Uric acid concentrations and fructose consumption are independently associated with NASH in children and adolescents.

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Abbreviations: NAFLD= Non-alcoholic fatty liver disease; NASH = Non-alcoholic steatohepatitis; MetS= metabolic Syndrome; IR= insulin resistance, T2DM= type 2 diabetes; UA= uric acid; ATP= adenosine triphosphate, AMP= adenosine monophosphate; IMP= inosine monophosphate; BMI= body mass index; LDL= low-density lipoprotein; AST= aspartate-aminotransferase,ALT=alanine-aminotransferases, GGT=gamma-glutamyl-transpeptidase; INR=International Normalized Ratio; OGTT= Oral Glucose Tolerance Test ; WHO= World Health Organization; PNFI= Pediatric NAFLD Fibrosis Index; NAS= NAFLD activity score ; CRN= NASH Clinical Research Network ;FCDBs= food composition database; INRAN= National Italian Institute of Food Research and Nutrition ; SINU = Italian Society of Human Nutrition; KHK=fructokinase.

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Abstract

Background/aims: Recent research suggests that dietary fructose intake may increase serum uric acid (UA) concentrations and both UA concentration and fructose consumption maybe increased in NAFLD. Currently, it is uncertain whether dietary fructose consumption and UA concentration are independently associated with non alcoholic steatohepatitis (NASH). Our aim was to investigate the factors associated with NASH in children and adolescents with proven NAFLD, and to test whether UA concentrations and fructose consumption are independently associated with NASH.

Methods: 271 obese children with NAFLD were studied. NASH was diagnosed by the NAFLD Activity Score ≥5 and the fatty liver inhibition of progression (FLIP) algorithm. Fructose consumption (grammes/day) was assessed by food frequency questionnaire, and UA (mg/dL) was measured in serum. Binary logistic regression with adjustment for covariates and potential confounders was undertaken to test factors independently associated with NASH.

Results: NASH occurred in 37.6% of patients. Hyperuricaemia (UA≥5.9 mg/dL) was present in 47% of patients with NASH compared with 29.7% of Not-NASH patients (p=0.003). Both UA concentration (OR= 2.488, 95% CI 1.87-2.83, p = 0.004) and fructose consumption (OR=1.612, 95% CI 1.25-1.86, p = 0.001) were independently associated with NASH, after adjustment for multiple (and all) measured confounders. Fructose consumption was independently associated with hyperuricaemia (OR=2.021, 95% CI 1.66-2.78, P = 0.01). These data were confirmed using the FLIP algorithm.

Conclusions. Both dietary fructose consumption and serum UA concentrations are independently associated with NASH. Fructose consumption was the only factor independently associated with serum UA concentration.

Lay summary

Currently, it is uncertain whether dietary fructose consumption and uric acid (UA) concentration are linked with non alcoholic steatohepatitis (NASH) in children and adolescents. Our aim was to test whether UA concentrations and fructose consumption are independently associated with NASH in children and adolescents with proven non alcoholic fatty liver disease (NAFLD).We show that both dietary fructose consumption and serum UA concentrations are independently associated with NASH and fructose consumption was independently linked with high serum UA concentrations.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is now largely regarded as the hepatic manifestation of Metabolic Syndrome (MetS) and NAFLD represents the most frequent chronic liver disease in children in Western countries [1]. NAFLD begins with the development of liver lipid accumulation and the condition progresses over time with the development of liver inflammation and fibrosis (non alcoholic steatohepatitis or NASH). Although it was initially thought that NAFLD was a relatively harmless condition in children and adolescents, recent evidence shows that NASH occurs in this young population [2]. The development of NASH may markedly affect life expectancy and quality of life in affected individuals and therefore it is crucial to understand the risk factors for NASH in children and adolescents in order to design effective interventions which can be used safely to treat this young group of patients.

Nutritional, metabolic and genetic factors contribute to the development of NAFLD and NASH is also an important independent risk factor for extra hepatic diseases such as type 2 diabetes and cardiovascular disease [3]. Risk factors for liver disease progression and the development of NASH are oxidative stress, systemic inflammation and insulin resistance [4]. Several studies in adults, have shown that hyperuricaemia is associated with insulin resistance (IR), type 2 diabetes (T2DM), MetS and NAFLD but whether hyperuricaemia is associated with NAFLD in the paediatric population is uncertain [5,6].

