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Effects of wine and grape polyphenols on blood pressure, endothelial function and sympathetic nervous system activity in treated hypertensive subjects

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Running head: Blood pressure effect of grape polyphenols in treated hypertensives

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Abstract

In a randomized double-blind crossover trial, the effect of 8 weeks supplementation with grape and wine polyphenols on functional and structural vascular parameters and autonomic activity was evaluated in 40 essential hypertensive patients treated with diuretic monotherapy. Ambulatory blood pressure, brachial artery flow mediated dilation (FMD) and pulse-wave velocity (PWV) were measured at baseline and after each 8-week intervention. Forearm resistance artery endothelial function and muscle sympathetic nerve activity (MSNA) response to mental stress and cold-pressor test were measured in two separate sub-groups. No statistically significant differences were found across time or between groups in either blood pressure, FMD, PWV, resistance artery endothelial function. The MSNA response to the two stressors was non-significantly attenuated after grape-wine polyphenol supplementation. These results do not support the hypothesis that daily consumption of a high dose of grape and wine polyphenols lowers blood pressure or affect vascular function in patients already on antihypertensive medication.

KEYWORDS: Wine, grape, polyphenol, cardiovascular disease, endothelial function, blood pressure

1. Introduction

Mediterranean dietary patterns may confer beneficial effects on the progression of cardiovascular disease (CVD)(Sofi, Abbate, Gensini, & Casini, 2010). These diets are particularly rich in polyphenols, which represent secondary plant metabolites purported to mediate these beneficial effects on human health (Rothwell et al., 2013; Vogiatzoglou et al., 2015). Wine is an important component of the Mediterranean diet and is rich in polyphenols. Epidemiological studies demonstrate that moderate wine drinkers show lower mortality rates than non-drinkers (O'Keefe, Bhatti, Bajwa, DiNicolantonio, & Lavie, 2014) and potential protective effects of grape derived polyphenols against certain types of cancer, diabetes, obesity and cardiovascular disease have also been reported (Shahidi & Ambigaipalan, 2015). Polyphenols are potent antioxidants and have been shown to have anti-inflammatory and anti-atherogenic properties, such as inhibition of peroxyl radical-induced DNA strand breakage, protection of low density lipoprotein from oxidative damage, inhibition of platelet aggregation and of the expression of adhesion molecules and of monocytes/macrophages adhesion to the endothelium (de Camargo, Regitano-d'Arce, Biasoto, & Shahidi, 2014; Denny et al., 2014; Dohadwala & Vita, 2009). Moreover, recent work has also suggested these compounds act as inhibitors of alpha-glucosidase and lipase activity (de Camargo, Regitano-d'Arce, Biasoto, & Shahidi, 2016). Consequently, these properties may contribute to the health benefits of increased polyphenol intake in humans.

Hypertension is one of the primary risk factors for CVD-related morbidity and mortality. Human intervention studies demonstrate that consumption of products rich in grape and wine polyphenols lower blood pressure, although the data are not entirely consistent (Botden et al., 2012; Chiva-Blanch et al., 2012; Dohadwala et al., 2010; Droste et al., 2013; Mellen, Daniel,

Brosnihan, Hansen, & Herrington, 2010; van Mierlo, Zock, van der Knaap, & Draijer, 2010; Ward et al., 2005). The blood pressure lowering effects of grape-derived polyphenols may be mediated by improvement in resistance artery function and/or decreases in peripheral artery vascular tone. For example, studies with wine and grape extracts have demonstrated improved endothelial function in conduit and resistance arteries (Botden et al., 2011; Siasos et al., 2014; Stein, Keevil, Wiebe, Aeschlimann, & Folts, 1999), possibly via nitric oxide dependent pathways (Botden et al., 2011). In addition, studies with tea and cocoa, i.e. prominent sources of dietary polyphenols, found improvement in indirect measures of sympathetic nervous system (SNS) activity patterns, possibly contributing to lowering of peripheral vascular tone (Steptoe et al., 2007; Wirtz et al., 2014).

To date, most previous studies investigating the blood pressure lowering effects of wine and/or grape extracts were conducted in healthy participants or in non-medicated hypertensives (Li, Zhao, Tian, Chen, & Cui, 2015). Such studies poorly translate to the majority of hypertensive patients who typically receive lifelong antihypertensive medication. Accordingly, the objective of the current study was to determine whether 8-week consumption of a polyphenol-rich grape-wine extract mix affect ambulatory blood pressure, endothelial function and muscle sympathetic nerve activity (MSNA) in drug treated patients with essential hypertension. We hypothesized that intake of a high daily dose of polyphenols lowers blood pressure, regardless of antihypertensive medication use, an effect mediated through improvement in resistance artery endothelial function and reduction in MSNA.

2. Methods

2.1. Participants

Fifty-one hypertensive patients on diuretic monotherapy were recruited from the outpatient clinic of the University of Pisa (starting December 2009). Inclusion criteria were office systolic BP values ≥140 mm Hg and/or office diastolic BP values ≥90 mm Hg, which were confirmed on repeated occasions within one month according to current guidelines, if untreated or controlled (BP<140-90 mmHg) by diuretic therapy (Mancia et al., 2013). Exclusion criteria were as follows: previous cardiovascular or cerebrovascular events, clinically significant arrhythmia, diabetes mellitus, smoking, clinically apparent liver disease or kidney damage, current treatment with statins and/or hormone replacement therapy, reported alcohol consumption > 28 units/week. The study protocol was approved by the local ethical committee of University Hospital of Pisa and was in accordance with guidelines in the Declaration of Helsinki. Patients gave their written informed consent to participation in the study after an explanation of its nature and purpose.

2.2. Experimental Design

This study adopted a randomized, placebo-controlled, double-blind crossover design with two 8-week intervention periods. At an initial screening visit, eligible patients were given dietary advices for a standard Mediterranean diet and informed to drink no more than two units of alcohol per day. Moreover, the patients were instructed to moderate their intake of polyphenol-rich products throughout the study (less than two daily cups of coffee and/or tea; avoid dark chocolate and red wine). For the 48-h preceding the experimental days, subjects were instructed to avoid consumption of all polyphenol-rich foods in order to fully exclude the impact of background dietary polyphenols.

Following a 4-week run-in period, patients were randomly allocated to either grape-wine extract or placebo treatments. After an 8-week intervention, patients were crossed over to the other treatment. The diuretic dose was kept stable throughout the run-in and intervention periods. Measurements were performed on three different occasions, at baseline and immediately after each 8-week treatment period. Invasive measurements (forearm resistance vessel endothelial function and muscle sympathetic nerve activity) were conducted before and after the first 8-week intervention period only. Therefore, data on these measures are available in two different subsets of the study population (Fig. 1).

2.3. Intervention

The grape-wine extract mix comprised of 870 mg of red wine extract (Provinols™; Seppic, France) and 540 mg grape juice extract (MegaNatural™ Rubired; Polyphenolics, USA). The total polyphenol content of the extract mix amounted to 800 mg (defined as gallic acid equivalents): 550 mg from the wine extract and 250 mg from the grape juice extract.

