**Effects of acute intake of grape/pomegranate pomace dietary supplement on
glucose metabolism and oxidative stress in adults with abdominal obesity**

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Abstract

A controlled acute, cross-over clinical study (NCT02710461) was performed in order to evaluate the effects on glucose metabolism of a grape/pomegranate pomace dietary supplement in subjects with abdominal obesity (aged 40-60, n=20). A standard 75 g oral glucose tolerance test was administered alone, together with or 10 h after the consumption of 10 g of the dietary supplement, rich in both extractable (0.4 g) and non-extractable (1.4 g) polyphenols. The dietary supplement did not ameliorate glucose or insulin at any sampling time. No improvement in antioxidant capacity was observed in plasma or urine, concordant with no increased urine polyphenol excretion. A tendency towards improved insulin sensitivity was observed when the product was consumed 10 h before glucose solution. These results suggest that a single realistic dose of grape/pomegranate pomace is not able to clearly improve glucose metabolism; chronic intake remains to be evaluated.

Keywords: Grape; pomegranate; pomace; metabolic syndrome; abdominal obesity; polyphenols.

30 **Abbreviations**

31 ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid).

32 ANOVA: analysis of variance.

33 AUC: area under the curve.

34 BMI: body mass index.

35 DF: dietary fiber.

36 EPP: extractable polyphenols.

37 FRAP: ferric reducing ability of plasma.

38 HDL: high-density lipoprotein.

39 HOMA: homeostatic model assessment.

40 HPP: hydrolyzable polyphenols.

41 IR: insulin resistance.

42 LDL: low-density lipoprotein.

43 MACAN: macromolecular antioxidants.

44 MetS: metabolic syndrome.

45 NEPA: non-extractable proanthocyanidins.

46 NEPP: non-extractable polyphenols.

47 OGTT: oral glucose tolerance test.

48 TPTZ: 2,4,6-tris(2-pyridyl)-s-triazine.

1. Introduction

Metabolic syndrome (MetS) is a health problem that is increasing around the world. It is a multiple risk factor for atherosclerotic cardiovascular diseases or type 2 diabetes, with a clinical diagnosis based on the concurrence of at least three factors (among abdominal obesity, hyperglycemia, hypertriglyceridemia, low HDL cholesterol and hypertension) (Alberti et al. 2009; Grundy, 2016). The onset and progression of MetS are associated with the development of insulin resistance (IR) and increased oxidative stress (Hopps et al. 2010). Despite its high incidence, MetS is still underdiagnosed (Reynolds et al. 2005), with abdominal obesity commonly the only factor subjects are aware of. The first steps towards prevention and treatment of MetS include diet modifications, in which increased consumption of certain bioactive compounds may play a beneficial role.

Polyphenols, which are widely present in plant foods, are known for their capacity to decrease oxidative stress. Moreover, increasing evidence suggests they are capable of ameliorating IR through different mechanisms, which have mostly been evaluated in *in vitro* and preclinical studies: direct inhibition of enzymes involved in the metabolism of carbohydrates; modification of the expression of glucose transporters; alteration of insulin synthesis, excretion and degradation (Martin et al. 2016; Medjakovic and Jungbauer 2013; Scazzocchio et al. 2015; Yang and Chan 2017). It may be expected that these beneficial effects are derived from the combined activity of intact compounds (extractable polyphenols; EPP) which may be absorbed in the small intestine and the activity of metabolites produced by the colonic microbiota (a fraction of EPP and all non-extractable polyphenols; NEPP), absorbed in the colon (González-Sarrías et al. 2017; Pérez-Jiménez et al. 2013; Rodríguez-Mateos et al. 2014). Therefore, the intake of both EPP and NEPP

would lead to prolonged circulation of bioactive compounds that may improve IR through a combination of mechanisms. Nevertheless, most studies of the role of polyphenols in MetS are based on food aqueous–organic extracts, which only contain EPP; whereas it has been reported that NEPP represent about 57% of the total polyphenols in fruit and vegetables (Pérez-Jiménez and Saura-Calixto 2015). Moreover, the specific contribution of intact polyphenols and microbial-derived polyphenol metabolites to the observed effects of polyphenols in modulating MetS remains as missing gaps in the study of dietary polyphenols.

Grape and pomegranate show a high and complementary content of EPP and NEPP (the latter also known as macromolecular antioxidants or MACAN). In particular, red grape is rich in anthocyanins and flavanols, including polymeric flavanols and proanthocyanidins, both in extractable and non-extractable forms (Chiva-Blanch and Badimon 2017; Pérez-Jiménez et al. 2009). Meanwhile, pomegranate is rich in anthocyanins and ellagitannins—polymers of ellagic acid—including some phenolic structures that are specific to this fruit (Fischer et al. 2011). Grapes are commonly processed for wine making, whereas pomegranate is used for juice production; both generate huge amounts of waste pomaces with a high content of polyphenols, which are present as the EPP and NEPP fractions (Pérez-Jiménez et al. 2009; Pérez-Ramírez et al. 2018; Touriño et al. 2008).

