Study Title: Long term treatment of overweight and obesity with polyglucosamine (PG L112), randomized study versus placebo in subjects following caloric restriction

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Abstract

Background

Short-term treatment of overweight and obesity with polyglucosamine (PG) was found to be more effective than placebo and orlistat in double-blind clinical studies.

Objective

To compare the efficacy of long-term (12 months) treatment for weight loss with PG and placebo (PL).

Methods

A double-blind randomized study in 100 subjects of both sexes with a BMI> 30 < 35: one group of fifty cases was treated for one year with PG at 1.6 g/day and a similar group with PL. PG is a combination of low molecular weight chitosan with organic acids. Subjects were instructed to reduce their caloric intake by 10 % and increase the physical activity level by 9 MET-hr/week. Dietary compliance was checked every three months using a weekly questionnaire (FIA: Food Intake Assessment) based on 25 different food servings. Body weight (BW), waist circumference (WC), blood pressure (BP), glucose, lipids, and hs-CRP were also monitored.

Results

Ninety-seven subjects completed the study (49 PG; 48 PL). The decrease in calories was similar in both groups as was the change in food servings (Anova $p > 0.05$). The BW and WC decreases were 8.0 kg and 10.2 cm respectively in the PL group, whereas they were 12.1 kg and 13.3 cm in the PG group (Anova $p < 0.001$). The decrease in BP, plasma lipids, glucose, and hs-CRP was more evident in the group treated with PG (Anova $p< 0.05$). The lipids intake was found to correlate directly with hs-CRP except for the extra virgin olive oil. In conclusion, PG was found to be more effective than PL in reducing BW, AC, glucose, BP, plasma lipids and hs-CRP in moderately obese subjects undergoing a 10 % caloric reduction

and slight increase of physical activity. Dietary monitoring using FIA was an effective tool in supporting dietary compliance.

The trial was also formally registered at ClinicalTrial.Gov with secondary IDs number U111111292405 [WHO] and retrospectively registered.

Keywords: polyglucosamine, PG L 112, overweight, obesity

Background

Excess weight and obesity are two of the biggest public health problems of the 21st century even though Medical Associations have been warning for some time that these conditions are becoming epidemic and often associated with many comorbidities such as cardiovascular diseases and cancer.

In 2014, over 1.8 billion adults were overweight [(1)] and about 3.4 million die each year as a result of this condition which is worsened by the fact that the necessary pharmacotherapy has many safety and efficacy limitations.

The decade-old statement that "obesity is now on everyone's plate"[(2)] is still highly relevant even though an oft-forgotten truth. The financial implication of excess weight is a real concern since it is not confined to developed countries any more. The economic impact of obesity epidemic is global and is now rapidly penetrating the world's poorest nations.

Physicians usually warn their patients about the risks that excess weight and obesity pose to their health. These problems can usually be tackled by reducing caloric intake, but what has

53 become a serious issue is the impact of food-related television advertisements (accounting
54 for about 75% of all TV commercials).
55 Nutritionists and dieticians know that food addiction is one of the hardest to quit. However,
56 the problem of overeating can be resolved with effective integrated tools. One of these is to
57 make the best eating choices by eating the right amount of high-quality food, and another is
58 the use of substances that prevent a certain amount of the ingested calories from being
59 absorbed. The pharmacological treatment is also an important option and to date there are
60 agents that were shown effective in the long-term treatment (1 year) allowing the
61 maintenance of at least 5 % reduction in body weight [(3)].
62 However, the problem of adverse reactions associated with the use of drugs still remains a
63 major concern. They are becoming increasingly popular for it is generally believed to have
64 fewer side effects [(4)].
65 One of the many treatments that has been proposed contains chitosan which is among the
66 most abundant polysaccharides found in insects, fungi, squid, oysters, krill, clams, and
67 shellfish.
68 It is a natural N-deacetylated derivative of chitin [(5)] which interacts with hydrophobic
69 compounds such as cholesterol, triglycerides, fatty acids and bile acids reducing their
70 absorption or reentry into the mucosal cells of animals and man.
71 In a review considering the clinical trials conducted up to 2008 [(6)], the authors' conclusion
72 was that despite some evidence that chitosan is more effective than placebo in the short-
73 term treatment of overweight and obesity, the poor quality of the trials indicate that the effect
74 of chitosan is minimal and unlikely to be of clinical significance.
75 However, there was one aspect not taken into consideration in the analysis of these trials
76 which is relative to the molecular weight (MW) of chitosan. It was not reported that the
77 activity in fat binding depends on this variable since high MW polymers tend to have a lower
78 fat binding affinity [(7)].
79 Recently, a new product that belongs to the class of Medical Devices, polyglucosamine (PG)
80 has been shown to have a very efficient fat binding capacity [(8)] without causing steatorrhea

[9,10]. PG is a low molecular weight chitosan (LMWC) used in fixed combinations with ascorbic and tartaric acids. Pharmacological studies have shown that PG reduces body weight, increases the glucose elimination in faeces [10], and the concomitant oral administration of [9-^{14}C] oleic acids in mini pigs has been found to lead to a consistent decrease in faecal fat content [11]. Gut bacteria seems to use the fats bound to PG and released in the large intestine as a source of energy, thus helping to restrict caloric intake and reduce steatorrhea[9,10].

In short-term double blind trials (between 12 and 24 weeks) designed to compare this product with placebo [12] or orlistat[13], PG was found to be very safe and significantly more reliable than placebo or orlistat in reducing BW, BMI, and waist circumference (WC) when used in combination with caloric restriction (500 cal/day) and increased physical activity (3 to 7 MET-h/week). The 5% decrease in body weight was achieved in a much shorter time than with placebo.

The aim of this trial is to investigate long-term treatment with PG (twelve months) and determine if the improved outcomes obtained in previous trials are confirmed.

As in every trial related to body weight reduction, compliance with caloric restriction was one of the most relevant aspects, since subjects tend to abandon the diet when the restriction is too drastic. For this reason, monitoring the subjects' diets and their continuity was considered fundamental, and the subjects were given dietary counselling to help change their eating habits permanently.

