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A serving of blueberry (*Vaccinium corymbosum*) improves peripheral vascular function but not metabolic and functional markers in older subjects: A randomized, controlled, crossover study

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A B S T R A C T

Evidence suggests that polyphenol-rich foods like berries may help counteract aging-related disorders such as vascular dysfunction and arterial stiffness. However, few intervention studies have been conducted in older adults. This study aimed to assess whether the consumption of blueberries may improve vascular function in older subjects. A randomized, controlled, crossover trial was conducted in a group of 20 volunteers over 60 years old. Participants consumed either a blueberry mousse (250 g, providing 480 mg of anthocyanins – ACNs) or a control product (250 mL of sugared water), with treatments separated by at least 1-week. Reactive hyperemia index (RHI), augmentation index (AIx), blood pressure, and heart rate were measured at baseline and 2 h post-consumption. Blood samples were collected at baseline and after 1, 1.5, 2 and 4 h from the intake to evaluate ACN bioavailability, metabolic, and vascular markers. Sixteen subjects completed the trial (9 males, 7 females; mean age 69 ± 5 years). Blueberry consumption significantly increased RHI compared to control (mean difference + 0.42, 95 % CI: 0.01–0.082, $p < 0.05$). Maximum serum ACN concentration was observed at 2 h (20.3 ± 7.4 ng/mL). No association was found between RHI improvement and total serum ACNs, but a significant positive correlation was detected with delphinidin and cyanidin-3-glucoside ($p < 0.01$). No effects on AIx, blood pressure, or other markers were found. In conclusion, blueberries may improve peripheral vascular function in older adults, potentially due to increased ACN levels. Further studies are needed to corroborate these findings and elucidate the mechanisms involved.

1. Introduction

Aging represents a natural physiological process leading to a time-dependent progressive loss of cellular functionality, predisposing people to an increased risk of frailty, morbidity, and mortality (Niccoli & Partridge, 2012). Several cellular and molecular hallmarks seem to drive this complex process, including genomic instability, telomere attrition, mitochondrial dysfunction, cellular senescence, altered intercellular communication, and chronic inflammation (López-Otín et al., 2023). It is estimated that by 2030, 1 in 6 people in the world will be aged 60 years or over, while the proportion of over 60 years, by 2050, will be up to one-third in developed countries and one-fifth in developing countries (WHO, n.d.). However, this increase in life expectancy is accompanied by an increase in age-related disorders and diseases, with important implications for societies and health care systems (United Nations, 2023).

Cardiovascular diseases (CVDs) still remain the most common cause of disability in older adults including death, although the death rate has dropped in the last 20 years (Saglietto et al., 2021). In this regard, the aging process plays a pivotal role in the deterioration of CV functionality, resulting in an increased risk of such chronic diseases (de Almeida et al., 2020). The biological mechanisms underlying these detrimental effects seem related to morphological changes of the vascular system and senescence processes. The main aged-associated morphological

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changes include luminal dilation and increase in arterial stiffness in large arteries, such as the aorta and carotids (Laurent, 2012). However, aging is also frequently associated with vascular and endothelial dysfunction in distal and small arteries due to pro-inflammatory activation of the endothelial cells, generally consequent to a state of unbalance between production of reactive oxygen species and buffering capacity of the antioxidant defense systems (i.e., oxidative stress), and cellular senescence, which indicates a specialized form of mitotic cell growth arrest that leads to impaired regeneration of endothelial cells (Herrera et al., 2010; Incalza et al., 2018).

Diet and dietary components can play an important role in modulating CV risk other than the aging process and related hallmarks (Mathers, 2015). Epidemiological studies suggest that diets rich in (poly)phenols and (poly)phenol-rich foods may represent an interesting potential strategy to counteract CVDs such as coronary heart disease, myocardial infarction, and vascular disorders, thus supporting an endothelium health-promoting effect (Del Bo' et al., 2019; Di Pietro et al., 2020; Najjar et al., 2021). However, the mechanisms explaining this relation have not been fully elucidated yet. Some studies have shown a direct effect of (poly)phenols at endothelial level by stimulating the synthesis of vasodilator agents (i.e., nitric oxide) and the reduction of vasoconstrictors (i.e., endothelin 1). However, (poly)phenols can also preserve endothelium by attenuating oxidative stress thanks to their capacity to act directly as radical scavengers or indirectly by upregulating antioxidant/xenobiotic response (Clifford et al., 2021). In addition, (poly)phenols can preserve endothelium by counteracting inflammation through the downregulation of nuclear factor kappa B (NFkB), the reduction of pro-inflammatory cytokines, and by increasing the expression of anti-inflammatory molecules (Martini et al., 2023; Tucci et al., 2022). Furtherly, (poly)phenols have shown to contribute to the modulation of blood pressure, platelet activity, lipid and glucose profile, reflecting an improvement of vascular response (Martini et al., 2023; Tucci et al., 2022).

Blueberries are, among berries, the most studied fruits. They contain a wide range of anthocyanins and phenolic acids, other than fiber, minerals and vitamins (Kafkas & Ürün, 2023; Krishna et al., 2023; Stevenson & Scalzo, 2012; Wood et al., 2019; Yücetepe et al., 2024). In addition, blueberries provide also dietary salicylates (range 0.57–27.6 mg/kg) hat may contribute to the beneficial effect (Wood et al., 2011; Yeasmin & Choi, 2020). The effect of blueberries and their components on cardiometabolic and vascular function has been largely investigated in numerous human trials (Venturi et al., 2023; Wood et al., 2019). These studies have shown the capacity of blueberries to improve, postprandially and/or after medium/long-term intervention, endothelial and vascular tone in smokers, subjects with CVD risk factors, metabolic syndrome and in subjects affected by disease conditions, while the evidence in older subjects is scarce. In fact, to the best of our knowledge only two studies have been carried out, showing opposite findings (Dodd et al., 2019; Wood et al., 2023).

Thus, the aim of the present single blind, randomized, controlled, acute crossover study was to evaluate whether the consumption of blueberries could improve markers of vascular function in a group of older subjects. To explain this possible modulation and identify the potential mechanisms of action, the analysis of a plethora of functional markers related to vascular health, together with the bioavailability of anthocyanins, as main bioactive contributors in such modulation, was carried out.

2. Methods

An accurate description of the methods used for this study is accessible by consulting the previously published study protocol (Del Bo' et al., 2022), however a brief description is reported below.

2.1. Participants enrollment

For this study, 20 older subjects of either sex were enrolled through advertisements by using notice boards, emails, phone calls, social networks, and grapevine, according to the inclusion and exclusion criteria reported in the protocol previously published (Del Bo' et al., 2022). Briefly, healthy subjects having at least 60 years of age were considered eligible for the participation. Also subjects under chronic drug treatment for mild hypertension, or chronic conditions but not related to vascular functions (e.g., gastroesophageal reflux disease) were considered eligible since representatives of this target population. Subjects with diabetes, history of thrombosis, myocardial infarction, amputation of hand or finger, potential allergies or aversion to the blueberries, as well as those with specific dietary advice, were excluded. Successively, subjects' eligibility was confirmed through a general anamnesis carried out by the medical staff of the International Center for the Assessment of Nutritional Status and the Development of Dietary Intervention Strategies (ICANS-DIS) of the University of Milan, through questions about health status and lifestyle, and an accurate clinical evaluation. All participants signed the informed consent form before being enrolled. The protocol was approved by the Ethics Committee of the University of Milan (ref: 121/20_Verbale_All-11) and registered at ISRCTN registry (https://www.isrctn.org as ISRCTN18262533).

