CLINICAL, OBSERVATIONAL, NO-PROFIT STUDY

TITLE: Evaluation of hepatic and osteo-metabolic complications related to fructose consumption in a cohort of overweight and obese children and adolescents.

PROTOCOL CODE: EO2022

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INTRODUCTION

Background

The increase in childhood obesity is a multifactorial phenomenon influenced by food systems, commercial factors, and social determinants; it has long-term consequences for the health of the individual and for the whole society. Italy is among the European countries where the problem of school-age overweight and obesity is most prevalent. 24% of girls and boys are overweight, 9.4% are obese, and the severely obese are the 2.4% of the population (1). The eating habits of girls and boys are far from those recommended for maintaining good health throughout life: despite the fact that the Mediterranean Diet is now known for its beneficial effects, due to the increase availability of ultra-processed and more palatable foods, children and adolescents are increasingly shifting to a Western-style diet (2,3). One in four children consumes sugary and carbonated beverages every day and consume fruits and vegetables less than once a day. Legumes enter the diet of 38% of children less than once a week, and nearly half of them eat sweet snacks more than three days a week. But the most worrying data come from the World Health Organization (WHO), which reports an estimated 38 million children under the age of 5 years who are overweight or obese, demonstrating a lowering of the age group from school to preschool (1.4). Fructose is a monosaccharide found naturally in fruits, vegetables, and honey; because of its high sweetening power and taste-enhancing ability, fructose is widely used in the food industry. High-fructose corn syrup, in particular, is one of the most widely used ingredients in the production of soft drinks, jams, breakfast cereals, and baked goods (3). According to the United States Department of Agriculture report, per capita consumption of fructose has gradually increased from 37 g/day to 49 g/day over the past 30 years (2).

Nonalcoholic hepatic steatosis (NAFLD), now also referred to as MAFLD (hepatic steatosis associated with metabolic dysfunction), is considered the hepatic manifestation of the metabolic syndrome and is currently the most frequent chronic hepatopathy in pediatric age in Western countries (5). Recent studies suggest that fructose consumption is implicated in the development of NAFLD both directly by providing metabolites that can be used for the synthesis of triglycerides and free fatty acids and indirectly through increased uric acid production. In the liver, in fact, fructose is rapidly converted by the enzyme phosphofructokinase C (PKF-C) to fructose-1-phosphate (FP). The depletion of phosphoric groups through the consumption of adenosine triphosphate (ATP) results in the enhanced activity of adenosine deaminase, which converts adenosine monophosphate (AMP) to inosine monophosphate (AMP) which is ultimately converted to uric acid. Finally, uric acid promotes the expression of enzymes involved in lipogenesis, thereby worsening hepatic steatosis (6,7).

In addition, consumption of foods high in fructose could be a risk factor for bone loss (8). Numerous studies conducted over the past 25 years, a period in which fructose use has increased exponentially, have shown that this sweetener tends to increase the incidence of fractures and osteoarthritis and decrease bone mineral density (BMD) and the apposition of new bone tissue (9,10). Fructose would act at the intestinal level by altering calcium and phosphorus absorption mechanisms. Intestinal absorption of calcium is mediated by the active form of vitamin D, 1,25-OH vitamin D, obtained by renal hydroxylation of 25OH-vitamin D. When the body's calcium reserves are sufficient, excess fructose does not would have any effect on intestinal absorption; under conditions of reduced dietary intake or increased requirement, such as occurs in developmental age, there is a compensatory increase in circulating levels of 1,25-OH vitamin D in order to increase intestinal absorption and reduce renal excretion of calcium (11). Excess fructose

would appear to act directly at the renal level by reducing the activity of la-hydroxylase and increasing the activity of 24-hydroxylase, leading to reduced production of 1,25-OH vitamin D and consequently reduced availability of calcium essential for the apposition of new bone tissue (12).
Reduced phosphate uptake, on the other hand, depends on the depletion of phosphate groups by a specific isoform of phosphofructokinase expressed by enterocytes (13); therefore, the reduction in the ATP/AMP ratio leads to inhibition of the Na/P transporter. This mechanism would also occur at the renal level where inhibition of this transporter promotes urinary excretion of phosphorus (14).
Recent studies show how an increase in dietary fructose appears to be responsible for the onset of several obesity-associated diseases; foremost among them NAFLD, which is increasing in tandem with obesity even in the pediatric population. The aim of this study is mainly to understand the effect of fructose on the molecular events contributing to pediatric development, as this could be an important target to address the clinical and social problems arising from its prevalence in pediatric age.