Material and Methods

Study design

This study was conducted at a single centre as a randomised, double-blind, placebo-controlled trial. The study was designed and implemented according to UNI EN ISO 14155:2012 and to the STROBE checklist [14] in conformity with the guidelines laid down in the Declaration of Helsinki and Good Clinical Practice (GCP). The Italian Personal Data

Protection Code and other applicable laws, regulations, mandatory standards and recommendations were also taken into consideration.

The patients were split randomly into two groups. In addition to a 10% calorie restriction and an increase in physical activity (9 MET-h/week), one group received treatment (PG) and the other, placebo (PL): 2 x 2 tablets before the two main meals for twelve months. All the procedures were approved by the Ethics Committee of the Rende Municipality: approval N14 according to section 48 of Italian legislative decree 267/2000 of 28 January 2010. The experiment was conducted on subjects who lived in the geographical triangle between Rende, Rovito and San Lucido during the MAP (Monitoraggio Alimenti e Patologie) study. The study was conducted between May 2014 and October 2016, and enrolment took three months. The trial was also formally registered at ClinicalTrial.Gov with ID number NCT02682277.

There was no need to make any important changes to the methods after the trial commenced.

Participants

187 subjects were analysed.

The inclusion criteria were as follows:

1. Aged between 25 and 65 years
2. BMI ranges > 30 to <35
3. Able to complete the FIA (Food Intake Assessment) questionnaire correctly

Only a hundred subjects were included in the trial: 50 men and 50 women.

The exclusion criteria were:

1. Inability to complete the FIA questionnaire and comply with the trial protocol criteria
2. Pregnancy or breast-feeding
3. Treatments for body weight reduction or metabolic syndrome
4. Alcohol abuse, drug abuse or drug addiction
5. Cancer diseases or malignant tumours
6. Known hypersensitivity reactions to crustaceans or any of the ingredients in the products

7. Pre-existence of chronic intestinal disease, such as constipation requiring medical treatment
8. Post-operative state after gastrointestinal surgery
9. Metabolic disorders or chronic malabsorption disorder
10. Subjects taking medications that decrease intestinal motility, such as opiates
11. Long-term use of medications, with the exception of antihypertensive drugs.

Patients undergoing treatment for hypertension were admitted, provided they continued taking the same type of medication during the trial.

Since the treatment continued for twelve months, any interruption of more than four weeks, pregnancy or missing more than two examinations were considered sufficient to exclude the case from the final assessment. If acute diseases occurred, all types of treatments were allowed.

The investigators informed the patients about the trial both verbally and in writing. A written informed consent was obtained on a form signed by both the trial participant and the investigators themselves. The patients were insured as specified on the consent form.

The FIA (Food Intake Assessment) questionnaire and dietary suggestions

The FIA is a typical FFQ (Food Frequency Questionnaire) which considers all the available foods registered by the INRAN (Istituto Nazionale Nutrizione Alimenti). The INRAN lists foods in term of their caloric content and all other food-related variables (e.g. carbohydrates, lipids, protein, water, vitamins and trace elements). The INRAN tables contain almost all the foods available in Italy[15].

The FIA consists of a questionnaire with 250 of the most common foods, which the subject had to complete on a daily basis for seven consecutive days. An extended version of the FIA was used for this trial: the number of food servings was increased from the usual 9[16] to 25. The amount of food had to be reported in grams and was subsequently transformed using an algorithm into “average portions” according to the INRAN tables.

All the foods were divided into 25 different food servings (see Table 2). For example, milk, eggs and cheese were counted as three different food servings and were not simply defined all together as dairy.

The first course was treated as a separate category, as it involved the preparation and cooking of food (e.g. pasta, together with a sauce made of sausages, pulses, vegetables, oil/butter, and cheese or other dressings).

Before the baseline assessment, all subjects were given instructions on how to complete the questionnaire, and those admitted to the trial were taught how to choose alternative foods with lower calorie content. A nutritionist was on hand for consultation during the baseline assessment and during the whole trial.

The subjects decided mostly for themselves which types of foods to replace and also plan their calorie restriction since the nutritionist only gave suggestions. In this way, the subjects were responsible for their own dietary change, and they were also given relevant information about common foods/servings with low calorie content. The main advantage of this method was that it was possible to analyse the ~~activity~~ efficacy?? of the product under trial (PG or PL) and - at the same time - limit any bias linked with dietary compliance. We knew from our previous experiment in the same geographical area that nine types of food servings cover more than 75% of weekly calorie intake: first course, biscuits (during breakfast and/or during the day), bread, cheese, vegetables, eggs, spirits, meat and processed meat. Apart from vegetables and eggs, the nutritionist made an effort to convince the subjects to reduce and/or replace the food servings which mainly contain carbohydrates or fats.

As regards the modifications made in the first course, the approach was to halve the quantity of pasta (e.g. from 100 to 50 g) and prepare the serving with three times the amount of the usual vegetables (from 20 to 60 g of any given boiled vegetable). In this way, it is possible to maintain more or less the same volume, but the calorie content can be lower by as much as 40%.

Cutting down the consumption of biscuits (croissants and rusks belong to the same category) was a more complex issue since they are part of the typical Italian breakfast,