2.2. Blueberry and control product preparation

Details about characterization of tested products are reported in Del Bo' et al. (Del Bo' et al., 2022). Briefly, the blueberry (BB) product consisted of a mousse prepared, fresh in the morning of each test day, by blending 250 g of frozen blueberries. Blueberries belonged to the cultivar Legacy of highbush blueberry (*Vaccinium corymbosum*) particularly rich in anthocyanins (ACNs; about 192 mg of anthocyanins/100 g of fresh product equivalent to 480 mg per portion). The portion was decided based on previous published studies also carried out in our laboratories, that tested blueberry as raw fruit or freeze-dried powder (portions ranging from 150 to 300 g) (Wood et al., 2023). The characteristics of the BB product, as well as its ACNs, are depicted in Fig. 1. Fifteen ACN glycosides were identified in BB product. Delphinidin-3-*O*-galactoside, cyanidin-3-*O*-arabinoside and malvidin-3-*O*-galactoside were the most abundant. The control product (C) consisted of 250 ml of water containing the same type and amount of sugars of the BB product (i.e., 16.8 g of total sugar, constituted by 8.0 g of fructose, 7.3 g of glucose, and 1.5 g of saccharose).

2.3. Experimental design

A single-blind, randomized, controlled, crossover trial was carried out in which the acute effect of a portion of blueberry (BB) versus a control product (C), was tested. The group of subjects enrolled were randomly divided (using a computer random number generator) into 2 sequences of intervention: B/wash-out/C, or C/wash-out/BB. The washout was lasted one week, which is sufficient time for the effects of the first treatment to dissipate before the second treatment, considering the metabolization timing of polyphenols. Details about the experimental design were previously reported (Del Bo' et al., 2022). Briefly, the study protocol included two phases; one phase focused on the study of BB polyphenols bioavailability and blood circulating levels of several metabolic and functional markers related to vascular function. Blood samples were collected at baseline (Time 0 h) and after 1 h, 1.5 h, 2 h, and 4 h from the intake of BB or C product. The second phase was devoted to the direct evaluation of blood pressure, heart rate and vascular functionality. This latter was carried out by using a non-invasive biosensor for the assessment of reactive hyperemia index (RHI), Framingham reactive hyperemia index (fRHI) and arterial stiffness (AIx and AIx@75). The evaluations were performed at baseline and after 2 h from the consumption of BB or C product. This division of the

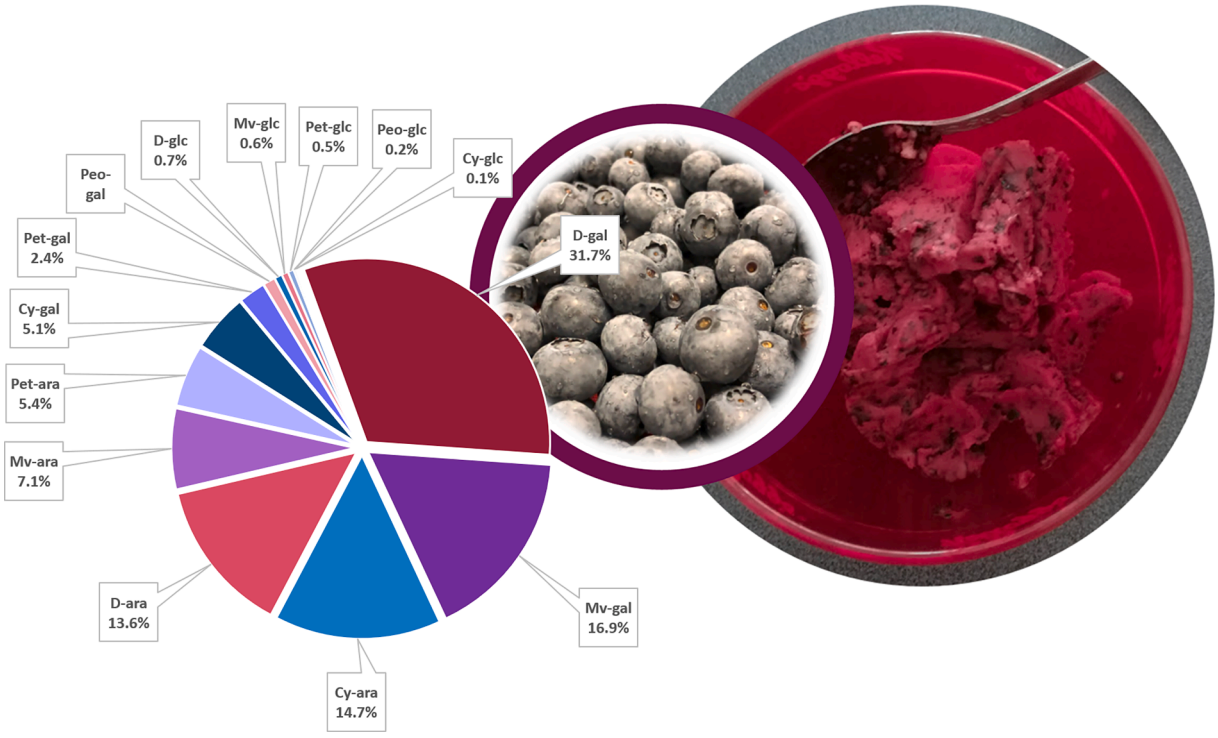

Fig. 1. Blueberries' anthocyanin profile in the test product. Cy = cyanidin; D = delphinidin; Mv = malvidin; Peo = peonidin; Pet = petunidin; −ara = arabinoside; −gal = galactoside; −glc = glucoside.

study in two phases was used to avoid interference between withdrawal by venous cannula and the evaluation of vascular function at the level of the brachial artery, in line with a study previously published (Del Bo' et al., 2013). The two phases were separated by 1 month of interruption. During the entire experimental period, subjects were asked to maintain their habitual lifestyle and dietary habits by excluding or limiting from their diet foods rich in (poly)phenols (e.g., berries, tea, coffee, chocolate, fruit juices). Further details are reported in the study protocol (Del Bo' et al., 2022). The day before the tests, a standardized dinner consisting of a light meal of pasta or rice, meat, fish or cheese and bread was consumed before 9p.m. by the volunteers. In addition, subjects participating at the phase 1 of the study were invited to consume a standardized light breakfast consisting of 200 mL of partially skimmed milk or 125 g of yogurt and 3 biscuits (i.e., shortbread) or 3 rusks. Each subject had to consume the same food selection at every test day. Breakfast was consumed early in the morning (before 7 a.m., at least 90 min before blood collection and at the same hour each test day.) to limit the possible noise deriving from a long-fasting period the day of the withdrawals. Further details about the experimental design are reported in Del Bo' et al. (Del Bo' et al., 2022).

2.4. Compliance

To ensure adequate compliance to the dietary instructions provided, a 24 h-dietary recall was registered by each participant the day before of each experimental day. In addition, a direct interview was scheduled by a dietician the day of the experiment to check dietary records and register eventual noncompliance.

