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Effects of two-month treatment with a mixture of natural activators of autophagy on oxidative stress and arterial stiffness in patients with essential hypertension: A pilot study

Giuliano Tocci [a], Giuseppe Biondi-Zoccai [b,c], Maurizio Forte [d], Giovanna Gallo [a], Giulia Nardoianni [a], Emiliano Fiori [a], Luca D'Ambrosio [b], Riccardo Di Pietro [e], Giulio Stefanini [f,g], Francesco Cannata [f,g], Erica Rocco [h], Beatrice Simeone [h], Gianmarco Sarto [h], Leonardo Schirone [b], Alessandra D'Amico [b], Mariangela Peruzzi [c,i], Cristina Nocella [i], Massimo Volpe [a,j], Speranza Rubattu [a,d,*]

[a] Department of Clinical and Molecular Medicine, School of Medicine and Psychology, Sapienza University of Rome, Italy
[b] Department of Medical-Surgical Sciences and Biotechnologies, Sapienza University of Rome, Latina, Italy
[c] Mediterranea Cardiocentro, Napoli, Italy
[d] IRCCS Neuromed, Pozzilli, Italy
[e] San Giovanni Addolorata Hospital, Rome, Italy
[f] Department of Biomedical Sciences, Humanitas University, Pieve Emanuele, Milan, Italy
[g] IRCCS Humanitas Research Hospital, Rozzano, Milan, Italy
[h] ICOT Istituto Marco Pasquali, Latina, Italy
[i] Department of Clinical Internal, Anestesiological and Cardiovascular Science, Sapienza University of Rome, Italy
[j] IRCCS S. Raffaele, Rome, Italy

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Abstract *Background and aims:* Trehalose, spermidine, nicotinamide, and polyphenols are natural substances that exert pro-autophagic and antioxidant properties. Their role in blood pressure (BP) regulation and preservation of vascular function in essential hypertension is unknown. The aim of this study was to evaluate the effect of a mixture of these agents on BP level, markers of oxidative stress, autophagy, endothelial function, and vascular stiffness in outpatients with grade 1 uncomplicated essential hypertension.

Methods and results: A single-centre, open-label, case–control, pilot study was conducted in adult outpatients (aged ≥18 years) receiving or not the mixture for two months along with the standard therapies. Both at baseline and at the end of the treatment the following clinical parameters were evaluated: brachial seated office BP level, central aortic pressure, pulse wave velocity, augmentation index (AI@75). Both at baseline and at the end of the treatment, a blood sample was drawn for the measurement of: H_2O_2, HBA%, levels of sNOX2-dp, Atg 5, P62, endothelin 1, and NO bioavailability. The mixture of nutraceuticals did not influence BP levels. Patients receiving the mixture showed a significant decrease of oxidative stress, stimulation of autophagy, increased NO bioavailability and no increase of the AI@75, in contrast to what observed in hypertensive patients not receiving the mixture.

Conclusions: The supplementation of the trehalose, spermidine, nicotinamide, and polyphenols mixture counteracted hypertension-related arterial stiffness through mechanisms likely dependent on oxidative stress downregulation and autophagy stimulation. These natural activators of autophagy may represent favourable adjuvants for prevention of the hypertensive cardiovascular damage.

* Corresponding author. Department of Clinical and Molecular Medicine, School of Medicine and Psychology, Sapienza University, S. Andrea University Hospital, IRCCS Neuromed, Rome, Italy.
E-mail address: speranzadonatella.rubattu@uniroma1.it (S. Rubattu).

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1. Introduction

The sustained increase in blood pressure (BP) levels above normal values is a common condition associated with cardiovascular damage and a higher occurrence of acute cardiovascular events, primarily ischemic stroke and acute myocardial infarction [1]. On the other hand, effective antihypertensive therapies achieving the recommended therapeutic BP targets have demonstrated to reduce the risk of both acute and long-term cardiovascular complications of hypertension [2,3].

Over the last decades, many studies highlighted the pathophysiological role of multiple mechanisms, which may either promote or counteract the hypertension-mediated organ damage (HMOD), independently of BP level [4,5]. A more thorough comprehension of these mechanisms may pave the way to the identification of new therapeutic tools, which may contribute to strengthen the cardiovascular protection exerted by the available pharmacological strategies used for the management of hypertension.

