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Stevia rebaudiana does not Affect Bone Turn-Over Factors after Nutritional Intervention

George Landis[1,2,3], Eva Kassi[2,4], Katerina Pavlaki[2], Vassiliki Efthimiou[3], Eleni Papanikolaou[2], Aimilia Mantzou[2], George P Chrousos[2,3,5], George I Lambrou[1,2,5*]

[1]Graduate Program "Metabolic Bones Diseases", National and Kapodistrian University of Athens, Medical School, Mikras Asias, Goudi, Athens, Greece

[2]Division of Endocrinology, Metabolism and Diabetes, National and Kapodistrian University of Athens, Medical School, Maiandrou, Ilisia, Athens, Greece

[3]Center for Adolescent Medicine and Department of Adolescent Health Care, Choremeio Research Laboratory, First Department of Pediatrics, University of Athens Medical School, Greece

[4]Department of Biological Chemistry, National and Kapodistrian University of Athens, Medical School, Mikras Asias, Athens, Greece

[5]First Department of Pediatrics, Choremeio Research Laboratory, National and Kapodistrian University of Athens, Thivon & Levadeias, Goudi, Athens, Greece

[*]**Corresponding author:** George I Lambrou, Department of Pediatrics, Choremeio Research Laboratory, National and Kapodistrian University of Athens, Thivon & Levadeias, 11527, Goudi, Athens, Greece. Tel: +302107467427, Email: glamprou@med.uoa.gr

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List of Abbreviations

Abbreviation	:	Explanation
ALP	:	Alkaline Phosphatase
ATP	:	Adults Treatment Panel
BAP	:	Bone-Specific Alkaline Phosphatase
BMD	:	Bone Mass Density
Cr	:	Creatinine
CRP	:	C - Reactive Protein
CVD	:	Cardiovascular disease
HDL	:	High Density Lipoprotein
IL-6	:	Interleukin-6
LDL	:	Low Density Lipoprotein
MetS	:	Metabolic Syndrome
NCEP	:	National Cholesterol Education Program
ODST	:	Overnight Dexamethasone Suppression Test
OPG	:	Osteoprotegerin
P1NP	:	N-Terminal Propetpide of Type I Collagen
RANKL	:	Receptor Activator of Nuclear Factor Kappa-B Ligand
SGOT	:	Glutamic-Oxaloacetic Transaminase
SGPT	:	Siamane Glutamate Pyruvate Transaminase
T2DM	:	Type 2 Diabetes Mellitus
TNF-α	:	Tumor Necrosis Factor-α
TRG	:	Triglycerides
uPAR	:	Urokinase Receptor
γGT	:	Gamma-Glutamyl Transferase

Abstract

Background: Metabolic Syndrome (MetS) also known as the syndrome X, increases the risk for the development of cardiovascular disease and Type 2 Diabetes Mellitus. Stevioside is a glycoside present in the leaves of the plant, *Stevia rebaudiana*, which has shown insulinotrophic and antihyperglycemic/antihypertensive effects in type 2 diabetic rats, while its effects on bone metabolism are still unknown. The present study focuses on the effects of stevioside supplementation on bone metabolism in a group of adult patients with MetS.

Materials and Methods: Seventeen patients were randomly allocated to two groups. Both groups received a six-month nutritional education program. Subjects in control group were allowed to consume a sugar containing dessert of their choice once a week whereas all subjects on stevia group (n=9) were provided with four stevia based snacks every week. Biometric, metabolic, biochemical and cytokine measurements were performed at the beginning of the intervention and six months after the nutritional education program. Bone formation was assessed by serum levels of Bone-Specific Alkaline Phosphatase (BAP) and N-terminal propetpide of type I collagen (P1NP).

Results: P1NP was significantly correlated to Body-Mass Index(BMI) in control and Stevia samples both before and after intervention, hinting towards an indirect mechanism of P1NP action between bone metabolism and total body metabolism. On the contrary, BAP was not found to be significantly correlated to BMI indicating a different mode of action as compared to P1NP.

Conclusion: This study is a first *in vivo* attempt to investigate the effects of *Stevia rebaudiana* supplementation on bone markers in human subjects with disturbed metabolic profile. Despite the limitations, this study amplifies that nutritional factors influence the pattern of bone turnover and at underlines the bone-fat cross talk as a promising research area in both bone disease and obesity understanding and treatment.

Keywords: Bone Metabolism; Bone-Specific Alkaline Phosphatase (BAP); Body-Mass Index (BMI); Metabolic Syndrome; N-Terminal Propetpide of Type I Collagen (P1NP); *Stevia rebaudiana*.