Several materials derived from grapes and pomegranates have been shown to have beneficial effects on the modulation of hyperglycemia and IR in studies using cell cultures or different animal models (Kerimi and Williamson 2016; Medjakovic and Jungbauer 2013). While clinical trials with grape polyphenols have shown contradictory effects regarding the regulation of MetS factors (Akaberi et al. 2016, Woerdeman et al. 2017). In

the case of grape pomace, the acute supplementation with a grape pomace extract to healthy subjects together with a standard meal improved post-prandial glucose and triglycerides (Costabile et al., 2018), a clinical trial of chronic supplementation with whole grape pomace to subjects at cardiometabolic risk observed an improvement in fasting glucose levels (Urquiaga et al. 2015) and whole grape pomace supplementation for 6 weeks improved fasting insulin in subjects with at least two MetS factors (Martínez-Maqueda et al. 2018). Regarding pomegranate, pomegranate juice supplementation did not modify insulin sensitivity in obese subjects (González-Ortiz et al. 2011), but it improved the glycemic response in healthy subjects, an effect not obtained with a pomegranate extract (Kerimi et al. 2017); this shows how related materials may originate different effects and the relevance of evaluating in clinical trials the potential effects of pomegranate pomace.

We previously reported the detailed profile of EPP and NEPP of a grape/pomegranate pomace dietary supplement, identifying more than 100 individual compounds (Pérez-Ramírez et al. 2018). The main EPP identified were malvidin 3-O-glucoside, gallotanins and gallagyl derivatives, whereas the main NEPP were vanillic acid and dihydroxybenzoic acid. Thus, the complementary profiles of grape and pomegranate pomaces, in terms of polyphenol classes and proportions of EPP and NEPP, suggest that their combined consumption may give rise to increased beneficial effects, and thus they are promising candidates for use in the development of dietary supplements or health-promoting food ingredients. Indeed, clinical studies have shown that both EPP and NEPP from grape pomace are partially bioavailable (Pérez-Jiménez et al. 2009) and that pomegranate polyphenols originate multiple absorbable metabolites (García-Villalba et al., 2016). Moreover, it may be expected that such a dietary supplement exerts beneficial effects when

consumed at nutritional doses, since a daily supplementation with 7.5 g of a product derived from grape pomace containing a similar dose of EPP and NEPP significantly improved cardiovascular risk markers in a previous clinical trial (Pérez-Jiménez et al. 2008). In the same way, it was recently reported that a dose of orange pomace containing 0.3 g of EPP (NEPP were not measured) significantly improved glycemic response (Chen et al. 2017).

Therefore, we hypothesize that the combined acute intake of EPP and NEPP from grape/pomegranate pomaces may improve glucose metabolism by a combination of effect of intact polyphenols and microbial-derived polyphenol metabolites, secondarily improving oxidative status. In order to test this, a controlled acute cross-over clinical study was conducted in adults with abdominal obesity (as core aspect of MetS) with a dietary supplement containing both EPP and NEPP, since their combined role in glucose metabolism has not been evaluated. It should be remarked that the doses in this study could be easily incorporated into a common diet.

2. Experimental

2.1 Reagents

Reagents were used for both the analysis of the dietary supplement and the determinations in the biological samples. Pepsin (2000 FIP-U/g) was obtained from Merck (Darmstadt, Germany). Amyloglucosidase (14 IU/mg) was from Roche (Manheim, Germany). Pancreatin, α -amylase (17.5 IU/mg), ABTS (2,2'-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), picric acid, (+)-catechin and creatinine were all obtained from Sigma-Aldrich (St. Louis, MO, USA). 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) was from Fluka Chemicals (Madrid, Spain).

Iron III-chloride-6-hydrate and 3,5-dinitrosalicylic acid were from Panreac (Castellar del Vallés, Barcelona, Spain).

2.2 Dietary supplement

A dietary supplement was prepared from pomegranate and grape pomaces. The pomegranate (*Punica granatum* L., cv Mollar de Elche) juice pomace (obtained by pressing the arils, after previous removal of the husk) was obtained from Vitalgrana S.A. (Catral, Alicante, Spain), whereas grape (*Vitis vinifera* L., cv Tempranillo) pomace was obtained from Roquesan Wineries (Quemada, Burgos, Spain). Both samples were collected fresh, at the moment of pomegranate pressing or wine devating. The samples were transported at -20°C, freeze-dried (CirTis, Gardinery, NY, USA) and ground in a mill (Retsch, Haan, Germany) to a particle size of 0.5 mm. The dietary supplement was obtained by mixing the pomegranate and grape pomaces in a 50/50 ratio, and it was stored at -20°C in vacuum-sealed packages protected from light.