Among the recently discovered molecular mechanisms, autophagy is a cytoprotective process able to mediate the degradation of proteins, recycling of damaged organelles and cytoplasmic material, as well as the removal of excessive ROS accumulation in conditions of nutrient deprivation and cellular stress [6]. Therefore, autophagy favours cell metabolism, homeostasis, and survival by supporting the cell adaptation to energetic and stressful changes. On the other hand, a defective autophagic activity contributes to the pathogenesis of several cardiovascular diseases [7]. The involvement of autophagy in the HMOD has been documented in animal models such as the spontaneously hypertensive stroke-prone rat. In this model, a defective auto/mitophagy process contributes to the development of cerebral and renal vascular damage [8]. Consistently, restoration of autophagy by natural activators, such as nicotinamide and trehalose, significantly prevented stroke and renal damage occurrence in this model [5,8].

Trehalose is a natural disaccharide synthesized by lower organisms such as yeasts, bacteria, insects, and plants, but not by mammals [9]. Spermidine, polyphenols, and nicotinamide are also potent natural activators of autophagy, as documented by the experimental evidence [10,11]. Previous studies showed that trehalose in combination with a mixture of spermidine, nicotinamide, and polyphenols (catechin and epicatechin) reduced platelet activation and oxidative stress in platelets isolated from patients with atrial fibrillation, metabolic syndrome, or smokers and also increased the production of nitric oxide, the process of angiogenesis and cell viability in human endothelial cells [12]. A recent study demonstrated that this combination of autophagy activators was also able to reduce oxidative stress and increase the walking distance in patients affected by peripheral artery disease [13]. Moreover, it reduced muscle injury biomarkers in endurance athletes [14]. Finally, other studies highlighted the clinical implications of antioxidant supplementation with several classes of antioxidants, and their potential role for protecting against cardiovascular risk factors [15].

Based on the available experimental and clinical evidence, the aim of the present study was to test the protective impact of a mixture of nutraceutical activators on circulating oxidative stress parameters, BP levels, and the ratio of the central augmented pressure to the central pulse pressure (augmentation index, AI@75) in a cohort of patients with uncomplicated grade 1 hypertension.

2. Methods

2.1. Study population

In this single-centre, open-label, case–control, pilot study, thirty-seven consecutive unrelated Caucasian adults who were referred for hypertension management to the Hypertension Unit of the Department of Cardiology, Sapienza University, Sant'Andrea Hospital, Rome, were enrolled between January and June 2021. Hypertension was diagnosed based on the presence of office seated systolic BP (SBP) values $\geq$ 140 mmHg and/or diastolic BP (DBP) values $\geq$ 90 mmHg (average of 3 repeated office measurements in the sitting position made by the same doctor with an oscillometric automatic sphygmomanometer) [16].

Hypertensive outpatients included in the study were all asymptomatic and in stable clinical conditions. Exclusion criteria included diabetes mellitus or known history of ischemic heart disease, peripheral artery disease, stroke or transient ischemic attack, and chronic renal failure.

At baseline, all patients underwent full physical examination, and clinic BP measurement. The presence of smoking habit, hypercholesterolemia, and obesity, as well as concomitant pharmacological drug treatment were also recorded. Then, all patients included in the study underwent non-invasive assessment of cardiac, vascular, and renal HMOD, in line with the clinical protocol adopted by our Hypertension Unit.

Blood samples were drawn for routine chemistry analysis, including glucose and lipid parameters, renal function with electrolytes. In addition, blood samples were drawn for the assessment of oxidative stress through the measurement of soluble NOX2-derivative peptide (sNOX2-dp), a marker of NOX2 activation, H_2O_2 production, and hydrogen peroxide breakdown activity (HBA) and NO generation, as assessed by serum levels of nitrite/nitrate

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(NOx). Moreover, endothelin-1 level, and two markers of autophagy (ATG5, P62) were determined. Thereafter, patients were randomly assigned to the oral administration of a mixture of natural activators of autophagy (10.5 g/twice day) for two months. At the end of the study, all biochemical and hormonal assessments were repeated, as well as office BP measurements and non-invasive evaluation of markers of vascular HMOD. The study protocol was approved by the local ethical board of Sapienza-University of Rome (Prot. N. 454/2020) and was conducted according to the principles of the 1975 Declaration of Helsinki. All patients provided written informed consent at baseline.