Introduction

Metabolic Syndrome (MetS) also known as the syndrome X, the insulin resistance syndrome and the deadly quarter is a constellation of metabolic disturbances which increase the risk for the development of Cardiovascular Disease (CVD) and Type 2 Diabetes Mellitus (T2DM) [1,2]. Worldwide prevalence of MetS ranges from <10% to as much as 84%, depending on the region, urban or rural environment, composition (sex, age, race, and ethnicity) of the population studied, and the definition of the syndrome used [3,4]. The increasing incidence of MetS has been mainly attributed to the parallel rising of obesity during the last two decades [5]. In addition, obesity and specifically central adiposity measured as intra-abdominal fat has been independently related to MetS features even among normal weight subjects with increased central adiposity [6]. On the other hand, while the last 20 years it was believed that obesity was related to higher Bone Mass Density (BMD) and thus lower fracture incidence, recent data from a multinational prospective study with 60,393 participants show a positive relationship between obesity and increased bone fracture risk [7]. Furthermore, adipose tissue is an active metabolic and endocrine organ [8] which produces cytokines and inflammation mediators including leptin, adiponectin, , proteins of the renin-angiotensin system and resistin which have been implicated in both MetS pathogenesis and bone metabolism [9-11]. Specifically, leptin is involved in energy homeostasis via hypothalamic pathways and through direct action on peripheral tissues including muscle and pancreatic β-cells thus it has been linked to obesity and insulin resistance. Low-dose leptin replacement has shown to decrease weight and increase BMD in leptin-deficient humans [12]. Animal studies have demonstrated improved insulin resistance after adiponectin administration and human studies have showed a negative correlation between adiponectin and insulin resistance [13]. Recent cross-sectional studies have suggested that insulin resistance and specifically hyperinsulinemia are negatively correlated to bone strength [14]. Similar findings have been reported by Verroken and colleagues who investigated the association between insulin resistance and bone geometry among non-diabetic men at the age of peak bone mass. They concluded that insulin resistance was inversely correlated to trabecular and cortical bone size independently of body composition, muscle size and sex hormone levels [15]. The linkage between MetS and bone metabolism or disease has been also approached via inflammation pathways. Recent research supports that increased levels of cytokines and inflammation mediators i.e. Tumor Necrosis Factor-α (TNF-α), interleukin-6, (IL-6), C - Reactive Protein (CRP), leptin and adiponectin which are produced by adipose tissue activate the NF-κB ligand (RANKL)/ receptor of NF-κB ligand (RANK)/ Osteoprotegerin (OPG) pathway which promotes osteoclasts differentiation and bone resorption [16]. Overproduction of leptin

and/or reduced secretion of adiponectin by adipocytes have been connected to recruitment and accumulation of macrophages to adipose tissue. Macrophages are responsible for almost all adipose tissue TNF-α expression and significant amounts of IL-6 which in turn contributing to the detrimental effects on bone metabolism in obesity [17]. It seems that MetS and bone metabolic diseases are not only featuring some common pathological mechanisms but also share alike therapeutic approaches. Healthy balanced nutrition and exercise are of major significance in MetS treatment, bone health and osteoporosis. Furthermore, dyslipidemia drugs used in MetS have been shown to increase BMD in postmenopausal women, whilst common antihypertensive therapy in hypertensive rats has been followed by osteoporosis improvement. Stevioside, a glycoside present in the leaves of the plant, *Stevia rebaudiana* (SR) has shown insulinotrophic effects in vitro and antihyperglycemic / antihypertensive effects in type 2 diabetic rats [18], thus has drawn attention as a potent therapeutic substance for T2DM and MetS. Although the very small number of human studies that have investigated the effects of stevia they all support the insulinotrophic and antihyperglycemic findings that have been demonstrated in animal models [19,20]. We investigated the effects of stevioside supplementation on bone metabolism in a group of adult patients with MetS.

Materials and Methods

Patients

Seventeen patients over 18 years of age (mean 52.7±7 years) were screened for MetS characteristics. MetS diagnosis was based on the 2004 revised National Cholesterol Education Program (NCEP) Adults Treatment Panel (ATP) III criteria according which MetS is diagnosed when 3 out of the 5 following characteristics are presented.

Inclusion Criteria

Inclusion criteria were: i) fasting plasma glucose levels≥100mg/dl, ii) blood pressure≥130/85mmHg, iii) triglycerides≥150mg/dl, iv) (High Density Lipoprotein) HDL-Cholesterol<50mg/dl for females or <40mg/dl for males and v) waist circumference >88 for females or >102 for males. Waist circumference was measured at the midpoint between the lowest rib margin and the iliac crest.

Exclusion Criteria

Patients on anti-depressive medication, glucocorticoids, hormonal replacement therapy, anti-lipidaemic, diabetic therapy and with diagnosed chronic diseases were excluded. In addition, all subjects were screened for Cushing's syndrome by measuring morning cortisol levels after Overnight Dexamethasone Suppression Test (ODST) with 1mg of dexamethasone. Subjects with morning cortisol values greater than 1.8μg/dl were excluded.

