STUDY PROTOCOL

Official title: Evaluation of the effects of taking bicarbonate-calcium water in premenopausal and postmenopausal women as prevention of osteoporosis

Date of Document: January 27th, 2021

Study design: experimental study, non-pharmacological trial

Background

Adequate calcium supplementation in association with the Mediterranean diet and lifestyle physically active can improve the quality and even life expectancy in the population, with a reduced risk of osteoporosis. Mineral water can be a good source of calcium,
highly bioavailable and easily assimilated. Calcium is naturally abundant in waters minerals that represent precious non-caloric sources. Mineral waters, assumed daily, with a high calcium content (over 150 mg/L) and a low sodium content
(less than 20 mg/L) can be considered excellent sources of this mineral.

Until a few years ago, calcium from water was believed to be poorly absorbable compared to that contained in other food sources, but several scientific studies have reversed this conviction, especially for calcium (calcium> 150 mg/L) and bicarbonate- calcium waters (bicarbonate > 600 mg/L). Furthermore, several studies have shown that the effects of sodium on the organism they are many and would also concern the bones,so much so that its effect hypercalciuric in the urine has led to hypothesize a possible roleof sodium in the pathogenesis of osteoporosis. Bicarbonates make water alkaline and affectwater markers bone resorption. In this scenario, the daily consumption of bicarbonate-calcium waters witha low sodium content is a major contributor to calcium intake and a
significant protective factor for osteoporosis.

Objective

Evaluate the effectiveness of regular consumption of Lete water classified as bicarbonate-calcium e low sodium for the prevention of osteoporosis in premenopausal and postmenopausal women
as part of a defined dietary protocol.

Materials

Subjects enrolled in the study:

120 women

Inclusion Criteria:

For premenopausal women age >40 years, for menopausal women period of onset of menopause1-10 years, with BMI of 19-30 Kg/m^2 .

Exclusion criteria:

Inclusion in other study protocols. Pharmacological therapies in progress: estrogen hormone therapy replacement, osteoporosis therapy, corticosteroid therapy, insulin therapy. Assumption of
calcium supplements. Presence of moderate to severe kidney and liver disease, already documented severe osteoporosis condition (T-score MOC <-2.5).
Waters:

- Lete bicarbonate-calcium water, low in sodium
- non-bicarbonate-calcium water: the ideal comparison water is low-mineral water from bottled with the same packaging as that of Lete water, with the addition of CO_2.

Methods

The subjects enrolled for the study will be divided into two main groups: premenopause A andpostmenopausal B and then subsequently randomly in the two subgroups and
respectively

- Group A1 (intervention group): 30 premenopausal subjects who will drink water

  The bicarbonate-calcium Lete

- Group A2 (control group): 30 premenopausal subjects who will drink water

  low mineral content

- Group B1 (intervention group): 30 postmenopausal subjects who will drink water

  The bicarbonate-calcium Lete

- Group B2 (control group): 30 postmenopausal subjects who will drink water

low mineral content

The study will be randomized single-blind.

As for the water to be taken, the bottles will be delivered at the expense of the company SGAM SpA in the following ways: the entire study takes a total of 6 months
considering that the recruited subjects (120) must drink 2 L of water per day 43200 L of

water, of which 21600 L Lethe bicarbonate-calcium water and 21600 L mineral water with addedCO2. For each subject, 240 1.5 L bottles are needed. As regards logistics,

the subjects they will receive home delivery of the assigned water and precisely 10 packs of 6 bottleseach for a total of 4 deliveries at set times.

Sample size

An analysis was performed to calculate the sample size and evaluate the power of theclinical trial statistics. The power of the (1-beta) test to be obtained, i.e., was considered as factors the probability of rejecting the null hypothesis when there is a real difference, the least difference between the treatments (control and intervention group), the variability of the measure and finally the level of significance alfa (5%), i.e. the probability of obtaining a statistically significant difference when in fact there is no difference. A priori information from the literature was used scientific.

