Effect of Annurca Apple Polyphenols on Intermittent Claudication in Patients With Peripheral Artery Disease

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Peripheral arterial disease (PAD) is an atherosclerotic process involving both modifiable and nonmodifiable risk factors. Prospective cohort studies show that patients with PAD have a 6-fold greater risk of death from cardiovascular disease than those without PAD. Currently, there is no effective treatment for PAD. The study was a randomized, placebo-controlled trial, involving 180 patients, aged 35 to 75. The subjects were divided into 2 groups. One group underwent 24 weeks of nutraceutical treatment consisting in the administration of 4 capsules of Annurca apple polyphenolic extract (AMS)/day. The placebo group was administered with identically appearing capsules containing only maltodextrin. Primary outcome measures were: walking autonomy, ankle-brachial index, acceleration time. In the AMS group, at the end of the treatment period, walking autonomy was increased on average by 69% ($p < 0.05$), while slighter effects were registered as regards ankle-brachial index (+25%; $p < 0.05$) and acceleration time (−3.6%; $p < 0.05$), when compared with baseline. Placebo group revealed no significant differences as regards variations of all outcomes measures ($p > 0.05$). Our preliminary results may indicate AMS product as a promising natural and safe tool for treatment of symptoms related to PAD. **(Am J Cardiol 2019;123:847−853)**

Peripheral arterial disease (PAD) is an atherosclerotic process that causes stenosis and occlusion of noncerebral and noncoronary arteries. It has an estimated worldwide prevalence of almost 10%, rising from 15% to 20% in people over 70 years of age,[1,2] and it affects around 27 million people in Europe and North America alone.[3] From a clinical point of view, PAD may be classified into 4 stages, according to the Lèriche and Fontaine classification.[4] Stage I patients are asymptomatic, with an ankle-brachial index (ABI) <0.90.[4,5] Stage II patients have manifest intermittent claudication (IC); this stage may be subdivided into stage IIa if walking autonomy (WA) is >200 m, and stage IIb if WA is <200 m.[4,6] Stage III is characterized by the appearance of rest pain.[4,6] In stage IV, ulcerations, ranging from trophic lesions to gangrene, are apparent.[4,6] It has been recently reported that cocoa catechins are the most effective polyphenolic compounds in acutely improving WA in patients with PAD.[7] This effect may be due to artery dilatation possibly related to an oxidative stress-mediated mechanism involving NOX2 regulation. Our research group have previously evaluated the antioxidant composition of Annurca apple, the only apple cultivar native to Campania region (Southern Italy), listed as a Protected Geographical Indication product [Commission Regulation (EC) No. 417/2006)].[8,9] By comparing experimental data with those from more common commercial apple cultivars, Annurca apple exhibited the highest polyphenolic concentration, specifically as regards catechin and epicatechin derivatives. It can be hypothesized that these compounds occurring in apples may have a similar mechanism of action to that of cocoa catechin derivatives as regards their effects on WA in patients with PAD. To clarify this aspect, the aim of the present work was to evaluate the effect of a nutraceutical product formulated using Annurca apple polyphenolic extract (AMS) on some PAD indicators, such as WA in IC, and hemodynamic parameters, manly ABI and acceleration time (AT).

Methods

Annurca (*M. pumila* Miller cv Annurca) apple fruits were collected in Valle di Maddaloni (Caserta, Italy) in October 2016 when fruits had just be harvested (green peel). Fruits were reddened, after the typical treatment for about 30 days, and then analyzed.[10] Large-scale production of AMS was accomplished by MB-Med Company (Turin, Italy). Apples were extracted with water at 35°C. After centrifugation, the extract was spray-dried in combination with maltodextrins, obtaining a fine powder with a maltodextrins/extract ratio 4:1. AMS consisted of Annurca apple extract microencapsulated in maltodextrins.