The polyphenol composition of the red wine- and grape juice extracts was determined in duplicate by means of high-performance liquid chromatography with diode array detection (HPLC-DAD) and HPLC with electrospray ionization mass spectrometry (HPLC-ESI-MS) using an Agilent HPLC series 1100 equipped with ChemStation software as previously reported (van Dorsten et al., 2010). For determination of anthocyanins a mobile phase consisting of water, formic acid and acetonitrile and a Betasil C18 column (Thermo Scientific, 150 x 2.1 mm i.d., 5 µm particle size), with a Guard Column Cartridge was used. The individual anthocyanins were

quantified via DAD using a calibration curve of cyanidin 3-O-glucoside (Roth, Karlsruhe, Germany), including a molecular weight correction factor (Chandra, Rana, & Li, 2001). The identification and peak assignment were accomplished by simultaneous HPLC-ESI-MS analysis in the positive ion mode (selected ion monitoring) as well as in scan mode. Increase of the fragmentor voltage resulted in cleavage of the pigments and release of the anthocyanidin aglycones, which were identified by comparison of their m/z ratios with those described in the literature (Wang, Race, & Shrikhande, 2003).

For determination of catechins, flavonols and stilbenes a mobile phase consisting of water, acetic acid and acetonitrile and a Synergi Hydro-RP column, (Phenomenex, 250 x 2mm i.d.; 5µm particle size) with a Guard Column Cartridge, was used. The individual phenolic acids, catechins, flavonols and stilbenes were quantified using a calibration curve of the corresponding standard compounds (gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, chlorogenic acid, gallocatechin, catechin, epicatechin, epicatechin-3-gallate, resveratrol (Sigma, St. Louis, USA); ellagic acid (Roth, Karlsruhe, Germany); myricetin, kaempferol-3-O-rutinoside (Extrasynthése, Lyon, France); quercetin-3-D-galactoside, quercetin-3-β-D-glucoside, quercetin-3-rhamnoside, quercetin (Fluka, Buchs, Switzerland)). The identification and quantification was accomplished by HPLC-ESI-MS analysis in the negative ion mode (selected ion monitoring).

Analysis of the extracts revealed that each daily dose of the wine and grape extract mix contained approximately 140 mg anthocyanidins and 40 mg flavanols along with small amounts of flavonols, phenolic acids and stilbenes with the remaining polyphenolic portion of the extracts consisting of unidentified polymeric proanthocyanidins. Detailed compositional information of

the extracts are reported in Table 1. Each daily dose was provided in six gelatine capsules (Capsugel Conisnap no. 0) which were taken at breakfast. Identical capsules containing microcrystalline cellulose (Avicel PH101, FMC Biopolymer) served as the placebo. Subjects were instructed to return all unused capsules at the end of each intervention period and compliance was determined by capsule counting. Average compliance was 88%.

2.4. Experimental Measures

2.4.1. Ambulatory blood pressure

At baseline and at the end of each 8-week intervention period, a 24-hour ambulatory blood pressure recording was performed using a Spacelabs monitor (Type 90 217; Spacelabs Medical Inc.) placed on the non-dominant arm. Blood pressure was recorded at 15-min intervals throughout the day and at 20-min intervals during the night (11 PM – 8 AM).

2.4.2. Forearm resistance vessel endothelial function

In a subset of 25 subjects (n=13 grape-wine, n=12 control), forearm resistance artery endothelial function was evaluated before and after the first 8-week intervention period by means of the isolated and perfused forearm technique as described previously (Virdis et al., 2001). Briefly, the brachial artery of the non-dominant arm was cannulated for vasoactive drug infusion at systemically ineffective doses. Forearm blood flow was measured in the experimental and contralateral forearm by strain-gauge venous occlusion plethysmography. Forearm blood flow was calculated using standard formulae and expressed as ml/100 ml forearm volume/min. To account for effects of potential arterial pressure variations, forearm vascular resistance (FVR) was calculated by dividing mean arterial pressure by forearm blood flow.

Endothelium-dependent vasodilation was assessed by a dose-response curve to intra-arterial acetylcholine (ACh; cumulative increase in infusion rates by 0.15, 0.45, 1.5, 4.5 and 15 mg/100 ml forearm tissue per min, 5 min each dose). To evaluate the NO availability, the response to ACh was repeated in the presence of the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA, 4 mmol/min) (Virdis et al., 2001). Because L-NMMA modifies blood flow, sodium nitroprusside (SNP; 0.2 mg/100 mL tissue/min for 5 min) was co-infused to neutralize the L-NMMA-induced vasoconstriction and restore baseline FVR. The role of reactive oxygen species (ROS) generation on endothelial function was investigated by repeating the ACh-infusion protocol under co-infusion of ascorbic acid (8 mg/100 mL forearm tissue/min) as well as during co-infusion of L-NMMA and ascorbic acid. L-NMMA and ascorbic acid infusion were started 10 min before ACh-infusion and continued throughout this protocol. A 30 min washout was allowed between each dose–response curve, whilst this washout was consistently prolonged to 60 min when L-NMMA was infused. Finally, endothelium-independent vasodilation was assessed with a dose-response curve to intra-arterial infusion of SNP (1, 2, and 4 mg/100 mL forearm tissue/min, 5 min each dose). To avoid making multiple comparisons, the responses to the vasoactive substances were expressed as the area under the curve (AUC) of change in FVR from baseline, expressed in arbitrary units. Analysis was performed by a single investigator (A.V.) blinded to the patient's allocation to treatment.

2.4.3. Muscle sympathetic nerve activity

In a subset of 16 subjects (n=8 grape-wine, n=8 control), multiunit recording of efferent postganglionic muscle sympathetic nerve activity (MSNA) of the peroneal nerve was obtained

using microneurography before and after the first 8-week intervention period. Briefly, a tungsten microelectrode with an uninsulated 1–5-μm-diameter tip (Medical Instruments, University of Iowa) was transcutaneously inserted in the peroneal nerve just posterior to the fibular head, as previously described (Bruno, Sudano, Ghiadoni, Masi, & Taddei, 2011). A reference electrode was inserted subcutaneously 1 to 3 cm from the recording site. The signal was integrated with a 0.1-s time constant, amplified with a gain of 50,000–80,000, band-pass filtered (700–2000 Hz), and acquired at 1000 Hz through a digital acquisition system (ACQ-16; Gould Electronics). MSNA was identified according to previously outlined criteria (Bruno et al., 2011; Delius, Hagbarth, Wallin, & Hongell, 1972). Obtained neurograms were recorded together with BP and heart rate by means of dedicated computer software (Ponemah; LDS). Recordings were considered acceptable if the signal:noise ratio exceeded the value of 3. MSNA responses were measured at rest and during 2-min of mental stress (serial 7 subtraction (Birkett, 2011)) followed by 2-min of cold pressor test. Data were quantified as bursts/min (burst frequency) and bursts/100 heart beats (burst incidence). MSNA was analysed by visual inspection by a single investigator (R.M.B.) blinded to the patient's allocation to treatment.