2.2. Randomization and blinding

The study treatment codes were assigned by an individual not involved in the study according to a randomly defined treatment sequence with the mixture of nutraceuticals or no-treatment and the key to the assignment scheme was kept in a sealed envelope and not opened until the end of the study. The randomization was computer-generated based on a random numeric sequence. The investigators and laboratory technicians were unaware of the treatment allocation. The composition of the mixture (obtained from Princeps srl., Piasco, Cuneo, Italy) has been previously reported [13,14].

2.3. Blood sampling and preparations

For each patient, blood samples were collected in the morning (from 8 to 10 AM) from the antecubital vein in a seated position in fasting conditions. Blood samples were collected in BD Vacutainer (Franklin Lakes, NJ, USA) without anticoagulants or with anticoagulants (trisodium citrate, 3.8%, 1/10 (v/v) or 7.2 EDTA). The blood was centrifuged at 300×*g* for 10 min at room temperature (RT). Serum and plasma samples were separated into aliquots and stored at −80 °C until analyses.

2.4. Clinic blood pressure monitoring

Clinic BP measurements were performed in the Hypertension Unit during the morning section (from 8:00 to 10:00 AM) by physicians certified by European Society of Hypertension specialists. Sequential BP measurements were performed in a quiet room, after 3–5 min of rest, on the same arm and with the participant in the sitting position, by using an automated, oscillometric device (Mobil-O-Graph PWA Monitor, I.E.M. GmbH, Stolberg, Germany). The average of three consecutive BP measurements (and heart rate) was collected at 1-min time interval and was considered as clinic systolic/diastolic bBP and clinic bPP. All clinic BP measurements were attended.

2.5. ECG and echocardiographic evaluations

All study patients had to be in sinus rhythm on the day of examination. A 12-lead surface ECG was obtained for all patients in the supine position using a Mortara Eli 350 ECG (Milwaukee, Wisconsin, USA) device. The 12-lead ECG was recorded at a paper speed of 25 mm/s and 1 mV/cm standardization. All ECGs were scanned at 600 dpi and conventional and new ECG parameters were measured on a high-resolution computer screen. Conventional ECG parameters for left ventricular hypertrophy (LVH) were defined according to standard criteria, by using Sokolow-Lyon, Cornell Voltage and Cornel Product indexes, as recommended by current hypertension guidelines [17].

Doppler echocardiographic examination was performed by a Philips Epic 7 ultrasound machine with a multifrequency transducer (2.5–4 MHz). Images were implemented using standardized acquisition methods. LV dimensions were measured at the end-diastole and end-systole, just below the mitral leaflets, through the standard left parasternal window. LV ejection fraction (LVEF %) was calculated according to the Simpson method. LV mass was calculated and then normalized by body surface area, and height ^2.7. Echocardiographic LVH was defined according to standard criteria.

Echocardiographic indexes of LV systolic and diastolic function were also assessed.

2.6. Pulse wave velocity (PWV) and augmentation index (Ai) evaluations

Sequential BP measurements were performed on the same arm with the participant in the sitting position, by using an automated oscillometric device (Mobil-O-Graph PWA Monitor, I.E.M. GmbH, Stolberg, Germany). After estimation of systolic/diastolic brachial BP, the cuff instantly reinflated and recorded systolic/diastolic central (aortic) BP and other vascular parameters, including AI@75, pulse wave velocity (PWV), and peripheral vascular resistances (PVR). Using this method, central BP is automatically calculated from the brachial BP, using a transfer function, while PWV is estimated from the time difference between the derived forward and reflected waves. Non-invasive estimation of these parameters of vascular function was performed according to validated protocols [18,19], in accordance with the recommendations from current guidelines [20].