191 together with milk, coffee, orange juice and jam. It was suggested to replace them with
192 omelettes made with one egg and 10 g of any jam. In this way, the volume was almost
193 exactly the same or even higher, but the caloric intake was about 30% lower. To decrease
194 cheese intake, the suggestion was again to make omelettes using one egg and 20g of any
195 cheese. On the whole, the volume of the serving increased, whereas about 30% fewer
196 calories were consumed.
197 The participants were advised to reduce the total amount of spirits consumed and/or replace
198 drinks containing 42-45% of alcohol with the same volume of those containing less than 30%
199 (e.g. limoncello, mirto, various amaros, dry or sweet marsala).
200 Recommendations were also made to cut down on pizza calories: cut the crust off the pizza
201 (corresponding to a quarter of the serving), which is usually the most burnt part. An Italian
202 pizza weighs about 325 g, and provides about 880 kcal. With the proposed suggestion, the
203 serving can be reduced to about 660 kcal.
204 On top of that the subjects were given a list of alternative recipes, particularly for first
205 courses, to help reduce the quantity of pasta eaten and increase the amount of vegetables
206 and pulses in the servings. In this way, it was also possible to minimise the amount of olive
207 oil and bread eaten during meals.
208 At the end, the FIA provided a kind of "dietary fingerprint", where the amounts in grams of
209 the 25 categories were transformed into average food servings.
210 For this reason, it was very important for the subjects to be able to complete the FIA and
211 correctly report the weight of the foods, so that they could be transformed into the number of
212 average weekly servings.
213 Before starting the treatment, all the data recorded by the subjects were checked twice. The
214 first time was after the training??? to allow them to become familiar with the questionnaire,
215 and the second time at the baseline examination. A data recording precision cut off value
216 was set as an admission criterion, based on the Mifflin-St. Jeor equation (MSJ). Only
217 subjects who recorded values with an MSJ score of at least 90% x 1.2 were admitted. The
218 1.2 factor was arbitrarily chosen to reflect minimal daily activity.

The data recorded by the subjects were inconsistent in about 13% of cases (25/187) because the calculated caloric intake was much lower than the quantity needed to maintain their metabolic rate at their given weight, sex and age.

Physical activity

Physical activity was measured using a simple questionnaire on daily activity, which was transformed into MET-h/day. Sedentariness (< 35 MET-h/day) was very frequent, and the subjects were asked to spend just one hour brisk walking every day, divided into four sessions to fit their daily activity. Considering that 1 h of slow walking corresponds to 2 MET-h and 1 h of brisk walking corresponds to 3.3 MET-h, the proposed increase in physical activity was equivalent to approximately 9 MET h/week. The time they spent walking was reported (in hours) for one specific week every three months, usually the week before their dietary check, and in the week before the end of the trial. Additional physical activity was not required, and if there had been any, it had to be reported together with their brisk walking time.

Other variables

Body weight (BW), waist circumference (WC), blood pressure (BP min, BP max), plasma lipids, glucose and hs-CRP were measured at baseline and after every three months (at 3, 6, 9 and 12 months).

BW was measured with the subjects wearing light clothing and no shoes after evacuation of urine and faeces and twelve hour-overnight fasting. If possible, the measurement was taken before blood sampling for laboratory analysis. A Tecnilab 2 scale with 50-gram accuracy was used. If the subject suffered from temporary constipation, the BW check was postponed until the problem had been solved with increased water intake. Waist circumference (WC) was measured at the umbilical line by two different investigators, and the average value was recorded.

BP was measured in both arms after the subject had been sitting for at least ten minutes using an A&D UA-851 digital blood pressure automated monitor, and the average value of the two measurements was recorded.

Plasma sampling for total cholesterol, LDL, HDL, glucose and hs-CRP was done after 12-hour overnight fasting and just after the BW measurement. A sample of fifteen mL of blood was obtained and divided into 3 aliquots of 5 mL each. All the samples prepared for analysis were kept at 4°C until the analysis was carried out. The laboratory tests were performed with a Beckman Coulter AU 500 analyser (Beckman Coulter, Brea, California USA).

Procedures - Treatment regimens and dosing schedule

The following products were used: polyglucosamine (formoline® L112, manufactured by Certmedica International GmbH, Aschaffenburg, Germany); this group was referred to as the PG group. The other group received a placebo consisting of excipients and Arabic gum as tablets that were identical to those of the PG group and was referred to as the PL group. Each patient took two tablets a day with the two meals containing the highest fat content, which meant four tablets with 400 mg strength of polyglucosamine PG L112 per tablet.

If the patients were getting other treatments that consisted of lipophilic medications, they were asked to take them at least two hours apart.

The trial medications provided by the manufacturer were packaged in identical blister packs. A double label with the randomisation code was affixed to the blister packs by an independent, certified service provider. One of the labels was attached to the patient's case report form by the investigator at trial admission.

Treatment started immediately after admission to the trial. The subjects were given three packs containing 48 tablets each, sufficient for twelve days of treatment. They were asked to return for more at the end of each month.

Each subject was told that they could stop taking part in the trial at any time, without giving any reason, and without any negative consequences.

They were asked to report adverse events or reactions at each examination, and their comments were recorded.

After enrolment, four examinations were performed at 3, 6, 9 and 12 months, and the FIA was carried out at baseline and 3, 6 and 9 months.

Sample size

The sample size was calculated based on changes in the hs-CRP levels for PL and PG and not changes in BW. The measurements were taken at baseline and 3, 6, 9 and 12 months. We assumed an autocorrelation of the covariance equal to 0.7, a difference of at least 20 % between the two groups, and a difference of at least 15 % during the period of observation. The experiment should also detect an interaction effect of the same dimension of the “factor time”.

For this aim the Geisser-Greenhouse corrected F test will be used.

Considering a baseline value of hs-CRP equal to 5 with a SD of 0.12, a 95% power with 0.05, α level of significance will be obtained by enrolling fifty subjects per treatment group. In case of 25% dropout rate, forty subjects will allow a potency of at least 80 %.

Randomisation

The randomisation list was prepared by using JMP software (SAS Institute) before enrolment started and sent directly to a certified clinical trial logistics company for the final packaging of the samples. The two products were assigned to consecutive patients in chronological order of enrolment. No randomisation number was omitted. Once a randomisation number was assigned, it could not be reassigned, even if the subject could not actually take part in the clinical trial. In this way, the trial was guaranteed to be blind throughout the entire study period.

Blinding

The participants were not aware of the treatment group they belonged to. The following people or groups were also blind: investigators, staff (nutritionists and technical staff), laboratory workers, sponsors and biostatisticians.

Statistical methods

The procedure used involved a mixed analysis of variance (*split-plot design, or between-within subjects Anova)*. The between factor was the two groups compared (PL and PG), while the within factor was the four or five examinations (baseline, 3, 6, 9 and 12 months). The procedure was not only correct methodologically (simultaneous analysis of data for each variable), but also provided detailed information and considerably reduced the uncontrolled variability of the responses, which led to greater sensitivity or power of the analysis itself. Tukey Test was applied to determine differences between baseline and 12 months, and also between products. Correlation coefficients were calculated between Hs-CRP and main components of food (protein, carbohydrates, lipids, sugars).