Recent data suggest a correlation between serum uric acid (UA) concentrations and increased consumption of sugary drinks [7] containing fructose and glucose as the disaccharide sucrose or sugar. An increased dietary intake of fructose may be important in the pathogenesis of NAFLD through

induction of de novo lipogenesis, inflammation, and insulin-resistance [8]. In the intestine, fructose intake alters the gut microbiome and enhances endotoxin translocation into the portal circulation via increased permeability of tight junctions [9]. In the liver, fructose is rapidly metabolized, consuming adenosine triphosphate (ATP), which may result in increased adenosine monophosphate (AMP) and inosine monophosphate (IMP) and conversion of IMP to uric acid [10].

Since it is plausible that dietary fructose intake and UA concentrations are potential risk factors for liver disease progression in NAFLD, the aim of our study was to investigate the factors associated with NASH in children and adolescents with proven NAFLD, and test whether UA concentrations and fructose consumption are independently associated with NASH. Additionally, because fructose consumption may increase UA concentrations, we tested whether fructose consumption was independently associated with UA concentrations in this population with NAFLD.

PATIENTS AND METHODS

Anthropometrical and biochemical measurements

Overweight/obese children and adolescents (defined by body mass index (BMI) with NAFLD, who were referred to the "Hepatometabolic Department" of the "Bambino Gesù" Children's Hospital, from January 2012 to November 2014 provided the data for the current study. In all patients, liver fat was initially identified by ultrasonography using established criteria, a bright hepatic echo pattern compared to echo response of the right kidney [11].Other causes of steatosis, were excluded in all subjects, including alcohol intake (≥ 140 g/week), total parenteral nutrition, and the use of drugs known to induce steatosis (e.g. valproate, amiodarone or prednisone). Patients with marked recent weight gain, diabetes and known genetic causes of dyslipidemia were excluded. Viral hepatitis (A, B, C, cytomegalovirus and Epstein-Barr virus), autoimmune or metabolic liver diseases, alpha-1-antitrypsin deficiency, Wilson's disease, and celiac disease were also ruled out by appropriate tests. Patients with systemic diseases, genetic syndromes, or chronically treated with drugs, were also excluded from the study.

Anthropometric and clinical parameters (weight, height, BMI, waist circumference and blood pressure) were measured in all children using standardized methods. The BMI Z-score (SDS) was calculated according to BMI reference tables from the WHO: overweight was defined by + 1 SD and obesity by +2 SDs, bearing in mind that the z-score is the deviation of the BMI value for an individual from the mean value of the reference population, divided by the standard deviation for the reference population[12]. Lipid profile (total cholesterol, LDL-cholesterol and triglycerides), uric acid (UA) and liver function tests (LFT's - aspartate- (AST) and alanine- (ALT) aminotransferases, gamma-glutamyl-transpeptidase (GGT), bilirubin, albumin and International Normalized Ratio (INR)) were measured by

standard methods. Moreover, in all children over the age of ten years an Oral Glucose Tolerance Test (OGTT) was performed, as already described according to the recommendations of the World Health Organization (WHO) [13,14].

Liver biopsy

According to the recent recommendation of the Hepatology Committee of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), all patients in the current study underwent liver biopsy in order to exclude other diseases, or to assess severity of liver disease(suspected by clinical and laboratory evaluation: marked and persistent hypertransaminasemia, hepatosplenomegaly, or the presence of a high Paediatric NAFLD Fibrosis Index (PNFI))[15,16]).

