Abstract

This is a prospective randomized, controlled, open-label study which was carried out over a two years period. Sixty postmenopausal women with metabolic syndrome were enrolled and treated orally with either 2 capsules of a nutraceutical (combination of 480 mcg corosolic acid, 38 mg glycyrrhizic acid and 2.4 mg procyanidin (methyl hydroxicalcone) or placebo. Duration of the administration of either the nutraceutical or placebo was three months. To all 60 women lifestyle changes were suggested, namely moderate aerobic physical activity and hypocaloric diet. The rationale of the study was that each of the three substances in the nutraceutical mixture is known to improve insulin sensitivity by acting at different levels along the complex pathways that characterize insulin action. Outcomes of the study were changes in the anthropometric measures, insulin resistance, lipid profile and thyroid function with corresponding measurements recorded at baseline and end of third month of follow up. Our data highlight the effectiveness of these ancient supplements with insulin-like and anti-adipogenesis, antioxidant and anti-inflammatory properties and they demonstrate a possible application of this nutraceutical combination in medical practice. The association of glucose uptake and anti-adipogenesis activity is not found in currently used insulin mimetic drugs and may indicate a good therapeutic potential. This is the first study which has analysed an alternative therapeutic approach in metabolic syndrome in menopause.

Introduction

The concept of **Metabolic Syndrome** (MS) has been much studied over the past few decades. Despite variations in diagnostic criteria, the syndrome is considered a cluster of metabolic abnormalities and a complex pre-disease state that predicts future development of type 2 diabetes mellitus (DM) and cardiovascular disease. It is defined by four clinical

manifestations: central obesity, glucose intolerance, dyslipidaemia and hypertension[1]. Several risk factors have been identified, mainly excessive calorie intake, sedentary lifestyle and genetic predisposition. Pathogenesis is largely attributable to insulin resistance while the onset of complications like cardiovascular disease and DM are closely linked to chronic, systemic proinflammatory and procoagulation states[2]. Menopausal transition is considered a vulnerable period for the onset of MS and this increased risk is attributed to decreasing oestrogen levels with an increased risk of insulin resistance. Menopause is a peculiar endocrine-metabolic condition characterizing a large fraction of a woman's life. As such, it raises a major public health concern with an enormous social impact. Menopause is usually associated with changes in body composition, due to the prevalence of androgens over oestrogens, and a decrease in physical activity. Adipose tissue, especially visceral fat, is an important source of inflammatory markers, which contributes to the development of a proinflammatory state. Conversely, it has been ascertained that high levels of physical activity and exercise have an anti-inflammatory effect[3]. Naturally-occurring compounds containing **Corosolic Acid**, **Glycyrrhizic Acid** and **Procyanidin** have been shown to improve insulin sensitivity and glucose control. These supplements are already individually used in oriental traditional medicine as a remedy for various illnesses and ailments, particularly for lowering glycemia, reducing body weight and treating metabolic diseases, such as DM. **Corosolic Acid (CA)** has been identified from the red leaves of *Lagerstroemia speciosa*, a common ornamental tree with attractive, colourful pink or purple flowers. Commonly known as crepe myrtle or Banaba, it grows widely in tropical countries, including the Philippines, India, Malaysia, China and Australia[4]. Despite growing in several countries, it is above all in the Philippines that the dried, shredded Banaba leaves are known to be used as a treatment for diabetes and kidney disease. It is not clear if Banaba plants grown in different countries are equally effective in the treatment of diabetes[5]. Unlike insulin, this compound stimulates glucose uptake and inhibits adipocyte differentiation; this