The hs-CRP variable was analysed using a multiple linear regression model or standard least square model in order to look for hs-CRP predictors.

Other model information was obtained by graphical estimation, and a prediction profiler was used to examine the response surface. These options were chosen because several effects were analysed using few observations, and the aim was to find a strong effect, rather than test for significance. The Chi-square or Fisher's Chi-square tests were used for frequency analysis.

Compliance

Dietary compliance was measured using an FIA questionnaire, and the treatment was checked every month by counting the remaining tablets. Physical activity was measured on the basis of the hours of brisk walking the subjects reported during the test week (the week before the FIA).

Results

In total the cohort of subjects consisted of 187 cases, and 87 subjects were excluded; 62 of them because their BMI was not in the range of the admission criteria, and 25 because they were found not reliable for the FIA compilation since were reporting a calorie intake at least 15 % less than the value based on of MSJ equation. Out of the 100 subjects admitted, only three participants dropped out (two in the PL group and one in the PG group), because they moved house. A total of 97 subjects completed the experiment: 50 males and 47 females (see Figure 1).

The general characteristics of the subjects are reported in Table 1.
No differences were found between the groups in terms of age distribution (Chi square = 0.2240), hypertension (Chi square = 0.749), smoking (Chi square = 0.7666), education (Chi square = 0.749) and physical activity (Chi square = 1.000).

Table 1

The treatments were well tolerated and no complaints of side effects were reported, apart from a few cases of constipation that were equally distributed between the two groups. This problem was solved by advising the patients to increase fluid intake.

The total calorie intake and main components of the food servings were recorded at baseline and at 3, 6 and 9 months. Since the data between the individual intermediate examinations did not change significantly, only the averages of the three examinations (average over twelve months) are shown in Table 2.

Table 2.

The weekly reduction in caloric intake was almost identical in both groups and slightly exceeded 10%. This was the aim of the diet. The average decrease in calories/week was 1667 kcal for PG and 1688 kcal for PL. This was mainly due to a decrease in carbohydrate intake (above 860 kcal/week for PG and PL), followed by lipids, alcohol and proteins, where the reduction in both groups ranged between 240 and 300 kcal/week. ???? The change in the main dietary components was significant within the groups ($p<0.05$) and very similar for both groups ($p>0.05$).

The decrease in protein consumption was almost identical in the two groups (49 g/week for PG and 50 g/week for PL) consisting of 7.5% and 7.8 % reduction respectively.

The reduction in both carbohydrate and alcohol intake were the most substantial in terms of percentage; carbohydrate intake was reduced to 221 and 215 g/week for PG and PL respectively, whereas the alcohol intake was 43 and 37 g/week respectively. In both cases the difference between treatments was not statistically significant ($p > 0.05$).

The water content in food was reduced to 499 mL in PG and 586 mL in PL, but again the difference between treatments was not statistically significant ($p> 0.05$).

Extra virgin olive oil (EXVO) was considered separately, but the difference at baseline was not statistically significant for either of the groups ($p>0.05$). Its intake during the trial in terms of calories increased slightly in both groups, but again the values were not significant ($p> 0.05$).

The drop in lipid intake was more consistent for PL than for PG (33 g/week and 27 g/week respectively) due to decreased cheese, processed meat and milk consumption (see Table 3) but the differences were not significant ($p>0.05$).

Fibre intake was also lower in both groups. However, this was compensated for in the PG treated group by the administration of 11.2 g of PG/week, as polyglucosamine is a polycation fibre.

The number of portions before and during the treatment is reported in Table 3.

Table 3

The "dietary fingerprint" of the two groups was not identical, however, looking at the overall diet these differences may be considered as marginal or in the range of normal variability. The differences between treatments were not shown to be statistically significant ($p>0.05$). Among the 25 servings, despite some modification in terms of percentages, 8 were found to be not significantly modified in both of the two groups: chocolate, dry fruit, pulses, meat, fish, yogurt, beverages and chips. Some of the servings (wine and beer) were reduced more consistently in the PL group, whereas there was a higher reduction in the servings of cake, ice cream, mozzarella and fruit in the PG group. However, with regard to all these servings, the results were found to be statistically non-significant ($p >0.05$) between both treatment groups.

Six food servings accounted for about 75% of the decrease in calories in both groups: bread, pizza, first course, cheese, biscuits and spirits. There was a significant decrease in the intake of other food servings (sugar, milk, processed meat, wine, beverages and cake), but they had a much smaller impact on the total weekly calorie count. Two food servings increased slightly but significantly: vegetables and eggs.

In the case of vegetables, the suggestion to cut back on pasta by adding more vegetables to the dish was incorporated by many participants. As regards eggs, the idea of making omelettes was also well received in many cases and was in line with the reduction in carbohydrates (at breakfast) and cheese consumption (during the main meals).

The primary variables changed in both groups. However, their change was significantly more substantial in the PG group (see Table 4). In the same table are reported the data pertaining to physical activity in terms of average MET-h /day.

Table 4.

The decrease in body weight with PG was 12.1 kg (-12.7%) compared to 8.0 kg (-8.4%) with PL ($p<0.05$). The BW change with PG was also more rapid ($p<0.05$), since the weight loss in the first six months was 8.9 kg compared to 5.6 kg in the PL group. The decrease was less evident in both groups (3.2 kg for PG and 2.4 kg for PL) in the second half of the experiment (6-12 months). However, the decrease in body weight in the PG group was again significantly more substantial ($p<0.05$ Tukey's test).

Only seventeen percent (8/49) of patients in the PL group had achieved 5R (5% body weight reduction) at three months, whereas 55% (27/49) were successful in the PG group; the difference was statistically significant (Chi square =16.04; $p<0.0001$). After six months, the percentages were 67% and 98% respectively (Chi square = 16.43; $p<0.0001$).