The composition of the dietary supplement was determined using standard methodologies: dietary fiber, by the indigestible fraction method (Goñi et al. 2009); soluble sugars, by the dinitrosalicylic method (Englyst and Cummings 1988) after extraction with ethanol/water (80:20, v/v); fat, using a Soxhlet System HT extractor with petroleum ether; and protein, using an FP-2000® (Dumas Leco Corp., St. Joseph, MI, USA) automated nitrogen analyzer. The polyphenol composition was determined as extractable polyphenols (EEP) and non-extractable polyphenols, divided into hydrolyzable polyphenols (HPP) and non-extractable proanthocyanidins (NEPA) (Hartzfeld et al. 2002; Pérez-Jiménez et al. 2008; Zurita et al. 2012). The main individual constituents in the EPP and the HPP fractions were determined by HPLC-ESI-QTOF analysis based on a methodology previously described

(Pérez-Jiménez and Saura-Calixto 2015). It should be remarked that the dietary supplement used in this study can be easily prepared from grape or pomegranate pomaces; moreover, previous studies have been performed with this kind of materials, showing that their polyphenol composition keeps rather constant independently of harvest year or collection place (Pérez-Jiménez et al. 2008).

Prior to the intervention study, analysis for standard microbiological (*E. coli* β -glucuronidase, *L. monocytogenes*, *Salmonella* spp., viable aerobes at 30°C and fungi/yeast) and metal (Pb, Cd, Ni, Zn, As and Sb) toxins was carried out to guarantee the safety of the product.

2.3 Participants

Subjects were recruited via poster advertisements, a mailing sent to university departments and personal contacts in Madrid, Spain. Inclusion criteria for the study were: abdominal obesity, defined as abdominal diameter ≥ 94 cm for men and ≥ 80 cm for women (IDF 2006); apparently healthy; not medicated for cardiometabolic pathologies; and aged between 40 and 60 years. Exclusion criteria were: pregnant or lactating; perimenopausal or postmenopausal; previously diagnosed with a chronic disease (cardiovascular diseases, inflammatory diseases, diabetes or cancer); any regular pharmaceutical treatment or nutritional supplements; participating in any other dietary intervention study. The initial inclusion criteria was to exhibit hyperglycemia and at least another MetS factor without associated medication, but practical problems during such recruitment made necessary to modify those inclusion criteria by abdominal obesity as core factor of MetS.

The primary outcome variable was the area under the curve (AUC) for glucose 0-120 min after an oral glucose tolerance test (OGTT). Power calculations were based on a 15%

reduction in glucose AUC, following previous nutritional clinical trials with other polyphenol-rich materials (Hlebowicz et al. 2009). A sample size of 12 was calculated as sufficient to detect this change with 95% power and an alpha value of 0.05, using published variances of this parameter (Hlebowicz et al. 2009). This number was increased to 20 to ensure statistical power was sufficient even if an important proportion of the subjects failed to complete the trial due to the discomfort that the OGTT may cause. Therefore, power size was considered only enough for the whole group; anyway, exploratory analysis was performed classifying data depending on sex to determine whether some particular tendency arose. Additional outcomes were insulin, uric acid and oxidative status. No modifications in the outcomes were performed during the study.

The flow diagram of subjects recruitment is shown in **Figure 1**. From, a total of 102 potential participants, 38 of them refused to participate on hearing the details, and 44 subjects did not fulfill the requirements. Therefore, 20 subjects (10 men and 10 women) were included in the study

2.4 Study design and nutritional intervention

This study was a controlled acute cross-over clinical trial. For logistic reasons, all treatments were applied in the same order and the same days. Due to the complex composition of the product, it was not possible to obtain a proper placebo. For this reason, the study design could not be randomized, placebo-controlled and there was no blinding for participants or care givers. In contrast, blinding was applied when performing the analytical determinations and processing the results. The design of the study made unnecessary to perform any interim analysis.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Ethics Subcommittee of the CSIC, Madrid, Spain (2015/12/21) and the Ethics Committee for Clinical Research of the University Hospital Puerta de Hierro-Majadahonda, Majadahonda, Spain (2015/11/23). It was registered in the Clinical Trials database as POME-GRAPE-1 (“Grape and Pomegranate Polyphenols in Postprandial Glucose and Related Parameters”) with the identifier NCT02710461. The study was conducted, including recruitment and follow-up, between February and June 2016. Written informed consent was obtained from all subjects.

Each subject underwent three nutritional interventions, performed every two weeks at the Unit of Human Nutrition of the ICTAN-CSIC: (i) the standard OGTT (treatment A); (ii) consumption of the pomegranate/grape pomace dietary supplement together with the OGTT (treatment B); and (iii) consumption of the pomegranate/grape pomace dietary supplement 10 h before the OGTT (treatment C). It should be noted that in treatment B, plasma was collected before glucose intake; therefore, the basal values for treatments A and B are equivalents. In all cases, it was observed that subjects consumed the glucose solution in a maximum of 10 minutes.