AIMS OF THE STUDY

The primary aim of this study is to evaluate the effects of fructose intake in a group of preschool children and overweight or obese adolescents; in particular, to investigate the impact of fructose consumption in the development of liver complications, such as NAFLD, and its degree of severity, as well as its impact in other obesity-associated diseases. In addition, the present study aims to describe the composition of bone metabolism according to the level of fructose consumption. Secondly, by means of omics analysis (metabolomics and metaproteomic), the aim is to characterize the microorganisms present in the gastrointestinal tract and their microbial activity in relation to dietary habits, fructose intake and clinical parameters. Finally, sociodemographic and cultural factors, in the children and adolescents considered in the study, and in their families, will be surveyed.

STUDY DESIGN

This study consists of two parts, the first phase involves the conduct of a cross-sectional study to assess the relationship between fructose consumption and the presence of NAFLD and the difference in several parameters, including anamnestic, clinical, auxological and hematochemical assays and the presence of the disease, and a second prospective part for the sociological evaluation one year after entering the study. It constitutes an extension of a study already approved by the University of Bari, approval of which is attached by the Independent Ethics Committee university hospital company "Consorziale Policlinico" with approval number CE/7331. The study involves the collaboration of three different units, respectively formed by the University of Eastern Piedmont (unit 1), University of Bari "A. Moro" (unit 2) and the University of Rome "Foro Italico" (unit 3). The first two units will enroll subjects with criteria of interest for the study. The University of Rome, on the other hand, will be concerned with the socioeconomic aspect of the study with the preparation of ad hoc questionnaires to be submitted to both children/adolescents and families.

Subjects

100 overweight or obese preschool children and adolescents visited at the Outpatient Clinic of Pediatric Endocrinology of the AOU Maggiore della Carità in Novara, at the Transition Endocrinology Outpatient Clinic of the SCDU Endocrinology and at the Outpatient Clinic of Pediatric Endocrinology "B.Trambusti" of the Giovanni XXIII Pediatric Hospital in Bari will be included in the study. Subjects will be selected continuously with adherence to the inclusion criteria from January 2024 until the end of December.

Inclusion criteria

- Children and adolescents with expression of informed consent according to art.2 quinquies of Legislative Decree n.196/2003;
- Preschool children of both sexes aged 3 to 6 years and adolescents of both sexes aged 12 to 16 years;
- Children diagnosed with overweight (defined by BMI > 97th percentile for children younger than 5 years; and BMI >85th percentile for children older than 5 years) or obesity (defined by BMI > 99th percentile for children younger than 5 years; and BMI > 97th percentile for children older than 5 years) (15,16).

Exclusion criteria

- Children and adolescents who are not in this age group;
- Children and adolescents with liver disease different than NAFLD;
- Children and adolescents with genetic type obesity or secondary obesity;
- Children and adolescents with skeletal fragility due to genetic causes;
- Children and adolescents who chronically use drugs capable of altering lipid metabolism and bone remodeling;
- Children and adolescents placed on a diet-therapeutic regimen with dietary styles other than the Mediterranean or Western diet (e.g., ketogenic diet, FoodMap diet, vegan/vegetarian diet) to avoid bias in the interpretation of the microbial signature (the microbial signature of the Western and Mediterranean diets is known in the literature);
- Failure of parents or adolescent (from age 12 years) to give informed consent;

Surgery

No type of surgery

DURATION OF THE STUDY

12-month recruitment and 12-month observation period for sociological survey.

