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Short communications

# Pomegranate vinegar beverage reduces visceral fat accumulation in association with AMPK activation in overweight women: A double-blind, randomized, and placebo-controlled trial

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Recent studies on animals have suggested that vinegar consumption may confer an anti-obesity effect through the activation of the AMP-activated protein kinase (AMPK) signaling pathway. However, mechanisms of action in humans remain largely unknown. A randomized, double-blind, placebo-controlled trial was performed to examine whether a pomegranate vinegar (PV) beverage alleviates adiposity in overweight subjects, with emphasis on AMPK activation. Seventy-eight overweight women (BMI ≥ 25) were randomly assigned to receive either PV (1.5 g acetic acid and 700 μg ellagic acid/200 mL/day) or a placebo for 8 weeks. The PV reduced visceral adipose tissue, as measured by computed tomography ($P = 0.037$), and enhanced AMPK phosphorylation ($P = 0.013$) compared with the placebo group. The PV tended to suppress downstream gene expression, such as that of sterol regulatory element binding protein-1c and acetyl coenzyme carboxylase, in adipose tissue. Together, these data suggest that PV is an excellent AMPK activator and may exert beneficial effects on adiposity.

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## 1. Introduction

Obesity is the most common metabolic disease caused by an imbalance between energy intake and expenditure. Two types of medications are now available for weight control: appetite suppressant/anorexics and inhibitors of specific nutrient absorption (Korner & Aronne, 2004). However, due to undesirable side effects associated with the currently available medications, much attention has been focused on food products that directly modulate energy metabolism (Seo et al., 2011).

Vinegar is a useful liquid that can be made from almost any fermentable carbohydrate source: initially, yeast ferments natural food sugars to alcohol; then, *Acetobacter*, an acetic acid bacterium, converts the alcohol to acetic acid (AcOH). AcOH is readily absorbed from the intestine and metabolized to acetyl-CoA in the liver, resulting in the feed-forward activation of AMP-activated protein kinase (AMPK) (Fushimi et al., 2006; Kondo, Kishi, Fushimi, & Kaga, 2009; Sakakibara, Yamauchi, Oshima, Tsukamoto, & Kadowaki, 2006). AMPK is a member of the metabolite-sensing protein kinase family and has been implicated in the regulation of enzyme activities involved in energy metabolism as well as in the regulation of gene expression related to lipogenesis and fatty acid oxidation (Warden et al., 2001). Thus, AMPK has been recognized as a promising target for the management of obesity.

As the public interest in vinegar has increased, vinegar beverages containing a combination of phytochemicals found in fruit have been gaining popularity in recent years (Costa, Garcia-Diaz, Jimenez, & Silva, 2013; Wakuda et al., 2013). We recently demonstrated that a pomegranate vinegar, made by mixing a vinegar base and a pomegranate extract, had higher potential for enhancing gene expressions related to fatty acid oxidation than acetic acid in HepG2 cells (Kim, Ok, Kim, Choi, & Kwon, 2013). In a following study, we confirmed the biological relevance of the *in vitro* findings in high-fat-diet-induced obese rats, suggesting a coordinated activation of AMPK in adipose tissue and the liver as an underlying mechanism (Ok et al., 2013). However, the effect of any type of vinegar on AMPK phosphorylation in adipose tissue has not been examined in humans to date.

In the present study, we investigated whether AMPK activation with pomegranate vinegar is involved in alleviating adiposity in overweight subjects by performing an 8-week, double-blind, randomized, placebo-controlled trial. To this end, we assessed objective measures of total and regional body fat using dual-energy X-ray absorptiometry (DEXA) and computed tomography (CT). We then determined the stimulation of AMPK phosphorylation and related gene expressions in abdominal subcutaneous adipose tissue biopsied from a subpopulation of the study subjects.

## 2. Subjects and methods

### 2.1. Test beverages

A pomegranate vinegar (PV) beverage and a placebo beverage were provided by Daesang Corporation (Seoul, Korea). The PV was standardized with ellagic acid at 350 μg/100 mL and acetic acid at 750 mg/100 mL based on the previous results of HPLC analysis for the chemical signature of pomegranate vinegar (Kim et al., 2013). The placebo contained lactic acid (765 mg/100 mL) as a replacement for the acetic acid, fructose (2.0 g/100 mL), low sugar sweet alternatives (2.0 g/100 mL), flavor (pomegranate), and color material to achieve identical appearance and smell. Test beverages were packed in pouches and labeled by an independent researcher for each subject according to the randomization schedule.