Study participants were recruited by the Samnium Medical Cooperative (Benevento, Italy). Patients were enrolled

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in September 2016. Patients aged 35 to 75 years were eligible for enrolment if they could have been assigned to Stage II of PAD Fontaine classification, and if they had shown evident signs of IC for 3 years at least. Exclusion criteria were: smoking, obesity (BMI >30 kg/m^2), serious hepatic disease (cirrhosis, hepatitis), serious renal disorders (serum creatinine >2.8 mg/dl), drug therapy or supplement intake containing apple polyphenols, heavy physical exercise (>10 hour/week), pregnant women, women suspected of being pregnant, women who hoped to become pregnant, breastfeeding, birch pollen allergy, use of vitamin/mineral or antioxidant supplements 2 weeks prior to entry into the study and donation of blood <3 months before the study. The subjects received oral and written information concerning the study before they gave their written consent. Protocol, letter of intent of volunteers, and synoptic document about the study were submitted to the Scientific Ethics Committee of AO Rummo Hospital (Benevento, Italy). The study was approved by the committee (protocol 104039 of 15/07/2016), and carried out in accordance with the declaration of Helsinki of 1964 (as revised in 2000). The subjects were asked to make records in an intake-checking table for the intervention study and side effects in daily reports. The study was a randomized, double-blind, single-center, placebo-controlled, trial conducted at the Samnium Medical Cooperative (Benevento, Italy).

The study duration was 24 weeks. A total of 180 eligible patients were randomly assigned to 2 subgroups: 90 patients, intervention; 90 patients, placebo. If a patient dropped out before the intervention period, he or she was replaced by the next eligible patient enrolled at the same center. The concealed allocation was performed by an Internet based randomization schedule, stratified by study site. The random number list was generated by an investigator with no clinical involvement in the trial. Patients, clinicians, core laboratories, and trial staff (data analysts, statisticians) were blind to treatment allocation. The decision process was performed according to a consensus document (unpublished standard operating procedure) before unblinding to define conclusive primary and secondary efficacy data from a clinical perspective. The intervention group underwent a nutraceutical treatment consisting in the administration of 4 capsules of AMS/day (2 capsules at lunch and 2 capsules at dinner, a total of 2,000 mg AMS in gastro-resistant capsules). The placebo group was administered with identically appearing capsules containing only maltodextrin. Subjects were allowed to take water freely. They were provided with a food diary on which annotate their daily dietary habits and were instructed to maintain their habitual patterns of physical activity throughout the entire study period. Both the examinations and the study treatment were performed in an outpatient setting.

Clinic visits were performed at weeks 0 and 24. All patients underwent femoral aorta Color Doppler Ultrasound using Xario 200 (Canon Medical Systems Europe B.V., Zoetermeer, The Netherlands) equipped with multifrequency matrix transducers at 4 to 10 MHz. All Doppler findings were analyzed by the same physician who was blinded to subjects' past histories. The ABI, to determine the ratio of the highest systolic pressure at the arm to the systolic pressure at the ankle, was recorded with a handheld 10 MHz Doppler instrument, while AT (time to peak systolic velocity, milliseconds) was measured with dedicated software analysis program. Both ABI and AT were measured at room temperature (about 25°C) after 30 minutes acclimatization. All patients were administered with a modified version of the WHO/Rose Questionnaire[11] for the subjective evaluation of the individual PAD condition before and after the intervention period. The questions regard a self-assessment of both the WA related to the IC state and specific underlying vascular abnormalities of lower limbs. Primary end points measured were the variations of the WA in IC, and hemodynamic parameters, such as ABI and AT, whereas key secondary outcomes were observation of underlying vascular abnormalities of lower limbs related to the IC state, such as paresthesia, cold limb, cold toes, and trophic lesions. We assessed safety from reports of adverse events as well as laboratory parameters concerning the hepatic and renal function, vital signs (blood pressure, pulse, height, weight, and body mass index), and physical or neurologic examinations. Safety was assessed over the entire treatment period at weeks 0, 4, 8, 12, 16, 20, and 24, including adverse events occurring in the first 3 weeks after cessation of treatments.