inhibition is time and concentration dependent. CA interacts with the GLUT 4 receptor indirectly, activating the peroxisome proliferator-activated receptor ϒ (PPAR-ϒ) system, up-regulating GLUT4 gene expression. CA also directly increases translocation from a low-density microsome membrane to the plasma membrane, permitting the entry of glucose[6]. This mechanism is quite different from the one used by other common antidiabetic drugs as it seems to be much faster. It is interesting to note that CA shows an insulin-like glucose uptake-inducing activity in adipocytes but does not show an insulin-like differentiation inducing activity in preadipocytes: this difference may be explained by the fact that these two activities involve two distinct signalling pathways. Pharmacological properties reported in literature include also antibacterial, antiviral activities, cytotoxic, anti-oxidant and anti-inflammatory activities. *Lagerstroemia speciosa* leaf powder extracts have been tested against *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli* successfully. The compound has good potential to be developed into a potent anti-human rhinovirus agent. With regard to anti-inflammatory activity, it has been examined for both acute and chronic models with a significantly reduced inflammation in a dose-dependent manner[7]. **Glycyrrhizic Acid (GA)**, a triterpenoid saponin found in abundance in the root of genus *Glycyrrhiza glabra*, commonly known as liquorice, has shown some compelling therapeutic activities in combatting MS. It is a non-selective inhibitor of 11β-hydroxysteroid dehydrogenase, an enzyme involved in the interconversion of inert and functional glucocorticoids. This inhibition could modulate the level of active glucocorticoids and, subsequently, inhibit its insulin-antagonistic property[8], and improve peripheral glucose uptake activating translocation of glucose transporter to the cell membrane: possessing an anti-hyperglycaemic effect, it may retard the development of insulin resistance[9,10]. Treatment with GA specifically induces upregulation of lipoprotein lipase (LPL) in myocardial tissue and skeletal muscle, which could promote free fatty acids (FFA) uptake in tissues. These therapeutic effects are linked to its inhibitory effect on stress hormones with

subsequently normalized gluconeogenic enzyme activities. GA is also a potential agonist for PPAR ϒ. PPARs are nuclear receptor proteins and transcriptor factors, which play critical regulatory roles in lipid metabolism and adipocyte differentiation. GA is also considered a modulator of vascular endothelium, acting as a protective factor, and evidence suggests that it reduces testosterone serum levels[11]. Common cinnamon species (*Cinnamomum verum, (or C. zeylanicum)*, *C. aromaticum*) have a long history of uses as spices, flavouring agents, preservatives and pharmacological agents. Several studies have examined the effects of cinnamon on glucose, insulin and lipid metabolism associated with MS. A review of the safety and efficacy of cinnamon on antioxidant activity, Helicobacter pylori infection, activation of olfactory cortex and brain, and oral candidiasis in human immunodeficiency virus has been published[12]. Extracts of cinnamon contained consistent amounts of **procyanidin (Pr)** that has insulin-potentiating properties and may be involved in the alleviation of the signs and symptoms of diabetes and cardiovascular disease related to insulin resistance and MS. Performed analysis in vitro show that aqueous cinnamon extracts potentiate insulin activity more than 20-fold and results in increased insulin sensitivity: women with MS may have adequate amounts of insulin but it is often not efficient. The activation of phosphorylation and the inhibition of dephosphorylation of the insulin receptor leads to increased phosphorylation of the insulin receptor, which is associated with increased insulin sensitivity[13]. Cinnamon renders insulin more efficient, increasing glucose uptake and GLUT4 expression in adipose cells and inhibiting retinol-binding protein 4 (RBP4), a novel adipokine that contributes to insulin resistance in plasma and adipose tissues. Retinol-binding protein 4 is increased in the sera of insulin-resistant patients, mediates insulin-resistance in muscle and increases glucose production in liver. Plasma RBP4 levels are inversely correlated with the expression of GLUT4 in adipose tissue. Cinnamon extract consumption also appears to regulate glucose uptake-related genes, such as Glut1, Glut4, glycogen synthesis 1, and glycogen synthase kinase 3β mRNA expression in adipose

tissue[14]. Proinflammatory cytokines, linked with obesity, insulin resistance and MS, stimulate the overproduction of intestinal apolipoprotein B48 containing lipoproteins. Procyanidin inhibits the oversecretion of apoB48. Treatment with cinnamon extracts decreases mRNA expression of inflammatory factors (IL1-β, IL-6, TNF-α)[15]. Therefore, PR has a lowering lipid effect and shows anti-inflammatory activity[16, 17].

Based on the results from the literature reviewed to date, the first outcome of this study was to show if the association of the above discussed supplements could really improve MS in post-menopause women.

In consideration of the great prevalence of thyroid diseases in women's life, the function of this gland was taken into consideration in this study. Thyroid hormone levels, and in particular FT3, were independently associated with subcutaneous fat in euthyroid postmenopausal women and this association was mainly evident in women with higher body mass index (BMI). Only TSH levels were positively associated with BMI in the postmenopausal period[18]. The correlation between MS with its peculiar pro-inflammatory state and thyroid diseases has not been completely elucidated in literature: women with Hashimoto's thyroiditis are more obese, have higher waist circumference, HOMA-index and higher prevalence of MS[19]. In this study, a secondary outcome was to evaluate if the natural compounds analysed could influence thyroid function in post-menopause women.