2.4.4. Brachial artery flow mediated dilation

Before and after both 8-week interventions, we examined brachial artery flow meditated dilation (FMD) using high-resolution ultrasound with a 10 MHz linear array transducer (MyLab25, ESAOTE, Florence, Italy), following recent guidelines as previously described (Thijssen et al., 2011). Endothelium-independent dilation was obtained by sublingual administration of 25 μg glyceryl trinitrate (GTN). FMD and the response to GTN were calculated as the maximal percentage increase in diameter. Analysis of changes in brachial artery diameter was performed

using a real-time computerized edge detection system, which is independent of investigator bias (Gemignani, Faita, Ghiadoni, Poggianti, & Demi, 2007; L. Ghiadoni et al., 2012) by a single investigator (L.G.) blinded to patient's allocation to treatment.

2.4.5. Arterial stiffness and wave reflection

Before and after both 8-week interventions, we assessed arterial tonometry according to international recommendations using procedures previously described (Plantinga et al., 2007). A hand held probe was placed on the radial artery and 10–15 subsequent images were recorded. Radial pressure waveform was transformed into aortic pressure waveform by pulse wave analysis (PWA) (SphygmoCor, AtCor Medical) using a validated transfer function. Two successive measurements were recorded and averaged. Augmented pressure was calculated as the difference between the second systolic peak and the first systolic peak, and augmentation index (AIx) was calculated as the ratio between augmented pressure and pulse pressure. Since AIx is correlated with heart rate, values have been normalized at a heart rate of 75 beats per minute. Aortic pulse wave velocity (PWV) was assessed with the same device, sequentially recording pressure waveforms at the femoral and carotid site. PWV was calculated as the ratio of the surface distance between the two recording sites (subtracting the carotid–sternal notch distance from the femoral–sternal notch distance) and wave transit time. Analysis was performed by a single investigator (R.M.B.) blinded to patient's allocation to treatment

2.5. Statistical analysis

All statistical analyses were conducted using JMP version 11.0 (SAS Institute Inc., Cary, NC, USA). Descriptive statistics are presented as means and standard deviation (SD). All data are

reported as LSmeans (95%CI), unless reported otherwise and was considered statistically significant at $P<0.05$. The change in outcome parameters of the invasive measurements (parallel-group study; FVR, MSNA) were analysed using a repeated measures ANCOVA with treatment as between-subject effect and period, ACh dose (for the FVR group) and stimulus (for the MSNA group) as within-subject effects and resting baseline measurement as covariate. The change in outcome parameters of the non-invasive measurements (FMD, PWV, PWA, blood pressure), which were performed before and after both interventions, were analysed using a mixed model with subject as random factor, treatment and period as fixed effects and the baseline measurement as covariate.

Power calculations indicated that: 40 subjects (20 per intervention arm) would be sufficient to detect an absolute difference of 1% in the FMD response between treatments in a crossover study design with a 80% power and a 5% significance; 20 subjects (10 per intervention arm) would be sufficient to detect a difference between treatments in the expected mean change of 20% in the percent L-NMMA inhibition on ACh-induced vasodilation in the forearm microcirculation (80% power, 5% significance in parallel group study design); 16 subjects (8 per intervention arm) are sufficient to detect a difference between treatments in the expected mean change of 10% in MSNA (burst/minute) (80% power, 5% significance in a parallel study design). In the laboratory performing the evaluation, the coefficients of variation of the latter variables is less than 5% (Bruno et al., 2011; Pedrinelli, Taddei, Graziadei, & Salvetti, 1986).

3. Results

Of the 51 subjects screened for inclusion, three were classified as screening failures as they all demonstrated blood pressure not ≤140/90 mmHg at the end of the run-in period. A further eight subjects did not complete the study procedures for personal reasons – five decided to not continue after Visit 0 and three more after Visit 1 (Fig. 1). No subjects were excluded during blind review. The Per Protocol and Intention to Treat study populations are thus equal and consist of 41 subjects that completed all study procedures of the first phase of the study and 40 subjects that completed all study procedures of the second phase of the study. Baseline characteristics are described in Table 2.

3.1. 24-hour ambulatory blood pressure, large artery endothelial function and stiffness

No statistically significant differences were found across time or between groups in either systolic- or diastolic blood pressure, FMD, GTN mediated dilation and PWV. Regarding PWA, no statistically significant changes were found across time or between groups in either central pulse pressure, augmentation pressure, AIx or AIx_{75} (Table 3).

3.2. Endothelium-dependent dilation in the microcirculation

In both the grape-wine and placebo groups, the decrease in FVR in response to ACh infusion was larger after the 8-week intervention period (Time effect, $P < 0.001$, Fig. 2). No Treatment effect or Time*Treatment*ACh Dose interaction was found, indicating that the change in FVR response to ACh-infusion was comparable between groups. Analysing the area-under-the-curve of the FVR response to ACh also revealed a change after the intervention period (Time effect P <

0.001) that was not different between the two groups (Treatment effect, P = 0.19, Time*Treatment interaction, P = 0.43, Fig. 3).

3.3. Endothelium-independent dilation in the microcirculation

FVR responses to SNP infusion were greater after the 8-week intervention period (Time effect, P = 0.02, Fig. 3). No Treatment effect or Time*Treatment interaction was apparent however (P = 0.16 and P = 0.28 respectively).

3.4. Nitric Oxide availability & ROS production

Decreases in FVR in response to ACh were inhibited through co-infusion of L-NMMA, whilst the magnitude of inhibition was increased after the 8-week intervention period (Time effect, P = 0.02, Fig. 3). This indicates a larger contribution of NO to resistance artery endothelial function after the intervention period. However, the magnitude of change across time did not differ between groups (Fig. 3).

Co-infusion of Vitamin C with ACh caused a larger decrease in FVR at baseline, indicating a role for ROS production to increase resting vascular tone. The intervention was associated with an attenuated decrease in FVR during co-infusion of Vitamin C and ACh ("Time effect", P = 0.01), whilst this effect was similarly present in both groups (Fig. 3). Finally, we found that co-infusion of Vitamin C potentiated the increase in FVR induced by L-NMMA. After the intervention, the potentiating effect of Vitamin C on L-NMMA was reduced (Time effect, P = 0.03), indicating that the improvement in L-NMMA responses after the intervention are, in part, mediated through decreased ROS production. Nonetheless, no Treatment effects or

Time*Treatment interactions were found for the co-infusion of Vitamin C and L-NMMA (Fig. 3).