A post hoc power analysis was performed using the primary end points measured, such as the variations of WA in IC, and hemodynamic parameters, such as ABI and AT, observed for all treatments (1-way repeated measures ANOVA, observed $F = 0.6596$, $\alpha = 0.05$, 1 group, $n = 50$, 5 measurements, observed correlation in measurements = 0.7977, Geisser-Greenhouse sphericity $\varepsilon = 0.4991$). Based on these values, the statistical power was 100%, indicating that the sample size was sufficient to detect statistically significant differences if they were indeed present. This resulted in $n = 50$ patients, which was increased to $n = 90$. Unless otherwise stated, all of the experimental results were expressed as mean ± standard deviation (SD) of at least 5 replications. Statistical analysis of data was performed by the Student's *t* test or 2-way ANOVA followed by the Tukey-Kramer multiple comparison test to evaluate significant differences between a pair of means. The statistic heterogeneity was assessed using Cochran's test ($p < 0.1$). The I^2 statistic was also calculated, and $I^2 > 50\%$ was considered as significant heterogeneity across studies. A random-effects model was used if significant heterogeneity was shown in the trials. Otherwise, results were obtained from a fixed-effects model. Percent change in mean and SD values were excluded when extracting SD values for an outcome. SD values were calculated from standard errors, 95% confidence intervals, p values, or *t* if they were not available directly. Previously defined subgroup analyses were performed to examine the possible sources of heterogeneity within these studies and included health status, study design, type of intervention, duration, total polyphenols dose, and Jadad score. Treatment effects were analyzed using PROC MIXED with treatment and period as fixed factors, subject as random factor and baseline measurements as covariates, and defined as weighted mean difference and 95% confidence intervals calculated for net changes in fecal and serum parameters, and blood pressure values. Data that could not meet the criteria of variance homogeneity (Levenes test) and normal distribution

(determined by residual plot examination and Shapiro-Wilks test) even after log transformation were analyzed by a nonparametric test (Friedman). The level of significance (α value) was 95% in all cases ($p < 0.05$).

Results

Analysis of AMS by LC-MS indicated that the main constituents were represented by [+]-catechin, [−]-epicatechin, and procyanidin compounds, followed by traces of chlorogenic acid, rutin, phloretin-2-*O*-xyloglucoside, and phloridzin (Table 1).

A total of 253 patients were screened for eligibility; 73 patients (29.0%) did not pass the screening stage; 180 patients were randomized. Figure 1 follows the CONSORT PRO reporting guideline[12] and reveals that within the assessment period, the following percentage of patients provided data for the primary end point: AMS, 100% (90 of 90 patients); placebo, 100% (90 of 90 patients). Table 2 shows the demographic and clinical characteristics assessed at the baseline visit of all 180 subjects randomized.

Compared with baseline, WA was increased on average by 69% in the AMS group after 24 weeks of treatment, whereas no significant differences were revealed in the placebo group ($p = 0.5$; Figure 2). Conversely, slighter effects

Table 1
Polyphenolic composition of Annurca nutraceutical formulation (AMS) determined by HPLC

Polyphenol	AMS
Chlorogenic acid	0.04 ± 0.001
p-Coumaroylquinic acid	ND
[+]-Catechin	0.3 ± 0.02
[−]-Epicatechin	0.3 ± 0.02
Procyanidin B_1	0.2 ± 0.01
Procyanidin trimer	0.3 ± 0.01
Procyanidin B_2	0.04 ± 0.001
Procyanidin trimer (isomer)	0.2 ± 0.02
Cyanidin-3-*O*-galactoside	ND
Rutin (Quercetin-3-*O*-rutinoside)	0.06 ± 0.002
Hyperin (Quercetin-3-*O*-galactoside)	ND
Isoquercitrin (Quercetin-3-*O*-glucoside)	ND
Reynoutrin (Quercetin-3-*O*-xyloside)	ND
Guajaverin (Quercetin 3-*O*-arabinopyranoside)	ND
Avicularin (Quercetin 3-*O*-arabinofuranoside)	ND
Quercetin-*O*-pentoside	ND
Quercitrin (Quercetin-3-*O*-rhamnoside)	ND
Phloretin-2-*O*-xyloglucoside	0.06 ± 0.003
Phloridzin (phloretin-2-*O*-glucoside)	0.06 ± 0.002

ND = not detected.
Results were expressed as μg/mg DW ± SD (n = 3).
Results were significantly different at a level of $p = 0.001$.