A large number of studies have demonstrated that MS correlates with chronic and low-grade inflammation with massive secretions of proinflammatory cytokines such as IL-6, IL-4, IL-10 [20]. For this reason, a further secondary outcome of the study was to evaluate a possible influence of these ancient compounds on high mobility group protein (HMGB1), a new potential biomarker of inflammation that positively correlates with insulin resistance. This new protein, acting as a "danger signal", migrates quickly during electrophoresis, hence 'high mobility' in its name, and correlates with conditions characterized by chronic, low-

grade, subclinical inflammation[21]. This is the first study which deals with this emergent biomarker of inflammation in menopausal women suffering from MS, treated with natural supplements.

Materials and Methods

This is a prospective randomized, controlled, open-label study, carried out in the Obstetrics and Gynaecology Unit, University Hospital "G. Martino", Messina, from September 2015 to August 2017. Permission was obtained from the Local Institutional Review Boards. Study behaviour and analysis was performed following the CONSORT criteria (www.equator-network.org) and the study was registered in a clinical trials database. A total of 58 women were selected from a cohort of women in post-menopause affected by MS referred to our

Department for a clinical check-up. After being thoroughly briefed about the aim of the study, each patient signed an informed consent for recruitment. Inclusion criteria were represented by women in amenorrhea for at least 12 months, with an age between 45 and 65 years old, affected by MS, diagnosed by ATP 2015 criteria (National Cholesterol Educational Program Adult Treatment Panel III)[22] which are: fasting glucose ≥ 100 mg/dl; triglycerides > 150 mg/dl; blood pressure ≥ 135/85 mmHg; abdominal obesity (waist circumference > 88 cm); good cholesterol HDL-c < 50 mg/dl. Exclusion criteria considered were: women in menopausal transition period, current treatment with hypoglycaemic drugs or anti-cholesterol drugs.

At enrolment (t0) and after 12 weeks (t1), blood pressure, waist circumference, weight and BMI were recorded for each participant. Moreover, a modified life-style, such as moderate aerobic physical activity (duration ranging from 20 to 50 minutes three times per week) and a hypocaloric diet was strongly suggested to all women. A blood sample to evaluate glycaemic (fasting glycaemia and insulin) and lipid profile (total cholesterol, HDL-c, triglycerides) was taken to confirm diagnosis of MS. Insulin-resistance was evaluated as HOMA-INDEX. In addition, thyroid function was evaluated (TSH, FT3, FT4, AbTg, AbTPO).

Participants were then randomly assigned into two groups using a computer-generated randomization table. Two soft gel capsules per day were administered to each participant of the treated group (TG) before breakfast and lunch, for 12 weeks. Each capsule contained corosolic acid 480 mcg, glycyrrhizic acid 38 mg and procyanidin (methyl hydroxicalcone) 2.4 mg. Low calorie diets and increased energy expenditure were indicated. No supplements were administered to the control group (CG), only a modified life-style and diet were carried out. After 12 weeks (t2), blood pressure, waist circumference, weight and BMI, glycaemic and lipid profiles, and thyroid pattern were re-evaluated.

Analysis of the biomarker HMGB1 was performed only in the TG before and after treatment

with supplements. Sera for the analysis of HMGB1 levels in the TG were collected from peripheral blood after being centrifuged for 15 min (4 C) at 4000 rpm, at the end of treatment. Serum was aliquoted and stored at 20 C. HMGB1 concentration was measured by performing an enzyme-linked immunosorbent assay using an HMGB1 ELISA Kit II (IBL by Shino-Test Corporation, Kanagawa, Japan. Range: 0–80 ng/ml; limit detection 0.2 ng/mL±2.6 SD for high sensitive range; limit of quantification:0.1 ng/mL with 20% for high sensitive range). The samples were diluted 1:5 and added to microtiter plates, which were then incubated for 24 h at 37 C. After washing, 100 mL/well of antihuman HMGB1 peroxidase conjugated monoclonal antibody were added and the plates were incubated at room temperature for 2 h. After washing, 3.30,5.50-tetramethylbenzidine was added to each well. The enzyme reaction was allowed to proceed for 30 min at room temperature. The chromogenic substrate reaction was stopped by the addition of stop solution (0.35 mol/L sulphuric acid) and the absorbance of each well was determined on a microplate reader (EIA) at 450 nm. The HMGB1 concentrations were deduced from a standard curve, with a lower limit of detection of 0.2 ng/mL.