The reduction in BMI was similar to the BW drop and statistically significant ($p<0.05$) for both treatments. In the first six months, weight loss in the PG group was -3 kg/m^2, followed by a slower weight loss rate which reached -4.3 kg/m^2 after twelve months. The drop in BMI was significantly lower in the PL group ($p<0.05$), and was marked by a flatter curve, which reached a decrease of only -2.8 kg/m^2 in twelve months.

The change WC reached -13.3 cm with PG and -10.2 cm in the PL group ($p<0.05$). In both cases, the most rapid decrease was recorded during the first six months.

The secondary variables also showed progressive improvement, and again the results in subjects treated with PG were better than in the PL group (see Table 5).

The physical activity was equally increased to 1.5 MET-H/day in both groups.

Table 5

All the variables were improved ($p<0.05$ Tukey's test) in both treatment groups, apart from HDL, where the increase was only significant for PG. Better results were obtained with PG ($p<0.05$) for TCh and hs-CRP from the third month, for TG from the sixth month, and for LDL from the ninth month onwards.

The minimum and maximum BP in the PL group decreased significantly (-5.1% and -5.8% respectively; $p<0.05$ Tukey's test). Although the decreases were -8.8% and -7.8% respectively in the PG group, no significant difference was measured between the two groups ($p>0.05$ Tukey's test).

As concerns plasma lipids, TG levels were the most affected by both treatments: -12.2% for PL and -17.3% for PG. No significant difference was found between the two treatments.

The average values of hs-CRP at baseline were close to 5 mg/L in both groups, and at the end of the year they were within the normal range (2.9 md/L for PL and 2.1 mg/L for PG). However, only 13 out of the 47 cases in the PL group had values within the normal range, compared to 48 out of the 49 cases treated with PG (chi square = 17.82 $p< 0.001$).

Correlations

This aspect was analysed by focusing on hs-CRP as the main variable, firstly considering the baseline data and then pooling the data of the two groups.

A good correlation was found between hs-CRP and total calories ($r^2 = 0.48$; $p<0.0001$), and when single calorie components were considered, protein and carbohydrates were not correlated, whereas lipids were seen to be very well correlated ($r^2 = 0.685$; $p<0.0001$). EXVO was treated separately from the other lipids, and no significant correlation was found ($r^{2=}$ 0.05; $p>0.05$).

Multiple correlation values were considered separately for PG and PL. None of the variables were found to be correlated to hs-CRP with PG. However, lipids again were shown to be directly correlated in the PL group, with the exclusion of EXVO.

Discussion

A strong point of this study was the dietary monitoring, but - at the same time - this was also its weak point, because only those who were actually able to understand the instructions provided to complete the rather complex FIA questionnaire would be selected.

In order to get reliable information about the results achievable with PG, we felt we had to be selective about the cases so as to avoid a large number from dropping out, which is common in this type of long-term research. Investigators who are familiar with this kind of trial know how difficult it is to keep subjects on a long-term diet. In the light of this, dietary compliance was considered a fundamental aspect.

Many food frequency questionnaires (FFQ) are available [16-20] which could have been adapted to the trial. However we chose FIA, since the investigators were more familiar with this kind of data recording [16,21,22].

The diet in this geographical area of South Italy is generally defined as a Mediterranean Diet [23]. However, the population is progressively abandoning the nutritional characteristics of a healthy diet [24], apart from the use of extra virgin olive oil. This means that to ensure the suitability of this FFQ in this study, it has to be adapted to the kind of food and cuisine distinctive of this particular region.

In the FIA system, the “cereals” are divided into various food servings (first course, bread, biscuits and pizza respectively) as they were eaten, and all the other servings (vegetables, pulses, meat and fish) were considered similarly. The validity of FIA compared to other methods is beyond the scope of this trial, but our experience has taught us that advising people to eat less carbohydrates, high-fat food or less cheese without giving precise information on the “final dish” they should eat leads to a high number of dropouts.

Another point that marked this trial was the focus on a decrease in calories per week, while maintaining the usual balance between carbohydrates, lipids and proteins, and in particular a reduced consumption of alcohol. The aim of all this was to make changes in amounts of foods consumed without changing the structure of the diet. This would help to prevent any drastic alteration in their eating habits, because the real objective was not to examine a diet but to demonstrate the effectiveness of PG while minimising the dropout rate as much as possible during the long-term trial.

This is the first time a long-term trial comparing PG with placebo has been conducted, and the results confirm what has been shown in previous short- term trials against placebo or other treatments [12,13,21,22].

Chitosans are a family consisting of a variety of polymers and one would expect their fat binding capacities and glucose, biliary salts and water affinity [19-21] to be different. However, attempting to predict their fat binding activity on the basis of their physicochemical characteristics is controversial [7,26].

Their activity is limited to the gut, since they are not absorbed as a result of their polyanionic character. This does not mean they cannot interfere -indirectly- with the metabolic pathways linked to cholesterol, glucose, triglycerides and other variables, as was shown in this trial.

Blood pressure sank as BW decreased, and the same happened with hs-CRP. The effect on blood pressure of both PL and PG groups was to be expected since this variable is linked to BW decrease. Weight loss was more substantial with PG, and the consequent blood pressure decrease was not linked to any particular mechanism of action of PG.

The change in cholesterol levels is well known and well documented for most chitosans; the European Agency (EFSA) allows chitosans to be advertised for cholesterol control, provided the daily dosage is at least 3 g, no matter what type of chitosan is concerned.

The dosage of PG in this study was much lower (1.6 g/day) and the cholesterol decrease was about 10%, most probably owing to the diet followed at the same time, which is known to decrease cholesterol levels.

The effect on triglycerides was also to be expected since dieting can decrease their levels due to a lower intake of food and alcohol. However, PG also has a triglyceride binding affinity in vitro [20].

The decrease in glucose was to be expected, due to the body weight reduction and also because an increase in the faecal excretion of glucose has been observed in rats fed with normal diet with added PG [10]. A similar change has also been seen in other clinical trials [16,21,22]. The mechanism of this effect is not known, but it may have something to do with the bacterial hydrolysis of short chain chitosan, which PG contains a minimal amount of [27].