### 2.2. Subjects and study design

Ninety healthy female subjects (20–65 years) with a BMI between 25 and 35 $kg/m^2$ were recruited from the general public by poster advertisements. At screening visit, all subjects were received a medical history review, physical examination and routine battery of blood tests. Exclusion criteria for subjects included participation in another clinical trial in the previous 4 weeks; pregnancy or lactation; current use of dietary supplements or medications affecting body weight and weight instability; hypertension, diabetes, or any other disease affecting the results of the study.

Seventy-eight eligible subjects were enrolled after providing written informed consent. Upon completion of the one-week run-in phase, subjects were randomly assigned to either the PV group or the placebo group for 8-week treatment phase. Randomization was performed using computer-generated random numbers and the group allocation was blinded for both investigators and participants.

During the 8-week treatment period, subjects were asked to take one pouch of PV or a placebo twice a day preferably after breakfast and dinner. Test beverages were dispensed by the investigator every 2 weeks; subject compliance in the consumption of the test beverages was assessed by counting returned pouches and questioning the subjects. The lifestyle changes of the subjects regarding diet and physical activity were monitored using a 3-day dietary record. The study protocol was approved by the Institutional Review Boards of CHA Bundang Medical Center and Ewha Womans University, where subjects were recruited and laboratory analyses were performed, respectively. This trial was registered in the International Clinical Trials Registry Platform of WHO with the following identification number: KCT0000276.

### 2.3. Outcome measures

Fasting blood samples were obtained at baseline and after 8 weeks. Plasma samples were analyzed for total cholesterol, triacylglycerols, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, glucose, and insulin using a Hitachi 7600 automated chemistry analyzer (Osaka, Japan). Insulin resistance was measured using the homeostasis model assessment [HOMA = (fasting insulin × fasting glucose)/22.5] (Emoto et al., 1999). Commercially available kits were used to assess adiponectin (DuoSet ELISA Development kit; R&D Systems, Minneapolis, MN, USA) and monocyte chemoattractant protein-1 (MCP-1) (Ready-Set-go; eBioscience, San Diego, CA, USA).

Visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) were measured at the fourth and fifth lumbar spine (L4–L5) level using a 16-slice CT scanner (Sensation 16, Siemens, Forchheim, Germany). Body composition was assessed using a Hologic QDR-2000 enhanced-array whole-body dual-energy X-ray absorptiometry (DEXA) scanner (Hologic, Waltham, MA, USA). The output (total fat mass and lean mass) was generated with the default settings of the Hologic enhanced-array whole body software version 12.1.

### 2.4. *Adipose tissue biopsy, RNA extraction, real-time polymerase chain reaction, and phosphorylation of AMPK in adipose tissue*

At baseline and after 8 weeks, adipose tissue was obtained from an abdominal region lateral to the umbilicus for a subpopulation of this study ($n = 13$ for PV and $n = 18$ for placebo) using a needle aspiration biopsy under local anesthesia. The adipose tissue samples were snap-frozen in liquid nitrogen and stored at –80 °C for later RNA extraction.

Total RNA was extracted using a TRIZOL reagent (Invitrogen Co., Carlsbad, CA, USA). Quantitative RT-PCR was performed in a Step-One-Plus RT-PCR System (Applied Biosystems) using the TaqMan method. The primer sets for target genes were sterol regulatory element binding protein-1c (SREBP-1c; Hs00231674_m1), acetyl-CoA carboxylase (ACC; Hs00167385_m1), and leptin (Hs00174877_m1). The relative amounts of these mRNAs were normalized to the amount of β-actin, and the relative amounts of all RNAs were calculated using the comparative Ct method (Schmittgen & Livak, 2008).

Protein in adipose tissue were separated by electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA). The membranes were probed with anti-p-AMPK (Cell Signaling Technology, Danvers, MA, USA), anti-AMPK (Cell Signaling Technology), and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoreactive antigen was then recognized using a horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology). Immunoreactive protein bands were visualized by the ChemiDoc XRS+ system (Bio-Rad) and calculated using the Image Lab™ software (Bio-Rad).