Biochemical and Cytokine Measurements

Whole blood was collected from fasting individuals through phlebotomy, practiced by a trained health professional at baseline 08.00-10.00. Blood serums were collected after centrifugation and stored at -80^{0}C until assayed. Insulin was measured with the method of two-site chemiluminescent immunometric assay (Immulite 2000 Siemens) with a detection limit of 2μIU/mL, and inter assay Coefficient of Variance (CV) 4.2%. The biochemical parameters glucose, total cholesterol, HDL-Cholesterol, (Low Density Lipoprotein) LDL-Cholesterol, Triglycerides (TRG), Glutamic-Oxaloacetic Transaminase (SGOT), Siamane Glutamate Pyruvate Transaminase (SGPT), Gamma-Glutamyl Transferase (γGT), Alkaline Phosphatase (ALP), Urea, Creatinine (Cr), Ca^{+2}, Mg^{+2}, P^{+4} were measured at the Cobas 8000 Roche analyzer. The plasminogen activator urokinase receptor uPAR was measured with ELISA (R&D SYSTEMS) with a detection limit of 33pg/ml. Bone formation was assessed by serum levels of Bone-Specific Alkaline Phosphatase (BAP) and N-Terminal Propetpide of Type I Collagen (P1NP), which were performed by outsourcing in an external reference laboratory.

Intervention Design

Seventeen patients were randomly allocated to two groups. Both groups received as 6 months nutritional education program delivered by a registered dietitian in a person to person consultation with a fifteen-day interval follow up. All subjects in control group (n=8) were allowed to consume a sugar containing dessert of their choice once a week whereas all subjects on stevia group (n=9) were provided with four stevia based snacks every week. The nutrition education program was based on the principles of Mediterranean Diet (MD) which has been shown to reduce and improve the risk factors of developing MetS [21]. The nutrition education program was aiming to increase the adherence of subjects to MD. Biometric, metabolic, biochemical and cytokine measurements were performed at the beginning of the intervention and at the end of it, that is 6 months after the nutritional education program.

Statistical Analysis

Statistical analyses were performed with the MATLAB® simulation environment (The Mathworks, Inc., Natick, MA). Examination of data normal distribution was performed with the Kolmogorov-Smirnov test, which manifested that the data came from a standard normal distribution, obtaining p-value<<0.001. For the present work, we have used two statistical approaches. The first included a paired two-tailed student t-test, in order to test for the mean differences between two groups. In addition, one-way ANOVA was performed in our data in order to compare multiple patient groups. Both statistical approaches manifested comparable results and data presented in the results section include those obtained from the paired two-tailed student t-test. Continuous

variables are expressed as mean±standard deviation unless indicated differently. Raw data as well as data mean, median, standard deviation, min and max values are available as supplementary data (please refer to **Supplementary_File_1.xlsx**).

Ethics Approval and Consent to Participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

Results

Subjects under investigation were divided into four groups: control samples that received a sugar-containing desert before and after a nutritional intervention (two groups) and case samples that received stevia-containing snacks before and after nutritional intervention (two groups). Waist/Hip ratio appeared to manifest significant difference in Stevia group as compared between the subjects before and after intervention (Figure 1A). Furthermore, no significant difference was observed with respect to other bone metabolic variables. In particular, no significant differences were observed in Mg^{+2} (Figure 1B), Ca^{+2} (Figure 1C), P^{+4} (Figure 1D), Alkaline Phosphatase (ALP) (Figure 1E), Bone-specific Alkaline Phosphatase (BAP) (Figure 1F), Serum type 1 Procollagen (P1NP) (Figure 1G), Urokinase Receptor (uPAR) (Figure 1H) and *Plasminogen Activator Inhibitor-1 (PAI1)* (Figure 1I).