2.5. Anthropometric parameters

Body weight and height were assessed during the screening visit following the international guidelines reported by Lohman et al. (Lohman et al., 1992). Body mass index (BMI) was calculated according to the following formula: BMI = weight (kg)/height $(m)^2$.

2.6. Blood pressure

Blood pressure was measured during the screening visit and during the phase 2 of the study. The evaluation included both systolic (SBP) and diastolic blood pressure (DBP) measured in a resting and seated position following the JNC 7 guidelines (Chobanian et al., 2003).

2.7. Blood sampling and analysis

Blood samples were drawn in Vacutainer tubes containing silicone gel or sodium EDTA for serum and plasma respectively. Tubes for serum were maintained at room temperature for at least 30 min. and centrifuged at 1088*g* × 15 min, 4 °C, while tubes for plasma were immediately centrifuged at the same conditions. Samples were divided in small aliquots and stored into specific vials at − 80 °C until analysis of several metabolic and functional parameters (Del Bo' et al., 2022).

2.8. Biochemical parameters

Glucose, insulin, lipid profile, C reactive protein, liver, and renal function were analyzed in serum, while c-peptide in plasma samples, at the first screening visit at fasting conditions. The analysis was carried out by a standardized validated protocol, using an automatic biochemical analyzer (YSI 2300 STAT Plus Glucose and Lactate Analyzer, Marshall Scientific, Hampton, VA, USA). Insulin sensitivity was assessed by considering fasting plasma concentrations of glucose and insulin, as indicated by Wallace and coworkers (Wallace et al., 2004). Further details are reported in Del Bo' et al. (Del Bo' et al., 2022).

2.9. Evaluation of arterial function and stiffness

The operational details for measuring arterial function and arterial stiffness are previously reported (Del Bo' et al., 2022). Briefly, Reactive Hyperemia Index – RHI, an index of vascular reactivity was measured at peripheral levels (fingertips) through Endo-PAT2000 (Itamar Medical Ltd, Caesarea, Israel), an operator independent and non-invasive device

based on plethysmographic probes. The methodology assesses the amplitude of reactive hyperemia due to the probes by measuring the changes in the pulsations in finger pressure that occur for some minutes following the induction of a reactive hyperemic response. This response is induced mechanically in the arm, by occluding for five minutes and then releasing the brachial artery with a cuff inflated between 200 and 220 mmHg. The same device allows also the detection of the Framingham Reactive Hyperemia Index, consisting in a variation of RHI proposed by Framingham Heart Study researchers, that imply variations related to the reduced length of the hyperemic response assessment, the absence of baseline correction, and natural logarithmic transformation (McCrea et al., 2012). Finally, the device also measures the heart rate, and calculates surrogate markers of arterial stiffness (i.e., Augmentation Index – AIx and Augmentation Index normalized on a heart rate of 75 bpm – AI@75).

2.10. Evaluation of serum vascular markers

Serum samples at each time point were used to quantify endothelin-1, total nitric oxide (total NO), vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) by ELISA kit. The determination was performed according to the instructions reported by the manufacturer. The quantification was carried out by measuring the fluorescence through a TECAN Infinite F200 plate reader. A 4-parameter algorithm was applied to generate the standard curve and to calculate final concentrations for each marker.

2.11. Evaluation of serum ACN concentrations

The extraction and analysis of serum ACNs were performed according to Martí et al., (Martí et al., 2010) by using a microelution solid phase extraction (SPE) and UPLC-ESI-MS/MS analysis. Details about the analysis were previously reported (Del Bo' et al., 2022). Slight modifications were carried out to optimize ACN extraction from serum. Specifically, microelution SPE (30 mm μElution Plates) were activated with 250 μL of methanol, followed by 500 μL of trifluoroacetic acid (TFA). Then, serum (300 μL) was mixed with 300 μL of a solution constituted by 20 % of 2,4,6-Trichloroanisole (TCA) to facilitate protein precipitation, and supernatant collected and loaded into the microplate. Then, the loaded plates were washed 2 times with 750 μL of 1 %TFA. The retained ACNs were eluted with 100 μL of 50 %methanol/1%TFA solution (50:50, v/v) and injected into the UPLC-ESI-MS/MS as previously reported (Del Bo' et al., 2022).

2.12. Evaluation of salicylate serum concentrations

The analysis of salicylates in serum samples was performed using stable isotope dilution and gas chromatography–mass spectrometry, according to Battezzati et al., (Battezzati et al., 2006). Details about the analysis were previously reported (Del Bo' et al., 2022).

2.13. Statistical analysis

The sample size was determined based on previous studies conducted in our laboratories and other research groups to test the efficacy of blueberry consumption on RHI (Del Bo' et al., 2013, 2014, 2017; Dodd et al., 2019; Rodriguez-Mateos et al., 2013; Stull et al., 2015). Fifteen subjects were considered sufficient to determine an increase of 0.30 in the RHI parameter after blueberry intake with alpha = 0.05 and 80 % statistical power. Considering a 25 % dropout risk rate, 20 subjects were enrolled. Data were analyzed on a per-protocol basis, meaning that participants who deviated from the study protocol, such as by missing required sessions or failing to follow standardized procedures, were excluded from the analysis. In case of missing data in one part of the intervention, data were entirely excluded from the analysis to minimize the risk of attrition and information bias. Reasons for dropout were documented and reported in the CONSORT flow diagram. All statistical analyses were performed with SPSS Statistics (SPSS 28.0.1.1, IBM, Armonk, USA). Normal distribution of data was verified by the Shapiro–Wilk test for all parameters. The presence of possible outliers was highlighted by creating boxplots for all parameters. Data are presented as means ± SD, with 95 % confidence intervals (CI) provided for the mean differences within each intervention (Δ C and Δ B), and for between-group mean difference. For the primary outcome and the other non-invasive markers of vascular function, paired samples Student's t-tests were used to compare pre- and post-intervention mean differences within each intervention. To assess the between-group differences, a univariate General Linear Model (GLM) was employed, accounting for time effects and time-treatment interactions. This approach helps to isolate the net effect of the intervention by accounting for the paired nature of the data. The significance level was set for both statistical tests at 0.05. For serum markers of vascular function and metabolic response, a repeated measures GLM was applied, with timepoints treated as a within-subjects factor and treatment as a between-subjects factor. This model accounted for the correlation among repeated measurements within subjects, providing a robust assessment of treatment effects over time. Additionally, independent samples Student's t-tests were applied to compare treatments at each timepoint (bilateral $p < 0.05$). Serum concentrations of total ACNs and single glycosides were linearly correlated with vascular function markers final values and variations by applying a two-tailed bivariate correlation and calculating Pearson's r. In this case, only $p < 0.01$ was considered. Finally, machine learning was applied to assess the profile of subjects who may be more responsive to blueberry intake, through predictive analytics that included all demographic and clinical parameters available for this sample of subjects. Using the machine-learning feature-selection approach, the Random Forest was performed to identify the most predictive features among the subjects enrolled that could affect the changes of the primary outcome of the study (RHI), considering every possible interaction between them, and also considering the non-linear relationships that classical models could not assess.