### 2.5. *Statistical analysis*

A power calculation based on a previous study (Kondo, Kishi, Fushimi, Ugajin, & Kaga, 2009a) predicted that with 39 subjects at each group and assuming a 20% drop-out rate among study subjects, there was an 80% chance of detecting a difference in VAT between the two groups with a two-sided alpha of 0.05. A Kolmogorov–Smirnov test was used to assess the normality of each variable, and log transformation was performed on skewed variables. Differences in demographic and baseline characteristics between the two groups were tested with Student's *t*-test. For each variable, the effect of the PV was expressed as the relative change between measurements obtained at baseline and those obtained at the 8-week follow-up within each group to take into account the possible differences at baseline between the two groups. Adjusted least-squares (LS) mean values were estimated using one-way analysis of covariance (ANCOVA). The effects of the PV on gene expressions and AMPK phosphorylation between the two groups were analyzed by Student's *t*-test. $P < 0.05$ was considered significant. SAS program package version 9.2 (SAS Institute, Cary, NC, USA) was used for all statistical evaluations.

## 3. Results

### 3.1. *Baseline characteristics*

Planned enrollment was 78 subjects. The intention-to-treat (ITT) efficacy analysis included all subjects who received at least one dose of the test materials. Because one subject was excluded due to myelolipoma, the ITT analysis included 77 subjects: 38 PV subjects and 39 placebo subjects (Fig. 1). The characteristics of the ITT population at baseline are shown in Table 1. The two groups were well matched for age and BMI, but there was a significantly unequal distribution of total fat mass ($P = 0.008$) and a tendency for differences in body weight, VAT, and SAT between the groups (i.e., higher in the PV group than in the placebo group). Accordingly, the results were analyzed as baseline-adjusted relative changes. From the 3-day dietary records completed during the intervention, no significant differences between the two groups were found in terms of relative changes in physical activity or total energy intake, either at baseline or during the intervention (data not shown).

### 3.2. *Biomarkers of adiposity*

Both group experienced a small decrease in bodyweight during the intervention, but no significant difference was observed in bodyweight and other plasma biomarkers between the two groups (Table 2). Non-significant tendencies for a difference in the changes in both total fat mass and lean mass were found between the two groups by DEXA. Drinking PV, however, resulted in a significant reduction in VAT compared with that measured for the placebo group ($P = 0.037$) by CT (Fig. 2). The adjusted mean relative changes from baseline in VAT were –13.37 $cm^2$ (10% decrease) in the PV group and –1.50 $cm^2$ (2% decrease) in the placebo group, a difference of 11.87 $cm^2$. Although the effect on the accumulation of SAT was not significantly different between the two groups, the reductions in terms of the ratio of VAT to SAT tended to increase in the PV group compared to the placebo group ($P = 0.067$).

### 3.3. *AMPK phosphorylation and mRNA expressions in adipose tissue*

Changes in the AMPK phosphorylation and mRNA expressions in abdominal subcutaneous adipose tissue were determined. AMPK phosphorylation was significantly increased in the PV group by a factor of 2.7 relative to that of the placebo group when baseline levels for each subject were normalized to 1 ($P = 0.013$) (Fig. 3A). Although no significant differences were identified between the two groups, drinking the PV resulted in decreases in mRNA expressions of SREBP-1c, ACC, and leptin in adipose tissue, whereas drinking the placebo resulted in increases (or smaller decrease) in each case (Fig. 3B).