519 Hydrolysis makes some glucosamine available. This in turn induces local insulin resistance
520 [28] which prevents some of the glucose from being absorbed by colonic enterocytes.
521 The effect of PG on hs-CRP was also to be expected since it has been shown before
522 (unpublished data), but the relevance of this effect is unknown. In our experiment, it seems
523 that this effect is relevant, because all the cases were out of the normal range before the
524 treatment with PG and only one case was greater than 3 mg/L at the end.
525 The activity of fibre on hs-CRP is controversial. An inverse relationship with fibre intake was
526 described [29] in the Women's Health Initiative Observational Study [30], but this has not been
527 confirmed by the same author. However, hs-CRP levels are considered a good predictor of
528 vascular events [31] and virtually all of the popular diets (Atkins, Ormish, Weight Watchers,
529 and Zone) reduce the C-reactive protein level by approximately 15 to 20% (it was however
530 not significant only in the case of the Zone diet) [32].
531 The subjects treated with placebo in our experiment showed a fairly substantial decrease in
532 hs-CRP levels (-41% compared to baseline values), even though their fibre intake was lower
533 than at baseline. This means that other factors may be important in determining the
534 inflammatory conditions revealed by hs-CRP.
535 Calorie intake was directly correlated with hs-CRP, and particularly with lipid intake,
536 according to the calculations made on the baseline data by pooling the PG and PL groups.
537 Among the lipids, it was interesting to note that EXVO was not responsible for the hs-CRP
538 increase, and this may reveal the importance of oxidized lipids. They are found in foods or
539 created during cooking processes, and may be absorbed by enterocytes to form oxidized
540 chylomicrons capable of spreading oxidation in vessels and tissues [32]. EXVO limits the
541 oxidative process in the gut owing to its antioxidant content. Moreover, PG also has an
542 antioxidant capacity, and one of its characteristics is its particular affinity for oxidised lipids
543 (since they are more polar). This affinity decreases the "explosion" of oxidative stress in the
544 gut.
545 The general conclusion is that PG may protect against inflammatory conditions caused by
546 lipids.

We can make one important observation by considering some comparable clinical trials carried out with PG.

Taking the two previous DB trials with at least three months of treatment at the same dosage as reference, in one trial the average BW loss was 5.6 kg [12] (with an increase in activity of 1 MET-h/day) and in the other 6.2 kg [13] (with an increase of 3 MET-h/day). In this trial, the weight loss in three months is 4.7 kg (with an increase of 1.3 MET-h/day) which is significantly lower. The reason for this discrepancy may be attributed to the degree of caloric restriction imposed: an average of < 250 kcal/day, instead of about 500 kcal/day in the two previous short-term experiments. However, this trial ended up with a BW reduction of 12.1 kg in twelve months, which may indicate that PG favours a substantial amount of weight loss even with relatively mild dietary restrictions.

The PL group lost only 8 kg, and the efficacy of the method that was followed and the time needed to reach satisfactory results is debatable.

According to the MSJ equation and considering a factor of 1.2 for daily activity, the average theoretical need for calories was 2033 ± 251.7 kcal/day at baseline for the PL group, whereas the calculated intake was much higher: 2315 ± 289.4 kcal/day. At the end of the trial, considering an average BW loss of 8.1 kg and a factor of 1.3 for daily activity, the theoretical requirement should have been 2259 ± 277.6 kcal/day, which is higher than the calculated intake of 2080 ± 254.7 kcal/day. This means that there is theoretically still room for further body weight reduction by continuing with the same diet. The same calculation for the PG group also indicates that by continuing with the same protocol, it should be possible to achieve a further BW decrease.

Based upon the present experimental conditions, assuming a linear regression, and considering the BW values between 3 and 12 months (4%) in the PL group, it would be theoretically possible to reach a BMI of 25 in about 4.5 years, whereas with PG this goal could be obtained in about half the time, i.e. after around 2.3 years. The same result is confirmed in the case of quadratic or logarithmic regression.

The only similar long-term experiment that we are aware of compared four different diets (Atkins, Ormish, Weight Watchers, and Zone) for a period of twelve months [33] and followed cohorts of forty subjects very similar to ours in terms of BMI (average between 34 and 35). Comparing diets with a similar daily caloric intake decrease (251 kcal/day for Zone and 244 kcal/day for Weight Watchers) to the one in this trial (244 kcal/day), the decrease in body weight was less than 4 kg, and all the other variables (PA, glucose, lipids, and hs-CRP) were much less affected than in the PL group of our trial. Moreover, the dropout rate was at least 35% of the trial enrolment. The reason for such a high number of dropouts was the subjects' dislike for the diet or their inability to comply. Only 4% abandoned our trial.

Even though comparing trials carried out in different countries has to be done with care, we may observe that our method, consisting of minimal dietary restriction and light physical activity together with an active involvement of the subjects can achieve better results than following common diets with more rigid rules.

Practically all the subjects treated with PL for one year achieved an 8.7 % reduction in BW. These results were not obtained with the common medications used for the treatment of obesity [3], whereas the use of PG consistently accelerated the achievement of this goal.

Conclusions

The treatment with PG for one year, combined with caloric restriction and light physical activity, was found to be significantly more effective than placebo, given the same experimental conditions. The use of the FIA questionnaire based on 25 different types of servings, and the adherence of the subjects to their own level of caloric restriction were found to be extremely important to help minimise the dropout rate.

List of abbreviations

WC = waist circumference; BMI= body mass index; BP =blood pressure; BW= body weight; FIA = food Intake assessment; FFQ = food frequency questionnaire; hs-CRP = high sensitivity C-Reactive Protein; LMWC = Low molecular weight chitosan; MET/ h= metabolic equivalents per hour; MSJ = Miffin St-Jeor equation; PL =placebo; PG =polyglucosamine;

Declarations

Ethics approval and consent to participate:

The trial was approved by the Ethics Committee of the Rende municipality: Approval N14, according to section 48 of Italian legislative decree 267/200 of January 2010.

Consent for publication: Not applicable

Availability of data material:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interest

None

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Authors' contribution

628 UC, GB, and DN were responsible for designing and conducting the trial; MR was

629 responsible for trial data analysis; UC, GB and MR wrote the paper.