3. Results

3.1. Recruitment phase workflow

A schematic flowchart of the protocol, reporting all the information from the recruitment until the end of the study, is shown in Fig. 2. A total of 26 subjects aged >60 years were initially recruited, starting from May 2021 and up to April 2022. After the screening 4 subjects were considered ineligible and other 2 subjects withdrew before the beginning of the study for personal reasons. Thus, 20 subjects were initially enrolled. Finally, 16 out of 20 subjects completed the study and were analyzed for the markers considered.

3.2. Baseline characteristics of the participants

The demographic and clinical characteristics of the subjects are reported in Table 1. Overall, subjects were healthy, with a mean age of 69 years. Anti-hypertensive drugs were the medications used by most of the volunteers (at about 70 %). This was in line with the characteristics of the target population. A high inter-individual variability was observed for most of the markers assessed, and particularly for BMI, blood pressure, total cholesterol, LDL-cholesterol, glycaemia and insulinemia. Eight out of 16 subjects showed vascular dysfunction (cutoff RHI < 1.67).

3.3. Effect of blueberry intervention on reactive hyperemia index, arterial stiffness and blood pressure

The effect of blueberry and control products on RHI (primary outcome of the study), arterial stiffness, heart rate and blood pressure

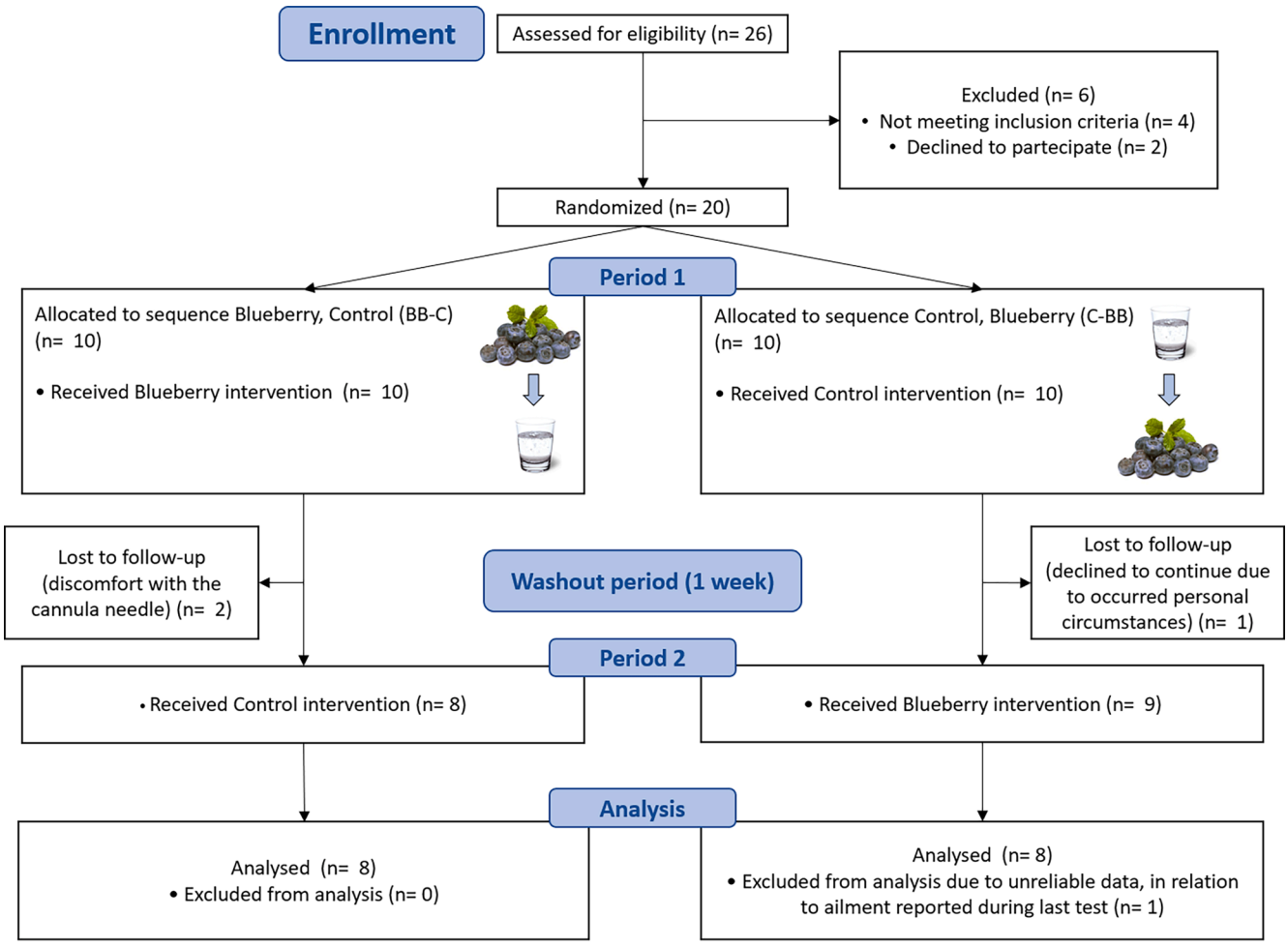

Fig. 2. CONSORT flow diagram.

are reported in Fig. 3 and Table 2.

In both interventions there was a significant increase in RHI at 2 h. The change in RHI was + 0.85 (95 % CI: 0.56–1.15) for BB and + 0.44 (95 % CI: 0.14–0.73) for C, both significant at $p < 0.05$. However, BB resulted in a significantly higher reactive hyperemic response at 2 h compared to C. The between-group mean difference was 0.42 (95 % CI: 0.01–0.082), with a significant time × treatment interaction ($p < 0.05$). Comparable results were obtained for the FRHI.

When examining AIx and AI@75 (indicators of arterial stiffness), a statistically significant increase in AIx was observed for both treatments. Regarding AI@75, the increase was registered only after BB treatment but not following the control. However, the difference between the deltas in the two interventions was not statistically significant.

Regarding heart rate, following both dietary treatments there was a significant decrease from the baseline. However, in line to the findings on arterial stiffness, there was no significant difference between the deltas of the two interventions.

Finally, no significant effect was registered for systolic and diastolic blood pressure following both the interventions, according to the comparison between the deltas, even though a significant reduction between baseline and final values was found for blueberry.

3.4. *Effect of blueberry on serum markers of vascular function and metabolic response*

In Fig. 4 are reported the effects of the intervention on markers of vascular function and metabolic response in this group of older subjects. Regarding vascular function variables, the Repeated Measures General Linear Model did not show any significant difference in the circulating levels of endothelin-1, NO, ICAM-1 and VCAM-1, between BB and C treatment. Conversely, a temporary increased in glycemia and insulinemia (marker of metabolic response) was registered at 1 h after the intake of BB but not C product (109.8 ± 16.9 mg/dL vs 95 ± 17.9 mg/dL, $p < 0.05$; and 21.3 ± 5.2 μU/mL vs 13.0 ± 7.1 μU/mL, $p < 0.001$ for glycemia and insulinemia, respectively). The increase observed dropped to baseline levels already at 1.5 h.