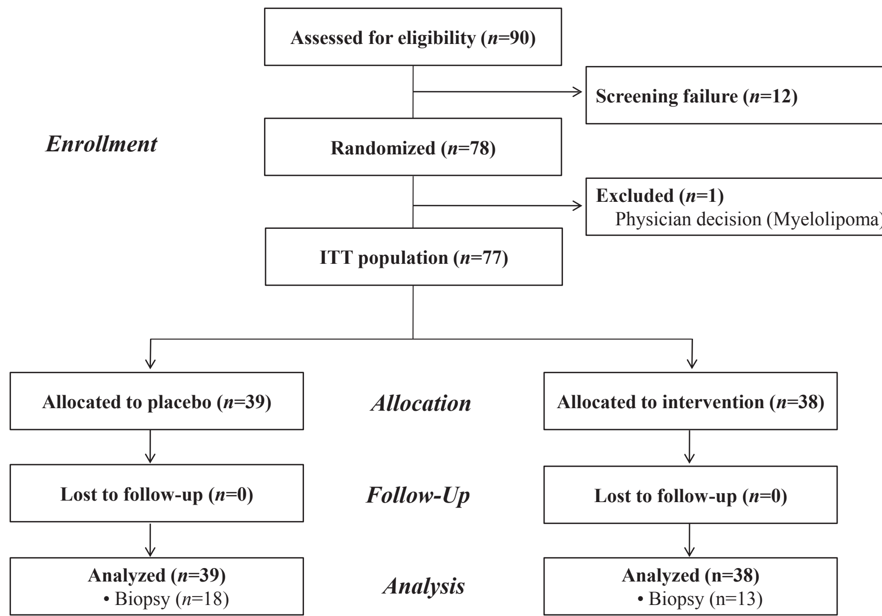



**Fig. 1 – Study design and disposition of subjects. ITT, intention to treat.**

## 4. Discussion

The hypothesis that PV ingestion is involved in the control of body fat was supported by recent *in vitro* and *in vivo* studies from our and other laboratories (Kim et al., 2013; Kondo et al., 2009; Ok et al., 2013). However, to the best our knowledge, this is the first observation of the molecular mechanism of PV-induced metabolic alterations in a double-blinded RCT of obese subjects.

Based on the results of Western blotting analysis, we clearly demonstrated that 8-week consumption of PV induced the activation of AMPK in abdominal subcutaneous adipose tissue biopsied from 31 obese subjects. The allosteric site of AMPK

**Table 1 – Baseline characteristics of intention-to-treat population[a].**

| | Placebo ($n$ = 39) | PV ($n$ = 38) | P-value[b] |
|---|---|---|---|
| Age (years) | 42 ± 2 | 41 ± 2 | 0.708 |
| Body weight (kg) | 70.4 ± 1.1 | 73.5 ± 1.4 | 0.081 |
| BMI (kg/m$^2$) | 28 ± 0.4 | 28.9 ± 0.3 | 0.061 |
| VAT (cm$^2$)[c] | 103.2 ± 4.5 | 106.2 ± 5.7 | 0.711 |
| SAT(cm$^2$)[c] | 269.3 ± 11.5 | 297.4 ± 13.3 | 0.113 |
| Total fat mass (kg)[d] | 26.8 ± 0.7 | 29.7 ± 0.8 | 0.008 |
| Lean mass (kg)[d] | 39.4 ± 0.6 | 39.7 ± 0.8 | 0.758 |
| Blood pressure (mm Hg): | | | |
| Systolic | 128.5 ± 1.4 | 128.82 ± 1.6 | 0.895 |
| Diastolic | 80.26 ± 1.4 | 81.26 ± 1.4 | 0.606 |
| Fasting plasma: | | | |
| Total cholesterol (mg/dL) | 195.0 ± 5.9 | 197.7 ± 5.2 | 0.732 |
| Triacylglycerols (mg/dL) | 121.4 ± 15.0 | 115.5 ± 8.0 | 0.959 |
| HDL-cholesterol (mg/dL) | 55.3 ± 2.2 | 57.8 ± 2.0 | 0.394 |
| LDL-cholesterol (mg/dL) | 108.5 ± 5.1 | 111.9 ± 4.7 | 0.563 |
| Glucose (mg/dL) | 97.13 ± 1.5 | 98.5 ± 1.5 | 0.508 |
| Insulin (uIU/mL) | 8.52 ± 1.01 | 8.02 ± 0.54 | 0.921 |
| HOMA-IR | 2.1 ± 0.3 | 1.96 ± 0.1 | 0.921 |

PV, pomegranate vinegar beverage; BMI, body mass index; VAT, visceral adipose tissue; SAT, subcutaneous abdominal adipose tissue.

[a] Mean ± SEM (all such values).

[b] Student's *t*-test was used to analyze the data.

[c] Determined by computed tomography.

[d] Determined by dual-energy X-ray absorptiometry.