2.7. Serum endothelin 1, nitric oxide, sNOX2-dp, H_2O_2 production, hydrogen peroxide scavenging activity, plasma ATG5 and P62 levels

All markers were evaluated by ELISA or colorimetric assays in serum samples. Briefly, human endothelin-1 was evaluated by a commercial ELISA kit (Elabscience). Values were expressed as pg/mL. Intra- and inter-assay coefficients of variation (CV) were both <10%. Nitric oxide (NO) production was evaluated by NO^{2-}/NO^{3-} determination. After the conversion of NO^{3-} to NO^{2-}, total nitrite is detected with Griess Reagents. Values were expressed as μM. Intra- and inter-assay CV were <10%.

sNOX2-dp, a measure of NOX2 activity, was detected with a previously reported ELISA method [21]. Values were expressed as pg/mL; intra- and inter-assay CV were <10%. Hydrogen Peroxide (H_2O_2) was measured by a colorimetric

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assay as described previously [22]. The final product was read at 450 nm and expressed as μM. Intra- and inter-assay CV were both <10%. Hydrogen peroxide (H_2O_2) break-down activity measures the antioxidant capacity of samples by HBA assay kit (Aurogene, code HPSA-50) and expressed in percentage.

The quantitative determination of autophagy markers ATG5 and P62 was performed by a sandwich enzyme immunoassay technique (Mybiosource, and FineTest, respectively). Values were expressed as ng/mL and intra-assay and inter-assay CV were ≤12% for both assays.

All assays were performed in a blind manner.

2.8. Statistical analysis

Descriptive analysis was based on reporting mean ± standard deviation for continuous variables, and count (%) for categorical variables. Bivariate comparative analysis was based on unpaired and paired (as appropriate) Student t test for continuous variables and Fisher exact test for categorical variables. Inferential analysis was based on a multilevel mixed-effects linear model, with an identity covariance matrix, forcing in the model each end point value, group, timing of sampling, and their interaction as fixed effects, with participant code as random effect. Statistical significance was set at the 2-tailed 0.05 level, without multiplicity adjustment. Computations were performed with Stata 13 (StataCorp, College Station, TX, USA).

3. Results

3.1. Baseline, blood pressure, echocardiographic and biochemical parameters

We included an overall sample of 37 hypertensive patients: 20 received the mixture and 20 were included in the control group. Three control subjects did not complete the study and were ruled out from the analysis. Demographic, clinical characteristics and ongoing therapies of the whole population as well as of case and control groups are reported in Supplemental Table 1. Echocardiographic parameters and biochemical parameters are shown in Supplemental Table 2 and Supplemental Table 3, respectively. Average values of ambulatory BP levels before and after the treatment are reported in Supplemental Table 4. No significant differences were found between the pre and post BP values in both groups.

3.2. Evaluation of oxidative stress

Levels of sNOX2-dp and H_2O_2 were significantly reduced upon the active treatment (Fig. 1A and 1B) whereas the HBA % increased (Fig. 1C), suggesting a decreased oxidative stress.

3.3. Markers of endothelial function

At the end of the study a significant rise in NO bioavailability was observed (Fig. 2A), in contrast to the reduction of endothelin-1 level, a potent vasoconstrictor (Fig. 2B). The latter suggests an improvement of endothelial function following nutraceuticals administration.

3.4. Evaluation of autophagy

The process of autophagy was stimulated by the administration of nutraceuticals, as documented by a rise of plasma Atg5 and a decrease of P62 levels in subjects undertaking the addition of the mixture (Fig. 3A and 3B).

3.5. Assessment of the pulse wave velocity and augmentation index (PWV-Ai@75)

A significantly different behaviour of the Ai was observed in hypertensive patients receiving the mixture of nutraceuticals as opposed to the control subjects (Fig. 4). In fact, these parameters of vascular function were increased in controls at the end of the study whereas they did not increase in subjects taking the mixture. The statistical comparison between the two groups was significant (Fig. 4). Furthermore, we evaluated the association between the changes of the autophagy marker P62 and the PWA-Ai@75 parameter and found a significant association (Fig. 5).

4. Discussion

The present investigation is a pilot study revealing for the first time that a short (2-month) administration of a mixture of trehalose, spermidine, nicotinamide, and polyphenols leads to a significant decrease of oxidative stress parameters and to a reduced arterial stiffness in hypertensive patients under standard anti-hypertensive therapy. The protective impact of the nutraceuticals added to the conventional pharmacological approach was also associated to a significant stimulation of autophagy. No effects were observed on the blood pressure levels at the end of the study in our hypertensive patients. The pharmacological treatment did not differ between treated and untreated patients, and it did not vary during the study in either group.