3.5. *ACN serum concentrations following BB and C products*

In Table 3 are reported the serum concentrations of ACNs registered at baseline and after 1, 1.5, 3 and 4 h following the consumption of the BB product. ACNs were absorbed rapidly, and their serum concentration increased already at 1 h (3.7 ± 2.9 ng/mL, corresponding to approximately 7.4 nmol/L, assuming the molar mass of delphinidin-3-*O*-galactoside), achieving the maximum serum concentration after 2 h from BB consumption (20.3 ± 7.4 ng/mL, corresponding to approximately 40.5 nmol/L). At 4 h, a significant decrease in serum concentration from the intake of BB product (16.1 ± 7.5 ng/mL, corresponding to approximately 32.2 nmol/L) was found. When considering the single ACNs, all the 15 glycosides identified in BB product were also detected in serum with differences among ACNs. The range varied from 0.53 ng/mL for petunidin-3-O-glucoside up to 2.70 ng/mL ± 1.71 for delphinidin-3-*O*-galactoside. A correspondence between the amounts of the single glycosides detected in the BB product and serum concentrations was found, especially for the most absorbed compounds delphinidin-3-*O*-galactoside (2.70 ± 1.71 ng/mL), cyanidin-3-*O*-arabinoside (2.18 ± 1.15 ng/

Table 1
Baseline characteristics of the study population included in final analysis (n = 16).

Variables	mean ± sd (min–max)
Age (years)	69 ± 5 (62–76)
Sex (F/M) (%)	7 (44 %)/9 (56 %)
Race/ethnicity (n) (%)	
Asian	0 (0 %)
Black or African American	0 (0 %)
White	16 (100 %)
Hispanic or Latino	0 (0 %)
Other	0 (0 %)
BMI (kg/m^2)	24.9 ± 2.4 (21.1–29.4)
Drug treatment*	11 (68.8 %)
RHI	1.78 ± 0.47 (1.11–2.81)
FRHI	0.36 ± 0.40 (−0.58–0.89)
AIx (%)	20 ± 20 (−40–46)
AI@75 (%)	12 ± 20 (−40–49)
HR (bpm)	62 ± 8 (50–76)
SBP (mmHg)	120 ± 8 (110–140)
DBP (mmHg)	77 ± 5 (70–90)
Glycemia (mg/dL)	101 ± 13 (77–124)
Insulin (μUI/mL)	12.6 ± 7.1 (3.1–30.8)
TG (mg/dL)	127 ± 55 (71–124)
TC (mg/dL)	211 ± 30 (154–259)
LDL-C (mg/dL)	123 ± 22 (86–158)
HDL-C (mg/dL)	51 ± 13 (33–73)
TC/HDL-C (ratio)	4.3 ± 1.0 (2.8–6.4)
LDL/HDL-C (ratio)	2.5 ± 0.6 (1.5–3.9)
CRP (mg/L)	1.5 ± 1.5 (0.2–4.2)
ALT (U/L)	14.8 ± 4.8 (7.0–24.2)
GGT (U/L)	27.1 ± 29.4 (9.6–130.8)
GOT (U/L)	21.4 ± 8.3 (13.8–46.7)
ET-1 (pg/mL)	2.2 ± 0.5 (1.6–3.7)
NO** (μM)	37.0 ± 24.3 (17.9–109.7)
VCAM-1 (ng/mL)	551 ± 184 (312–1012)
ICAM-1 (ng/mL)	18.7 ± 7.1 (8.0–33.8)

Values are reported as mean ± standard deviation (min − max), apart from sex, race/ethnicity, and drug treatment which are reported as number of subjects (percentages). AIx = augmentation index; AI@75 = augmentation Index normalized to an heart rate of 75 bpm; ALT = Alanine transaminase; BMI = body mass index; CRP = C-reactive protein; DBP = diastolic blood pressure; ET-1 = endothelin-1; FRHI = Framingham reactive hyperemia index; GGT = Gamma-glutamyl transferase; GOT = glutamic oxaloacetic transaminase; HDL = high-density lipoprotein; HR = heart rate; ICAM-1 = intercellular adhesion molecules-1; LDL = low-density lipoprotein; NO = Nitric oxide; RHI = reactive hyperemia index; SBP = systolic blood pressure; TC = total cholesterol; TG = triglycerides; VCAM-1 = vascular cell adhesion protein-1; Minimum and maximum is reported in parenthesis. * Refers mainly to anti-hypertensive drugs. **Measured indirectly as a sum of nitrites and nitrates.

mL) and malvidin-3-*O*-galactoside (2.07 ± 1.31 ng/mL), while higher variation was found for ACNs detected in smaller quantities (e.g. Malvidin-3-*O*-glucoside) (Table 4). ACNs were not detected following the consumption of the C product (data not shown).

3.6. Salicylate serum concentrations following BB and C products

In Table 5 are reported the serum levels of salicylates measured overtime following the consumption of BB and C products. Overall, the intervention with BB and C products did not affect salicylate serum concentration that remained unchanged overtime.

3.7. Correlation between BB bioactive serum concentrations and RHI

A linear correlation between total ACNs or single glycosides at the different timepoints and final values of RHI at 2 h or RHI Δ change was found indicating a positive correlation for delphinidin-3-o-glucoside at both t1 (1 h) and t2 (1.5 h), and cyanidin-3-O-glucoside at t1 (1 h). Correlation coefficients are reported in Fig. 5.

3.8. Machine learning approach to identify higher responders to BB intervention

In relation to the wide confidence interval within which the effect of BB product on RHI improvement falls, and the non-negligible degree of heterogeneity among the characteristics of the recruited subjects, a machine learning approach was tested to identify the profile of subjects who may be more responsive to the dietary intervention. All available demographic and clinical parameters were included in this analysis.

Results obtained in this sample of subjects seem to indicate that higher BMI or fasting glycemia could be the most important factors associated with RHI improvement imputable to blueberry consumption, followed by VCAM1 and GOT (see Fig. 6).

4. Discussion

This study aimed to evaluate the effects of a portion of blueberries on markers of vascular function and stiffness in a group of older subjects. To the best of our knowledge, this is the first study evaluating the acute effects of blueberry intake on different markers related to vascular function in this target population while a single study tested the effects only on markers of vascular stiffness (Dodd et al., 2019). Recently, Wood and colleagues documented that the daily intake of 26 g of wild blueberry freeze-dried powder (providing 300 mg of anthocyanins) for 12 weeks could lead to improvement in vascular function, measured as FMD, in a group of 54 older subjects (Wood et al., 2023). In our experimental condition, the administration of 250 g of a blueberry mousse, providing 480 mg of anthocyanins, led to an improvement in vascular function, measured as RHI, by 29.4 % after 2 h from the consumption. The present findings are in line with those obtained by our research group in a previous study carried out in young subjects with vascular function impairment (Del Bo' et al., 2017) in which a 26.6 % net increase of RHI was observed following the consumption of 300 g of blueberry mousse (providing 348 mg of anthocyanins). Similarly, Rodriguez-Mateos et al., reported an acute FMD increase in healthy male young adults, after the consumption of 3 buns of baked blueberry-containing products or a blueberry drink (containing the equivalent of 240 g of fresh blueberries) (Rodriguez-Mateos et al., 2014). However, other studies reported that a comparable amount of blueberry and polyphenols failed to affect RHI in subjects with normal vascular function (Del Bo' et al., 2013).