Study

Study of the ability to assimilate calcium by drinking bicarbonate water with

a high calcium and low sodium vs control (low mineral water) in women in pre

and postmenopausal for a period of 6 months. After the end of the study there willbe a period offollow-up for study monitoring.
Subjects will follow a Mediterranean diet of 25-30 kcal/kg of ideal bodyweight/day (possibly free from acidic foods, controlling calcium intake
<700 mg/day, of 1 g/weight ideal protein per day, carbohydrates with a lowglycemic index 60%, lipids mainly of type
unsaturated 40%). The effects of water intake will be evaluated in relation to the presence of bicarbonates also on the functionality of the digestive and locomotor systems. During recruitment i subjects will complete a FFQ Food Frequency Questionnaire to establish their daily intake
of calcium in the diet.

The patients will be recruited by the associations Amos Paternopoli, Amos Montoro,Amdos Forino,Amos Solofra, Noi in Rosa, Amos Valle del Sabato.
The study includes measurements at 0, 3 and 6 months. Operational center of the study: Research LaboratoryUniversity nutrition Nutriketo_Lab, "San Giuseppe Moscati" Hospital of Avellino.

The two groups will be subjected to the indicated study protocol and will carry out:

- Recruitment test C (clinical, anthropometric, instrumental and blood chemistry tests)
- Test I after set times (Mediterranean diet, with 2 L Lete water (30 women in group A1 and in group B1) or with 2 L mineral water (30 women in group A2 and in that
  B2), with 500 mL with meals three times a day and the remainder during the day.

The following tests will be performed:

Clinical examinations: Physiological and pathological history, physical examination and nutritional

evaluation,

to investigate the patient's clinical conditions and the functionality of the digestive and locomotive.systems

Instrumental examinations: computerized bone mineralometry (MOC), articular ultrasound to evaluate the degree of elasticity, BIA/BIVA for the qualitative and quantitative evaluation of the body composition;

Metabolomic analysis of serum, urine, and saliva samples by spectroscopy nuclear magnetic resonance to investigate change in glycemic, lipidic and bone metabolism.

Processing of results

Statistical analysis, data visualization and predictive analysis will be carried out through Statgraphics software (Statgraphics Technologies, Inc., The Plains, Virginia). Features of the population included in the study will be analyzed by descriptive techniques. In
all evaluations made the results will be expressed as arithmetic mean and deviation

standard. The statistical analysis of the parametric data will be performed with the Student's T test for measurements paired and unpaired, comparing data at the start and at the end and then between the control and the intervention group. P-values <0.05 will be considered statistically significant.

Study monitoring

The study will be performed in accordance with the protocol and international Good Clinical guidelines Practice, and with the current regulations on clinical trials and those of reference for
natural mineral waters.

Ethical aspects

The current version of the Declaration of Helsinki (2013), the reference for the ethical aspects of this clinical trial will be respected by all who are engaged in this research.
The protocol, informed consent and any other material will be reviewed by the

Ethics Committee.

All eligible subjects must be informed of the purpose of the study, possible risks, and

benefits of the proposed interventions, and must obtain an information documentstating the nature,the purpose and course of the study.
The data of the study subject will be confidential and used in accordance with current legislation onprotection of sensitive data and privacy legislation.

Outcomes

The intake of an easily assimilable dose of calcium from a resource such asbicarbonate water calcium and sodium levels, may be able to determine

improvements in turnover markers
bone and also joint functionality and elasticity, decreasing the risk of bone fractures.

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Study sponsor: SGAM SpA

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Laboratory tests will be performed on 120 patients at 0, 3 and 6 months.

- Vitamin D
- Albumin
- Bicarbonates
- Football (S)
- Football (U)
- Ionized Calcium
- Creatinine -S/U/DU/LA-
- Creatinine clearance
- Emocrome
- Urine test
- Alkaline phosphatase (ALP)(S)
- Phosphorus (S)
- Phosphorus (U)
- Magnesium S/U/DU/(SG)ER-
- Osteocalcin*
- P1NP N-terminal propeptide of type I procollagen (S)
- Parathyroid hormone (PTH)

- Sodium S/U/DU/(SG)ER-
- C-terminal collagen telopeptide (CTX) (S)
- Total cholesterol
- HDL
- LDL

The MOC exam (Computerized Bone Mineralometry) will be performed on 120 patients at time 0 and

6 months.