3.5. Muscle sympathetic nervous activity

Resting MSNA burst frequency and burst incidence did not change in either grape-wine or placebo groups after the 8-week intervention period (Fig. 4). The increase in MSNA burst frequency in response to mental stress and cold pressor test was comparable after the 8-week interventions. However, MSNA burst incidence during mental stress and the cold pressor test was attenuated after the 8-week intervention in the grape-wine group, whilst MSNA burst incidence during these tests increased after placebo. These differences were not statistically significant though (Fig. 4, Time*Treatment interaction, P = 0.06, Time*Treatment*Stimulus interaction, P= 0.24). The increase in mean arterial pressure in response to mental stress and cold pressor test was comparable after the 8-week interventions. However, a statistically significant Time*Treatment interaction was found for the increase in heart rate in response to mental stress and cold pressor test. More specifically, heart rate during mental stress and the cold pressor test increased after the 8-week intervention in the grape-wine group, whilst the increase was attenuated during these tests increased after placebo (Table 4).

4. Discussion

The aim of this study was to determine whether a high daily intake of grape and wine polyphenols affected blood pressure, endothelial function and MSNA in treated hypertensive subjects. We observed that 8 weeks supplementation with a mixture of grape and wine extracts, providing a daily dose of 800 mg polyphenols, did not result in changes in 24-hour ambulatory blood pressure compared to placebo. Furthermore, we found no effects of dietary intake of

polyphenols on resistance- or conduit artery endothelial function, arterial stiffness or measures of resting SNS activity. However, we found an attenuated increase in MSNA during sympathetic stimulation in those who received daily grape-wine polyphenol supplementation. Taken together, these data suggest that neither ambulatory 24-h blood pressure nor measures of vascular function or tone are affected by grape and wine polyphenol intake in patients receiving antihypertensive medication. Possible beneficial effects of polyphenols may however be present by attenuating increases in autonomic stress reactivity, which is a possible determinant of poor cardiovascular outcome (Chida & Steptoe, 2010).

A recent, well-controlled study in untreated mildly hypertensive subjects, found small reductions in blood pressure and endothelin-1 after 4 weeks' twice daily consumption of grape and wine polyphenols (Draijer, de Graaf, Slettenaar, de Groot, & Wright, 2015). Moreover, a recent meta-analysis of 10 studies (including mostly un-medicated subjects) indicated a small (1.5 mmHg) reduction in systolic blood pressure at an average dose of grape derived polyphenols close to that of our study (Li et al., 2015). In the current "diseased" study population, it is plausible that a longer treatment duration may be needed to elicit a blood pressure lowering effect. Our observations are however in agreement with several previous studies, all which have found no effects of grape and/or wine polyphenols on blood pressure or measures of vascular function and stiffness in both healthy subjects and those with elevated CVD risk (Botden et al., 2012; Droste et al., 2013; Mori et al., 2016; Ras et al., 2013; van Mierlo et al., 2010; Ward et al., 2005) .

Effects on blood pressure and vascular structure and function have been ascribed various different individual polyphenols and polyphenol classes present in grape-derived foods, with

flavanols (e.g. catechin, epicatechin, epigallocatechin gallate) having the most robust evidence at this time (Kay, Hooper, Kroon, Rimm, & Cassidy, 2012). Isolated anthocyanins, stilbenes and flavonols have to varying extents also been associated with either beneficial effects on blood pressure, endothelial function or both, although the evidence is not entirely consistent (Rodriguez-Mateos et al., 2013; Wong et al., 2013; Zhu et al., 2016; Zhu et al., 2011). As such we opted to utilise a mixture of two grape products in order to cover the spectrum of grape-related polyphenols normally found in the diet.

Polyphenols are thought to improve endothelial function by increasing bioavailability of NO. Specifically, polyphenols may stimulate activity of endothelial nitric oxide synthase (eNOS) and prevent superoxide-mediated NO breakdown (Fitzpatrick, Hirschfield, Ricci, Jantzen, & Coffey, 1995; Grassi et al., 2008). We specifically chose to include only subjects on diuretic monotherapy to avoid direct vascular effects of most other commonly prescribed anti-hypertensive agents (Ghiadoni, Taddei, & Virdis, 2012). Thiazide diuretics, which were prescribed to the patients in our study, are known to have no effects on endothelial function (Chung, Beevers, & Lip, 2004; Klingbeil et al., 2003; Yamanari, Nakamura, Miura, Yamanari, & Ohe, 2009). Therefore it seems unlikely that the use of diuretics would have obscured any potential effects of grape and wine polyphenols on measures of endothelial function in our study.

Polyphenols are rapidly metabolized and eliminated from the circulation with peak plasma concentrations usually occurring a few hours after intake (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). Accordingly, several studies have found that intake of grape derived polyphenols resulted in increases in brachial artery FMD 30 to 120 minutes after intake (Li,

Tian, Zhao, Chen, & Cui, 2013). FMD and resistance artery endothelial function in our study was measured several hours after intake of the last dose of polyphenols. It is plausible that a transient improvement in endothelial function might have been missed due to the single daily dose regime and timing of the measurements. To our knowledge only one other study investigated the effects of red wine polyphenols on resistance artery responses to infusion of endothelium-dependent and independent agonists (Botden et al., 2011). In contrast to our findings, increases in both ACh and SNP mediated vasodilation were seen after daily consumption of red wine for 3 weeks in healthy young women. Time of wine ingestion (acute and the evening prior to measurements) differed from our study. This study did not include a control group however, so it is unclear what portion of the observed effects could be explained by the alcohol content of the wine.

The polyphenol content of the background diet may also explain the lack of effects on blood pressure or endothelial function in the current study. Several prospective follow-up studies have found non-linear dose-response relationships between flavonoid intake and CVD risk, with low risks already occurring at relatively low levels of intake (Cassidy et al., 2011; McCullough et al., 2012; Mink et al., 2007). Moreover, dose-response studies of tea and cocoa flavonoids have found that the relative increases in FMD become smaller with increasing doses of flavonoids (Grassi et al., 2015; Grassi et al., 2009). Given that the average polyphenol content of the Italian diet is relatively high (Vogiatzoglou et al., 2015), it is plausible that additional grape and wine polyphenols would not have caused any demonstrable hemodynamic or vascular effects.

It is well established that the SNS is involved in regulation of blood pressure and vasomotor tone (Bruno et al., 2012). Studies of tea and cocoa polyphenols have found effects on indirect measures of SNS activity (Steptoe et al., 2007; Wirtz et al., 2014). This led to us hypothesize that grape and wine polyphenol consumption would affect resting SNS activity as well as the extent of SNS activation in response to various stimuli. We found no change in resting SNS activity in response to grape and wine polyphenol consumption. However, the extent of SNS activation by mental stress and the cold pressor test was attenuated in the subjects receiving grape and wine polyphenols. This finding may be of clinical significance as elevated sympathetic and cardiovascular reactivity to stressful stimuli has been associated with the development of hypertension and cardiovascular disease (Matsukawa et al., 1991; Park, Middlekauff, & Campese, 2012; Steptoe & Marmot, 2005).