Blueberries represent a source of different bioactives with potential vasoactive properties, other than polyphenols, including vitamins, minerals, and salicylates, however several studies have attributed the beneficial effect on vascular function mainly to polyphenols and their metabolites (Rodriguez-Mateos et al., 2019). In this regard, Rodriguez-Mateos et al. documented that the positive modulation in endothelial function induced by blueberries was similar only to the modulation obtained after the administration of blueberries' pure anthocyanins, while no effect was observed when consuming a product constituted by blueberry sugars, fiber, minerals or vitamins (Rodriguez-Mateos et al., 2019). Within the same study, a dose–response relationship was found between FMD and serum anthocyanins (0–2 h) and their metabolites (6–8 h) (Rodriguez-Mateos et al., 2019). Studies using radio-labeled ACNs indicated that the parent compounds represent a minority (2 %) compared to the total metabolites found in the circulation following blueberries consumption, and consequently it was supposed that ACNs' bioactivity is more likely to be mediated by their phenolic metabolites (Cutler et al., 2017).

In our study, we found a significant increase in ACNs absorption already at 1 h from blueberry consumption, with a maximum peak in the range of nanomolar concentrations at 2 h, especially for malvidin, cyanidin and delphinidin glycoside (the most representative ACNs in the blueberry), while we did not measure metabolites in consideration of the time-points considered (0–4 h). In addition, we found that delphinidin and cyanidin-3-glucoside were significantly correlated with an

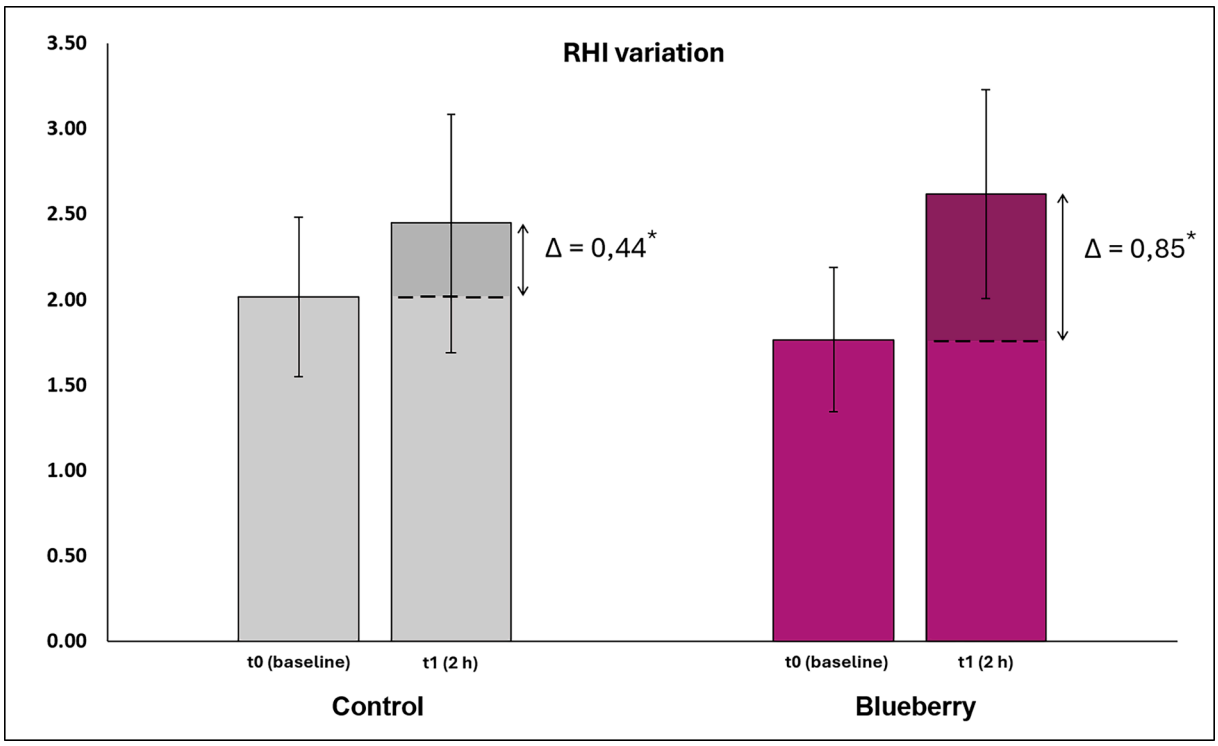

Fig. 3. RHI variations following control and blueberry consumption. C, control product; BB, blueberry product. Dotted lines and color differences highlight the deltas between the final RHI values and the baseline values within the two arms of the study. * Indicates that the comparison between these two deltas (between-group mean difference) result statistically significant (p-value time × treatment; $p < 0.05$).

Table 2
Effect of intervention on physical and mechanical markers of vascular function.

Variable	Pre-C mean (SD)	Post-C mean (SD)	Δ C (CI 95 %) p-value	Pre-B mean (SD)	Post-B mean (SD)	Δ B (CI 95 %) p-value	Between-group mean difference (CI 95 %)	p-value time × treatment interaction	p-value for time
RHI (unit)	2.01 (0.46)	2.45 (0.63)	0.44 (0.14; 0.73) $p < 0.05$ in percentage +24.1 % (+5.7 %; +42.5 %)	1.76 (0.42)	2.62 (0.61)	0.85 (0.56; 1.15) $p < 0.001$ in percentage +53.5 % (+35.1 %; +71.9 %)	0.42 (0.01; 0.82) in percentage +29.4 % (+3.4 %; +55.5 %)	$p < 0.05$ in percentage $p < 0.05$	$p < 0.001$
FRHI (unit)	0.53 (0.30)	0.91 (0.34)	0.38 (0.18; 0.58) $p < 0.001$	0.34 (0.40)	1.06 (0.39)	0.72 (0.53; 0.92) $p < 0.001$	0.34 (0.07; 0.62)	$p < 0.05$	$p < 0.001$
AIx (%)	19.9 (20.0)	33.7 (16.7)	13.8 (2.7; 24.8) $p < 0.05$	18.9 (15.4)	37.4 (23.9)	18.4 (8.0; 28.9) $p < 0.01$	4.7 (−9.9; 19.2)	$p = 0.516$	$p < 0.001$
AI@75 (%)	11.7 (17.8)	22.5 (16.6)	10.8 (−0.3; 21.9) $p = 0.56$	11.8 (16.1)	27.3 (22.4)	15.4 (5.5; 25.3) $p < 0.01$	4.6 (−9.6; 18.9)	$p = 0.513$	$p < 0.001$
HR (bpm)	62 (9)	58 (6)	−4 (−8; 0) $p < 0.05$	62 (8)	59 (6)	−4 (−6; −1) $p < 0.05$	0 (−4; +5)	$p = 0.826$	$p < 0.01$
SBP (mmHg)	119 (7)	118 (6)	−1 (−3; 1) $p = 0.06$	120 (7)	117 (6)	−3 (−6; 0) $p < 0.05$	−2 (−4; +1)	$p = 0.242$	$p = 0.01$
DBP (mmHg)	76 (3)	76 (4)	0 (−2; +2) $p = 0.89$	78 (6)	75 (4)	−3 (−5; 0) $p < 0.05$	−3 (−5; 0)	$p = 0.098$	$p = 0.070$

Pre-C = Pre-control treatment; Post-C = Post control treatment; Pre-B = Pre-blueberry treatment; Post-B = Post-blueberry treatment; RHI = Reactive Hyperemia Index; FRHI = Framingham Reactive Hyperemia Index; AIx = Augmentation Index; AI@75 = Augmentation Index normalized for a heart rate of 75 bpm; HR = Heart rate; SBP = Systolic blood pressure; DBP = Diastolic blood pressure.

improvement in RHI; delphinidin and its glycosides, have already been reported to exert an *in vitro* and *in vivo* effect in vascular health (Chen et al., 2019). Parallelly, we have also measured circulating levels of salicylates reporting no modulation in their serum levels following the blueberry intake. Thus, we may speculate that polyphenols, in particular ACNs, could be the main bioactive compounds responsible for the observed effects on vascular function in line with the observations previously reported (Rodriguez-Mateos et al., 2019).