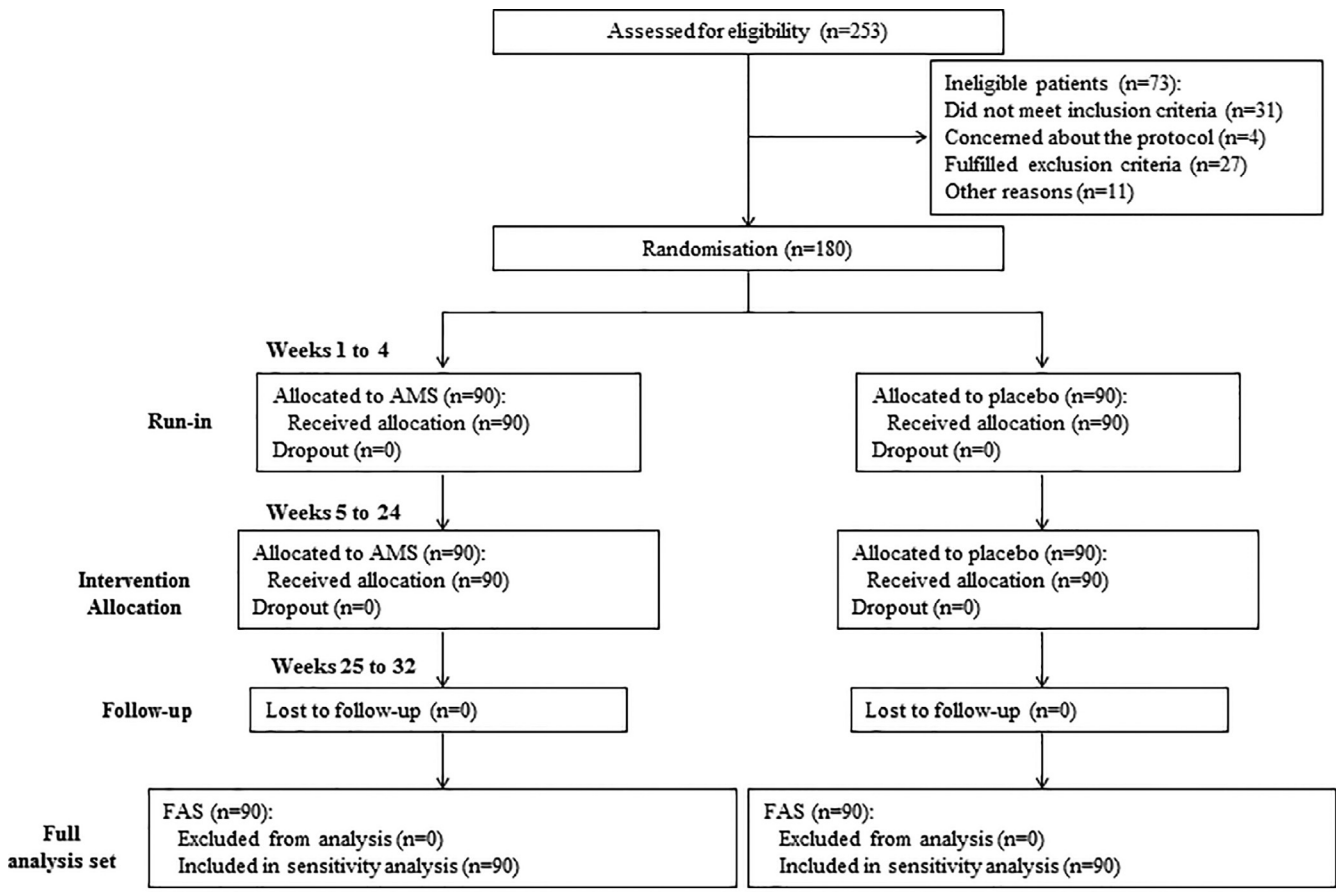

Figure 1. Study flowchart, according to the consolidated standards of reporting trials (CONSORT). The diagram shows enrolment and primary efficacy end points based on patient diaries, from prescreening to data collection; and the extent of exclusions, loss to follow-up, and completeness of diary documentation available across the entire trial period.
AMS = Annurca nutraceutical formulation; FAS = full analysis set.

Table 2
Baseline characteristics of PAD subjects administered with placebo and AMS during treatment period (n = 180)

Parameters	Placebo (n = 90)	AMS (n = 90)
Age (years)*	70.5 ± 10.2	71.5 ± 9.4
Male sex	68 (75%)	62 (69%)
White ethnicity	90 (100.0%)	90 (100.0%)
BMI (kg/m^2)*	22.8 ± 3.1	23.7 ± 4.0
Hypertension	70 (78%)	68 (75%)
Diabetes mellitus	24 (27%)	20 (22%)
Former smoker	44 (49%)	37 (41%)
Dyslipidemia	75 (83%)	70 (78%)
CHD	20 (22%)	24 (27%)
Previous stroke	11 (12%)	15 (17%)
Pharmacological treatments		
ACE-inhibitors	61 (68%)	57 (63%)
Oral antidiabetic drugs	18 (20%)	16 (18%)
Insulin	20 (22%)	17 (19%)
Statin	70 (78%)	64 (71%)
Antiplatelets	74 (82%)	68 (75%)
Oral anticoagulants	6 (7%)	9 (10%)