Liver biopsies were performed in all children using an automatic core biopsy device (Biopince, Amedic, Sweden) with an 18-G needle, 150 mm long, and the ability to cut tissue with lengths of up to 33 mm with precision [17]. Biopsies were at least 18 mm in length and were assessed by a single liver pathologist who was unaware of the patient's clinical and laboratory data. Biopsies were routinely processed (formalin-fixed, paraffin-embedded) and analysed by different staining. The main histological features, commonly described in NAFLD/NASH, including steatosis, inflammation (portal and lobular), hepatocyte ballooning, and fibrosis were scored according to the Scoring System for Non-Alcoholic Fatty Liver Disease developed by the NIH-sponsored NASH Clinical Research Network (CRN) [18]. Steatosis was graded on a 3-point scale: grade 0 = steatosis involving < 5% of hepatocytes; grade 1 = steatosis involving up to 33%; grade 2 = steatosis involving 33-66%; and grade 3 = steatosis involving > 66%. Lobular inflammation was graded on a 3-point scale: grade 0 = no foci; grade 1 = less than 2 foci per 200x field; grade 2 = 2-4 foci per 200x field; grade 3 = more than 4 foci per 200x field. Hepatocyte ballooning was graded from 0 to 2: 0 is none, 1 is a few balloon cells, 2 is

many/prominent balloon cells. The stages of fibrosis were quantified on a 4-point scale: stage 0 = no fibrosis; stage 1 = perisinusoidal or periportal (1a = mild, zone 3, perisinusoidal; 1b = moderate, zone 3, perisinusoidal; 1c = portal/periportal); stage 2 = perisinusoidal and portal/periportal; stage 3 = bringing; and stage 4 = cirrhosis.

The presence of NASH was defined according to the NAFLD activity score (NAS). Cases with NAS of 5 or greater were diagnosed as NASH.

The Fatty Liver Inhibition of Progression (FLIP) algorithm, conceived by Bedossa et al., for the diagnosis of NASH was also used to test the robustness of the results obtained with the NAS. The FLIP algorithm is another histological classification for diagnosing NASH andis based on the semi-quantification of 3 features: steatosis, ballooning and lobular inflammation as evaluated according to the steatosis, activity and fibrosis (SAF) score [19]. The FLIP classification uses steatosis as a criterion for entry into the weighted algorithm for hepatocellular ballooning and lobular inflammation. For any biopsy with at least steatosis grade1, the algorithm includes nine possibilities for diagnosing NASH [20] .The presence of at least one component of the three characteristics (steatosis, ballooning, lobular inflammation) defines NASH.

Assessment of dietary fructose consumption

A food frequency questionnaire (FFQ) was administered to all patients who underwent liver biopsy, as previously reported [21]. Briefly, the frequency intake of a particular food was defined as follows: ‘every day of the week’, ‘sometimes’, and ‘never’. The questionnaire included numerous subsections (breakfast, morning snack, lunch, afternoon snack, dinner, etc.) that examined the intake of specific foods and portions. The daily intake of all dietary components, for each patient was calculated using the food composition database (FCDBs), shown in the book of LARN – IV Edition, published by the National Italian Institute of Food Research and Nutrition (INRAN) and Italian Society of Human

Nutrition (SINU). This database was used to assess the intake of fructose consumption per day (INRAN) [22].

The study design conformed to the ethical guidelines of the Declaration of Helsinki (as revised in Seoul, Korea, October 2008) and was approved by the local Ethics Committee for our Hospital (ID Prot. 323/12).

Statistical analysis

Statistical analyses were performed with STATISTICA (version 2010, Chicago, IL, USA). Normally distributed date are described as mean ± standard deviations (SDs) and non-normally distributed data are expressed as median and IQRs. Categorical variables were analysed using χ^2 tests. Pearson's and Spearman's correlation coefficient tests were used to test univariate associations between exposures and outcomes. Binary logistic regression was used to test associations between NASH/not NASH as the outcome and UA concentration, fructose consumption and age, sex, anthropometric and biochemical parameters as exposures. NASH was diagnosed by a NAS score ≥ 5 (=1) and Not-NASH for NAS< 5 (=0), in the binary logistic regression analysis (and the regression model was repeated using NASH diagnosed by the FLIP algorithm). Subsequently, binary logistic regression was performed to investigate the association between fructose as the exposure and hyperuricaemia (UA≥5.9mg/dL or UA<5.9 mg/dL) [23] as the outcome; with other covariates and potential confounders as exposures in the model. Logistic regression analyses were undertaken using SPSS (IBM SPSS Statistics for Windows, version 20.0, Armonk, NY).