630 Authors information: all the authors approved the text

631 Acknowledgements: No other than authors

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Table 1. General characteristics of the subjects under treatment with PG and PL.

Mean values, SD, [number of subjects]

Treatments	Age years	SD	BW kg	SD	Ht cm	SD	Hypertension	Smoking	Education Degree [a]	Physical activity <35 MET-hr/day
PG [49]	47.0	7.75	95.3	6.69	1.68	0.06	8/49	12/49	19/49	16/49
PL [48]	46.4	4.42	95.0	8.27	1.67	0.09	9/48	14/48	17/48	17/48
PG M [26]	46.5	7.59	100.3	3.99	1.72	0.03	8/26	10/26	10/26	8/26
PG F [23]	47.6	8.07	89.3	3.62	1.63	0.04	0/23	2/23	9/23	8/23
PL M [24]	46.9	8.39	101.5	3.66	1.74	0.04	9/24	11/24	9/24	7/24
PL F [24]	45.9	10.51	89.6	6.78	1.61	0.06	0/24	3/24	8/24	10/24

[a] bachelor or University degree

Table 2. Main food components intake in the week before the treatment and during one year of treatment in groups treated with PG or PL. Mean values, SD, [number of cases], and % variation vs Baseline

Variable	measure	Baseline				Average of the 3, 6, 9 months [a]				% variation Vs Baseline	
		PG [49]	SD	PL [48]	SD	PG [49]	SD	PL [48]	SD	PG [49]	PL [48]
Total Kcal	Kcal	16359	1813.3	16277	1984.4	14663	1605.4	14658	1758.6	-10.2§	-10.5§
Water	mL	7052	959.2	7165	1042.7	6563	748.3	6579	1046.6	-6.2§]	-8.2§
Proteins	g	650	78.3	643	73.1	601	66.7	593	66.7	-7.5§	-7.8§
Lipids[b]	g	609	79.4	602	68.1	582	74.8	570	71.7	-6.1§	-5.3§
Carbohydrates	g	1836	195.0	1825	228.3	1615	193.6	1610	216.4	-11.5§	-11.8§
Fiber	g	131	16.2	130	15.5	135	4.6	124	15.9	+3.1§¥	- 4.6§
Alcohol	g	149	82.5	150	88.2	106	75.6	113	82.9	-28.9§	-24.7§
EXVO	g	352	42.7	347	47.8	343	46.8	340	47.8	-2.6	-2.0
% of total Kcal											
Carbohydrates	%	44.8	3.34	44.7	3.40	43.5	3.50	43.8	3.30	-1.8	-2.0
Lipids	%	33.7	3.22	33.4	2.43	35.2	2.21	34.9	2.66	+3.1	+4.5
Protein	%	16.2	2.75	15.8	1.18	16.2	1.59	16.3	2.09	0	+2.8
Alcohol	%	6.3	3.26	6.2	3.33	4.9	3.10	5.2	3.60	-20.6§	-16.1§
EXVO	%	19.5	2.75	19.6	2.96	21.1	2.74	21.0	2.47	+8..2	+7.1

[a] Averages of the observation taken at 3,6, and 9 months

[b] Also contains EXVO (extra virgin olive oil)

§ Tukey test $p < 0.05$ Baseline vs 12 months; ¥ Tukey test $p < 0.05$ PG vs PL

Table 3. Number of servings at baseline and during one year of diet in subjects treated with PG or PL. Mean values, SD, [number of cases], and % variation vs Baseline

Type of serving	Baseline				Average of the 3, 6, 9 months [a]				% variation vs Baseline	
	PG [49]	SD	PL [48]	SD	PG [49]	SD	PL [48]	SD	PG	PL
sugar [b]	6.3	2.08	6.4	2.61	4.9	2.51	5.4	2.92	-25[§]	-23[§]
chocolate	1.6	1.48	1.7	1.44	1.6	1.32	1.8	1.43	-8	+1
milk	5.5	1.86	5.3	1.99	4.9	2.00	4.6	2.31	-8	-16[§]
biscuits	5.1	1.76	5.0	2.01	4.6	1.76	4.5	1.43	-11[§]	10[§]
bread	11.8	2.69	11.9	2.62	9.5	2.18	9.7	2.69	-19[§]	-18[§]
first dish[c]	9.0	1.52	8.9	1.85	8.4	1.34	8.4	1.72	-7[§]	-4
pizza	1.0	0.89	1.0	0.80	0.8	0.71	0.7	0.67	-29	-18
vegetables[d]	5.4	1.51	5.4	1.68	5.8	1.57	5.8	1.62	+10[§]	+11[§]
fruit	11.2	3.34	11.1	3.26	10.0	3.12	11.4	3.03	-11[§]	+3
dry fruit	1.4	0.98	1.2	0.91	1.4	1.06	1.1	0.97	-1	-13
pulses	1.9	1.03	1.8	0.82	2.0	0.99	1.7	0.94	-5	+4
meat	3.1	1.04	2.9	0.95	3.3	0.96	3.0	0.96	+2	+5
processed meat[e]	4.1	2.06	4.2	1.56	3.7	1.81	3.8	1.32	-6	-11[§]
fish	1.6	1.02	1.7	0.78	1.6	0.95	1.7	0.76	-5	-6
cheese	4.9	1.66	4.9	1.92	3.6	1.43	3.9	1.68	-26[§]	-22[§]
mozzarella	1.5	1.36	1.4	1.35	1.2	1.19	1.2	1.32	-19[§]	-11
yogurt	3.1	3.43	3.4	3.49	3.2	3.02	3.0	3.05	+2	-13
wine	7.5	5.09	8.4	5.44	6.6	4.50	7.0	5.01	-13	-16[§]
beer	1.2	1.89	1.2	1.89	1.0	1.52	0.8	1.54	-14	-48[§]
spirits	3.3	3.03	3.1	3.28	1.6	2.29	1.10	2.55	-52[§]	-65[§]
beverages	1.6	2.27	1.7	2.82	1.3	1.71	1.1	1.71	-18	-34
ice cream	1.3	1.43	1.3	1.67	1.1	1.18	0.7	1.22	-47[§]	-17

eggs[f]	2.2	1.95	1.8	2.03	2.8	1.85	2.8	1.90	+30[§]	+58[§]
chips	0.4	0.73	0.50	0.85	0.4	0.75	0.5	0.84	0	0
cake	1.8	1.59	1.8	1.57	1.3	1.31	1.6	1.46	-29[§]	-11

[a] Averages of the observation taken at 3,6, and 9 months

[b]: contains also candies; [c]: it refers to the complete dishes (containing also boiled vegetables/pulses, meat, oil/ butter, cheese added to the any pasta or rice, polenta, gnocchi, tortellini); [d] contains also oil; [e]: contains also hamburgers, cheese burgers, big burgers; [f]: contains also omelette and mayonnaise.