ACE = angiotensin-converting enzyme; BMI = body-mass index; CHD = coronary heart disease; PAD = peripheral artery disease.
* Values are means ± SD (n = 5).

were registered as regards ABI (+25%; p = 0.035; Figure 2) and AT (−3.6%; p = 0.018; Figure 2), in the AMS group, when compared with baseline, while again placebo group revealed no significant differences (p = 0.2). A number of 53 and 36 subjects on 90 individuals, belonging to the placebo and AMS groups, respectively, showed at baseline specific vascular abnormalities of lower limbs related to their IC state (Table 3). Most of the placebo subjects (81.1%) revealed paresthesia, with low degree of severity (72.1%), and at the end of the treatment period none of them showed significant variation of their symptoms. Analogously, subjects complaining cold limbs, cold toes, and trophic lesions, reported unchanged states at the end of the trial. All of the AMS subjects (100%) revealed paresthesia, with low degree of severity (71.8%), but at the end of the treatment period 26 on 36 subjects (72.2%) declared full disappearance of the symptoms. None of the AMS subjects reported cold limbs and cold toes, whereas only 3 subjects complaining trophic lesions, with high degree of severity, declared unchanged symptoms at the end of the treatment period.

Although no specific toxicity studies have been performed herein, mutagenicity tests, acute/subacute toxicity studies have long since demonstrated the safety of polyphenol content of apples both in mice and human beings.