Hypertension is known to increase the cardiovascular risk through the promotion of vascular damage favoured by increased oxidative stress and endothelial dysfunction. An impaired endothelium-dependent vasorelaxation is a typical feature of the hypertensive vascular damage [23]. Endothelial dysfunction predisposes to atherosclerosis, and it also promotes the atherosclerotic plaque vulnerability with consequent higher susceptibility to acute events [24]. Among the molecular mechanisms underlying the hypertensive vascular damage, an increased oxidative stress plays a major role [25]. A higher level of oxidative stress, in turn, may impair autophagy [26]. The latter is a cellular protecting process toward extrinsic and intrinsic cellular insults [6]. A reduced autophagy contributes to further injury in endothelial cells from human atherosclerotic lesions [27]. Furthermore, autophagy restoration by natural activators, such as nicotinamide and trehalose, significantly prevented

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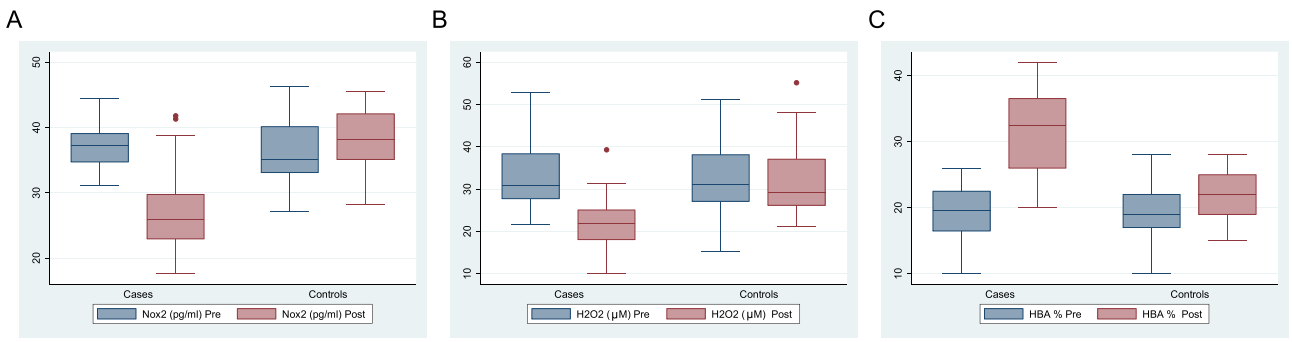

Figure 1 Parameters of oxidative stress. Comparison of sNOX2-dp (pg/ml) (A), H_2O_2 (μM) (B) and HBA (%) (C) according to timing of sampling and group ($p < 0.001$ for interaction at multilevel mixed-effects linear model).

stroke and renal damage occurrence in the animal model of spontaneously hypertensive rat [5,8]. Trehalose is also able to improve resistance artery endothelial function in healthy middle-aged and older adults [28].

In our current study we tested the hypothesis that a supplementation with antioxidants and autophagy activators could counteract, through the decrease of oxidative stress and the stimulation of autophagy, the promotion of vascular damage in human hypertension. We selected the Ai parameter as an index of arterial stiffness predisposing to the subsequent vascular damage [29,30]. Previous experimental evidence demonstrates that trehalose protects the cardiovascular system, through autophagy stimulation, from common health problems, including the high salt induced vascular damage in hypertension [5] and the ventricular remodeling in ischemic heart disease [31]. Polyphenols are known to counteract the endothelial dysfunction through a reduction of oxidative stress [32,33]. Nicotinamide was shown to decrease, through the correction of mitochondrial dysfunction, the occurrence of cerebrovascular events in a model of hypertension and higher predisposition to stroke [8]. Spermidine also exerted beneficial effects through autophagy stimulation in several contexts and reduced cardiovascular ageing [11].

As a result, we observed that, after 60 days of administration of a mixture of trehalose, nicotinamide, spermidine and polyphenols, the group of treated hypertensive patients showed a reduced oxidative stress status, as proven by the reduced levels of NOX2 and H_2O_2 compared to patients not taking the mixture. Consistently, the HBA level, as a marker of increased antioxidant status, was higher in the hypertensive patients at the end of the mixture administration.

As expected, the mixture of nutraceuticals improved autophagy, as documented by the increased plasma Atg5 and decreased P62 levels. An important observation was made with the measurement of NO and endothelin levels. Whereas NO was significantly increased by the treatment, the endothelin level decreased.