Timing of data collection

At the time of recruitment, patients afferent at the Outpatient Endocrinology Clinic of the AOU Maggiore della Carità in Novara, at the Transition Endocrinology Outpatient Clinic of the Endocrinology SCDU, and at the "B. Trambusti" Pediatric Endocrinology Outpatient Clinic of the Giovanni XXIII Children's Hospital will undergo:

Questionnaires to assess the amount of fructose introduced in the diet:

- Questionnaire to assess adherence to the Mediterranean diet in children/adolescents, KIDMED;
- Questionnaire to assess food frequency in children, IDEFICS;
- 24h recall questionnaire, to collect information regarding meals taken in the previous 24 hours The amount of macronutrients will be obtained through dedicated software "Dietosystem Professional Food Therapy Software" from DSMedica. The questionnaire is added with specific questions and figures to identify the portions of foods and put them in relation to the amount of fructose intake;
- Calculation of the Dietary Inflammatory Index (DII) using a list of foods. This parameter makes it possible to define the extent to which a dietary pattern is able to promote the synthesis of pro-inflammatory molecules (17,18);
- Sociological questionnaire prepared ad hoc by Unit 3 to assess diet and fructose intake in relation to family socioeconomic status.

Anamnestic evaluation

- The patient's mother will be asked about the gestational age, mode of delivery and anthropometric data of the child at birth.
- Detection of current drug therapy (if any).

Instrumental assessment

- Quantitative and qualitative analysis of bone composition using quantitative ultrasonography (QUS). This instrument provides different parameters related to bone composition, specifically speed of sound (SOS) assessing bone density, broadband ultrasound attenuation (BUA) assessing trabecular structure, ultrasound peak amplitude assessing trabecular size, number of peaks assessing connectivity of the mineralized matrix structure, and fast wave energy and amplitude assessing elasticity.
- US ultrasound of the abdomen to evaluate hepatic steatosis, perivisceral and subcutaneous fat. The presence of hepatic steatosis (NAFLD) will be classified from stage 1 to stage 3 according to literature (19).

Clinic-auxological evaluation

- Anthropometric measurements, such as weight, height and BMI assessment according to STANDARD DEVIATION SCORE (SDS). Waist and hip circumference measurements.
- Measurement of blood pressure and heart rate.
- Analysis of body composition by bio-impedance analysis (BIA)

Laboratory evaluation, hematochemical assays

Subjects will undergo the following assessments after at least 12 hours of overnight fasting:

- OGTT (glucose-loading curve; glucose 1.75 g/kg per os at time 0', maximum dose 75 g), with basal and every 30 min blood glucose and insulin draws.
- Basal blood draw for c-peptide, AST, ALT, gamma-GT, fractional and total bilirubin, azotemia, creatinine, eGFR, HbAic, fructosamine, total cholesterol, HDL, triglycerides, TSH, FT3 and FT4, leptin, adiponectin, DPP4, uric acid.
- Basal blood sampling for PCR, advanced glycation products (AGEs), AGE receptor (RAGE) and advanced protein oxidation products (AOPPs),
- Basal blood sampling for assay of intermediate products of fructose metabolism (fructose-lysine)
- Calcium, phosphorus, 25-OH vitamin D, PTH, osteocalcin, RANKL, osteoprotegerin, bone isoenzyme alkaline phosphatase, telopeptide C-terminal collagen type I (CTX), intact protocollagen amino terminal type 1 (P1NP), IL-6, IL-1beta, biochemical parameters needed to assess bone metabolism.

They will, in addition, be performed:

- A urinalysis and 24-hour urine collection for evaluation of fructose metabolites in urine.
- Fecal sample for metabolomics and metaproteomic analysis.

ANALYSIS BY BIOCHEMICAL, HORMONALAND MASS SPECTROMETRY PARAMETERS

Biochemical parameters according to clinical practice will be analyzed at the laboratories of Maggiore della Carità Hospital in Novara and at the laboratories of Giovanni XXIII Children's Hospital in Bari by estimation with Siemens Advia 2400 Healthcare Diagnostics, Siemens ADVIA chemistry XPT and in accordance with the respective protocols of reagent use.

Metaproteomic and metabolomics analysis will be conducted on stool, plasma and urine samples stored during the study for omics signature designation. Metaproteomic analysis with UPHLC-MS/MS mass spectrophotometer will be performed on stool samples to identify the presence of microorganisms in the gastrointestinal tract to be then correlated with the diet followed and clinical parameters of the patients. Peptides identified by the analysis will be entered into the UniProtKB, KEGG database and used for phylogenetic analysis.