In the present study, an explorative machine-learning approach was used to identify predictors of intervention efficacy, considering all relevant data collected within this trial. The model applied may indicate that a dysfunctional phenotype characterized by elevated BMI, higher fasting blood glucose and elevated VCAM-1 represented the most relevant predictor, once corrected for all the other factors included. However, from a mechanistic perspective, the analysis of serum markers failed to identify clear factors involved in such modulation. In fact, while a transient increase in glucose response was registered at 1 h, but not at 2 h, following blueberry intake (see later), no effect was reported for

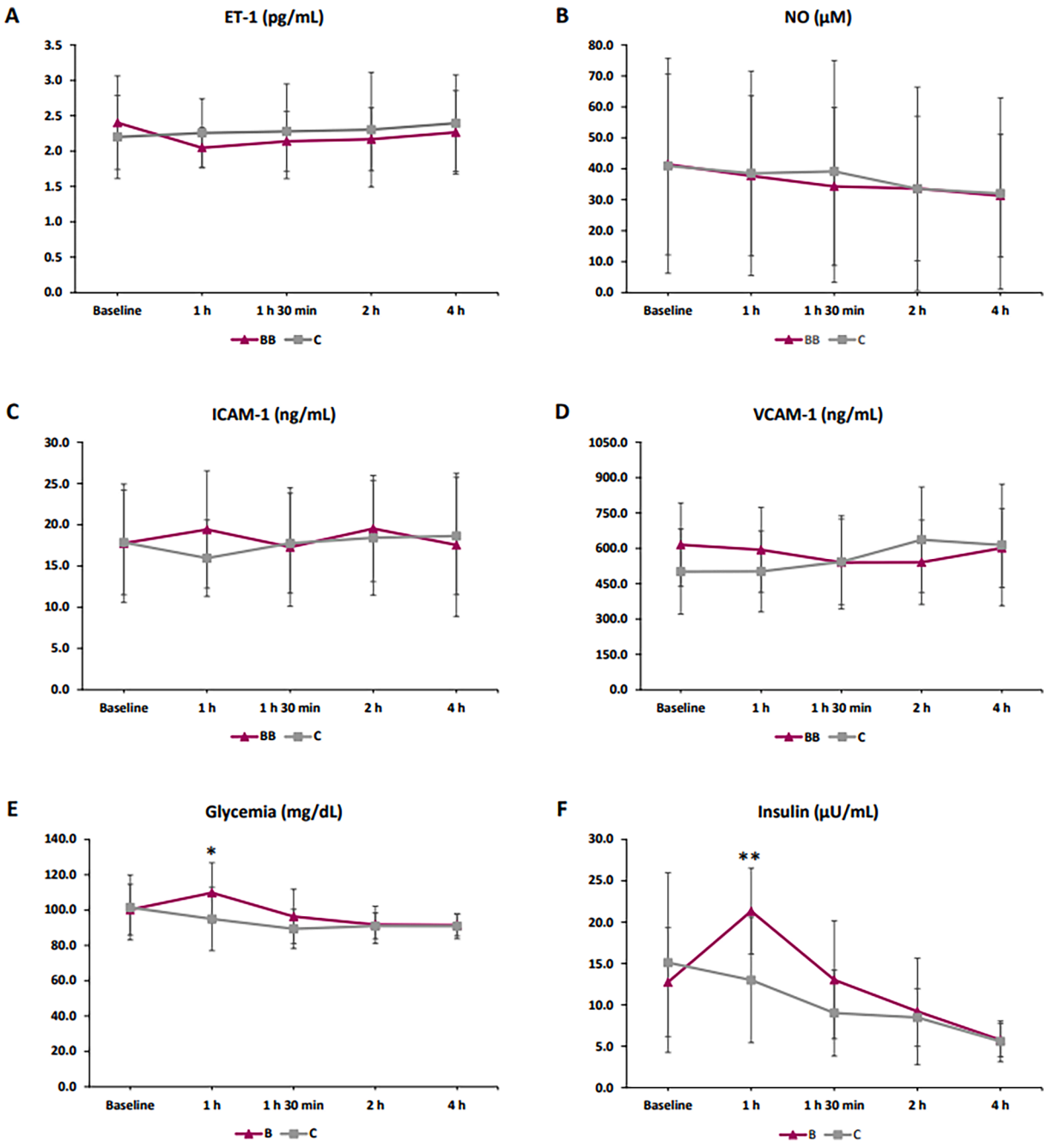

Fig. 4. Serum concentration changes of vascular function and metabolic markers registered at different timepoints following the two interventions (BB and C product). Values are expressed as mean concentration at each timepoint. Error bars indicate the standard deviation (SD). Each sub-figure (A-F) represents a different marker. A: ET-1 = Endothelin-1; B: Total NO = total nitric oxide; C: ICAM-1 = Intercellular adhesion molecule 1; D: VCAM-1 = Vascular cell adhesion molecule 1; E: Glycemia; F: Insulin. * Statistically different ($p < 0.05$) according to independent-samples T test between treatments within the same time points. ** Statistically different ($p < 0.01$) according to independent-samples T test between treatments within the same time points.

endothelin-1, VCAM-1, ICAM-1 and NO. This latter is released by endothelial cells representing one of the main mechanisms underlying the endothelium's ability to modulate vascular tone, leading to the production of cGMP in muscle cells and consequent relaxation (Mónica et al., 2016; Vanhoutte et al., 2017). The effect of blueberries on NO production/modulation has been evaluated in numerous studies. Considering acute studies, we already reported no effects of blueberry intervention on NO in the healthy young subjects group (Del Bo' et al., 2013). We have also documented similar results after 6-week intervention with wild blueberry in a group of men with cardiovascular risk factors (Riso et al., 2013). Our observations were in line with those reported by Curtis et al. (Curtis et al., 2019) that failed in detecting an increase in NO production after 6 months of daily blueberry consumption. We may speculate that quantification of NO production levels is difficult to achieve both in acute and chronic conditions in relation to its very short half-life (5–30 s), differently from other more stable markers (i.e. cGMP levels) that may provide indirect information related to NO production. However, Stote and colleagues found a significant increase in NO production after 7-day consumption of wild blueberries drink (240 mL) in women presenting cardiovascular risk-factors (mild-hypertensive postmenopausal women or risk for type 2 diabetes) (Johnson et al., 2015; Stote et al., 2017). Also, Johnson and coworkers found that the consumption of 22 g of freeze-dried blueberry powder for 8 weeks improved NO concentration in postmenopausal women with pre and

Table 3
Mean (±SD) total and individual serum concentrations of ACNs following the intake of the BB product.