All changes observed in our study are consistent with previous evidence obtained with the same mixture in patients affected by PAD [13] and in endurance athletes [14]. In fact, the combination of nutraceuticals inhibited NOX2-derived oxidative stress, activated autophagy, increased NO availability, and reduced endothelin level in all these experimental contexts. The modulation of both NO and endothelin levels points to an improvement of endothelial function upon the treatment.

Finally, all described biological changes may have contributed to the reduction of Ai in patients receiving the mixture, as compared to the control patients, observed at the end of the study. Reduced oxidative stress, increased autophagy, and improved endothelial function may have positively modulated the arterial

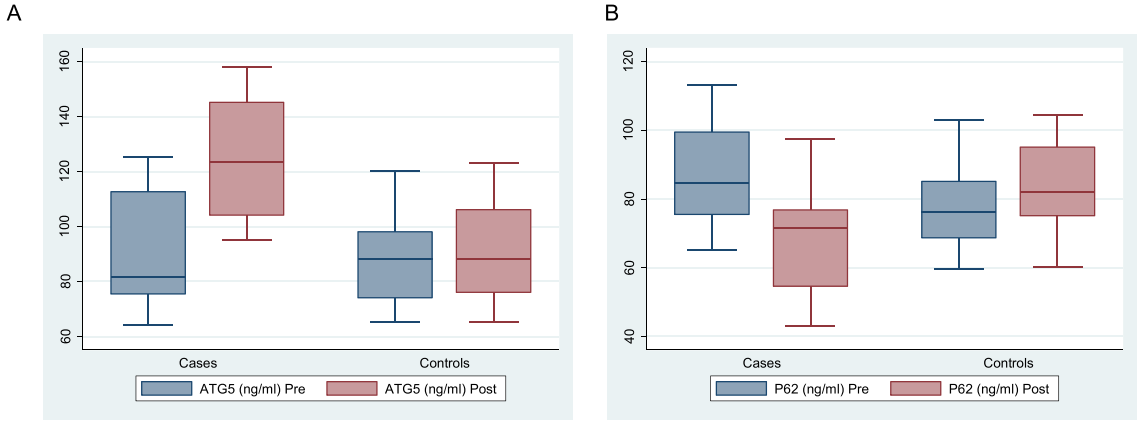

Figure 2 Levels of autophagy. Comparison of ATG5 (ng/ml) (A) and P62 (ng/ml) (B) according to timing of sampling and group ($p < 0.001$ for interaction at multilevel mixed-effects linear model).

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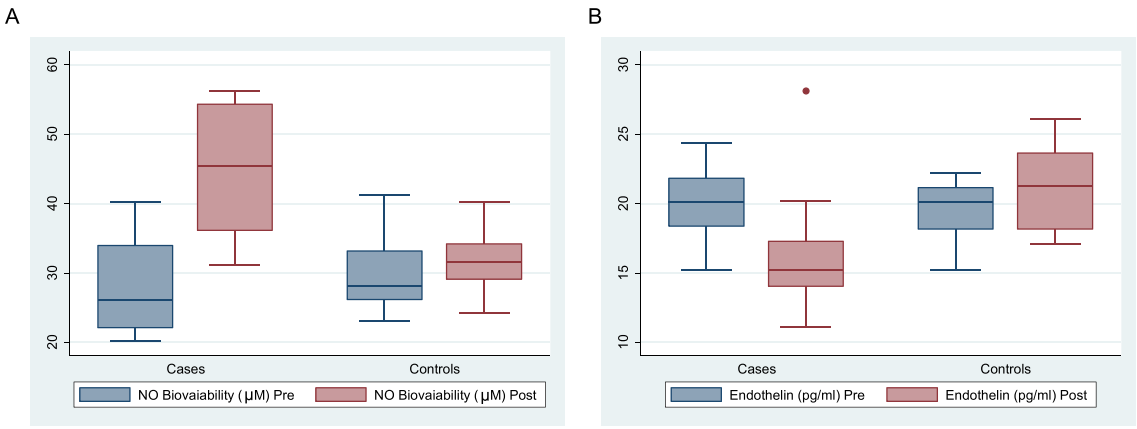

Figure 3 Parameters of endothelial function. Comparison of NO bioavailability (μM) (A) and endothelin (pg/mL) (B) according to timing of sampling and group ($p < 0.001$ for interaction at multilevel mixed-effects linear model).