Metabolomic analysis will be carried out on all three types of samples; metabolomics conducted on fecal samples will allow assessment of the functional activity of microorganisms, characterizing the intermediate phenotype that mediates host-microbiome interactions. Using one-dimensional gas chromatograph coupled with mass spectrometer (GC-MS-SPME), the metabolic profile and changes in metabolite levels in association with fructose intake will be identified. The identification of the main SCFAs will be carried out with the same instrument and then confirmed by injecting the appropriately treated samples into the instrument, comparing the retention time and the corresponding spectra obtained from GC-MS analysis. This method allows a metabolomic signature to be defined in relation to fructose consumption.

SAMPLE SIZE AND STATISTICAL METHODS

The study plans to enroll 100 children afferent to the centers previously listed. Given a first-type error of 0.05, a power of 80%, a variance of equal fructose consumption in the groups of children with and without NAFLD of 9.53 (pooled variance obtained from data reported in the article by Nier et al. (20), a ratio of 1:3 between children with and without NAFLD (expected prevalence in overweight and obese children ~24% (21)), the sample size will allow to highlight as statistically significant a minimal difference in the averages of fructose consumption between healthy and diseased children of 2.02 grams.

Statistical analysis

Evaluation of the relationship between fructose and NAFLD
Descriptive statistics will be calculated in order to summarize the sociodemographic, perceptual, anthropometric, dietary, clinic-auxological, anamnestic and laboratory characteristics of the children included in the study.
Categorical variables will be summarized by absolute frequencies and percentages while numerical variables as mean and standard deviation (SD) or median and interquartile range (Q1-Q3) if not normally distributed according to the Shapiro Wilks test and quantile plot observation. The chi-square test or the corresponding nonparametric Fisher's test will be used to test the association between categorical variables and the presence of NAFLD while the t-test or the Mann- Whitney test will be used to test the difference in fructose consumption between children with NAFLD and those without the disease as well as the presence of differences between the two groups for the other numerical variables. The positive false discovery rate method will be used to account for the presence of multiple tests. Based on the number of NAFLD events observed, stratified analysis by age group (preschool age vs. adolescents) and center will be evaluated. Regarding the assessment of bone metabolism, mean and standard deviation (SD) or median and interquartile range (Q1-Q3) of the parameters provided by the QUS overall and separately for high and low fructose consumption (below vs above median consumption) will be calculated.

Microbiota analysis
Initially, an exploratory analysis of the relative abundance of the genera of bacteria in the microbiota will be carried out. The relative abundance of each species detected in the fecal samples will be represented by heatmaps. Next, a beta-diversity analysis will be performed comparing the complexity of genera

between subjects. Specifically, three matrices will be created based on weighted and unweighted Bray-Curtis and Unifrac distances that will be used to perform a principal coordinate analysis to highlight the presence of subject clusters based on the complexity of their microbiota.
The "Linear discriminant analysis effect size" (LEfSE) method will be used to identify taxa that best discriminate between NAFLD-infected and non-infected patients. The LefSE method is an algorithm-based approach that performs the nonparametric Kruskal-Wallis and Wilcoxon rank-sum test to identify bacterial taxa whose relative abundance is statistically different between groups. Then discriminant analysis is applied considering only those bacterial taxa found to be significantly different between the two groups.

EXPECTED RESULTS

The present study is expected to gain new knowledge on the effect of fructose in relation to the onset of obesity-associated liver disease (NAFLD) and bone metabolism, with the identification of new biological, immunological, and omics markers that can be studied in the future as disease targets in the pediatric population. In addition, due to the scientific and sociological interest, the present study aims at the identification of sociodemographic and cultural determinants related to fructose intake, useful for the creation of preventive campaigns aimed at the promotion of a healthy lifestyle, with a change in eating habits that incentivize a healthy diet and a reduction of obesity status in the population. The results obtained, if statistically significant, will ultimately aim to develop effective preventive communication to promote a healthier lifestyle and diet in the population.

ETHICAL ASPECTS

The study was designed and will be conducted in accordance with international and national ethical standards on biomedical research with human beings, specifically:

- *Ethical principles for medical research involving human subjects* (Declaration of Helsinki -World Medical Association, current version);
- European Union *Standards* of *Good Clinical Practice* (ICH/GCP);
- *Convention on Human Rights and Biomedicine* (Oviedo Convention of 04/04/1997);
- Italian codes of ethics for health professions and specific current national regulations on clinical trials.