During the two weeks before each dietary intervention, the subjects answered three 24 h dietary recalls. Moreover, starting 72 h prior to each intervention, the subjects were required to refrain from consumption of wine, coffee, tea, cocoa, whole bread, virgin oil olive, nuts, legumes and polyphenol-rich fruit and vegetables such as berries or artichoke, among other foods and beverages. The OGTT test consisted of ingesting 75 g of glucose in 200 mL of water; the dietary supplement was administered as 10 g of pomegranate/grape

pomace suspended in 250 mL of commercial apple-flavored water with non-caloric sweeteners (Fontvella, Aguas Danone S.A., Barcelona, Spain). During each intervention, blood samples were obtained 0, 15, 30, 60, and 120 min after ingestion of the glucose solution. A drop of venous blood immediately collected was used for glucose determination. Then, blood samples were centrifuged and plasma was recovered. Urine corresponding to the period of 0-3 h after the ingestion of the glucose solution was also collected. Plasma and urine samples were stored at -80°C until analysis.

2.5 Methods

2.5.1 Biochemical outcomes

Venous blood glucose was determined at 0, 15, 30, 60, and 120 min with a *Free Style Optimum Neo* blood glucose meter from Abbott (Chicago, IL, USA). Plasma insulin was determined with an ELISA kit (Millipore, MA, USA). The areas under the curves (AUCs) were estimated using the trapezoidal function (Allison et al. 1995).

IR was estimated with the homeostatic model assessment (HOMA-IR) and HOMA-beta cell function using the following equations: $HOMA-IR = [glucose \text{ (mg/dL)} * insulin \text{ (}\mu\text{U/mL)}] / 405$; $HOMA-beta = [insulin \text{ (}\mu\text{U/mL)} * 360] / [glucose \text{ (mg/dL)} - 63]$. Meanwhile, insulin sensitivity was estimated in the same samples using the Quicki index: $Quicki = 1 / [\log insulin \text{ (}\mu\text{U/mL)} + \log glucose \text{ (mg/dL)}]$.

Plasma and urine uric acid was determined using an enzymatic-colorimetric kit (Spin React, San Esteve de Bas, Girona, Spain).

2.5.2 Evaluation of oxidative status

To evaluate oxidative status, the urine polyphenol content was measured; while antioxidant capacity—estimated using the ferric reducing ability of plasma (FRAP) and 2,2'-azino-bis(3-ethylbenzothiazoline-sulfonic acid (ABTS) assays—was evaluated in both plasma and urine.

All urine measurements were normalized using the creatinine concentration, measured via the Jaffe reaction. Briefly, 65 μ L of urine was mixed with 1.215 mL of 25 mM HCl and 2.1 mL of 0.004% picric acid. After vortexing, 0.64 mL of NaOH was added, the mixture was vortexed and incubated for 90 min in darkness. Absorbance was measured at 500 nm and replaced in a calibration curve of creatinine.

To evaluate the polyphenol content of urine, the Folin-Ciocalteu assay was used after solid-phase extraction (SPE) (Roura et al. 2006). Urine polyphenols were extracted with SPE cartridges (3 mL/60 mg, Oasis, Waters, Milford, MS, USA) activated with 2 mL of 1.5 M formic acid and 2 mL of 5% methanol. Polyphenols were eluted from urine samples (1 mL) with 1 mL of 0.1% formic acid in methanol. Then, polyphenols were measured with Folin-Ciocalteu reagent by mixing 160 μ L of the polyphenol extract with 140 μ L of Folin, 2.3 mL of water, and 420 μ L of 200 g/L sodium carbonate. The samples were incubated for 1 h at room temperature, then 910 μ L of water was added, and absorbances were measured at 750 nm. The results were expressed as mg equivalents of catechin/g creatinine, using a calibration curve of catechin in synthetic urine (Roura et al. 2006).

The ABTS and FRAP antioxidant capacities were measured in plasma and in the urine polyphenol extract. The ABTS^{•+} radical was prepared by mixing ABTS with 2.45 mM potassium persulfate and overnight incubation in darkness. Then, it was diluted in 5 mM phosphate buffer with 0.145 M NaCl (adjusted to pH 7.4) to obtain an absorbance of $0.70 \pm$

0.02 at 730 nm. The samples (10 μ L) were mixed with 3 mL of the ABTS^{•+} radical, and absorbance was measured every minute for 6 min (Pulido 2003; Re et al. 1999). Plasma results were expressed as μ M equivalents of Trolox/L; urine results as μ M Trolox/g creatinine.

The FRAP reagent was prepared by mixing 2.5 mL of 10 mM TPTZ in 40 mM HCl with 2.5 mL of 20 mM iron (III) chloride hexahydrate and 25 mL of 0.3 M acetate buffer (adjusted to pH 3.6). Then, 30 μ L of sample was mixed with 900 μ L of the FRAP reagent and 90 μ L of distilled water, and absorbance was measured 30 min later at 595 nm (Benzie and Strain 1996; Pulido et al. 2000). Plasma results were expressed as μ M equivalents of Trolox/L; urine results as μ M Trolox/g creatinine. Uric acid correction was made by subtracting the uric acid value from the FRAP results, since uric acid may interfere with this antioxidant capacity.