The renin–angiotensin system may interfere with the sympathetic function and inhibition of angiotensin converting enzyme activity (ACE) has been shown to affect MSNA (Grassi, 2016). Isolated polyphenols and polyphenol-rich foods have shown ACE inhibitory activity both *in vitro* and *in vivo* (Guerrero et al., 2012; Parichatikanond, Pinthong, & Mangmool, 2012; Persson, Persson, Hagg, & Andersson, 2010). Structure-activity relationship studies have found that the presence of: 1) a catechol group in the B-ring, 2) a double bond between C2 and C3 at the C-ring, and 3) a ketone group in C4 at the C-ring are important determinants of the level of ACE inhibitory activity (Guerrero et al., 2012). A number of the polyphenols found in wine and grape extracts are known to directly interact with the sympathetic and central nervous systems which might explain the MSNA effects observed in the present study (Lee, Seo, & Lim, 2009; Shinohara et al., 2007; Wasowski & Marder, 2012). Notably resveratrol has been demonstrated

to inhibit agonist-induced catecholamine synthesis and secretion by inhibiting nicotinic acetylcholine receptor-ion channels in the adrenal medulla and sympathetic neurons in *in vitro* studies (Shinohara et al., 2007). It is also noteworthy that AIx was differentially modified (although in a non-significant manner) in the two intervention arms in the presence of similar values of AP and HR. This difference might be explained by the sympathoinhibitory effect of polyphenols suggested by the attenuation of autonomic reactivity to stress. Longer treatment might induce a greater reduction of AIx, contributing to a possible BP-lowering effect. These observations should be interpreted with caution though as the sub-group in which MSNA was measured was small (n=16) and the differences did not reach statistical significance.

Limitations: We did not measure circulating or urinary levels of polyphenol metabolites. Therefore, we cannot comment on the bioavailability of the polyphenols from the encapsulated extracts provided in our study. However, after a previous 4-week intervention with the same type and dose of grape and wine extracts provided in capsules, we found significant elevations in the subject's urinary excretion of a wide range of phenolic acids (van Dorsten et al., 2010). This suggest that at least a portion of the polyphenols from the grape and wine extracts or their metabolites formed in the body's tissues or the colonic microflora will typically reach the circulation. The subgroups in which we measured FVR and SNS activity were quite small. However, both techniques are highly reproducible and sensitive enough to reveal subtle changes (Bruno et al., 2011; Pedrinelli et al., 1986). Strengths of this study include the double-blind crossover design, long duration and the use of accurate 24-hour ambulatory blood pressure measurements combined with gold-standard measures of resistance artery endothelial function and SNS activity in the same subjects. We observed a consistent absence of effects of grape and

wine polyphenols on resting blood pressure and across a range of integrated vascular and endothelial parameters related to its regulation. These observations support the robustness of our findings.

In summary, this study does not support the hypothesis that 8 week once-daily consumption of a high dose of grape and wine polyphenols lowers resting blood pressure in subjects receiving antihypertensive medication. The potential of grape and wine polyphenols to attenuate over-responsiveness of the SNS should be confirmed in larger, well-controlled studies set up for this purpose. Future studies should also determine which subclasses of polyphenols common to different foods can lower blood pressure and improve endothelial function. It should also be determined whether potential vascular and hemodynamic effects vary by subject's health- and treatment status.

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Disclosures

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Figure captions

Fig. 1. Enrolment, randomization and experimental design of the study

Fig. 2. Change in forearm vascular resistance induced by increasing doses of acetylcholine at before (Visit 0, closed symbols) and after 8 weeks' intervention (Visit 1, open symbols) with grape-wine extract (A) or placebo groups (B). P-values refer to a 3-way repeated measures ANCOVA with treatment (grape-wine vs placebo) as between-subject effect, period (Visit 0 vs 1) and stimulus as within-subject effects and resting baseline measurement as covariate. Data are presented as LSmeans (95% CI).

Fig. 3. Change in forearm vascular resistance expressed as area under curve at baseline (grey bars) and after 8 weeks (black bars) in the grape-wine and placebo groups. Data are presented as LSmeans (95% CI).

Fig. 4. Change in MSNA burst frequency (A) and burst incidence (B) response to mental stress and cold pressor test before (Visit 0, closed symbols) and after 8 weeks intervention (Visit 1, open symbols) with grape-wine extract or placebo. P-values refer to a 3-way repeated measures ANCOVA with treatment (grape-wine vs placebo) as between-subject effect, period (Visit 0 vs 1) and stimulus as within-subject effects and resting baseline measurement as covariate. Data are presented as LSmeans (95% CI).

714 **Table 1.** Polyphenol content of the red wine and grape juice extracts

	Wine (mg/g)	Grape juice (mg/g)	Total in 870 mg wine + 540 mg grape juice
Anthocyanins	**21.50**	**225.85**	**140.66**
delphinidin 3,5-diglucoside	0.00	3.59	1.94
cyanidin 3,5-diglucoside	0.00	1.78	0.96
delphinidin 3-glucoside	0.47	3.23	2.15
petunidin 3,5-diglucoside	0.00	10.32	5.57
cyanidin 3-glucoside	0.11	1.29	0.79
peonidin 3,5-diglucoside	0.00	46.05	24.87
malvidin 3,5-diglucoside	0.00	82.75	44.69
peonidin 3-glucoside	1.76	5.40	4.45
malvidin 3-glucoside	9.26	11.66	14.35
delphinidin 3-coumaroyl-5-diglucoside	0.00	3.53	1.91
cyanidin 3-coumaroyl-5-diglucoside	0.00	0.93	0.50
petunidin 3-coumaroyl-5-diglucoside	0.00	7.33	3.96
delphinidin 3-coumaroylglucoside	0.35	1.09	0.90
peonidin 3-coumaroyl-5-diglucoside	0.00	5.37	2.90
malvidin 3-coumaroyl-5-diglucoside	0.00	34.51	18.63
petunidin 3-coumaroylglucoside	0.46	0.70	0.78
peonidin 3-coumaroylglucoside	0.80	0.65	1.04
malvidin 3-coumaroylglucoside	5.36	5.66	7.72
petunidin 3-glucoside	1.29	0.00	1.12
peonidin 3-acetylglucoside	0.15	0.00	0.13
malvidin 3-acetylglucoside	1.49	0.00	1.30
Phenolic acids	**7.89**	**5.22**	**9.68**
caffeic acid	1.27	0.30	1.26
p-coumaric acid	0.68	0.46	0.84
ferulic acid	0.07	0.09	0.11
gallic acid	1.46	2.02	2.36
protocatechuic acid	0.62	0.30	0.70
p-hydroxybenzoic acid	0.20	0.08	0.21
vanillic acid	0.39	0.19	0.45
syringic acid	1.02	1.00	1.42
caftaric acid	0.60	0.18	0.62
coutaric acid	0.79	0.09	0.73
fertaric acid	0.52	0.30	0.61
ellagic acid	0.24	0.19	0.31
chlorogenic acid (5-O-caffeoylquinic acid)	0.03	0.03	0.05
Flavanols	**45.18**	**0.40**	**39.52**
catechin	12.85	0.13	11.25