**Table 2 – Changes from baseline to week 8 in anthropometric and plasma biochemical biomarkers of adiposity[a].**

| | Placebo ($n$ = 39) | PV ($n$ = 38) | P-value[b] |
|---|---|---|---|
| Body weight (kg) | −0.68 ± 0.41 | −0.94 ± 0.42 | 0.728 |
| Body mass index (kg/m²) | −0.27 ± 0.16 | −0.45 ± 0.16 | 0.521 |
| Adiponectin (μg/mL) | −0.24 ± 0.18 | −0.26 ± 0.18 | 0.930 |
| MCP-1(pg/mL) | 9.42 ± 5.21 | 13.5 ± 5.31 | 0.660 |
| Total cholesterol (mg/dL) | −2.86 ± 5.87 | −6.78 ± 5.99 | 0.705 |
| Triacylglycerols (mg/dL) | −23.17 ± 11.10 | 2.75 ± 11.34 | 0.190 |
| HDL-cholesterol (mg/dL) | 1.95 ± 1.85 | −1.65 ± 1.89 | 0.273 |
| LDL-cholesterol (mg/dL) | 0.76 ± 4.79 | −1.39 ± 4.88 | 0.799 |
| Fasting plasma glucose (mg/dL) | −0.90 ± 1.93 | −0.71 ± 1.97 | 0.957 |
| Fasting plasma insulin (uIU/mL) | −2.28 ± 1.57 | 2.27 ± 1.60 | 0.106 |
| HOMA-IR | −0.59 ± 0.39 | 0.57 ± 0.40 | 0.100 |

PV, pomegranate vinegar beverage; BMI, body mass index.
[a] LS mean ± SEM values are relative changes adjusted for baseline levels.
[b] Differences between the two groups were evaluated by ANCOVA.

can bind AMP and ATP competitively, implying that the key signal that activates AMPK *in vivo* is rising AMP coupled with falling ATP (Hardie, 2003). Acetic acid, a main component of PV, is known to be metabolized to acetyl-CoA with the consumption of ATP and results in the elevation of the AMP/ATP ratio and the phosphorylation of AMPK in hepatocytes (Fushimi et al., 2006; Li et al., 2013). In our previous animal study, we have demonstrated that the coordinated stimulation of AMPK occurred in adipose tissue and in the liver of high-fat-diet-fed obese rats by PV drinking (Ok et al., 2013). It has been generally accepted that AMPK activation switches off anabolic pathways by suppressing key gene expressions such as those of SREBP-1c and ACC (Hardie, 2011). In this RCT, although not statistically significant, we observed a differential modification of gene expression in adipose tissues between the two groups: mRNA expression levels of SREBP-1c and ACC were consistently decreased by drinking the PV, whereas consumption of the placebo had no such effect. Leptin is produced