2.6 Statistical analysis.

Data are shown as mean values $\pm$ standard error. Analysis of covariance (ANCOVA) was carried out using age and BMI as covariables followed by pairwise adjusted for multiple comparisons. Differences with $P < 0.05$ were considered significant. Outliers (>1.5 QR) and extreme values (>3.0 QR) were identified using box-and-whiskers plots, and extreme values were excluded from the results. Statistical analysis was performed using SPSS software.

3. Results

3.1 Composition of the dietary supplement

The approximate composition and polyphenol content of the grape/pomegranate pomace dietary supplement used in this study are shown in **Table 1**. It contained 18% polyphenols in total; interestingly, ~80% of them were NEPP, mostly as NEPA. It also had a content of ~50% total dietary fiber, mostly as insoluble dietary fiber.

In this study, we aimed to evaluate acute effect of the grape/pomegranate pomace dietary supplement on adults with abdominal obesity, using nutritional doses; in contrast to studies focused on polyphenol doses that could not easily be incorporated into normal diets. The dose of the dietary supplement used in this study (10 g) contributed with about 1.4 g of NEPP and 0.4 g of EPP. The main individual constituents of the EPP fraction were the anthocyanins malvidin 3-O-glucoside (117 mg/100 g dw) and delphinidin 3-O-glucoside (51.9), the gallotannin monogalloyl hexoside (91.4) and the ellagitannins punicalagin (115.6) and punicalin (64.98). In the NEPP fraction, the major identified constituents were non-extractable proanthocyanidins (12,360 mg/100 g dw), the hydroxycinnamaldehyde feruladehyde (61.8), the gallic acids methyl gallate (52.1) and vanillic acid (40.77) and the hydroxycinnamic acid ferulic acid (20.1). Nevertheless, most NEPP constituents could not be identified, due to the transformations that take place during the hydrolysis needed to release them. The detailed quantitative phenolic characterization of the dietary supplement has been published elsewhere (Pérez-Ramírez et al. 2018). Finally, it should be highlighted that the dietary supplement only provided 21.2 kcal/dose.

3.2 Subject characteristics

Twenty subjects (10 men and 10 women) were included in the study. All of them completed the three treatments in the study, without reporting any side effect. The baseline characteristics of the subjects included in the study are shown in **Table 2**. They all showed

abdominal obesity, with 35% being overweight and 40% obese. Also, 50% exhibited a waist-to-height ratio above recommendations (WHO 2008) (> 0.80 for women and > 0.90 for men). Regarding the other factors recognized as constituting MetS (IDF 2006), 25% of the subjects showed high systolic pressure (< 130 mm Hg), 50% high diastolic pressure (> 85 mm Hg), 15% high triglycerides (> 150 mg/dL), 20% low HDL-cholesterol (< 50 mg/dL for women and 40 mg/dL for men), 60% high LDL-cholesterol (> 130 mg/dL) and 55% high total cholesterol. Indeed, mean values for the subjects corresponded to borderline diastolic hypertension (85 mm Hg) and hypercholesterolemia (total cholesterol, 203 mg/dL).

3.3 Glycemic and and insulin responses

Figure 2 shows the glycemic response (blood glucose, AUC) of subjects who consumed only the glucose solution during OGTT (treatment A), subjects who consumed the glucose solution with 10 g of the grape/pomegranate pomace dietary supplement (treatment B) and subjects who consumed the glucose solution 10 h after consumption of 10 g of the grape/pomegranate pomace dietary supplement (treatment C). **Figure 3** shows insulin response (plasma insulin, AUC) of all the treatments. No significant differences were observed in any of these parameters.

IR and insulin sensitivity were calculated at baseline in treatment A, as a control, and treatment C, where dietary supplement intake had been 10 h before; treatment B was not included because, at basal time, it was equivalent to treatment A. No significant differences were observed in the insulin sensitivity indexes: HOMA-IR values were 1.32 (SEM 0.21) for treatment A and 1.18 (SEM 0.15) for treatment C; Quicki values were 3.61 (SEM 0.09) for treatment A and 3.78 (sem 0.11) for treatment C; HOMA-Beta values were 51.39 (SEM

5.62) for treatment A and 40.07 (SEM 4.10) for treatment C. Nevertheless, a non-significant tendency towards higher Quicki values in treatment C than in treatment A was observed. This was derived from the fact that basal plasma insulin was 5.27 (SEM 0.76) $\mu\text{U/mL}$ in treatment A and 4.52 (SEM 0.51) $\mu\text{U/mL}$ in treatment C, i.e., 14% lower, 10 h after intake of the grape/pomegranate pomace.

Similar results were obtained when subjects were divided by sex (data not shown).

3.4 Plasma and urine uric acid levels

Data for plasma uric acid during the OGTT and urine uric acid are shown in **Table 3**; no significant differences between the three treatments were found in any of the measurements. Similar results were obtained when subjects were divided by sex (data not shown).