Results

Anthropometric, biochemical and fructose consumption characteristics of children and adolescents with NAFLD

We included in the present study 271 consecutive obese adolescents with NAFLD (155 males, mean age 12.5 years) who underwent liver biopsy. In our population, 37.6% (n=102) of patients had NAS≥5 (NASH) and 62.4% (n=169) did not have NASH (NAS<5). **Table 1** shows the differences in anthropometric and biochemical characteristics between the NAS≥5 and Not-NASH (NAS<5) groups. Subjects in the NAS≥5 group had higher WC, transaminase levels, total cholesterol, triglyceride and UA concentrations and also fructose consumption. Furthermore, the NAS≥5 group showed higher significant TNF-α values compared with the NAS<5 group. There were no differences between the groups for IL-6 and IL-1β concentrations.

Dietary behaviour

The FFQ showed that breakfast was the meal that was most likely to be skipped in our population. 143 (52.76%) children never ate breakfast, 70 (25.8%) ate breakfast infrequently (sometimes) and 58 (21.4%) ate breakfast regularly (ever day). Milk was consumed at breakfast by all children. Morning and afternoon snacks were regularly consumed by 257 (94.8%) and 241 (88.9%) of children, respectively. The most consumed morning snacks were crackers, pizza and salty food, an evening snack consisted of biscuits, yogurt or other snacks. Lunch and dinner were regularly consumed by all patients. The foods eaten every day were cereals 127 (46.8%), vegetables 116 (42.8%) and fruit 108 (39.8%), whilst the foods consumed at least 1-2 time per week were meat 249 (91.8%), fish 131 (48.3%) and eggs 121 (44.6%).

90% of children ate vegetables, such as green salads and tomatoes one or more times per day. 89% reported drinking sodas and soft drinks one or more times a week. All children consumed extra virgin olive oil, at least 5-10 ml day. **Table 1** shown the differences in fructose consumption (gr/day) and carbohydrate consumption (gr/day) between the two groups with NAFLD (NAS≥5 vs. NAS<5).

Histological features of NAFLD

Table 2 describes the histological differences of patients stratified by NAS. 102 (37.6%) children had NASH (NAS≥5) and 169 (62.4%) were classified as Not-NASH (NAS<5).The NAS ≥5 group had higher levels of steatosis (S3= 45% vs. 10.6%), inflammation (17.6% vs. 6.5%) and fibrosis (F2-F3= 21.6% vs. 7.7%) compared to the Not-NASH group. To test the independence of associations between NAS and anthropometric, biochemical parameters and fructose consumption, we undertook regression analysis with NASH/Not NASH as the binary outcome (**Table 3**). This analysis showed that the following factors were independently associated with NAS≥5: WC, HOMA-IR, triglycerides, fructose consumption (OR=1.612, 95% CI 1.25-1.86, p = 0.001) and uric acid (OR= 2.488, 95% CI 1.87-2.83, p = 0.004).

To validate the NAS findings obtained from classifying patients into NASH and not NASH, we also stratified patients into NASH and not NASH groups using the FLIP algorithm. The FLIP algorithm classified 19 (7%) patients as not having NAFLD, 156 (57.56%) had NAFLD, and 96 (35.42%) had NASH (**Supplementary Table 1**). We repeated the logistic regression analysis shown in Table 3, to determine which factors were independently associated with NASH determined by the FLIP algorithm. These data (**Supplementary Table 2**) showed that WC, HOMA-IR, triglyceride concentration, fructose consumption and uric acid were independently associated with NASH and the data were very similar to that obtained with the NAS.

Table 4 shows univariate correlations between anthropometric and biochemical parameters with both UA concentrations and fructose consumption. These analyses showed that UA concentration was positively correlated with fructose consumption and UA concentration was also correlated with BMI, HOMA-IR, fasting insulin, triglycerides and TNF-α concentrations. Consumption of fructose was correlated with WC, HOMA-IR, ALT, triglycerides, IL-6 and TNF-α concentration. Conversely, fructose consumption was not correlated with daily carbohydrate intake

Because the univariate analyses showed correlations between fructose consumption and UA concentration, we tested whether fructose consumption was independently associated with hyperuricaemia in regression analysis (**Table 5**). These data show that fructose consumption was independently associated with UA concentration (OR=2.021, 95% CI 1.66-2.78, p = 0.01).