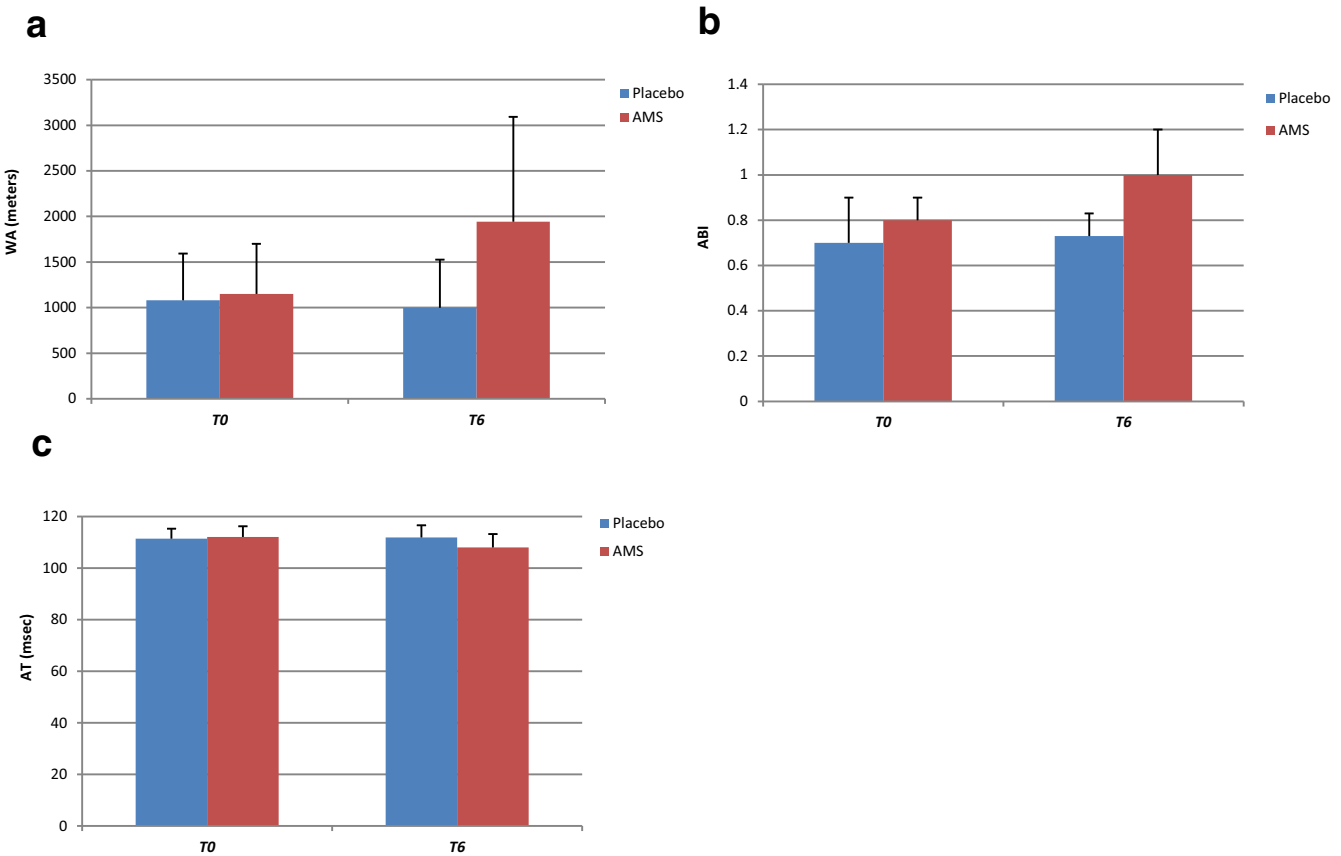

Figure 2. *(A)* Walking autonomy (WA), *(B)* ankle-brachial index (ABI), and *(C)* acceleration time (AT) of PAD subjects administered with placebo and AMS during treatment period (Placebo, n = 90; AMS, n = 90).
Values are means ± SD (n = 5); p <0.05.
AMS = Annurca nutraceutical formulation; T0 = beginning of week 0; T6 = end of week 24.

Table 3
Number of patients who have answered to the questions included in the modified WHO/Rose Questionnaire, for the self-assessment of specific underlying vascular abnormalities of lower limbs related to the IC state, at the beginning and at the end of the placebo and AMS intervention periods

	Placebo							
	T0			Tot. *T0*	*T6*			Tot. *T6*
	Degree				Degree			
	Low	Medium	High		Low	Medium	High	
Paresthesia	31	9	3	43	29	10	4	43
Cold limb	3	0	0	3	0	2	1	6
Cold toes	7	0	0	7	0	5	2	7
	AMS							
	T0			Tot. *T0*	*T6*			Tot. *T6*
	Degree				Degree			
	Low	Medium	High		Low	Medium	High	
Paresthesia	28	7	1	36	6	3	1	10
Cold limb	0	0	0	0	0	0	0	0
Cold toes	0	0	0	0	0	0	0	0

IC = intermittent claudication; *T0* = week 0; *T6* = week 24.

Specifically, the Commission Regulation (EC) No. 258/1997 established 1,000 mg as maximum polyphenolic extract daily intake in humans. Accordingly, the AMS dose adopted for the trial was a fully compatible amount with that regarded as safe in humans. Other safety assessments, such as vital signs, blood pressure, or electrocardiographic findings, were all periodically monitored and baseline values did not change substantially during and at the end of the trial.

The major strengths of the clinical trial herein presented reside in the originality of the study and in the evaluation of the treatment effects in real-world settings. The positive results, herein reported, can inform physicians about a novel treatment/intervention, which can represent a valuable support and/or alternative to the clinical practice. Conversely, the main limitations of our study include the short-term assessment for the treatment of a long-term condition and the choice of exclusively white race at the stage of the recruitment.

Discussion

The present study demonstrated for the first time that the intake of a novel nutraceutical product, based on AMS by PAD subjects for 24 weeks, was able to provide a significant improvement of WA and hemodynamic parameters, such as ABI and AT.

Previous authors reported that the short-term consumption of dark chocolate by patients with severe atherosclerosis was associated with a significant increase of WA.[8] This effect was ascribed to cocoa polyphenols, because the intake of milk chocolate by patients of a control group registered no effect. Loffredo et al had previously documented an acute increase of artery dilatation after dark chocolate intake in smokers.[13] Such positive effect was attributed to a vasodilating property due to a sequence of events, including downregulation of NOX2-mediated oxidative stress and eventually enhanced NO generation.[13] These changes are biologically plausible because cocoa polyphenols have antioxidant capacity that has been documented in humans by reduction of plasma markers of oxidative stress.[14,15] Specifically, the upregulation of NO generation is likely to play a major role in the WA changes, thanks to its well-known vasodilating capacity.

