ASSESSMENT OF NUTRIENT PROFILE, NUTRITIONAL STATUS, DEVELOPMENT AND SEVERITY OF CHILDREN WITH AUTISTIC SPECTRUM DISORDER IN COMPARISON WITH TYPICALLY DEVELOPING CHILDREN IN ADDIS ABABA, ETHIOPIA

August 19, 2022

Table of contents

STUDY PROTOCOL WITH SAP

1. SCOPE

This study protocol specifies objective, design and methods for the assessment of **assessment of nutrient profile, nutritional status, development and severity of children with autistic spectrum disorder in comparison with typically developing children in Addis Ababa, Ethiopia**

2 DEFINITION OF TERMS

a) **AL-cup:** the type of glass and non-glass cups used exclusively for laboratory purposes. For instance in the analysis of fat.
b) **Desiccator:** a glass container or other apparatus holding a drying agent for removing moisture from sample and protecting them from surrounding moisture.
c) **Digestion**: heating of a sample in extreme temperatures using various reagents that corresponds to the type of analysis.
d) **PH**: measure of level of acidity and Alkalinity (base) on a scale of 1 to 14. Lower PH indicating higher acidity while higher PH indicates the alkalinity of a substance.
e) **Cruisable:** ceramic bowl container used in the analysis of samples that involve direct heat exposer or of stored in an oven with elevated temperature
f) **Titration:** a slow addition of a solution of a known concentration (titrant) to a known solution of unknown concentration until the reaction reaches neutralizations, which is often indicated by a change of color.
g) **Muffle furnace:** a type of jacketed enclosure that is used to heat a material to a significantly high temperature while keeping it contained and fully isolated from external contaminants.
h) **Spectrophotometry:** a method of measuring how much a chemical substance absorbs light by measuring the intensity of light as a beam of light passes through sample solution.

i) **Colorimetery:** a method of determining the concentration of a chemical agent with the aid of a color reagent and its light absorption.

j) **Thimble:** apparatus of fat analysis equipment (soxhlet extractor) that is usually made of thick filter paper which retains the solid to be extracted.

2. OBJECTIVES

2.1. General objectives

The general objective of this study is to assess the nutritional status, nutrient profile, ASD severity, and development of children diagnosed with ASD as compared to typically developing controls in Addis Ababa, Ethiopia.

2.2. Specific objectives

The specific objectives of this study are to:-

1. Evaluate the nutrient profile of diets using proximate analysis.
2. Determine the nutritional status (i.e. stunting, wasting, underweight and overweight)of children diagnosed using anthropometric measurements.
3. Assess the dietary diversity score (DDS) of children using food frequencyquestioners (FFQ).
4. Examine the urine samples of children to identify the urine iodine (UI) level.
5. Assess the parents and caregivers perceptions about the Eating behaviour of ASD children using questioner.
6. Evaluate the ASD severity level and developmental growth of children with ASDin correlation to UI level.

3. MATERIALS

a) Weight scale
b) Height board measurement
c) Middle Upper Arm Circumference tape
d) CARS standard version rating booklet

e) Food collection Bag
f) Urine collection cup
g) Proximate and urine analysis equipments and chemicals (hexane, H_2SO_4, Ethanol, digester, soxhlet extractor, etc.)

4. SUBJECTS

Selection of 26 children with Autistic Spectrum Disorder (ASD) (case group) and the corresponding 26 typically developing children (control group), 52 children in total, shall be made on the basis of:

4.1.Inclusion criteria

a) Children that are already diagnosed with ASD for the "case" group,
b) Children fulfilling the matching criteria to the case group (i.e. age and sex) for the control group.

4.2.Exclusion criteria

a) Use of special supplements,
b) Use of heavy medication,
c) Children with critical chronic disease such as cancer, HIV AIDs.

4.3.Test conditions

The subjects shall:

a) Be of school age;
b) Legal guardians of the child need to Sign a consent form for the willingness of his/her participation;
c) Give the necessary information and specimen agreed upon in the given consent forms.