3.5 Plasma and urine antioxidant capacity

Figure 4 shows plasma ABTS and FRAP antioxidant capacity during the OGTT. Treatment A showed FRAP plasma values significantly lower ($P < 0.05$) than treatments B and C at all sampling times, as well as significantly lower ($P < 0.05$) ABTS values than treatment B at all sampling times. As described above, basal values for treatments A and B represent the same situation, so the higher values at the beginning of treatment B than those for treatment A mean that subjects exhibited higher basal antioxidant capacity values. Moreover, these were not modified during the OGTT, indicating no effect of the dietary supplement. As regards to treatment C, plasma antioxidant capacity values (FRAP assay) were significantly higher than those for treatment A at all sampling times, but since they

were similar to basal values in treatment B (a situation prior to supplementation) we cannot state that this increase was derived from microbial-derived polyphenol metabolites.

Similarly, although both urine ABTS and FRAP values were higher in treatments B and C than in treatment A, this was not statistically significant either (**Table 4**). Neither were any significant differences observed for polyphenol excretion between the treatments (**Table 4**).

Similar results for these parameters were obtained when subjects were divided by sex (data not shown).

4. Discussion

In this controlled cross-over clinical study, we evaluated the acute effect of a grape/pomegranate pomace dietary supplement rich in EPP and NEPP, in adults with abdominal obesity: a core characteristic of MetS. The experimental approach was based in three different OGTT to each subject in a cross-over design. The first OGTT evaluated the normal glycemic response of the subjects during an OGTT. The second OGTT aimed to evaluate the effect of those polyphenols which exert their action as intact compounds (0-2 h after consumption); this would correspond mostly to local actions throughout the digestive tube inhibiting the activity of intestinal enzymes involved in the degradation of carbohydrates (Williamson 2013). Finally, the effects of NEPP and a fraction of EPP that exert their action as microbial-derived polyphenol metabolites were tested in the third OGTT (10-12 h after consumption); it has been reported that these microbial-derived metabolites are able to stimulate insulin secretion (Fernández-Millán et al. 2014; Scazzocchio et al. 2015). The specific characteristics of the product (rich in both dietary fibre and several polyphenol fractions) did not allow the preparation of a proper placebo, which is a limitation of this clinical trial. It should be remarked that the dose used here (1.4

g of NEPP and 0.4 g of EPP), incorporated into a common diet, would represent about 30% of current mean intake of EPP and 100% of NEPP according to Spanish and French polyphenol dietary intake (Arranz et al. 2010; Pérez-Jiménez et al. 2011). Therefore, it approaches daily intake of polyphenols to the highest consumers, but always within nutritional doses.

Based on this background, the hypothesis this study set out to test was that acute intake of this (EPP- and NEPP-rich) dietary supplement would ameliorate glucose and insulin responses, exerting an antioxidant effect, and that intact polyphenols and microbial-derived polyphenol metabolites exert a differential beneficial effect. Also, we hypothesized that EPP and NEPP would be the main hypoglycemic compounds, since the DF in the dietary supplement was mainly insoluble DF, whose effects on glycemia are mostly through long-term mechanisms (Isken et al. 2010), while short-term effects on glycemia have mostly been attributed to soluble DF (Galisteo et al. 2008), nearly absent from the dietary supplement.

However, the results of the clinical trial did not confirm this hypothesis. The grape/pomegranate pomace dietary supplement showed no effect on glycemic response, insulin or insulin resistance during the OGTT. Moreover, no significant effect was observed on plasma or urine antioxidant capacity. Neither were uric acid levels affected by the supplementation, but they also remained constant during the OGTT in the control group. Although postprandial increases in uric acid have been reported, concomitant with a decrease induced by polyphenol-rich beverages, this happened when the meal contained fat, (Miglio et al. 2014) which was not the case in this study.

These data are in contrast with the results of studies in animal models, where both grape and pomegranate pomace aided glucose metabolism (Kerimi and Williamson 2016; Medjakovic and Jungbauer 2013). However, to the best of our knowledge, only one clinical trial explored the effect of whole grape pomace on insulin sensitivity (Urquiaga et al. 2015). That study found an improvement, but it was a chronic study, so it cannot be directly compared with this one. Similarly, an acute study with a grape pomace extract found an improvement in insulin secretion and sensitivity when the product was provided with a standard meal, which cannot be either directly compared with our glucose load (Costabile et al., 2018).

Acute studies with another polyphenol-rich product, cinnamon, did report beneficial effects on postprandial glucose and insulin. For instance, this was observed after supplementing healthy subjects with either 3 or 6 g of cinnamon, consumed with rice pudding (Hlebowicz et al. 2007; Hlebowicz et al. 2009). Interestingly, that effect was maintained 12 h after cinnamon intake, probably due to the action of microbial-derived polyphenol metabolites (Solomon and Blannin 2007). In contrast, no modifications in OGTT results were observed when consuming the closely related spice Ceylan cinnamon (Wickenberg et al. 2012), thus showing that small differences in polyphenol profiles may significantly affect the derived physiological effects. Acute supplementation of overweight men with orange pomace before OGTT also caused a significant decrease in insulin AUC and the time to reach the maximum concentration of blood glucose (Chen et al. 2017); but in contrast to the dietary supplement tested here, orange pomace has a high proportion of soluble DF, which may have contributed to the effects observed. Finally, when comparing control OGTT results with those after an acute intake of berries—rich in polyphenols—a significant decrease in

glucose was observed 15 minutes after the berries were consumed, while there was a significant increase after 90 min; overall, meaning an improvement in glycemic profile (Törrönen et al. 2012). This illustrates the complexity of the processes by which polyphenols may regulate glucose metabolism.