epicatechin	12.17	0.10	10.64
procyanidin B1	9.97	0.05	8.70
procyanidin B2	6.78	0.02	5.90
procyanidin C1	1.19	0.00	1.04
gallocatechin	0.62	0.05	0.56
epigallocatechin	0.20	0.00	0.17
epicatechin-3-O-gallate	1.41	0.06	1.25
Flavonols	**4.82**	**9.48**	**9.31**
hyperoside (quercetin-3-O-galactoside)	0.13	0.10	0.17
miquelianin (quercetin-3-O-glucuronide)	1.78	2.49	2.89
isoquercitrin (quercetin-3-O-glucoside)	0.66	1.39	1.32
qercitrin (quercetin-3-O-rhamnoside) + astragalin (kaempferol-3-O-glucoside)	0.12	0.11	0.16
quercetin	0.47	1.17	1.04
kaempferol	0.05	0.16	0.13
myricetin-3-O-glucoside	0.94	2.45	2.14
myricetin	0.67	1.61	1.46
Stilbenes	**0.97**	**0.15**	**0.92**
polydatin 1*	0.37	0.06	0.35
polydatin 2*	0.36	0.07	0.35
trans-resveratrol	0.24	0.02	0.22

717 **Table 2.** Subject characteristics from hypertensive patients included in the trial. Data are

718 presented as mean ± SD.

Characteristics		
N	40	
Gender, females/males	4/36	
Age (years)	46.8	± 9.0
Weight (kg)	80.4	± 8.2
Body Mass Index (kg/m^2)	26.1	± 2.1
Systolic blood pressure (mmHg)	141.1	± 8.0
Diastolic blood pressure (mmHg)	87.9	± 5.0
Plasma glucose (mg/dL)	93.0	± 10.7
Total cholesterol (mg/dL)	194.0	± 38.0
HDL cholesterol (mg/dL)	52.0	± 12.0
LDL cholesterol (mg/dL)	119.6	± 33.5
Triacylglycerol (mg/dL)	104.0	± 51.6

719

720 **Table 3**. Hemodynamic and vascular measurements at baseline and the end of the grape-wine
721 and placebo intervention periods. Data are presented as raw unadjusted means ± SD.

	Baseline	**Grape-Wine**	**Placebo**	**P-value***
24-h SBP (mmHg)	134 ± 9	131 ± 9	131 ± 9	0.9
24-h DBP (mmHg)	81 ± 8	79 ± 8	79 ± 8	0.7
24-h HR (bpm)	68 ± 9	68 ± 9	67 ± 8	0.5
Day-time SBP (mmHg)	138 ± 10	136 ± 10	135 ± 9	0.8
Day-time DBP (mmHg)	85 ± 8	83 ± 8	83 ± 8	0.8
Day-time HR (bpm)	71 ± 9	72 ± 10	71 ± 9	0.3
Night-time SBP (mmHg)	127 ± 9	125 ± 10	125 ± 9	0.6
Night-time DBP (mmHg)	75 ± 8	73 ± 8	74 ± 8	0.4
Night-time HR (bpm)	63 ± 9	62 ± 8	62 ± 8	0.7
Baseline BAD (mm)	4.4 ± 0.8	4.5 ± 0.8	4.4 ± 0.8	0.07
FMD (%)	4.8 ± 2.6	5.0 ± 2.7	5.2 ± 3.1	0.6
GTN (%)	7.8 ± 3.8	7.2 ± 4.6	7.2 ± 3.1	0.9
PWV (m/s)	7.9 ± 1.1	7.8 ± 1.1	7.7 ± 1.2	0.6
Central PP (mmHg)	45.8 ± 10.2	44.2 ± 8.8	44.2 ± 8.7	0.9
AIx (%)	27.9 ± 20	23.1 ± 12.5	24 ± 11.3	0.5
AIx_{75} (%)	21.4 ± 13.6	18.3 ± 11.7	21.0 ± 14.8	0.2
AP (mmHg)	13 ± 7.6	11.8 ± 8.4	12.2 ± 7.6	0.7

722 SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; BAD, Brachial Artery Diameter;
723 FMD, Flow Mediated Dilation; GTN, Glycerol Trinitrate induced dilation; PWV, Pulse Wave
724 Velocity; PP, pulse pressure; AIx, Augmentation Index; AIx_{75}, Augmentation Index corrected for

heart rate of 75 bpm; AP, Augmentation Pressure; * P-value refers to a mixed model with subject as random factor, treatment and period as fixed effects and the baseline measurement as covariate.

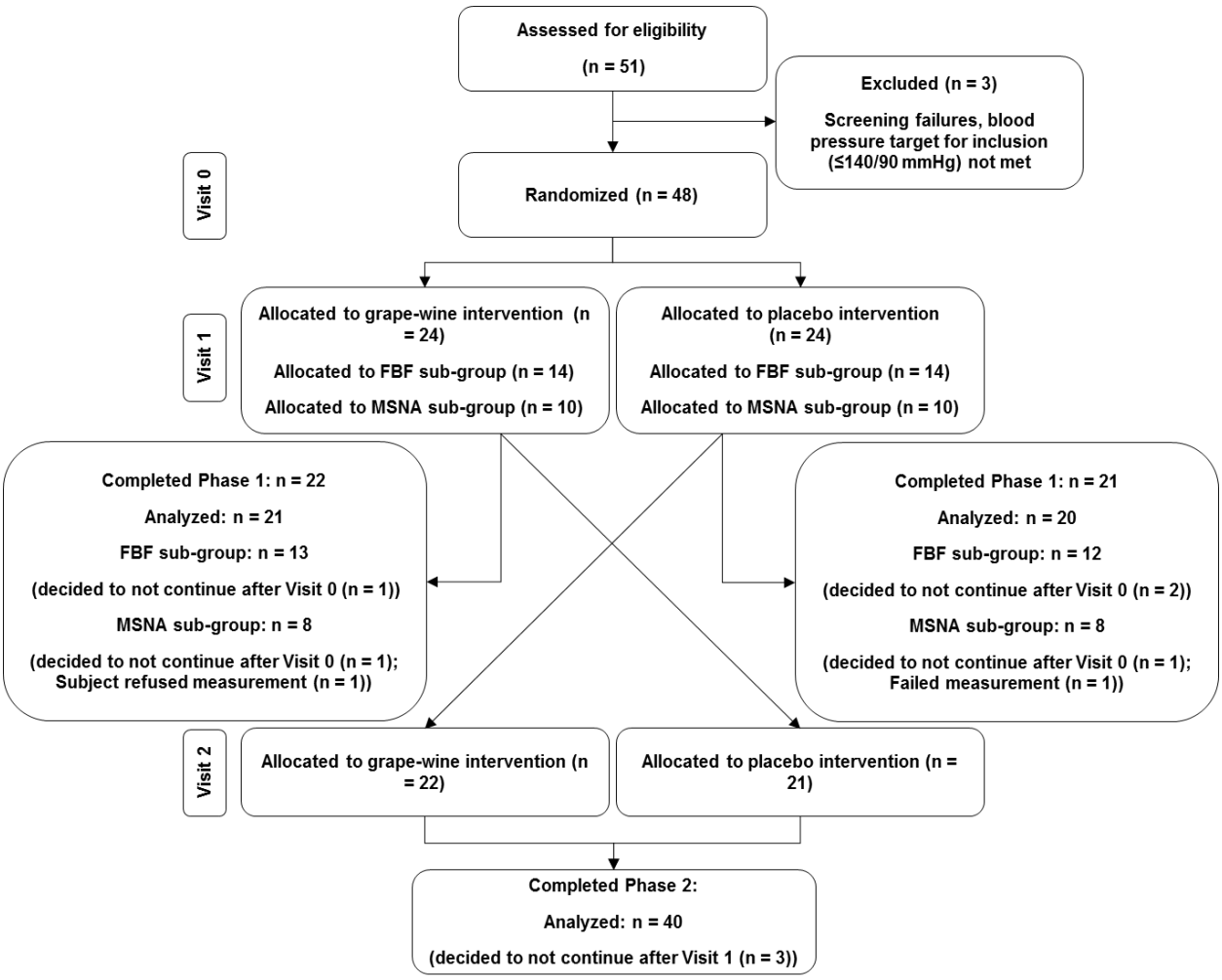
728 **Table 4**. Heart rate and blood pressure responses to mental stress and cold pressor test at
729 baseline and the end of the grape-wine and placebo intervention periods. Data are presented as
730 raw unadjusted means ± SD.