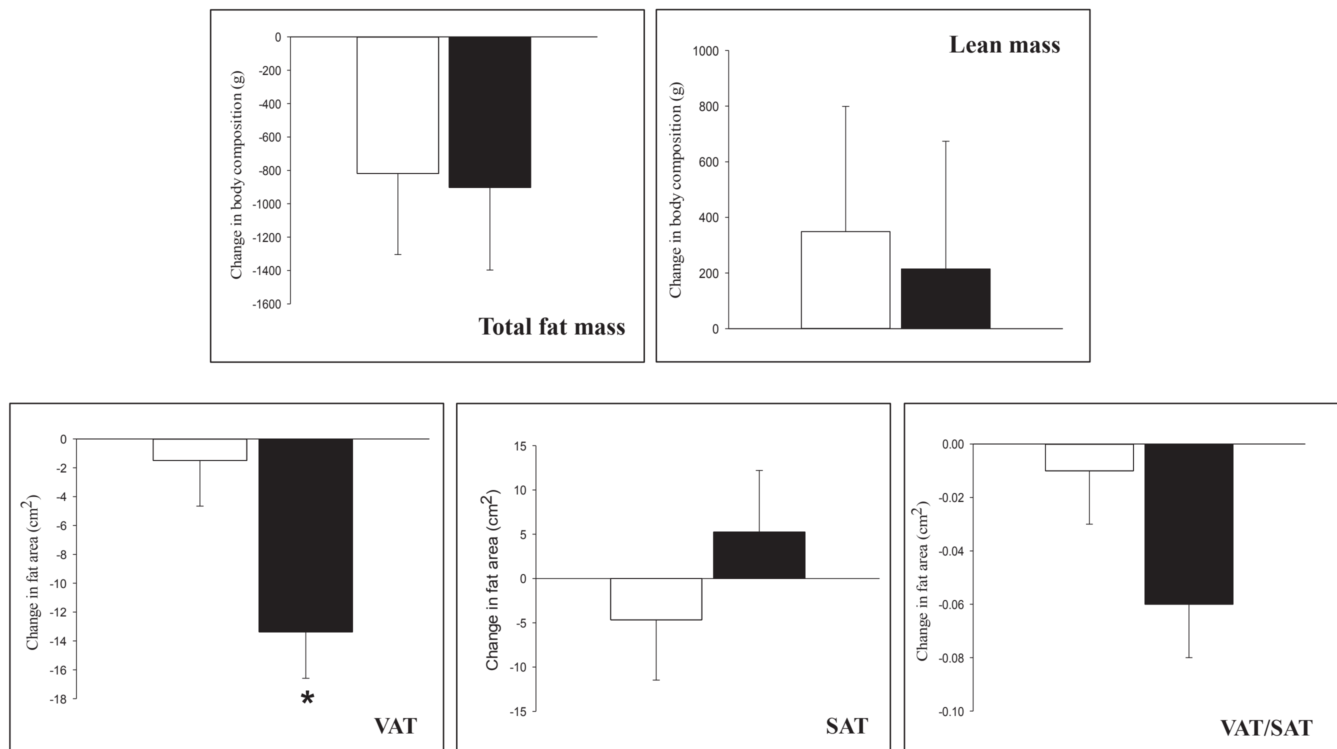
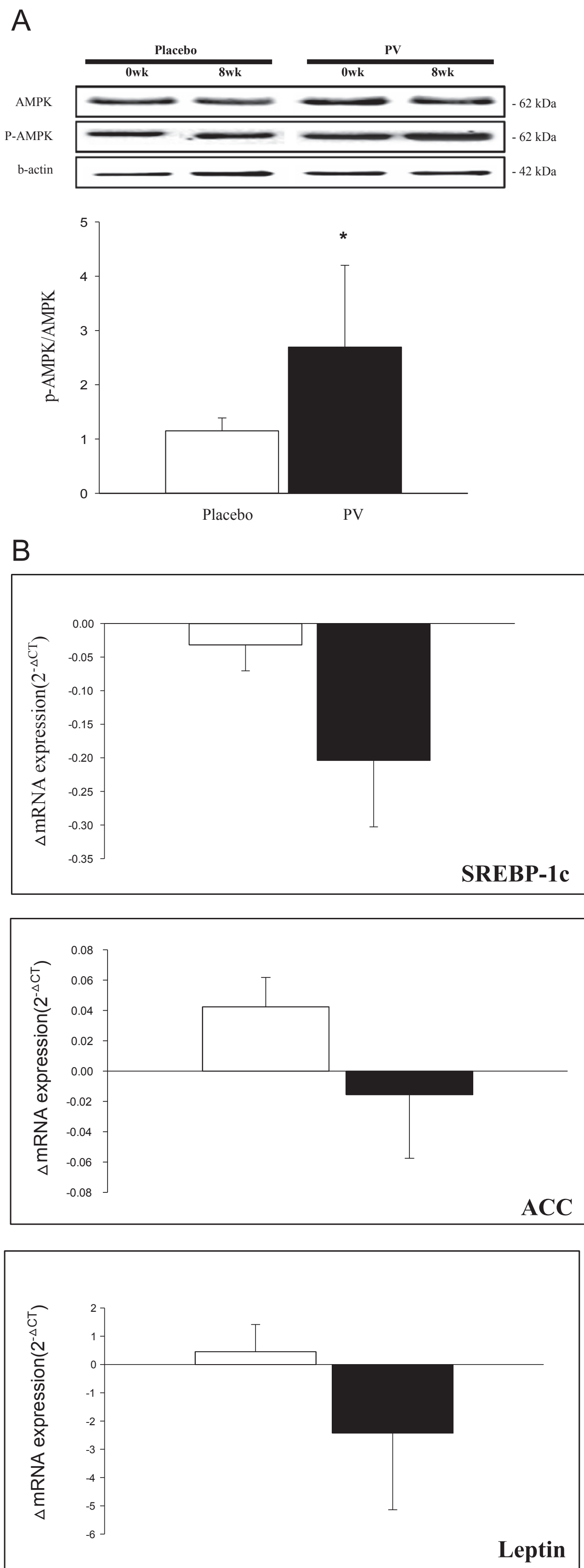