Indeed, in the present clinical trial, no modification was observed in urine total polyphenols; a parameter directly related to polyphenol intake (Medina-Remón et al. 2009). This indicates that the dose used was not capable of inducing significantly increased polyphenol circulation at the selected times, thus explaining the lack of an effect on the biochemical parameters and antioxidant capacity measurements. Since the approach adopted in this study was to provide a maximal dose that could easily be incorporated into a common diet, we do not consider it advantageous to test higher ones. Nevertheless, a complete kinetic study would be needed in order to determine whether the doses used here would lead to an increased circulation of polyphenol metabolites at some moment after intake, as it was indeed observed in a previous study with a product derived from grape pomace (Pérez-Jiménez et al. 2009). Moreover, it cannot be ruled out that the dose tested here did in fact induce slight though non-detectable modifications in polyphenol levels (maybe at times later than the 10 h tested here) that, in combination with the other polyphenol-rich products consumed in a real situation, would lead to significant increases in circulating polyphenol metabolites. Moreover, it has recently been suggested that subtle polyphenol effects, repeated daily over a lifetime, could ultimately explain the beneficial effects of polyphenols observed in epidemiological studies but not always confirmed in clinical trials (De Pascual-Teresa and Clifford 2017).

So, although the grape/pomegranate pomace dietary supplement tested here did not seem to cause any physiological modifications in subjects with abdominal obesity after a single intake, the effects of a chronic intake remain to be evaluated. We should note that a tendency towards an improvement in insulin sensitivity in fasting state (as measured by HOMA and QUICKI indexes) was observed for treatment C, as compared to treatment A; this tendency could be strengthened after a prolonged intake of the dietary supplement. It should also be highlighted that chronic intake of grape pomace at realistic doses did improve insulin sensitivity and lipid profile in previous studies (Pérez-Jiménez et al. 2008; Urquiaga et al. 2015). And other studies found an effect of polyphenol-rich diets in glucose metabolism in subjects at high cardiometabolic risk (Bozzetto et al. 2015), in agreement with epidemiological data (Vitale et al. 2017; Vitale et al. 2018). Indeed, this connects with the limitations of this study: exclusively acute approach and focused on a particular situation (abdominal obesity) which may not completely define MetS. It cannot be established at which point current results would be applicable to other populations, in particular considering that obese subjects may exhibit differences in the metabolic fate of polyphenols (Novotny et al. 2017).

In conclusion, the results of this controlled acute cross-over clinical trial show that ingestion of a grape/pomegranate pomace dietary supplement does not affect glycemic or insulin responses, and neither does it affect the plasma or urine antioxidant capacity of adults with abdominal obesity. This was observed when the dietary supplement was consumed together with the glucose solution of the OGTT, or when it was consumed 10 h prior to the OGTT. These results suggest that a single intake of this dose of both EPP and NEPP does not exert acute health-beneficial effects, either from intact polyphenols or from

microbial-derived polyphenol metabolites. However, and especially considering the non-significant tendency towards an improvement in insulin sensitivity observed, further studies are necessary to evaluate the potential effects of chronic intake of grape/pomegranate pomaces on glucose and insulin metabolism, as well as on other cardiometabolic risk factors.

Acknowledgments

The authors are grateful to the Analysis Services Unit of ICTAN for microbiological, mineral, and chromatographic analyses. Dr. Laura Barrios, from the *Subdirección General de Apoyo a la Investigación* (SGAI) of the CSIC is also thanked for her assistance with statistical treatment. Vitalgrana S.A. and Roquesan Wineries are thanked for providing the raw materials of the dietary supplements. The authors are grateful to the volunteers for their participation in the study.

Disclosure of interest

The authors report no conflict of interest.

Funding

This work was supported by the Spanish Ministry of Science and Innovation (MINECO-FEDER) under grant AGL2014-55102-JIN and by CONACyT (international scholarship, I.F.P.-R.).

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TABLES

Table 1. Chemical characterization of the grape/pomegranate pomace dietary supplement

Component	Content [*]	
	mg/100 g fw	DS
Proximal composition		
Carbohydrates		
Simple sugars	20.46	1.56
Soluble dietary fiber	0.34	0.03
Insoluble dietary fiber	48.65	2.32
Protein	10.51	0.08
Fat	9.67	0.08
Ash	3.89	0.14
Humidity	5.74	0.72
Caloric value (kcal/g)	2.12	
Polyphenol composition		
Extractable polyphenols [†]	3.54	0.23
Non-extractable polyphenols ^{‡§}	14.34	0.86
Total polyphenols	17.88	0.89

^{*} The dose used in the study was 10 g.