The analysis shall:

a) Be conducted by

- The researcher as per guidelines and procedures described for all nutritional analysis.
- Registered nurse for urine sample collection
- Registered physician for CARS diagnosis.

b) Have no risk involved since it doesn't involve any penetration to the human muscle

c) Be done with the willingness of the participant and their legal guardians (he/she can stop the participation anytime)

5. PROCEDURE

5.1. Height

A child height board will be used to measure height of the children. The children will be measured with minimal clothing and no shoes and socks must be worn. The feet must be together, arms to the side, legs straight and shoulders relaxed, the head must be in the Frankfort horizontal plane (looking straight ahead). An average of 3 readings will be taken and read to the nearest 0.1 cm.

5.2. MUAC (Middle Upper Arm Circumference)

MUAC is the circumference of the left upper arm and is measured at the mid-point between the tips of the shoulder and elbow using a MUAC tape. To measure:

Bend the left arm, find and mark with a pen the olecranon process and acromium.

- Mark the mid-point between these two marks.
- With the arm hanging straight down, wrap a MUAC tape around the arm at the midpoint mark.
- Measure to the nearest 1 mm

5.3. Weight

The children will be measured using an electronic weight scale with minimal clothing and no shoes and socks must be worn. The scale will be placed on a hard, flat (level) surface up on measurement and scale reading

5.4. Urine collection

60 ml. urine samples will be collected from each child by a trained clinical practitioner (nurses), at the child's period of urinary stimulation, using sample collection cups.

5.5. CARS diagnosis

CARS is scale that is frequently used in the diagnosis of ASD in children. It consists of 15 items that cover different symptoms of ASD, and provides a reliable comparison of an affected child's behaviors and skills against the expected developmental growth of a healthy child. The diagnosis will be conducted by a physician by combining parent reports (direct question and answer) and direct observations of children made by the clinician.

5.6. Food sample collection

50gm sample of food will be collected in sample collection bags from each meal consumed throughout the day by the researcher and legal guardians.

6. ANALYSIS

6.1. Proximate analysis

A) **Moisture content:** An Al-cup will be dried at 104°cfor 30 minutes and cooled in a desiccator, and record the weight (W1). 3 g of the sample will then be added in the Al-cup and weighed (W2). The sample will then be dried for two hours at 139 °C. Then, the final dried sample will be weighed (W3) and recorded after cooling in a desiccator. The net moisture content of the sample will then be calculated using the following formula. %MC= ((W2-W1)-W3) ×100/ W1-W3 (%MC = percentage of net moisture content, W1= weight of Al-cup, W2= weight of sample before drying, W3= weight of sample after drying.

B) **Crude protein:** The amount of protein present is calculated using the Kjeldahl method of protein analysis. The food sample will be weighed (0.5-1g) into a digestion flask. The sample will then be digested by heating it in the presence of sulfuric acid, a catalyst, copper (0.5g), and potassium sulfate (100g). Digestion will convert any nitrogen in the food into ammonia, and other organic matter to $C0_2$ and H_20. After the digestion has been completed the

digestion flask will be connected to a receiving flask by a tube. The solution in the digestion flask will be made alkaline by adding sodium hydroxide, which converts the ammonium sulfate into ammonia gas. The ammonia gas that is formed will be liberated and mixed with boric acid. The low pH of the solution will convert the ammonia gas into the ammonium ion, and will simultaneously convert the boric acid to the borate ion. The nitrogen content will then be estimated by titration of the ammonium borate formed with standard hydrochloric acid using a suitable indicator to determine the end-point. The following equations will be used to determine the nitrogen concentration of a sample and corresponding crude protein percentage. %N = {(Vs - Vb) *molecular weight of N * N Hcl}/sample weight*10, % protein = 6.25* %N (Vs= sample volume, Vb= blank volume, N Hcl = normality of Hydrochloric acid.)