**Fig. 2 – Relative changes in fat accumulation. Seventy-seven volunteers drank 200 mL of PV ($n$ = 38) or placebo ($n$ = 39) daily for 8 weeks. Total fat and lean mass were measured using a whole-body DEXA scanner. VAT and SAT were measured at the fourth and fifth lumbar spine (L4–L5) level using a 16-slice CT scanner. The vertical bars represent placebo (□) and PV (■), respectively. The data are percentages of relative changes from baseline to week 8 and were compared with ANCOVA (*$P$ < 0.05). CT, computed tomography; DEXA, dual-energy X-ray absorptiometry; PV, pomegranate vinegar beverage; VAT, visceral adipose tissue; SAT, subcutaneous abdominal adipose tissue.**




primarily by adipocytes and associated with the control of bodyweight and energy homoeostasis (Friedman & Halaas, 1998). We also found that the pattern of mRNA expression of leptin in adipose tissue was different among the two groups studied: PV drinking resulted in a tendency of decrease in mRNA expression of leptin in adipose tissue, whereas mRNA expression of leptin was tended to increase in the placebo group. This finding supports leptin's postulated role as an extracellular signaling protein, but the difference between groups was not statistically significant. The lack of statistical significance in mRNA expression levels might be due to the low statistical power resulting from the relatively small number of subjects.

In humans, fat is organized into subcutaneous and visceral compartments. In the current study, we demonstrated that subjects in the PV group experienced significant decreases in VAT, as determined by CT scan at week 8. The amount of VAT loss that occurred with an intervention of PV in this study was greater than that observed in a previous study (–13.37 *vs.* –7.0 $cm^2$) (Kondo et al., 2009a). Numerous studies have shown that the regional distribution of body fat is a more important variable than the magnitude of generalized obesity in the relationship between obesity, metabolism, and health (Després et al., 1990), showing that VAT was more strongly associated with an adverse metabolic risk profile than SAT. Therefore, a decrease in VAT may have a positive effect on metabolic risk factors: dyslipidemia, increased blood pressure, and impaired glucose tolerance (Alberti, Zimmet, Shaw, & Group, 2005; Eckel, Grundy, & Zimmet, 2005; Isomaa et al., 2001; Kondo et al., 2009a). Adipose tissue can also be distinguished into two types functionally: white adipose tissue for storage of excess energy and brown adipose tissue for production of heat. Recent evidence highlights brown adipose tissue as a potential relevant target for human obesity (Frühbeck, Becerril, Sáinz, Garrastachu, & García-Velloso, 2009). Further study is needed to determine the effects of PV on brown adipose tissue.

In our previous study, using HPLC analysis, we revealed that PV contains arrays of phytochemicals including ellagic acid and punicalagin (Kimet al., 2013). However, we were unable to identify if ellagic acid or punicalagin works as a major causative compound. Activity-guided screening of fractions may be needed for this purpose. However, given that ellagic acid and punicalagin are unique compounds in PV, we can use them as marker compounds for the purpose of standardizing PV. In this

**Fig. 3 – Relative changes in p-AMPK/AMPK protein [A] and expression of mRNA [B] in abdominal subcutaneous adipose tissue. Western blotting of AMPK and p-AMPK and quantitative RT-PCR for mRNA of SREBP-1c, ACC, and leptin genes were performed for a subpopulation of the study: PV (*n* = 13) or placebo (*n* = 18). The vertical bars represent placebo (□) and PV (■), respectively. The blots were quantified, and the p-AMPK/AMPK ratio was determined for each treatment. Transcript levels were determined using the comparative Ct method and normalized for the β-actin. The data are relative changes from baseline to week 8 and compared with Student's *t*-test (*$P < 0.05$). AMPK, AMP-activated protein kinase; PV, pomegranate vinegar beverage.**

study, we especially chose ellagic acid because some publications have suggested that ellagic acid in pomegranate suppresses resistin secretion in white adipose tissue and murine 3T3-L1 cells (Koh et al., 2011; Lei et al., 2007; Makino-Wakagi et al., 2012). With this limitation, it is worth noting that the findings of this study clearly support the results obtained from our and other laboratories in which acetic acid, plain vinegar, or PV was used as a test material, suggesting acetic acid as an active component.

## 5. Conclusions

The present study represents, to the best of our knowledge, the first attempt to investigate the molecular mechanism underlying the effects of PV on alleviating adiposity in obese but otherwise healthy subjects. The limitations of our study included an adequate but small sample size, the duration, and the subsequent lack of power to observe significant differences between the two groups. Despite the limitations of this study, the findings obtained are in agreement with those of previous studies on animal models and cells, adding strong evidence to support the hypothesis that PV is an excellent AMPK activator and reduces VAT. Together with those of previous studies, the results suggest that PV may exert beneficial effects on adiposity and related metabolic disorders in overweight and obese individuals.

## Conflicts of interest

The authors declare no conflicts of interest.

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