Table 4. Primary variables and physical activity at different times following 12 months treatment with PG or PL.

Mean values, SD, [N number of cases] and % variation vs Baseline

Period	Treatment	BW kg	SD	%	WC cm	SD	%	BMI kg/m^2	SD	%	MET/h day	SD	%
Baseline	PG [49]	95.2	6.73	-	115.1	8.65	-	33.9	1.03	-	35.4	1.12	-
	PL [48]	95.5	8.07	-	115.2	8.71	-	34.1	1.03	-	35.0	0.83	-
3 months	PG [49]	90.5	6.59	-4.9§	106.7	7.11	-7.3§	32.2	1.01	-5.0§	36.9	1.22	+4.2§
	PL [48]	91.6	7.37	-4.1§	109.4	6.84	-5.0§	32.7	1.07	-4.1§	36.6	1.09	+4.6§
6 months	PG [49]	86.3	6.52	-9.3$^{§¥}$	104.8	7.70	-8.9$^{§¥}$	30.8	1.05	-9.1$^{§¥}$	36.9	1.32	+4.2§
	PL [48]	89.9	7.06	-5.9§	107.9	6.54	-6.3§	32.1	1.14	-5.8§	36.5	1.15	+4.3§
9 months	PG [49]	84.1	6.49	-11.6$^{§¥}$	103.0	8.09	-10.5$^{§¥}$	30.0	1.08	-11.5$^{§¥}$	36.9	1.26	+4.2§
	PL [48]	88.5	7.04	-7.3§	106.1	6.70	-7.9§	31.6	1.15	-7.3§	36.5	1.15	+4.3§
12 months	PG [49]	83.1	6.27	-12.7$^{§¥}$	101.8	7.89	-11.6$^{§¥}$	29.6	1.06	-12.7$^{§¥}$	36.9	1.26	+4.2§
	PL [48]	87.5	6.94	-7.8§	105.0	7.02	-8.8§	31.3	1.23	-8.2§	36.5	1.15	+4.3§

% = Percent variation vs Baseline; § Tukey test $p < 0.05$ vs Baseline; Tukey test $p < 0.05$ PG vs PL

Table 5. Secondary variables modification at different times following 12 months treatment with PG or PL

Mean values, SD, [N number of cases] and % variation vs baseline

Variable [Measure]	Treatment	Baseline		3 Months			6 Months			9 months			12 months		
		Mean	SD	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%
BP diastolic [mm Hg]	PG [49]	75	5.9	72	5.4	-4.0	72	4.5	-4.0	70	4.2	-7.7§	68	3.9	-8.8§
	PL [48]	75	7.1	74	6.4	-1.3	74	6.4	-4.0	71	4.8	-5.3	71	4.0	-5.8§
BP systolic [mm Hg]	PG[49]	139	10.2	134	3.7	-6.6	132	6.6	-5.0	128	5.7	-7.9	128	4.9	-7.8§
	PL[48]	137	10.1	134	8.8	-3.6	132	5.2	-5.0	130	4.5	-5.1	130	4.0	-5.1§
Total Ch [mg/L]	PG[49]	197	10.2	187	7.9	-5.0§¥	184	8.1	-6.6§¥	179	8.4	-9.1§¥	174	7.6	-9.6§¥
	PL[48]	199	14.7	196	13.6	-1.5	194	12.1	-2.5	192	11.1	-3.5§	190	11.4	-4.6§
LDL [mg/L]	PG[49]	110	15.1	104	12.2	-5.4§	101	11.1	-8.8§	96	11.2	-12.7§¥	93	12.6	-12.9§¥
	PL[48]	112	18.5	109	16.6	-2.7	109	14	-2.7	107	12.0	-4.5	107	12.0	-5.3§
HDL [mg/L]	PG[49]	46	9.7	46	7.9	0.0	47	6.6	+2.2	48	5.7	+4.3	48	4.7	+5.4§
	PL[48]	46	9.4	48	8.0	+4.3	48	6.5	+4.3	48	5.9	+4.3	48	5.1	+3.2
TG [mg/L]	PG[49]	206	20.6	187	10.2	-9.2§	174	9.4	-15.5§¥	170	9.1	-17.3§¥	170	7.9	-17.3§¥
	PL[48]	204	23.2	197	20.6	-3.4	187	20.4	-8.3§	182	18.4	-10.7§	179	17.9	-12.2§
Glucose [mg/L]	PG[49]	98	5.2	94	4.6	-4.1§	92	3.6	-6.1§	90	4.1	-8.2§	88	3.9	-8.9§
	PL[48]	99	5.3	96	4.7	-3.0§	96	4.5	-3.0§	95	5.1	-4.0§	96	3.6	-4.1§
Hs-CRP [mg/L]	PG[49]	5.0	1.18	3.6	0.60	-28.0§¥	3.2	0.47	-36.0§¥	2.9	0.43	-42.0§¥	2.1	0.51	-58.3§¥
	PL[48]	4.9	1.11	4.6	0.97	-6.1§	3.7	0.69	-24.4§	3.4	0.61	-30.6§	2.9	0.35	-40.6§

% = Percent variation vs baseline; § Tukey test $p < 0.05$ vs Baseline; ¥ Tukey test $p < 0.05$ PG vs PL

Figure 1

Patients recruitment for the single centre, randomized, double blind, placebo controlled clinical investigation of PG112 in overweight and obese subjects.

Figure 1