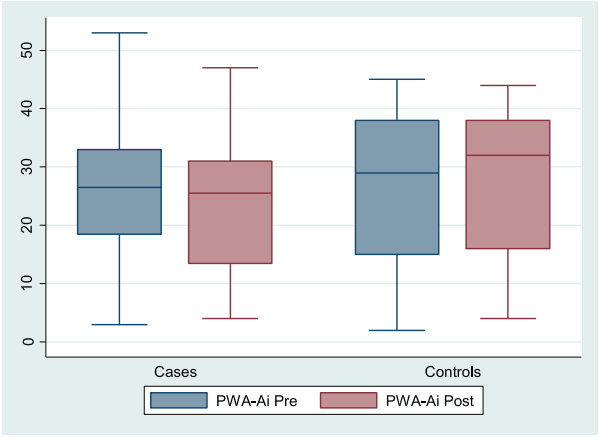

Figure 4 Assessment of the pulse wave velocity and augmentation index. Comparison of PWA Augmentation index (PWA-Ai) according to timing of sampling and group ($p = 0.018$ for interaction at multilevel mixed-effects linear model).

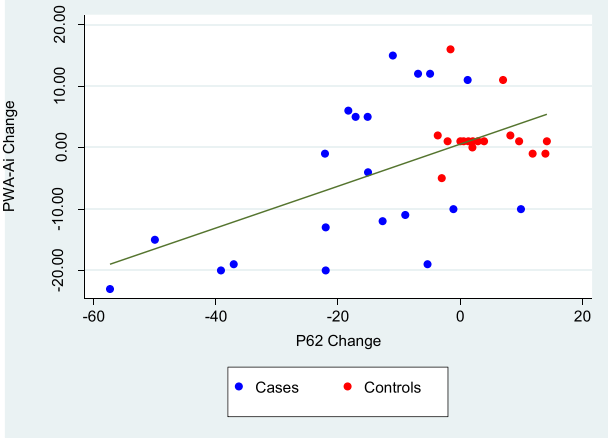

Figure 5 Correlation between autophagy and PWA augmentation index. Scatterplot highlighting the association between changes in P62 and in PWA Augmentation index (PWA-Ai) ($p = 0.012$ at multilevel mixed-effects linear model).

stiffness that increased in the hypertensive patients upon the standard anti-hypertensive treatment only. Interestingly, a significant correlation was detected between a marker of autophagy, p62, and the Ai parameter. Of note, the evidence of a significant difference in PWA-Ai@75 behaviour between the two groups of patients was achieved after only a two-month treatment with the mixture. Furthermore, the lower PWV-Ai observed at the end of the study was independent from age and was not accompanied by any change in BP levels.

Two main limitations of the study need to be highlighted. Firstly, the size of the patient's cohort was small; secondly, the length of the treatment was short. A longer time of administration in larger cohorts may allow to detect more relevant differences regarding vascular function and structure, as well as the clinical effects, in future studies.

In conclusion, the present investigation is a pilot study useful to provide evidence to be confirmed in larger cohorts and upon longer treatment. The originality of the present study mainly relies on the first detection of a biological effect of a mixture of natural activators of autophagy in human uncomplicated essential hypertension. In fact, a parameter of arterial stiffness did not increase in patients receiving the mixture differently from what observed in patients upon a standard pharmacological treatment only. All observed changes took place in the absence of any variation of BP levels. The natural antioxidant and autophagy stimulators may represent a valuable supplement to reinforce the standard anti-hypertensive treatment to better counteract the vascular damage development by acting through alternative pathways.

Availability of data

The data supporting this study's findings are available from the corresponding author, SR, upon reasonable request.

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Author contributions

Conceptualization, data analysis, statistics: GT, GBZ, SR, FC; Biomolecular analyses. MF, CN, AdA, LD, LS, GSa, GN, EF, RdP, ER, BS; Statistics. GBZ, GG; Writing manuscript GT, MF, SR, MP, MV; Conceptualization and providing valid criticism: GSt, MV, SR; Fundings: GSt, SR. All the authors discussed the data and approved the final version of the manuscript.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.numecd.2023.07.026.

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