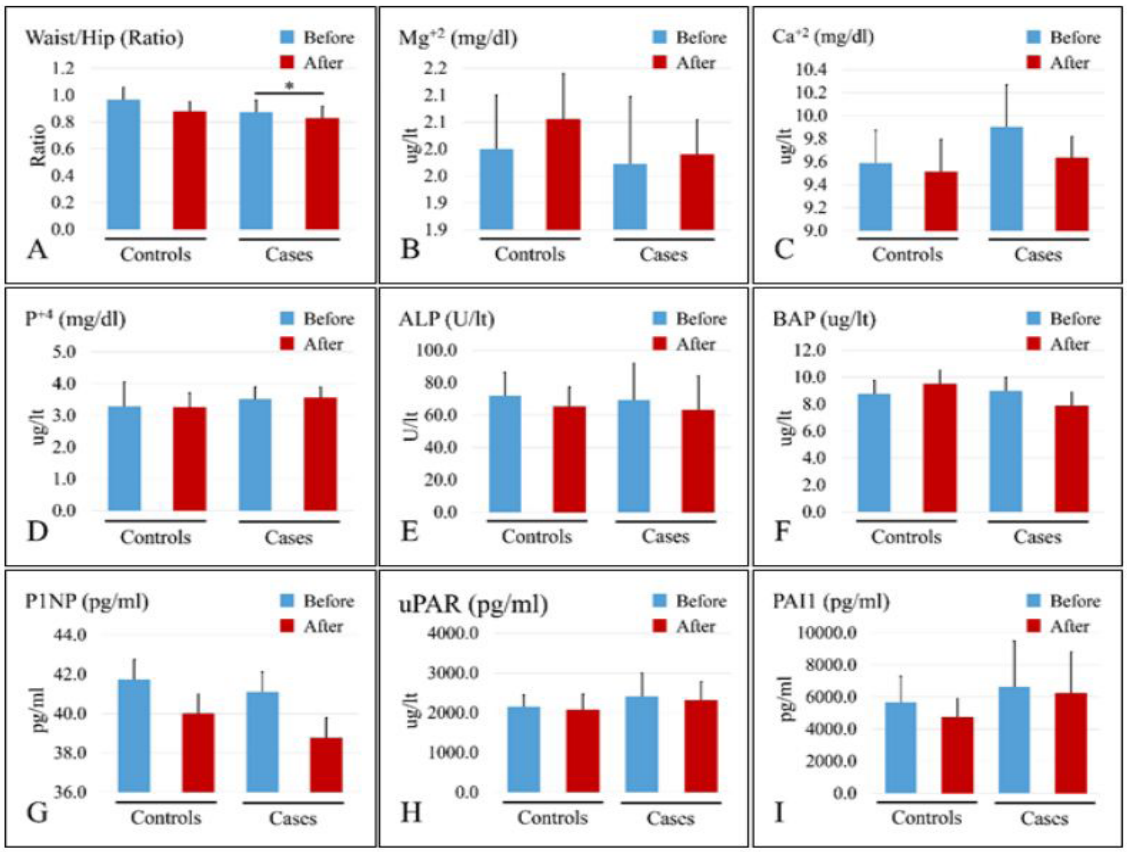

Figure 1: Histograms of metabolic and bone metabolic factors in subjects treated with *Stevia rebaudiana*. In particular, following metabolic variables are presented: waist to hip ratio (A), Mg^{+2} (mg/dl) (B), Ca^{+2} (mg/dl) (C), P^{+4} (mg/dl) (D), Alkaline Phosphatase (ALP) (U/lt) (E), Bone Alkaline Phosphatase (BAP) (ug/lt) (F), Serum type 1 procollagen (P1NP) (pg/ml) (G), Urokinase Receptor (uPAR) (pg/ml) (H) and Plasminogen activator inhibitor-1 (PAI1) (pg/ml) (I). Control group (indicated as "Controls") included subjects that received a nutrition educational program (indicated as "Before") and allowed to consume a sugar-containing dessert after intervention (indicated as "After"). Study group (indicated as "Cases") included subjects that received a nutrition educational program (indicated as "Before") and allowed to consume four Stevia-based snacks after intervention (indicated as "After").

In order to unravel further relations in bone metabolic factors we have searched for correlations between estimated variables both in metabolic and bone metabolic factors. In particular, significant positive correlations appeared to be present between BMI and P1NP (rho=0.707), waist/hip ratio and BAP (rho=0.751) and significant negative correlations were observed between waist/hip ratio and BAP (rho=-0.538). Correlations are summarized in Table 1.

		P1NP	BAP	PAI1 pg/mL	uPAR pg/mL	BMI	w/h ratio
BAP	Before	.258					
	After	.039					
PAI1 pg/mL	Before	.089	-.146				
	After	.414	.457				
uPARpg/mL	Before	-.215	.170	.181			
	After	.348	-.182	.325			
BMI	Before	-.025	-.148	.286	.291		
	After	**.707****	.144	.468	.292		
w/h ratio	Before	.071	.074	.070	**-.538***	-.104	
	After	.115	**.751****	.204	-.301	-.012	
*** P<.001; **P<.01 ; *P<.05							

Table 1: Spearman Rho Correlation Coefficients.

Based on the aforementioned Spearman correlation results we have attempted to investigate regression correlations between studied variables. In particular, in the control group a reversal was observed between P1NP and Ca^{+2} as the two variables were negatively correlated before intervention (R^2=0.14) and positively correlated after intervention (R^2=0.37) (Figure 2A). In the Stevia group, it appeared that correlation was similar before intervention (R^2=0.1742) while it changed after intervention (R^2=0.0037), indicating that in both cases intervention played a significant role in bone metabolic factors physiology. Similarly, Ca+2 appeared to interact in a similar way with BAP as correlation changed before and after intervention (Figure 2C, 2D).