Statistical Analysis

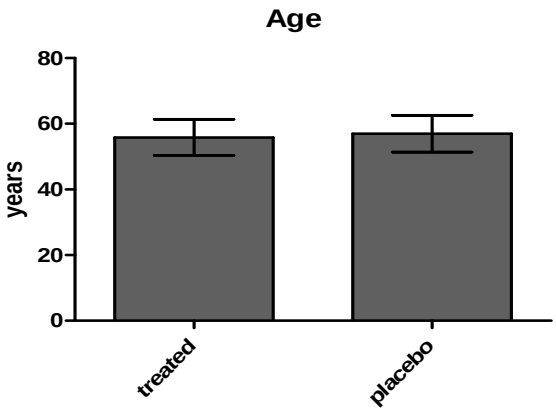
Descriptive statistical analyses were performed to evaluate basal demographic and clinical characteristics. All results were expressed as mean with standard error mean (SEM) for continuous variables. All variables were evaluated at basal time and after 12 weeks of treatment with the Wilcoxon test for non parametric values, and absolute values were evaluated in all subjects to verify differences between the groups using ANOVA after a log 10 transformation. A p value of 0.05 or less was considered statistically significant. All

analyses were performed using SAS software, version 9.1 (SAS Institute, Inc., Cary, NC, USA).

Results

A total of 56 women represented the study group and were randomly divided into two equal groups: the treated group (TG) and the control group (CG), made up of 28 participants. Two patients, from the initial 58, were excluded from the study as they did not undergo check-up after 12 weeks.