Nevertheless, there are still uncertainties on whether NO is directly implicated in the improvement of WA. In fact, although some studies contribute to confirm that the administration of NO sources seems to enhance WA and claudication pain-free time, supporting the hypothesis that NO increased peripheral tissue oxygenation in the area of hypoxia,[16,17] a large prospective, double-blind, placebo-controlled study with prolonged administration of an NO-donating agent (NCX 4016) did not improve walking distance in PAD patients.[18] Thus, the present question still remains an open debate.

Two drugs are currently available in the UK for the treatment of IC: cilostazol and naftidrofuryl oxalate. A meta-analysis of 26 studies assessing the efficacy of these drugs found that both offer modest improvement in WA with minimal adverse effects. However, there is little follow-up data beyond 6 months and cost effectiveness remains questionable.[19] As a result, the new NICE guidelines recommend only naftidrofuryl for use in patients with PAD and suggest that it be reserved for those who have failed to improve with structured exercise programmes and do not wish to be referred for angioplasty or surgery. Treatment with naftidrofuryl can be started in primary care, but its use should be reviewed after 3 to 6 months and discontinued if symptoms have not improved.[20] Although antiplatelet agents and vasodilators (such as nifedipine) may be useful for reducing overall cardiovascular risk, there is little evidence that these drugs offer any benefit in treating the symptoms of claudication. Thus, the need of alternative remedies, possibly natural, and with a low incidence of side effects, is still in great demand.

Our AMS nutraceutical formulation is mainly composed of catechin-derived compounds (Table 1), which are the same polyphenols occurring in cocoa products. Thus, it

could be hypothesized a common mechanism of action on WA and hemodynamic parameters in PAD subjects. We previously demonstrated that diverse apple species differ in the amount of polyphenolic compounds and that Annurca apple, a cultivar native to Southern Italy, was the richest.[8] We formulated gastric-resistant capsules due to previous knowledge about gastric digestion effects on some polyphenolic components.[21] Moreover, the specific technological formulation of AMS product, consisting of Annurca extract microencapsulated in maltodextrins, would possibly account for a better solubility of apple polyphenols in the intestinal aqueous medium and, thus, an improved availability to intestinal absorption of AMS constituents, as corroborated by our in vitro results.[22] This would partially explain the very significant physiological effects of AMS product on WA and ABI, although its lower provision of total polyphenols (about 3.0 mg/die, corresponding to what provided by 2,000 mg AMS; Table 1) respect to what assumed through cocoa product (about 32 mg/die, corresponding to what provided by 40 g dark chocolate) in the study of Loffredo et al.[7]

The positive effects of AMS on vascular abnormalities of lower limbs, observed in a significant number of enrolled subjects (Table 3), may find the same explanation to that of the previously mentioned primary efficacy outcome measures. Undoubtedly, the vasodilating capacity of the antioxidant catechin-derived compounds would allow an improvement of peripheral circulation, with decrease or disappearance of specific symptoms, in prevalence, paresthesia.

In conclusion, results indicated AMS nutraceutical product as effective in improving WA, hemodynamic parameters, and vascular abnormalities of lower limbs, in PAD subjects. It could be hypothesized that the polyphenolic constituents of this formulation would exert a vasodilating effect, thanks to their antioxidant capacity able to counteract NOX2-mediated oxidative stress and enhance NO release. These effects would be similar to what already demonstrated by previous authors in PAD patients, for the same polyphenols occurring in cocoa products. AMS product could be expected to better vehicle antioxidant polyphenols to the intestinal aqueous medium, thanks to its polar microencapsulated formulation.

Contributors

All authors were responsible for study concept and design. GCT, DC, MDA, RC, CA, and EN acquired the clinical data. CA designed, acquired and analyzed the Doppler experiments. All authors analyzed and interpreted the data and drafted the manuscript. All authors critically revised the manuscript for important intellectual content. EN obtained funding. EN provided administrative, technical, or material support. All authors supervised the study. All authors, external and internal, had full access to all of the data. EN is the guarantor.

Competing Interests

All authors declare: EN has received research grants from Regione Campania under POR Campania FESR 2007-2013 - O.O. 2.1 (FarmaBioNet); no other relation of activities that could appear to have influenced the submitted work.

Ethical Approval

The study was approved by the ethics committee at the Hospital AO Rummo of Benevento, Italy.

Transparency Statement

The lead author (the manuscript's guarantor) affirms that this manuscript is an honest, accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

Data Sharing

No additional data available.

Disclosures

The authors have no conflicts of interest to disclose.

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