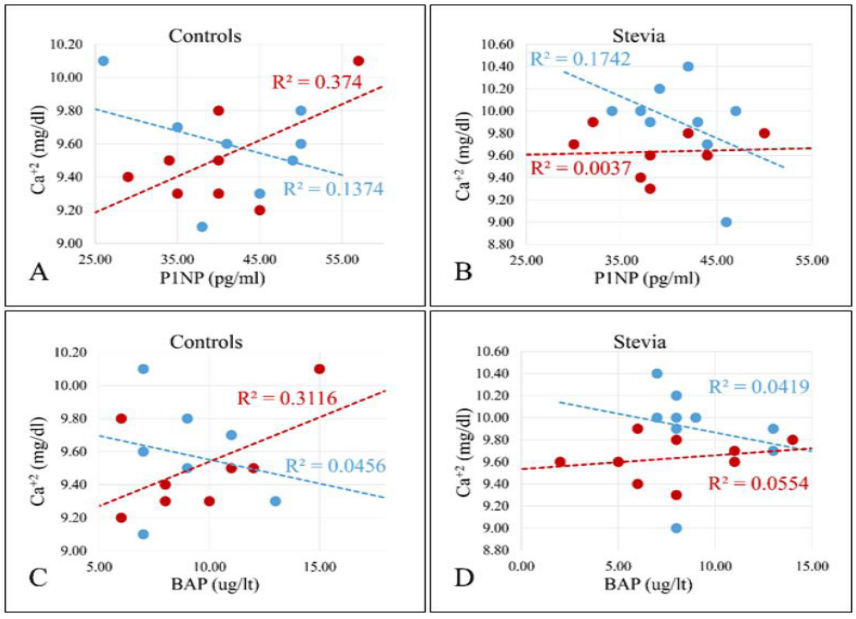

Figure 2: Regressions of metabolic and bone metabolic factors in subjects treated with *Stevia rebaudiana*. In particular, Ca^{+2} levels is depicted with respect to Serum type 1 procollagen (P1NP) in control samples (R^2=0.14 before intervention and R^2=0.374 after intervention) **(A)** and Serum type 1 procollagen (P1NP) in Stevia receiving group (R^2=0.17 before intervention and R^2=0.0037 after intervention**) (B).** In addition, Ca+2 levels is depicted with respect to Bone Alkaline Phosphatase (BAP) in control samples (R^2=0.045 before intervention and R^2=0.312 after intervention) **(C)** and Bone Alkaline Phosphatase (BAP) in Stevia receiving group (R^2=0.042 before intervention and R^2=0.055 after intervention) (D) (Blue dots, indicate subjects before the intervention and red dots indicate subjects after the intervention).

At the same time no significant correlation was observed in control group with respect to the relation of phosphorus and BAP (Figure 3A, 3C). Yet, in the subjects group that received Stevia, a reversal pattern was revealed before and after intervention with respect to both phosphorus P1NP and BAP (Figure 3B, 3D).