The two groups were homogenous for anagraphic characteristics: the treated and the control groups were comparable for age at enrolment (55.8 years +/- 5.5 and 56.9 +/- 5.6 years (**fig.1**). The time lapse from the last menstruation was 81.4 +/- 61 months in the TG and 95.6 +/-66.7 months in the CG (**fig.2**). No statistically significant differences were found in the two groups regarding anthropometric variables, such as BMI and waist circumference (**fig 3-4**).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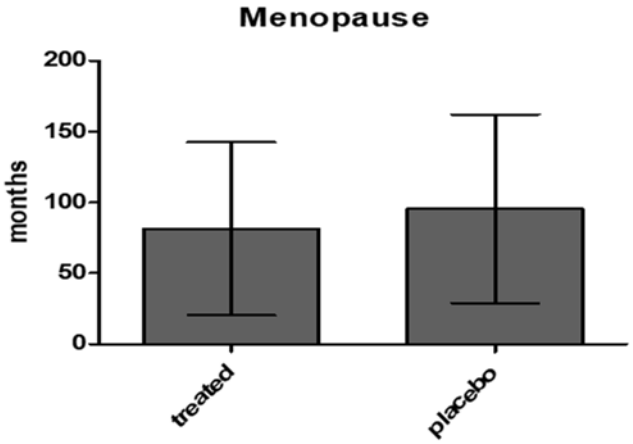
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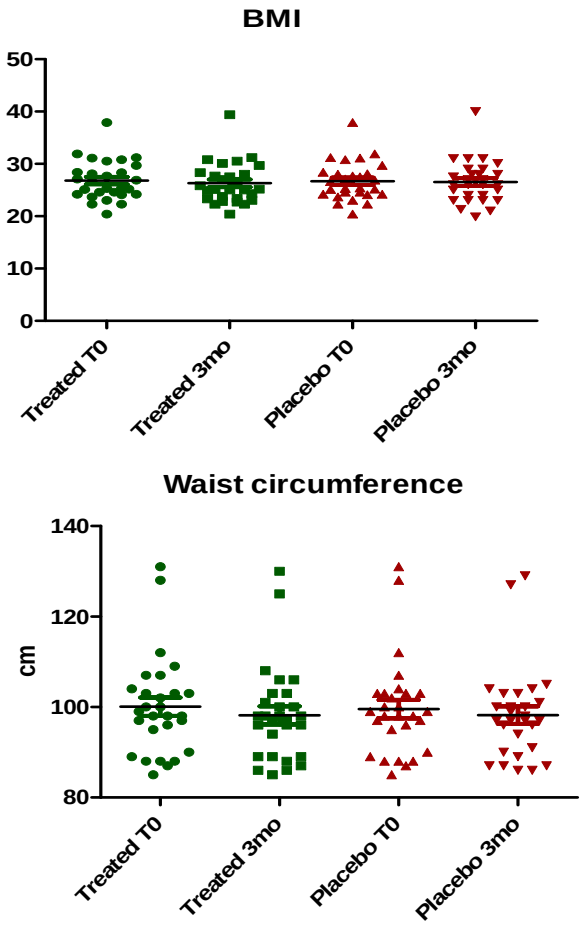
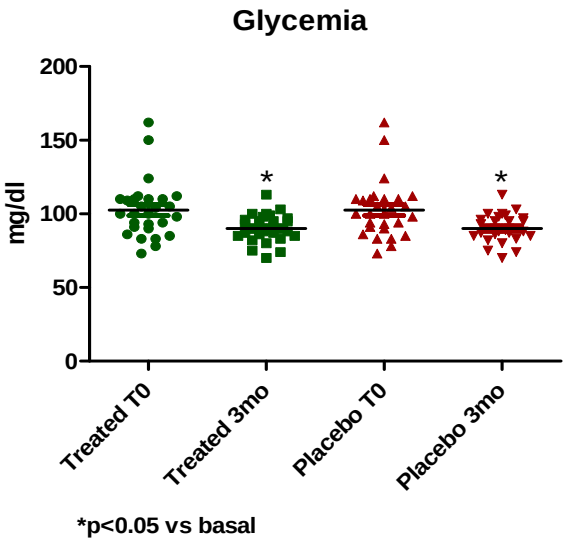
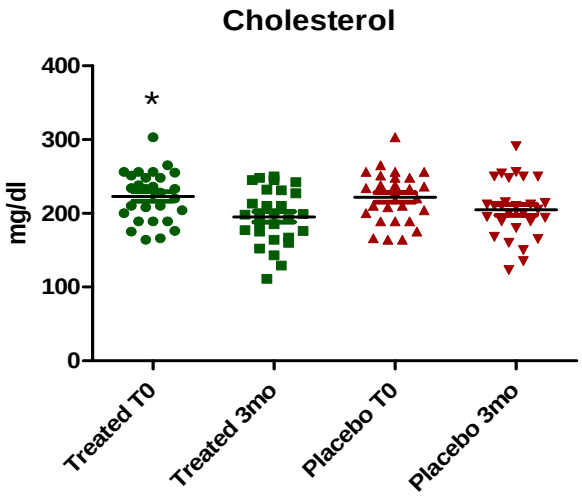