Discussion

Our novel data shows that in children and adolescents with NAFLD, serum UA concentration and dietary fructose consumption are independently associated with NASH, using two different histological scoring systems for classifying patients as having NASH. Furthermore, fructose consumption was independently associated with hyperuricaemia and hyperuricaemia occurred more frequently in patients with NASH, than in patients who did not have NASH. In each of the regression models, we were able to adjust for a range of potential confounders. That we are able to show the associations are independent of a comprehensive range of factors, gives confidence that these associations are unlikely to be due to confounding. Additionally, we demonstrate these findings in a considerable number of children who all underwent liver biopsy (n=271 children and adolescents); the findings are also biologically plausible, and thus it seems reasonable to conclude that the results are not due to chance, bias (or confounding as mentioned above).

Numerous studies have shown that high UA levels are associated with metabolic syndrome and NAFLD but to date, to the best of our knowledge, no studies have tested the independence of associations between UA concentrations, fructose consumption and NASH confirmed by biopsy [24,25]. There is a growing body of evidence that UA may have a role in NAFLD, and our data are consistent with studies that have identified hyperuricemia as an independent predictor of fatty liver disease [26]. In this cross-sectional study, the authors show that in adults higher values of UA are associated with greater risk of NAFLD, both in obese (OR=2.55, 95% CIs 1.87-3.50) and in non-obese subjects (1.69, 95% CIs 1.37,2.08), ($p<0.05$) [26]. Ouyang et al correlated the hyperuricemia in hepatic steatosis with the elevated consumption of fructose in association with increased expression of fructokinase (KHK) in the liver [27]. It is known that the increased consumption of fructose induces an upregulation of expression of both Glut 5 and KHK. KHK, up-regulated by the concentration of fructose, is also regulated by the intracellular production of UA [28]. Fructose is absorbed in the intestinal lumen, and is then transported to the liver, where it rapidly enters glycolysis and is phosphorylated to fructose-1-phosphate by KHK. The phosphorylation of fructose also stimulates adenosine monophosphate (AMP) deaminase to convert AMP in inosine monophosphate (IMP) and then IMP is converted to uric acid [11]. Patients with NAFLD, who have a history of high fructose exposure, have a high concentration of UA, because they show a higher hepatic ATP depletion in response to fructose. These studies suggest how high levels of UA may be linked to both fructose consumption and hepatic steatosis via up-regulation of KHK [29].

In our study, fructose consumption was significantly higher in the NASH group compared with the Not-NASH group (70.4 g/day vs.52.6 g/day; $p=0.002$). Additionally, hyperuricaemia was independently associated with fructose consumption, which is in accordance with several studies that

have shown that UA concentrations are related to excessive consumption of fructose [30]. Numerous studies have demonstrated that hyperuricemia is associated with insulin- resistance and is a feature of the MetS and NAFLD [31] and reassuringly our data shows that HOMA-IR was independently associated with hyperuricaemia, after adjustment for covariates and potential confounders. Huang et al. shown that hyperuricemia is associated with ALT, LDL-C, fasting glucose and NASH (NAS >5), but mostly was independently associated with greater odds of advanced lobular inflammation of NAFLD and progression to NASH [5]. With regard to this finding [5], recently it has become evident that UA is biologically active and can stimulate the production of inflammatory mediators and a high level of UA also inhibits the bioavailability of endothelial NO causing a reduction of the vasodilatation [32]. Thus, it is plausible that uric acid may influence risk of NASH by promoting liver inflammation and affecting liver microvascular responses.

There are strengths and limitations to our study that should be considered. We have studied 271 children and adolescents who have undergone liver biopsy to assess the severity of NAFLD. All subjects have completed a dietary questionnaire to assess their fructose consumption and this assessment may not truly reflect all dietary consumption of fructose. However, any misclassification bias would tend to attenuate the strength of our findings, and would bias our results towards the null.

In conclusion, in a cohort of children and adolescents with a histological diagnosis of NAFLD and histological confirmation of NASH, we show for the first time that UA concentrations and dietary fructose consumption are independently and positively associated with NASH. Our data also show that dietary fructose consumption (and also HOMA-IR) were positively and independently associated with hyperuricaemia.

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