C) **Ash content:** A cruisable will first be weighed on electronic balance (W1). Afterward, 3g of food sample will be measured using an electronic balance (W2). The samples will then be put on the hot plate and charred and will be placed in a muffle furnace for 2 hours at 550°C. The final weight will then be measured after cooling it down (W3). The ash content will then be calculated using the formula: Ash % = W3 – W1 × 100 W2

D) **Fat content (AOAC, 2000):** Al-cup will first be heated for 30 minutes at 106°C, cooled on a desiccator, and weighed on electronic balance (W1). 1-5g of food sample will then be added on a thimble and weighed (W2). The thimble and Al-cup will be slot in the corresponding positions of the fat extraction unit. Subsequently, 70% of diethyl ether will be added to the sample in the thimble using a dispenser. The hot plate temperature will then be set at 500°Cfor 20 minutes for soaking followed by the extraction step for 40 minutes. The third step will be the evaporation step. After the extraction step is complete, the extracted fat will be placed in an oven for 30 minutes at 106°C. The fat content will then be determined using the following formula (W3) % of fat= W3-W1 ×100 W2

E) **Total dietary fiber:** duplicate portions will be incubated with pancreatic α- amylase and amyloglycosidase for 16 hr. at 370°C in a sealed 250ml bottle in shaking water. During this step, non-resistant starch will be solubilized and hydrolyzed to glucose and maltose by the combined action of the two enzymes. The reaction will then be terminated by PH adjustment and temporary heating. Protein in the sample will be digested with protease. For the measurement of high molecular weight dietary fiber, Ethanol or industrial methylated sprits

will be added and the insoluble and perceptible soluble dietary fiber will be captured, washed with ethanol and acetone, dried, and weighed. The final result will then be obtained by using the formula: Total dietary fiber (%) = (HMWDF+LMWSDF)/1000 (HMWDF = high molecular weight soluble dietary fiber LMWSDF= low molecular weight soluble dietary fiber)

6.2.Urine iodine analysis

Analytical methods consist of an initial digestion step to get rid of interfering substances, followed by the Sandell–Kolthoff reaction, of which the reading is obtained spectrophotometrically or colorimetrically. The digestion step will be held to remove Interfering substances in urine. The sample will be digested using the appropriate strong acid (e.g. ammonium persulphate). The oxidizing step will be carried out in a heated environment. The Sandell–Kolthoff reaction is a two-step reaction in which the rate of reaction will be measured during the reduction of the yellow-coloured ceric ions by arsenic in the presence of iodide to form colourless cerous ions and elemental iodine.

6.3.Statistical analysis plan

Given the variability of the type of data generated from each assessment, data analysis and interpretation shall be done in the respective the nature of each data.

Anthropometric measurement data shall be analyzed using corresponding index of Weight for height, height for age, weight for age and MUAC for age and interpreted using international standards. ENA 2020 shall be used to record the measurements and convert to respective Z-scores.

The Data generated from CARS diagnosis will be on a scale from one (normal behavior) to four (severely abnormal behavior) for each category of questions. The scores between 30 and 37 indicate mild to moderate ASD, while scores between 38 and 60 indicate severe ASD. These results shall be analyzed by SPSS version 26 for descriptive statistics and correlations

In case of urine iodine analysis, the decrease in yellow color over a fixed period is measured by a colorimeter with an appropriate filter or with a spectrophotometer and plotted against a standard

curve constructed with known amounts of iodine. The resulting reading of iodine content shall then be compared among case and control groups of the study. SPSS version 26 shall be used to for descriptive statistics, significant difference and correlations

The data generated from proximate analysis of food samples shall be recorded for each analysis and calculated for average. The final figure represents the amount of nutrient present per 100 gm. thus amounts consumed by children. This data shall then be compared among case and control groups of the study. SPSS version 26 will also be used for descriptive statistics, significant difference and correlations