMI), a medical history and use of concomitant medications.

**Baseline visit**
The baseline visit occurred in the same day as the Screening visit, or as late as 8 weeks after the screening visit. The baseline visit consisted on the following: obtaining subject’s medical history, assessing use of concomitant medications, physical exam, a CT scan, a DXA scan, clinical laboratory assessments (blood and urine), and the Youth/Adolescent Food Frequency questionnaire. Contact information was confirmed.

**History, physical exam and laboratory assessments**

**Contact information**
Basic contact information was obtained for each subject, their parent or legal guardian, and for additional person (to call in case of emergency). This contact information was located in the subject’s study file.
Initial medical history

A complete medical history of the subject was ascertained at the Screening visit. This included a chart review and an interview for a comprehensive medical history (including menstrual history, review of systems and family health history), as well as basic demographic information.

Initial physical exam

The initial physical exam took place at the Baseline visit and included height, weight and Tanner staging.

Concomitant medication review

Concomitant medications are defined as over-the-counter and prescription medications, vitamin and mineral supplements, and herbal supplements. Assessment for use of concomitant medications took place at screening and baseline visits and all medications were recorded.

Clinical Laboratory assessments

Blood and/or urine specimens were collected at Baseline visit.

Fasting insulin concentrations

These methods include glucose clamps and related procedures that involve the infusion of insulin. Because of the risk associated with glucose clamps, this procedure was not used in this study. Numerous algorithms utilize insulin and glucose data obtained during either oral or intravenous glucose tolerance tests in order to calculate surrogate measures of insulin production and insulin sensitivity. The ratio of glucose to insulin and data from oral glucose tolerance testing (OGTT)
have been used as markers of insulin sensitivity, but show limited overall correlation with more precise measures as clamp studies.

Fasting insulin concentrations can be combined with fasting glucose concentrations using an algorithm to estimate insulin sensitivity. Of the available algorithms, the homeostasis model assessment (HOMA) and the quantitative insulin sensitivity check (QUICKI) have the most extensive published validation data.74,81–84

**Radiology studies**

Each subject underwent CT and DXA scanning during the study. The CT and DXA scans were read centrally and results of these scans were not provided directly to subjects.

**Computed Tomography (CT)**

A 2-slice CT scan was obtained to characterize abdominal fat distribution as visceral or subcutaneous, and one slice was obtained to assess fatty liver infiltration of the liver. Abdominal two-slice CT scan was obtained on each subject at baseline visit. The CT scans were GE, Siemens, or Philips equipment, depending on the center and they were analyzed at Harvard Medical School with a standard software program (Photoshop CS2, Adobe Systems, San Jose).75 Pregnancy was ruled out by a urine pregnancy test BEFORE the CT scan was performed.74

**Clinical Laboratory Assessments**

**Safety Labs**
Urine pregnancy test

A pregnancy test was obtained at baseline visit. All females that had begun menstruating had a urine pregnancy test. Pregnancy tests results must be negative prior to performance of the CT and DXA scans.

Other Labs

Oral Glucose Tolerance Test

Subjects were also assessed for 3-hour glucose tolerance test (OGTT; 75g of glucose). The OGTT included 7 measurements of glucose and insulin, and 6 measurements of C-peptide.

Glucose and insulin were measured as part of an extended OGTT at 0 minutes (immediately before), and at 15, 30, 60, 90, 120 and 180 minutes after consumption of a standard oral glucose load.

Insulin and IGFBP-1 were measured by two-site immunochemiluminometric assays with sensitivities of 0.6 μU/ml and 1 ng/ml respectively. The ranges for the IGFBP-1 are pre-pubertal (fasting) 30-1000 ng/ml and pubertal (fasting) 20-200 ng/ml.74

C-peptide were measured at 0 minutes (immediately before), and at 15, 30, 60, 120 and 180 minutes after consumption of an oral glucose load. C-peptide was not measured at 90 minutes.

If the OGTT results in either a fasting plasma glucose ≥126 mg/dl or a 2-hour plasma glucose ≥200 mg/dl, the subject was instructed to return to the lab for a standard 2-hour GTT (with fasting and 2-hour post prandial glucose
measurements). If either results of the 2-hour GTT were elevated, a diagnosis of diabetes was confirmed. The Principal or Lead Site Investigator was responsible for confirming that the subject was referred to diabetes treatment.

Insulin was measured by two-site immunochemiluminometric assays with sensitivities of 0.6 μU/ml.

- Hemoglobin A1c (Hgb A1c)
- Lipid Panel (TG)

Triglycerides, total cholesterol, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) were measured.

**Fasting amino acids.** Fasting plasma samples for amino acids were stored frozen at −70°C until analyzed by using ion-exchange liquid chromatography (Amino Analyzer L-8800; Hitachi, Tokyo, Japan) at the Children’s Hospital of Boston Chemistry Laboratory, Boston.

**Laboratory Specimen Handling and Analysis**

Most laboratory assays were performed at Esoterix Clinical Trial Services, Calabasas Hill, CA. Laboratory assays were performed at Esoterix Clinical Trial Services, Calabasas Hill, CA. This laboratory also provided the reference range data used for the analyses.74,75

**DATA HANDLING AND RECORD KEEPING**

Data collection and Handling

Source Documents
Study data was extracted from source documents. Source documents included original documents and data (included but not limited to hospital records [physical or electronic], clinical and office charts, worksheets developed for this study, laboratory notes, memoranda, subjects’ calendars or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions, microfiches, photographic negatives, microfilm or magnetic media and x-rays, wherever the foregoing bay be kept). Source documents were provided to a sponsor audit committee or monitor (to ensure compliance to the protocol), for IRB review, or to regulatory inspectors if requested.