In the comparison between the two groups, statistical significance was achieved for different metabolic variables in TG; the metabolic parameters glycaemia (**fig.5**), total cholesterol (fig.6) and HDL–cholesterol (**fig.7**) were positively influenced by supplementation with nutraceuticals. A statistically significant decrease was noted for glycaemia and total cholesterol values in the TG ($p < 0.05$), while a statistically significant increase was found for HDL-c in the same group ($p < 0.0005$).



*p<0.05 vs basal



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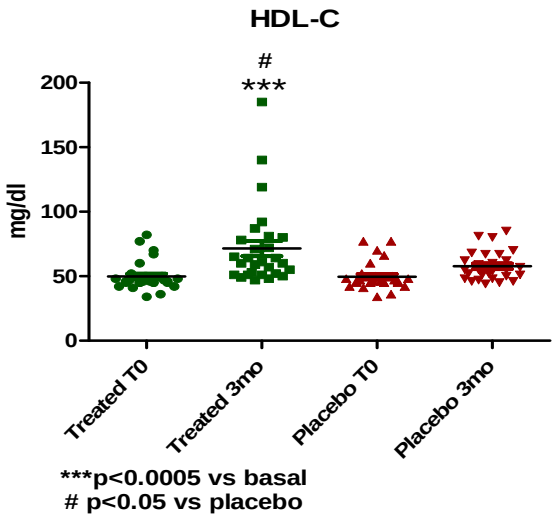
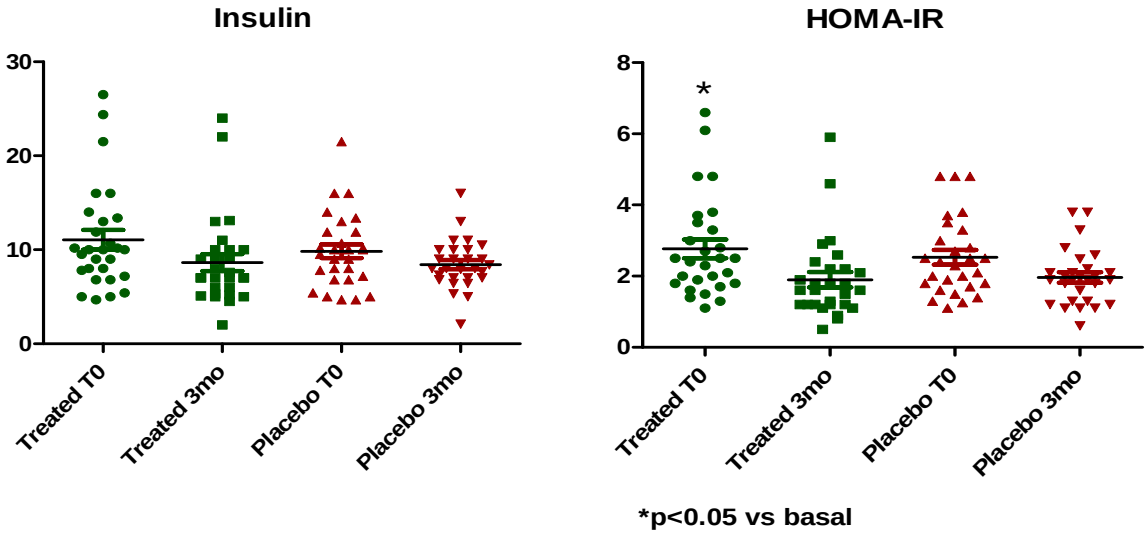


No statistically significant variations were recorded in the two groups for triglyceride levels; on the contrary, a significant reduction of total cholesterol was seen in TG after supplementation ($p < 0.05$). A decrease of insulin was seen in TG, but this reduction did not reach statistical significance (**fig.8**). The positive influence of the administration of these compounds on metabolic profile was highlighted by the statistically significant reduction of insulin-resistance evaluated as HOMA-INDEX ($p < 0.05$) and this could represent the most significant finding of the study (**fig.9**).



No variations were evidenced in TG regarding thyroid and blood pressure values. No adverse effect correlating with the administration of these natural compounds was recorded during the study: with regard to GA, the dosage used did not trigger noticeable changes in the serum electrolyte and angiotensin II levels of morphology of the liver and kidney, signifying no toxicity to the hepatic and renal tissues at such a dosage.

Table 1: General characteristics and outcomes

	suppl t0	placebo t0	suppl t3	placebo t3	p1	p2	p3	p4
Age	55.8 ± 5.5	56.9 ± 5.6						
MaM	81.4 ± 61	95.6 ± 66.7						
BMI	26.8 ± 3.7	26.7 ± 3.7	26.3 ± 3.9	26.9 ± 3.7	ns	ns	ns	ns
WC	100.1 ± 10.9	99.6 ± 10.7	98.2 ± 10.5	98.2 ± 10.3	ns	ns	ns	ns
Glucose	102.5 ± 19.3	104.9 ± 22.3	90.1 ± 9.4	97.0 ± 14.2	ns	ns	< 0.05	< 0.05
Insulin	11.1 ± 5.6	9.8 ± 3.9	8.6 ± 4.8	8.4 ± 2.6	ns	ns	ns	ns
HOMA	2.8 ± 1.4	2.5 ± 1.1	1.9 ± 1.1	1.9 ± 0.8	ns	ns	< 0.05	ns
Triglyc	171.0 ± 58.4	169.6 ± 59	134.4 ± 48.7	149.4 ± 57.3	ns	ns	ns	ns
Tot chol	222.8 ± 34.1	221.8 ± 34.3	195.3 ± 37.4	204.8 ± 39.3	ns	ns	< 0.05	ns
HDL chol	49.8 ± 11.3	49.6 ± 10.7	71.5 ± 31	57.7 ± 11.3	ns	<0.05	<0.0005	ns
TSH	1.1 ± 0.6	1.2 ± 0.6	1.4 ± 0.8	1.5 ± 0.8	ns	ns	ns	ns
T3	3.6 ± 1.3	3.3 ± 0.7	3.7 ± 1.4	3.3 ± 0.9	ns	ns	ns	ns
T4	1.2 ± 0.6	1.2 ± 0.6	1.1 ± 0.5	1.1 ± 0.5	ns	ns	ns	ns
AbTG	44.2 ± 45.5	28.0 ± 40.2	45.5 ± 91.2	30.1 ± 43	ns	ns	ns	ns
AbTPO	8.3 ± 9.1	9.2 ± 10	6.2 ± 4.6	6.5 ± 5.2	ns	ns	ns	ns