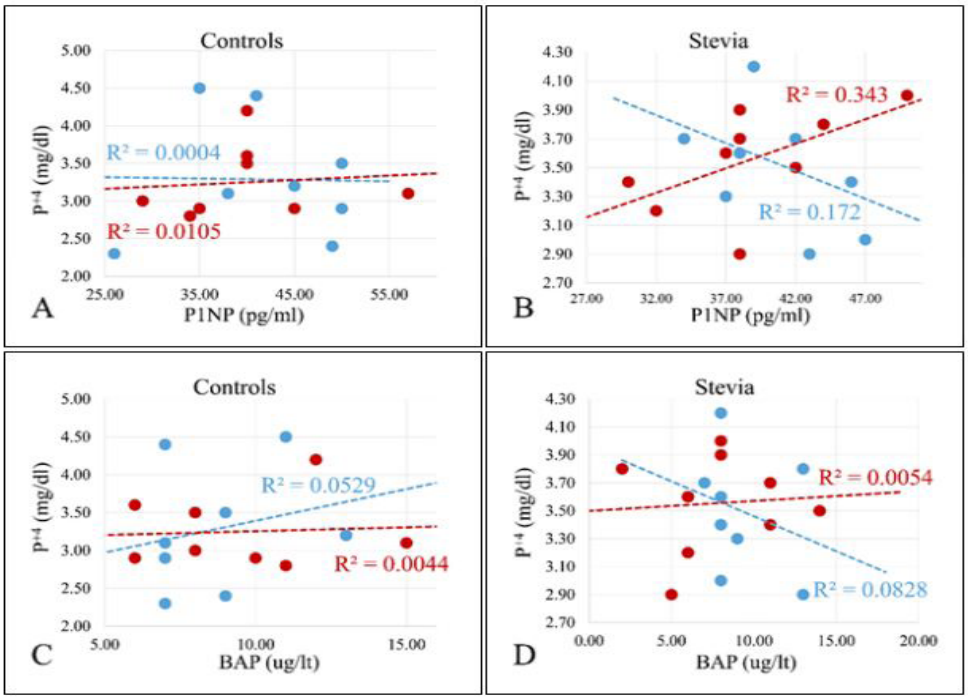

Figure 3: Regressions of metabolic and bone metabolic factors in subjects treated with *Stevia rebaudiana*. In particular, P^{+4} levels is depicted with respect to Serum type 1 procollagen (P1NP) in control samples (R^2=0.0004 before intervention and R^2=0.015 after intervention) **(A)** and Serum type 1 procollagen (P1NP) in Stevia receiving group (R^2=0.172 before intervention and R^2=0.343 after intervention) **(B).** In addition, P^{+4} levels is depicted with respect to Bone Alkaline Phosphatase (BAP) in control samples (R^2=0.05 before intervention and R^2=0.0044 after intervention) **(C)** and Bone Alkaline Phosphatase (BAP) in Stevia receiving group (R^2=0.0054 before intervention and R^2=0.083 after intervention) **(D)** (Blue dots, indicate subjects before the intervention and red dots indicate subjects after the intervention).

Interestingly, no particular changes were observed in relation to P1NP, BAP and ALP both before as well as after intervention in both groups (Controls and Stevia) (Figure 4). It appeared that regression curves remained similarly aligned in all cases with respect to ALP vs. P1NP and BAP.

Examining the relation between P1NP and BAP it was found that no significant correlation existed between the two variables. Yet, it appeared that in the case of control samples regressions manifest similar slopes both before and after intervention, while in the case of Stevia samples there was a reversal in that pattern between before and after intervention measurements (Figure 5).

Finally, it was interesting to observe that P1NP was significantly correlated to BMI in control and Stevia samples both before and after intervention (Figure 6A, 6B). These finding hints towards an indirect mechanism of P1NP action between bone metabolism and total body metabolism. On the contrary, BAP was not found to be significantly correlated to BMI indicating a different mode of action as compared to P1NP (Figure 6C, 6D).

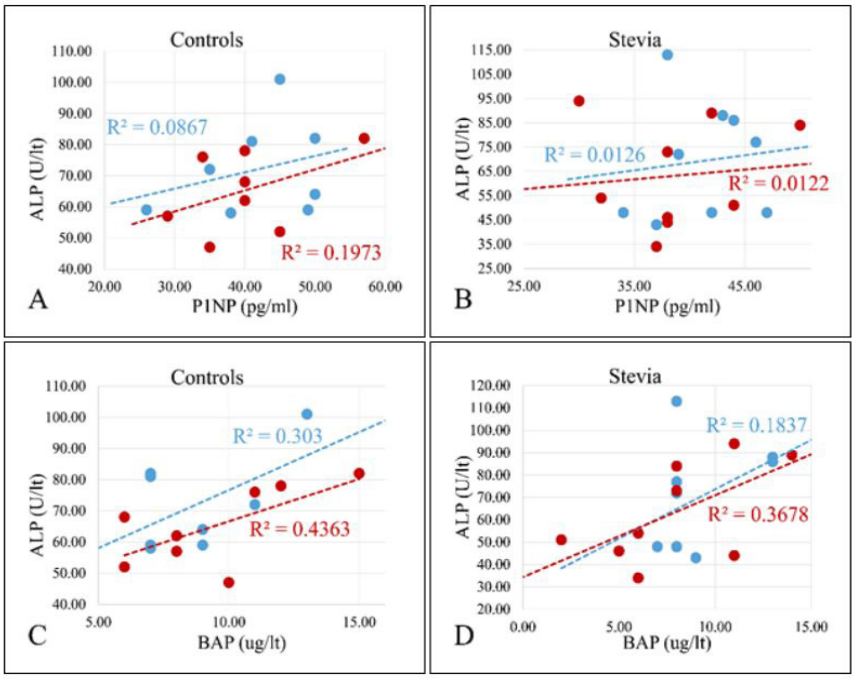

Figure 4: Regressions of metabolic and bone metabolic factors in subjects treated with *Stevia rebaudiana*. In particular, Alkaline Phosphatase (ALP) levels is depicted with respect to Serum type 1 procollagen (P1NP) in control samples (R^2=0.087 before intervention and R^2=0.19 after intervention) **(A)** and Serum type 1 procollagen (P1NP) in Stevia receiving group (R^2=0.013 before intervention and R^2=0.0122 after intervention) **(B).** In addition, ALP levels is depicted with respect to Bone Alkaline Phosphatase (BAP) in control samples (R^2=0.303 before intervention and R^2=0.4363 after intervention) **(C)** and Bone Alkaline Phosphatase (BAP) in Stevia receiving group (R^2=0.184 before intervention and R^2=0.368 after intervention) **(D)** (Blue dots, indicate subjects before the intervention and red dots indicate subjects after the intervention).