	Grape-Wine		**Placebo**		**ANCOVA P-value***	
Heart rate (beats/min)	*Visit 0*	*Visit 1*	*Visit 0*	*Visit 1*	*Time*Treatment*	*Time*Treatment *Stimulus*
Resting	62 ± 7	61 ± 10	69 ± 13	66 ± 12		
MS'2	71 ± 11	74 ± 12	81 ± 13	78 ± 10	**0.03**	0.66
CPT'2	67 ± 17	71 ± 13	76 ± 15	74 ± 13		
MAP (mmHg)	*Visit 0*	*Visit 1*	*Visit 0*	*Visit 1*	*Time*Treatment*	*Time*Treatment *Stimulus*
Resting	100 ± 9	100 ± 12	104 ± 11	103 ± 12		
MS'2	114 ± 13	114 ± 14	115 ± 14	114 ± 13	0.72	0.99
CPT'2	118 ± 15	122 ± 27	119 ± 16	121 ± 14		

731 MAP, Mean Arterial Pressure; MS'2, 2 min mental stress; CPT'2, 2 min cold pressor test. *P-
732 value refers to a repeated measures ANCOVA with treatment as between-subject effect, period,
733 and stimulus as within-subject effects and resting baseline measurement as covariate.

734

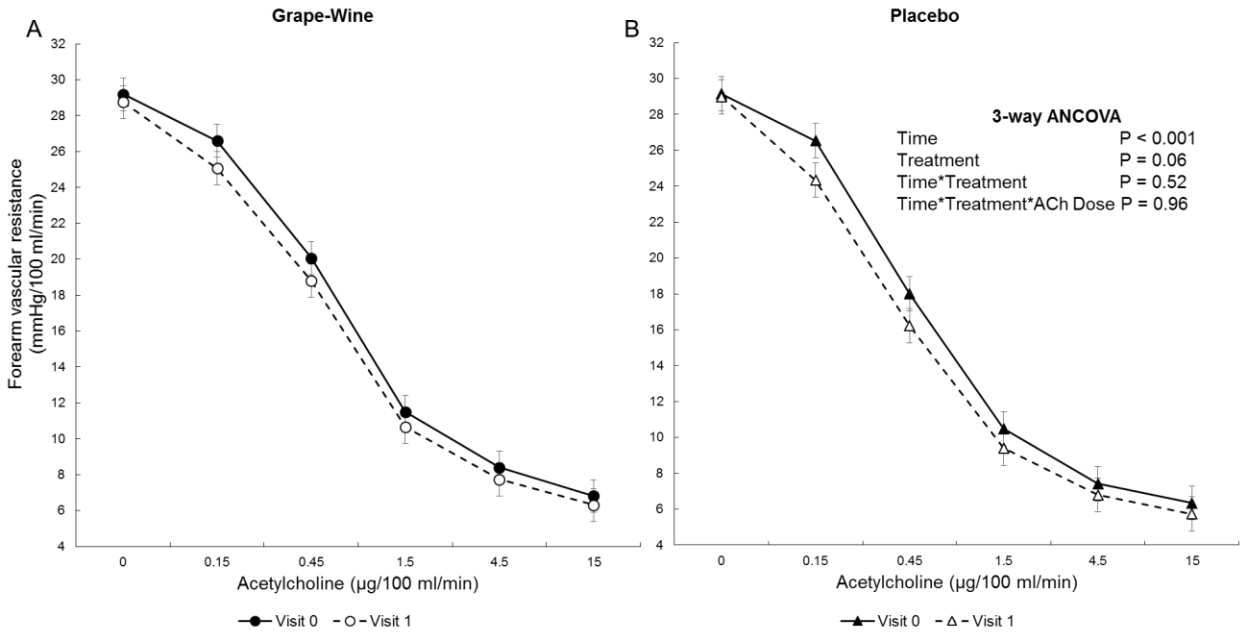
735 **Fig. 1.**



736

737

738 **Fig. 2.**



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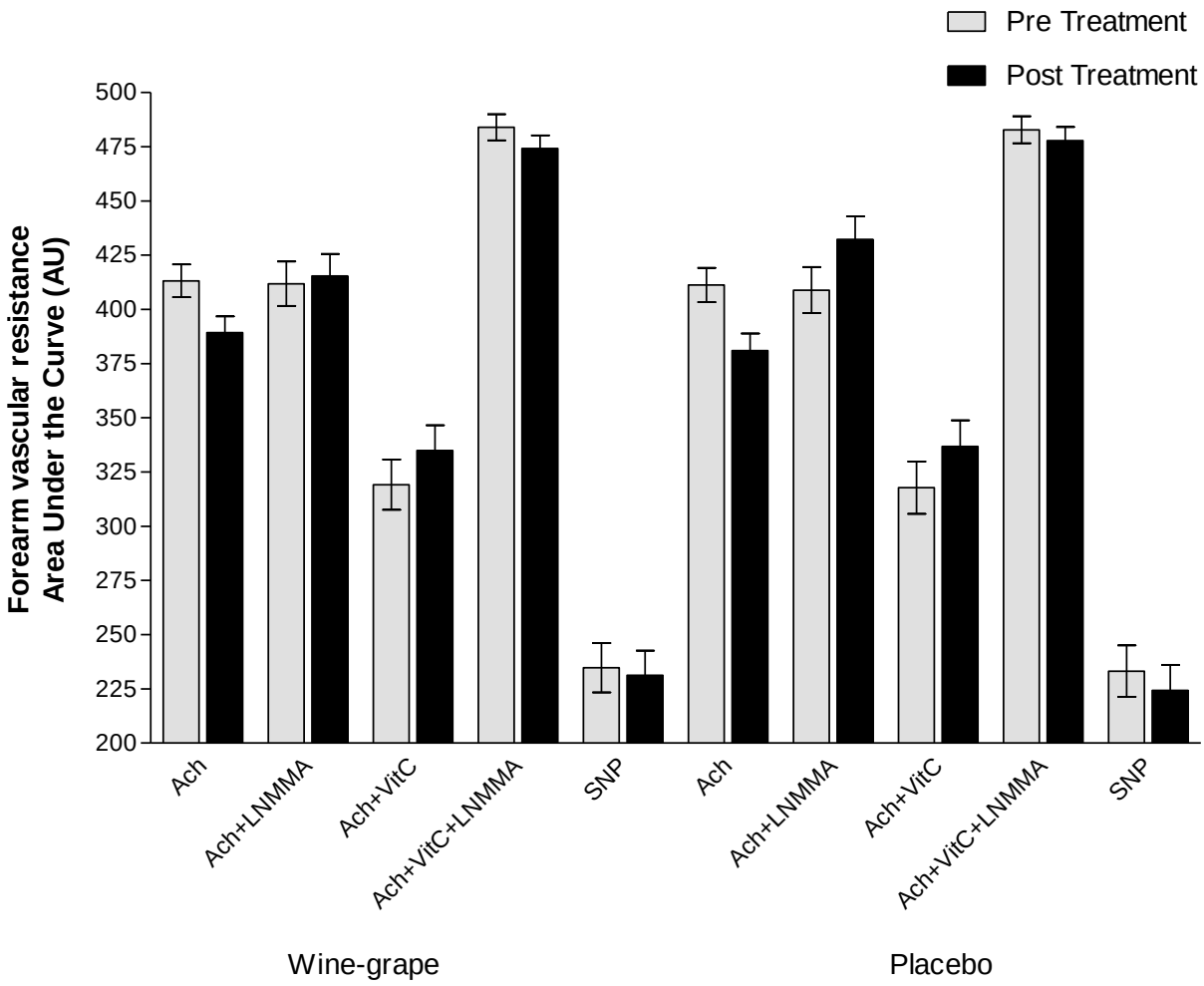
740

741 **Fig. 3.**



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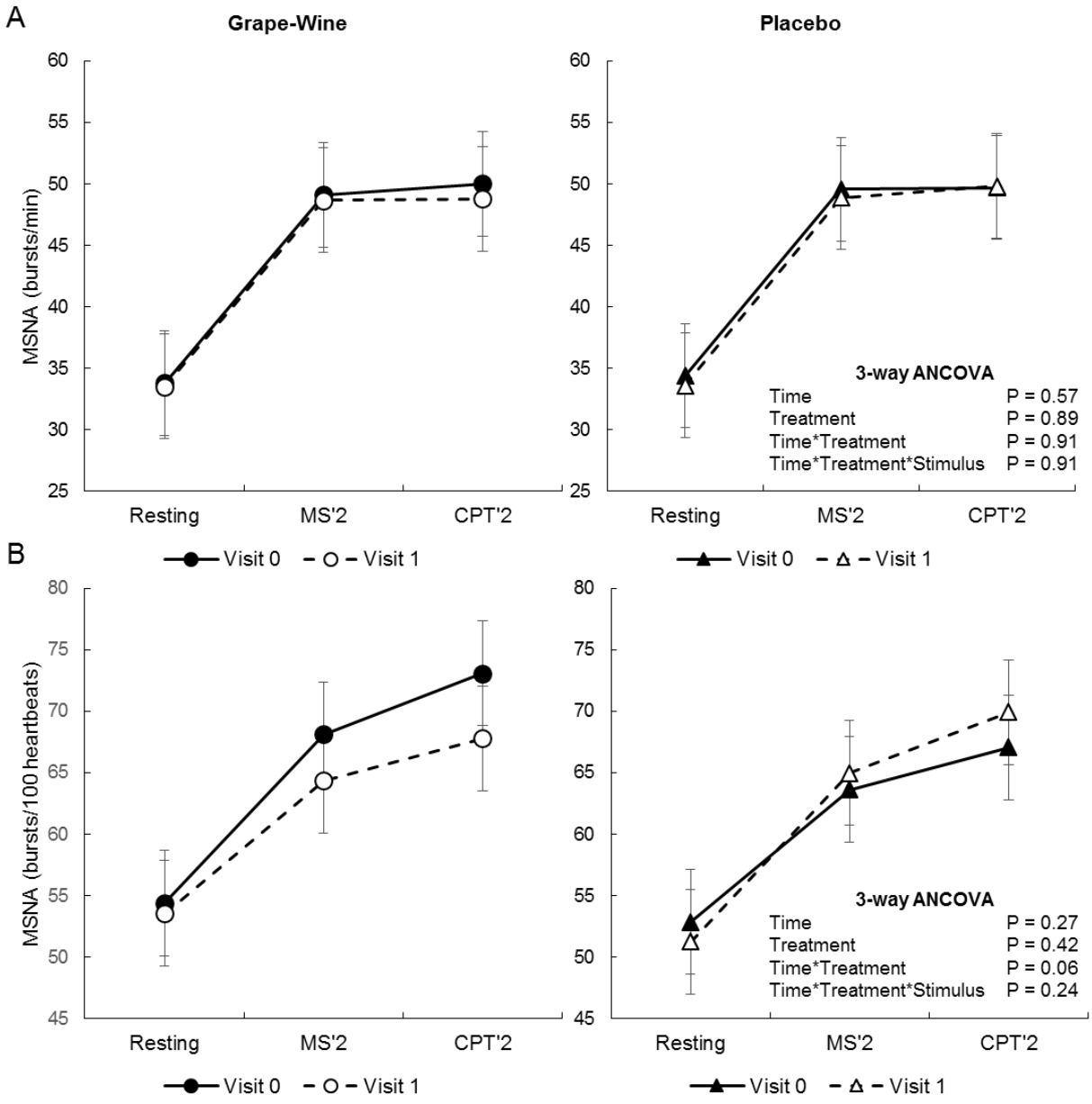
743

744 **Fig. 4.**


A
Grape-Wine
Placebo
MSNA (bursts/min)
60
55
50
45
40
35
30
25
Resting
MS'2
CPT'2
Visit 0
Visit 1
3-way ANCOVA
Time P = 0.57
Treatment P = 0.89
Time*Treatment P = 0.91
Time*Treatment*Stimulus P = 0.91
B
MSNA (bursts/100 heartbeats)
80
75
70
65
60
55
50
45
3-way ANCOVA
Time P = 0.27
Treatment P = 0.42
Time*Treatment P = 0.06
Time*Treatment*Stimulus P = 0.24

745