[†] Polyphenol classes (decreasing content): anthocyanins, ellagitannins, gallotannins, hydroxycinnamic acids, flavanols, hydroxybenzoic acids, flavonols.

[‡] Polyphenol classes (decreasing content): proanthocyanidins, hydroxybenzoic acids, ferulaldehydes, hydroxycinnamic acids, ellagitannins, hydroxyphenylpropanoic acids, flavanones.

[§] A fraction is included in dietary fiber.

Table 2. Baseline subject characteristics.

Parameter	Total (n=20)	
	Mean	SEM
Age (years)	48	1
Height (cm)	2.00	0.02
Weight (kg)	88	3
Body mass index (kg/m ²)	29.7	0.6
Abdominal circumference (cm)	105	2
Waist circumference (cm)	99	2
Hip circumference (cm)	109	1
Waist-to-hip ratio	0.91	0.02
Waist-to-height ratio	0.57	0.01
Systolic blood pressure (mm Hg)	121	2
Diastolic blood pressure (mm Hg)	85	2
Glucose (mg/dL)	84	2
Triglycerides (mg/dL)	102	10
Total cholesterol (mg/dL)	203	7
HDL cholesterol (mg/dL)	55	4
LDL cholesterol (mg/dL)	128	6

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Table 3. Plasma and urine uric acid in clinical trial on acute effects of a grape/pomegranate pomace dietary supplement

Treatment	Plasma										Urine	
	0 min		15 min		30 min		60 min		120 min			
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Treatment A	5.59	0.68	4.99	0.54	4.95	0.40	5.02	0.56	4.41	0.41	2,748.3	169.4
Treatment B	5.13	0.49	4.61	0.49	4.69	0.44	4.42	0.51	4.08	0.48	2,160.9	227.3
Treatment C	4.80	0.33	4.53	0.28	4.61	0.32	4.53	0.29	4.36	0.32	2,654.8	169.5

Treatment A, control OGTT; Treatment B, dietary supplement (10 g) + OGTT; Treatment C, dietary supplement (10g) + OGTT 10 h later. Plasma values, mg/mL; urine values, mM/g creatinine. No statistical differences were found at any sampling time.

Table 4. Urine polyphenols and antioxidant capacity (AC) in clinical trial on acute effects of a grape/pomegranate pomace dietary supplement

Treatment	Total polyphenols*		AC- ABTS assay [†]		AC-FRAP assay [‡]	
Treatment A	145.69	13.90	6,750.49	1,250.11	1,895.96	282.11
Treatment B	136.19	9.90	6,858.86	712.08	3,768.79	1,167.87
Treatment C	127.72	9.47	7,364.98	846.57	3,925.88	1,160.07

Treatment A, control OGTT; Treatment B, dietary supplement (10 g) + OGTT; Treatment C, dietary supplement (10g) + OGTT 10 h later. ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); FRAP, ferric reducing ability of plasma. *mg eq. catechin/g creatinine, [†]μM Trolox/g creatinine, [‡]μM Trolox/g creatinine adjusted with urine uric acid values. No statistical differences were found at any of the sampling times.

FIGURE CAPTIONS

Figure 1. Flow chart for screening and enrolment of study participants.

Figure 2. Glycemic response of participants in clinical trial on acute effects of a grape/pomegranate pomace dietary supplement: a) blood glucose and b) area under the curve, AUC.

Treatment A: control Oral Glucose Tolerance Test (OGTT); Treatment B: dietary supplement (10 g) + OGTT; Treatment C: dietary supplement (10g) + OGTT 10 h later. Data are presented as mean values and error bars represent standard error. Different letters indicate significant ($p < 0.05$) difference between treatments.

Figure 3. Insulin response of participants in clinical trial on acute effects of a grape/pomegranate pomace dietary supplement: a) plasma insulin and b) area under the curve, AUC.

Treatment A: control Oral Glucose Tolerance Test (OGTT); Treatment B: dietary supplement (10 g) + OGTT; Treatment C: dietary supplement (10g) + OGTT 10 h later. Data are presented as mean values and error bars represent standard error. Different letters indicate significant ($p < 0.05$) difference between treatments.

Figure 4. Plasma antioxidant capacity of participants in clinical trial on the acute effects of a grape/pomegranate pomace dietary supplement: a) ABTS assay and b) FRAP assay.

Treatment A: control Oral Glucose Tolerance Test (OGTT); Treatment B: dietary supplement (10 g) + OGTT; Treatment C: dietary supplement (10g) + OGTT 10 h later. Data are presented as mean values and error bars represent standard error. ¹Adjusted with

22 plasma uric acid values. Different letters indicate significant ($p < 0.05$) difference between
23 treatments. ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; FRAP, ferric
24 reducing ability of plasma.

Figure 1.

Figure

2.

Figure 3.

Figure 4.