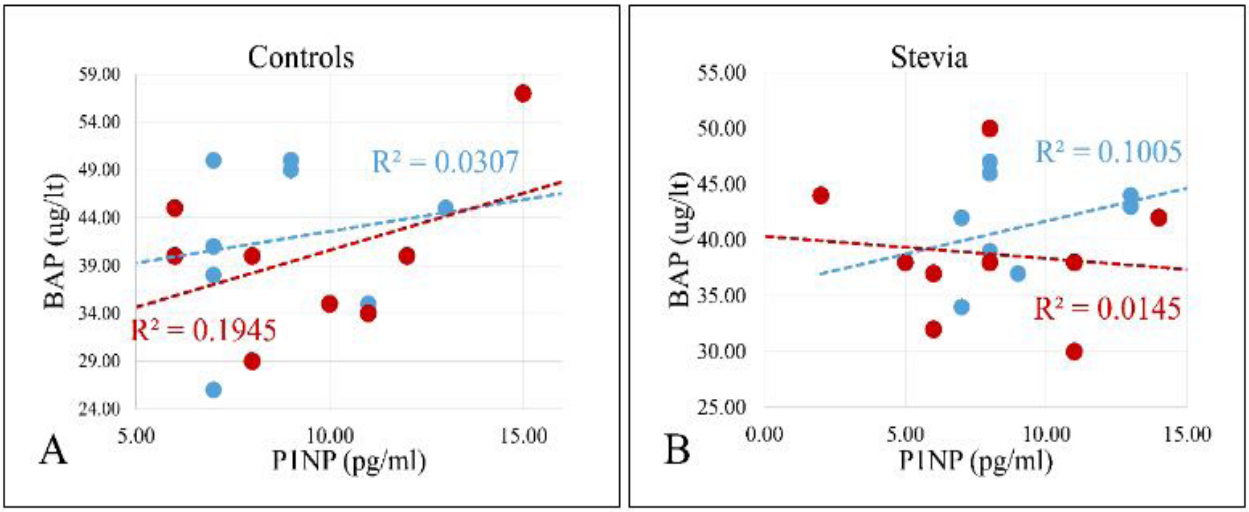

Figure 5: Regressions of bone metabolic factors in subjects treated with Stevia rebaudiana. In particular, Bone Alkaline Phosphatase (BAP) levels is depicted with respect to Serum Type 1 Procollagen (P1NP) in control samples (R^2=0.03 before intervention and R2=0.19 after intervention) (A) and in Stevia receiving group (R^2=0.1 before intervention and R^2=0.014 after intervention) (B) (Blue dots, indicate subjects before the intervention and red dots indicate subjects after the intervention).