Management of informed consent

An information sheet explaining the type of study, purpose, procedures, sample and data collection, and possible benefits and risks of the research is available. This information will be presented to interested persons, who will have the freedom to give consent or to be able to withdraw it when the study has already begun.

Management of sensitive data

The following European and national regulations are kept in mind:

- *Regulation* (*EU*) *2016/679* of the European Parliament and of the Council of April 27, 2016 and subsequent amendments published in the Official Journal of the European Union 127 of May 23, 2018 (GDPR);
- *Legislative Decree No. 196* of *June 30, 2003* (Personal Data Protection Code), as amended by Legislative Decree No. 101 of August 10, 2018 on "Provisions for the adaptation of national legislation to the provisions of Regulation (EU) 2016/679."
- Art.8 of Legislative Decree 101/2018 of September 19, 2018, for the processing of data in minors under the age of 16, for the expression of informed consent. The Legislative Decree harmonizes the Privacy Code (d.gls. 196/2003) with the EU Regulation 2016/679.

Before the start, the study will be registered on ClinicalTrail.gov and AEA RCT Registry.

DATA PROCESSING METHODS AND BIOLOGICAL SAMPLES

The study involves the collection of information related to lifestyles and diseases of the subjects involved. The biological samples and the attached personal/sensitive data collected will be immediately pseudonymized by random assignment of alphanumeric codes associated with the study and will be entered into the REDCap Management Software. The decoding of said alphanumeric codes will be in the possession of the responsible research investigator. Therefore, it will only be possible for the responsible research investigator to link the information and data obtained from the searches to a specific subject. Personal data will be processed for the purposes set out in the project, in accordance with the principles of lawfulness, fairness, transparency, purpose limitation, minimization and accuracy of data (Art. 5 GDPR) in paper and electronic form by individuals authorized to process the data. Data availability, management, access, storage and usability is guaranteed by the adoption of technical and organizational measures to ensure adequate levels of security (Articles 25 and 32 GDPR). Only researchers involved in the study and authorized by the responsible research investigator will have access to the data.
Biological samples will be stored at UPO Biobank according to certified quality and security standards. The biological samples will be pseudonymized, and only UPO Biobank officials authorized to process the data, and only in cases of necessity, will be able to link the pseudonymization code of the samples to the sensitive data of the study participant. Researchers who will perform analysis on the collected samples will, therefore, have no way of associating them with the participant's identity.
Aliquots of the biological samples, in pseudonymized form, may be released to the managers of the facilities that will perform the laboratory investigations. Any remainder will be returned to UPO Biobank. Participants will also be offered, completely freely and optionally and through the signing of an appropriate informed consent (UPO Biobank Participation Pact), to provide consent for the use of biological samples taken for research purposes to UPO Biobank population studies.
Personal data collected within the scope of this project will be kept at the experimental center, for a period of 15 years after the conclusion of the research or for a longer period, if necessary, in compliance with legal obligations, to which the Owner is bound. Research results will be made public or used for scientific communications/publications, only in anonymous and aggregated form.
The data controllers, each for their respective areas of responsibility, are The A.O.U Maggiore della Carità di Novara, DPO: Dr. Alessandra Gaetano (dpo@innovasrl.it; privacy@maggioreosp.novara.it) and Universita del Piemonte Orientale, DPO: lawyer Antonio Perrini(dpo@uniupo.it)

COSTS

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Participating centers

1. Endocrinology of Transition of the SCDU of Novara, University of Eastern Piedmont. Responsible: Prof. Flavia Prodam
2. Pediatric Endocrinology, Novara Hospital, University of Eastern Piedmont. Responsible: Prof. Flavia Prodam (in collaboration with Prof. Ivana Rabbone and Prof. Simonetta Bellone).
3. Pediatric endocrinology "B.Trambusti"- Ospedale Pediatrico Giovanni XXIII. Responsible: Prof.ssa Maria Felicia Faienza.
4. University of Roma "Foro Italico". Responsabile: Prof.ssa Francesca Romana Lenzi.

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