MaM = Months after menopause

P1: supplement t0 vs placebo t0

P2: supplement t3 vs placebo t3

P3: supplement t0 vs t3

P4: placebo t0 vs t3

With regard to HMGB1 protein, matching serum HMGB1 levels in TG before and after treatment, data evidenced significantly lower levels after treatment (4.6 ± 2.2 vs 2.6 ± 1.3 ng/ml, $p < 0.001$).

Discussion

Metabolic syndrome has developed into a worldwide epidemic. Ironically, this dramatic increase can be attributed to rapid economic development and correlated to changes in lifestyle within the last 50 years. High calorie food and a sedentary life-style are major causes for obesity that contribute to insulin-resistance and type 2 diabetes. Insulin resistance is defined as defective insulin signalling and a decreased insulin efficiency in inducing glucose transport from the blood into key target cells, such as muscle and fat cells[5]. Menopause is a risk factor for cardiometabolic diseases, including MS, type 2 diabetes, and cardiovascular diseases. The prevalence of MS in post-menopause is due to loss of the protective role of oestrogens and increased circulating androgens resulting in changes to body fat distribution and development of abdominal obesity. Excessive visceral adipose tissue plays an important role due to synthesis and secretion of bioactive substances such as adipocytokines, proinflammatory cytokines, reactive oxygen species, prothrombotic and vasoconstrictor factors. MS may also impact risk assessment of breast cancer, osteoporosis, chronic kidney

disease and quality of life during menopausal transition[23]. In the past 10 years, many studies regarding natural compounds with metabolic effects have attracted significant scientific attention. For the first time, our study proposes an association of three natural compounds as a therapeutic approach for MS in a peculiar target of patients: women in post-menopause. Our study underlines the importance of a modified life style and this was strongly recommended in both groups. The supplementation of the natural compounds containing corosolid acid, glycyrrhizic acid and procyanidin may in fact only alleviate high calorie diet-induced glucose and lipid metabolic dysregulations. Our results show that the association of these natural compounds determines metabolic effects, ameliorating some characteristics of MS, with benefits achieved only after 12 weeks of treatment. The significant reduction of glycaemia values and total cholesterol and the increase in HDL-cholesterol could be considered important targets even if no case of a resolution of MS was recorded. These supplements led to increased insulin sensitivity as evidenced by a statistically significant reduction of HOMA INDEX. The short period of treatment could be considered the reason for lack in resolution of MS; perhaps a longer therapy could modify these metabolic parameters more. Data regarding the absence of changes in blood pressure and thyroid function highlight that the association of these supplements could also be utilized as a therapeutic approach in women suffering from hypertension and thyroid diseases without causing adverse events.

Increased diagnosis of MS has stimulated the exploration of new laboratory tests for early detection and therapies of the syndrome: the statistically significant reduction of HMGB1 values in patients in the treated group suggest that these natural compounds may decrease the antioxidant status characteristic of MS, procrastinating the onset of complications such as cardiovascular diseases and diabetes. HMGB1 could be considered a new, effective tool for monitoring metabolic profile in MS. The association of corosolic acid, glycyrrhizic acid

and procyanidin may actually represent an alternative choice to antidiabetic drugs for improving glucose uptake. Commonly used antidiabetic drugs up-regulate both glucose transport and lipid biosynthesis in adipocytes: weight gain is a frequent side effect, damaging the psycophysical wellness of women in menopause; therefore, drugs with glucose-lowering activity while lacking adipogenic activity are highly desirable[24]. To our knowledge, this is the first study which has analysed an alternative therapeutic approach in MS in menopause. Our data highlight the effectiveness of these ancient supplements with insulin-like and anti-adipogenesis, antioxidant and anti-inflammatory properties, to evaluate a possible application in medical practice. The combination of glucose uptake and anti-adipogenesis activity is not found in currently used insulin mimetic drugs and may indicate a good therapeutic potential.

Further studies appear necessary in this field, and a larger study sample is required to confirm our results.

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