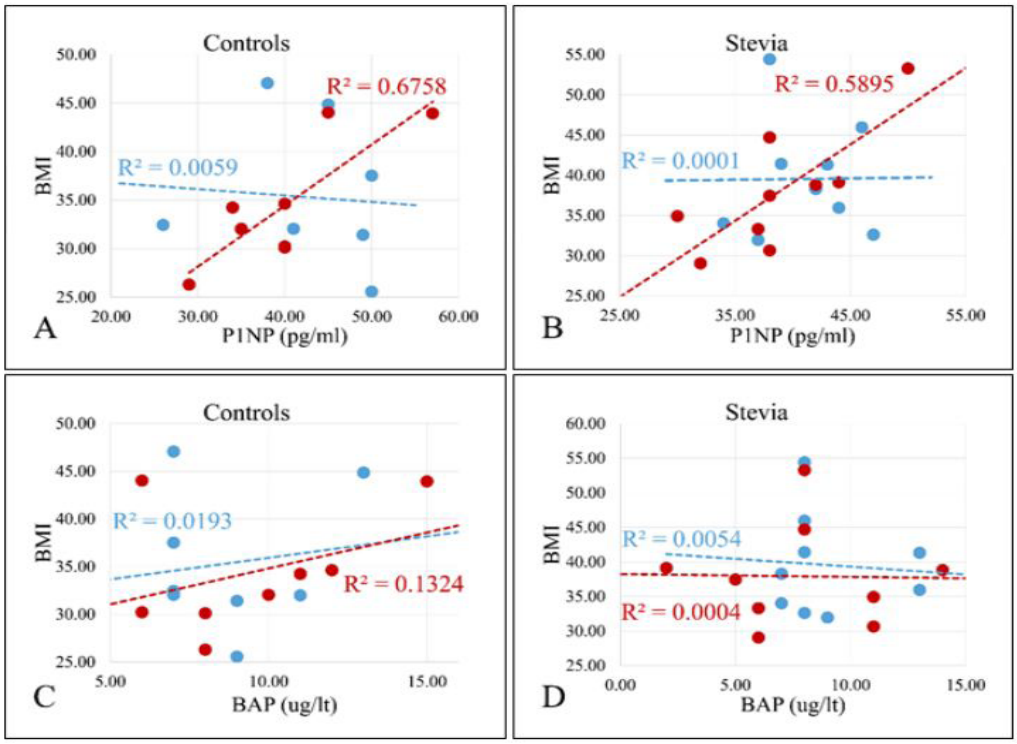

Figure 6: Regressions of bone metabolic factors in subjects treated with *Stevia rebaudiana*. In particular, Body-Mass Index (BMI) levels is depicted with respect to Serum type 1 procollagen (P1NP) in control samples (R^2=0.006 before intervention and R^2=0.68 after intervention) **(A)** and in Stevia receiving group (R^2=0.0001 before intervention and R^2=0.59 after intervention) **(B).** In addition, Body-Mass Index (BMI) levels is depicted with respect to Bone Alkaline Phosphatase (BAP) in control samples (R^2=0.02 before intervention and R^2=0.13 after intervention) **(C)** and in Stevia receiving group (R^2=0.0054 before intervention and R^2=0.0004 after intervention) **(D)** (Blue dots, indicate subjects before the intervention and red dots indicate subjects after the intervention).

Discussion

In the present work, we have attempted to investigate the effects of nutritional intervention and *Stevia rebaudiana* supplementation in bone metabolism factors. To the best of our knowledge this is the first work on this topic. There are no previous reports concerning the effects of *Stevia rebaudiana* on bone metabolism and bone turnover factors and thus there is no previous experience on Stevia supplementation. In the present work, we have found that nutritional supplementation did not have any short-term effects (during a six-month period) on bone turnover factors. Impaired skeletal characteristics are known to be closely related to obesity and metabolic syndrome [22]. Yet, the precise mechanisms are still to be elucidated. Based on our observations it appeared that nutritional intervention had an effect, on bone turnover factors and although it did not appear to be significant there was a clear change in the pattern that bone metabolic factors interacted. In a recent report, it has been found that P1NP and metabolic factors are affected by age and in particular, manifest higher values in young individuals as compared to adults [23]. Further on, in the same study it appeared that these changes were affected by nutritional habits and vitamin D intake. In another report, it has been found that metabolic syndrome as well as nutrition did not directly affect P1NP and BAP levels, while it affected calcium and phosphorus levels in the subjects under study [24].

P1NP is a bone formation marker produced by osteoblasts during the synthesis of procollagen type 1 [25]. It has been suggested that bone also acts as an endocrine organ and in that context, it may affect the metabolic activity of the host [26, 27]. Special interest has been given to the metabolic effects of osteocalcin, which is a product of osteoblasts, in glucose metabolism [28-30]. Our findings suggest that P1NP may also play an indirect role in body energy regulation, yet in vitro research is required to investigate the plausible mechanisms.

Despite the fact that this is the first study investigating the effects of Stevia on bone metabolic factors there are some limitations that need to be addressed. Firstly, the small subjects' number of the study is a crucial limitation. Secondly, the lack of imaging techniques i.e. Dual X-Ray Absorptiometry (DEXA) or Magnetic Resonance Imaging (MRI) which was not incorporated in the study design does not allow us to draw clear conclusion

regarding the quality and architecture of bone. Nevertheless, this study was not targeted to osteoporosis which would have justified the use of imaging techniques. Lastly, our decision to exclude osteocalcin and bone reabsorption markers from our measurements has also narrowed our holistic approach of bone remodeling.

Conclusion

In conclusion, this study is a first *in vivo* attempt to investigate the effects of *Stevia rebaudiana* supplementation on bone markers in human subjects with disturbed metabolic profile. Despite the limitations, this study amplifies that nutritional factors influence the pattern of bone turnover and at underlines the bone-fat cross talk as a promising research area in both bone disease and obesity understanding and treatment.

Declarations

- **Ethics Approval and Consent to Participate**: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. Please also refer to the "Materials and Methods" section.
- **Availability of Data and Material**: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. Please also refer to the "Materials and Methods" section.
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- **Authors' Contributions:** GL: collected nutritional data, drafted the manuscript, performed data analysis. EK: collected clinical data. KP: performed physical examinations, collected clinical data. VE: performed statistical analysis, EP: patient management. AM: performed laboratory tests. GPC: provided data, provided funding, proof-edited the manuscript, supervised the study and gave final permission for submission. GIL: performed data analysis, drafted the manuscript and gave final permission for submission.

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