**Study Data**

Study data, extracted from source documents, includes all information developed in the performance of this study as compiled and delivered to the sponsor in accordance with the study protocol, including, but not limited to, the case report forms generated by each participating member institution.

**Confidentiality of Source Documents and Study Data**

An unambiguous subject identification code was used in lieu of the subjects’ name on all study data compiled and delivered by the participating institution to the sponsor (or other party contracted by the sponsor). This subject identification code included the subjects’ initials, the site number and subject number. A key for this code was maintained at the site and kept separate from study files. All source documents and study data were kept confidential.

**Study Data Maintenance, Management and Ownership**
All the study data is owned by the sponsor (the Elizabeth Glaser Pediatric Research Foundation). All study data was maintained and managed in compliance with FDA and ICH/GCP standards and as agreed upon by the Network and participating institutions. Also, in accordance with the Network Agreement, participating institutions will have access to all study data collected at every participating institution, to be utilized for research and educational purposes, but each participating institution may only publish and present any data collected by that institution as described in the publication policy below.

**Gender and Ethnic Diversity**

The female to male ratio was 1:1. The ethnic distribution was expected to reflect the distribution at each site. Each institution recruited according to the populations served. The estimated distribution for each institution is described in the individual IRB applications.

**Risk and Benefits**

**Risks**

Risks to subjects included the following:

1) **Radiation exposure:** ***The risk refers to the complete study, which includes 3 CT scans and 3 DXAs.***

2) **Phlebotomy:** Blood draw amounts will fall below the acceptable range designated by site IRB's. Risk include minimal temporary discomfort, possibly a small amount of bruising, and rarely infection. Phlebotomy was performed by pediatric-trained personnel with proper technique for
minimizing risks of hematoma and infection. EMLA® or ElaMax® cream was offered to all subjects to minimize discomfort. There is a slight risk of an allergic reaction to the EMLA® or ElaMax® cream.

3) Questionnaires: Information is straightforward and non-threatening.

4) Confidentiality: Was strictly maintained.

Benefits

The main benefit was a close evaluation of health status.
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CURRICULUM VITAE

LAURA LARA-CASTOR

Year of birth: 1990

Contact information: laura.lara119@gmail.com

PROFESSIONAL GOALS

To become a world class nutrition researcher with a special concentration in obesity and associated comorbidities. Highly interested in policies and community based interventions directed at promoting healthier environments.

PROFESSIONAL SKILLS

General. Responsible, organized, determined, good at problem solving, good social skills, excellent in team and independent work, passionate about nutritional research.

Academic. Strong training in biostatistics, proficient nutritional science background, intermediate proposal writing training.


ACADEMIC BACKGROUND

Graduate and undergraduate studies

2015 MS Master in Science in Nutrition and Metabolism. Boston University, US.

Ongoing Thesis: Cross-sectional analysis for the diagnosis of Non-alcoholic Fatty Liver Disease in obese adolescents using non-invasive methods.

GPA: 3.77. Expected graduation date: Jan 2017

2012 LCN Bachelor’s in Nutritional Sciences. UDLAP, México.

Final grade: 9.7 (scale 0 to 10)

PROFESSIONAL EXPERIENCE

2014 Researcher Assistant

National Institute of Public Health (INSP) within the Nutrition and Health Research Center (CINyS). Cuernavaca, Morelos, MEXICO. August 2013 to December 2014.

- Screening of patients of with diabetes in a low income community of Mexico (Food frequency questionnaire, background questionnaire, healthy life style recommendations).

- Submission of research protocols for grants.

-Analysis of a database of women from an amateur race to evaluate the measurement error in the self-reported BMI compared to direct measurements.
2013  **Clinical Nutritionist**

Vital Alternative Equilibrium (EVA) Puebla, Puebla, MEXICO. January 2013 to April 2013
- Nutritional assistance to adult patients, mainly focused on weight loss.

2012  **Clinical Nutritionist Practitioner**

INNCMSZ. Distrito Federal, MEXICO. May 2012 to July 2012.
- Nutritional evaluation and supervision of diets for hospitalized geriatric patients.

2012  **Clinical Nutritionist - Volunteer.**

- Nutritional evaluation and supervision of diets for hospitalized patients.

**AWARDS AND SEMINARS**

*Awards*

2013  1st place poster of my bachelor’s thesis project presented in the 6th International congress of SOMEICCA A.C. in Cancún, México.

2012  **Magna Cum Laude** - Universidad de las Américas Puebla.

*Seminars and conferences attended*

2016 Epidemiology and Genetics. May 9th. HSPH. Boston, MA, USA.

2015  7th Scientific Conference of Metabolic Syndrome. August 13 to 15th. Hospital
Siglo XXI. Ciudad de México. México.

2015  BNORC Annual Symposium. July 24th. BUSM. Boston, MA, USA.

*Poster presentation*

2015  “Accuracy of self-reported height and weight in Mexican women from an amateur running race”. Poster presentation. BNORC Annual Symposium: July 24th. BUSM. Boston, MA, USA.

**ADDITIONAL PROFESSIONAL ACTIVITIES and VOLUNTEERING**

2014 (to date) Management of websites to inform and promote healthier lifestyles:

  https://www.instagram.com/nutricion.it/

  https://www.facebook.com/NutricionIdealParaTi/

2016 Staff Assistant at *The Danielsen Institute* at Boston University – Interaction with clients, problem solving and administrative skills.

2016 Volunteer in a 7 weeks mentoring program for high school students.