Anthocyanin	Time after ingestion					overtime absorption
	baseline	1 h	1.5 h	2 h	4 h	p value
Cy-ara	n.d.	0.33 ± 0.52a	1.79 ± 1.22b	2.18 ± 1.15b	1.54 ± 1.31b	<0.001
Cy-gal	n.d.	0.13 ± 0.24a	1.36 ± 0.66b	1.66 ± 0.64b	1.42 ± 0.88b	<0.001
Cy-glc	n.d.	0.07 ± 0.14a	0.40 ± 0.32a,b	0.74 ± 0.39b	0.61 ± 0.70b	<0.001
D-ara	n.d.	0.27 ± 0.38a	1.13 ± 0.93b	1.84 ± 0.88c	1.07 ± 0.93b	<0.001
D-gal	n.d.	1.31 ± 0.78a	2.52 ± 1.46b	2.70 ± 1.71b	2.53 ± 1.58b	<0.05
D-glc	n.d.	0.06 ± 0.09a	0.33 ± 0.25b	0.74 ± 0.48c	0.54 ± 0.42b,c	<0.001
Mv-ara	n.d.	0.35 ± 0.34a	1.52 ± 0.67b,c,d	1.77 ± 0.66c	1.24 ± 0.76d	<0.001
Mv-gal	n.d.	0.75 ± 0.82a	1.96 ± 1.44b	2.07 ± 1.31b	1.80 ± 1.54b	<0.05
Mv-glc	n.d.	0.12 ± 0.22a	0.40 ± 0.44a	1.10 ± 0.52b	0.69 ± 0.37b	<0.001
Peo-ara	n.d.	0.04 ± 0.08a	0.20 ± 0.23a	0.80 ± 0.59b	0.57 ± 0.44b	<0.001
Peo-gal	n.d.	0.04 ± 0.09a	0.30 ± 0.22b,d	0.69 ± 0.38c,d	0.49 ± 0.42d	<0.001
Peo-glc	n.d.	0.04 ± 0.08a	0.37 ± 0.41b	0.62 ± 0.56b,c	0.74 ± 0.60c	<0.001
Pet-ara	n.d.	0.07 ± 0.19a	1.41 ± 0.49b	1.62 ± 0.58b	1.54 ± 0.93b	<0.001
Pet-gal	n.d.	0.05 ± 0.12a	0.40 ± 0.36b	1.20 ± 0.59c	1.02 ± 0.44c	<0.001
Pet-glc	n.d.	0.04 ± 0.08a	0.27 ± 0.30b	0.53 ± 0.36c	0.32 ± 0.27b	<0.001
Total	**n.d.**	**3.67 ± 2.92a**	**14.37 ± 7.34b**	**20.27 ± 7.35c**	**16.10 ± 7.45b,c**	<0.001

Serum concentration (ng/mL) of total anthocyanins and single glycoside in 250 g of fresh blueberries, corresponding to the portion provided to the 16 subjects that completed the study. Cy = cyanidin; D = delphinidin; Mv = malvidin; Peo = peonidin; Pet = petunidin; −ara = arabinoside; −gal = galactoside; −glc = glucoside. Values are reported as (ng/mL).

Table 4
Amount of the single ACN provided by the BB portion and relative serum concentration at 2 h from the consumption.

ACNs	mg/250 g of blueberries (portion)	serum concentration (ng/mL)
D-gal	152.5	2.70 ± 1.71
Mv-gal	81.5	2.07 ± 1.31
Cy-ara	70.6	2.18 ± 1.15
D-ara	65.7	1.84 ± 0.88
Mv-ara	34.3	1.77 ± 0.66
Pet-ara	26.2	1.62 ± 0.58
Cy-gal	24.5	1.66 ± 0.64
Pet-gal	11.3	1.20 ± 0.59
Peo-gal	5.1	0.69 ± 0.38
D-glc	3.2	0.74 ± 0.48
Mv-glc	2.7	1.10 ± 0.52
Pet-glc	2.4	0.53 ± 0.36
Peo-glc	0.9	0.62 ± 0.56
Cy-glc	0.6	0.74 ± 0.39
Peo-ara	0.2	0.80 ± 0.59

Cy = cyanidin; D = delphinidin; Mv = malvidin; Peo = peonidin; Pet = petunidin; −ara = arabinoside; −gal = galactoside; −glc = glucoside.

stage 1 hypertension (Johnson et al., 2015). So far, the effects of blueberry feeding on NO has been documented in women but not in men, and this should be further investigated. Regarding the effect on VCAM-1, ICAM-1 and ET-1, few studies have found a significant reduction following bilberries, cranberries, and strawberries, but not with blueberries, and mainly in diabetic and obese subjects or in individuals with an inflammatory state (Basu, Du, et al., 2010; Basu, Fu, et al., 2010; Lehtonen et al., 2011; Riso et al., 2013; Ruel et al., 2008). In the present study, despite being older, our subjects were healthy and did not show an altered inflammatory state thus, the lack of effect on such markers could be in part explained by the healthy conditions of the volunteers.

The role of blueberry in the modulation of arterial stiffness is not univocal. In the present study an increase (time effect) in arterial stiffness was observed following the administration of both control and blueberry treatment. However, this increase was not different when comparing the two treatments. In older subjects, arterial stiffness is caused by impaired regulation of vascular tone but also by structural changes in the artery walls (Merz & Cheng, 2016; North & Sinclair, 2012; Tucci et al., 2022). Thus, it is possible to hypothesize that increased vasodilation can acutely exacerbate measured levels of arterial stiffness of arterial vessels undergone such structural changes, for reasons related to the mechanics of such physiological and anatomical processes. Here, we found a statistically significant bivariate linear correlation between arterial stiffness markers and those of vascular reactivity (data not shown) thus, this hypothesis is worthy of further investigation. Other studies evaluated the effect of blueberry on arterial stiffness. For example, Dodd et al. (Dodd et al., 2019) did not find any significant effect after an acute consumption of a flavonoid-rich blueberry beverage (30 g of highbush blueberry powder providing at about 580 mg of ACNs and procyanidins) in a group of 18 healthy older subjects. Also, Curtis et al. did not find a modulation of markers of arterial stiffness (i.e., pulse wave velocity – PWV, and AIx) after an acute intake of a blueberry drink and high-energy meal in subjects with metabolic syndrome (Curtis et al., 2022). Similar findings were also obtained by our research group after the intake of 300 g of blueberries in healthy subjects. Considering other chronic studies, Wood and colleagues did not report a modulation of markers of arterial stiffness (PWV nor AIx) after 12 weeks of intervention with blueberries in older subjects (Wood et al., 2023). Conversely, Johnson et al. showed a significant improvement in brachial-ankle PVW, but not carotid-femoral PVW following 8 weeks of daily intake of 22 g of freeze-dried blueberry powder in a large sample of postmenopausal women. Curtis and coworkers found a positive modulation in AIx@75, but not PWV after 6 months of blueberry intake (26 g) in a group of adults (including older subjects) with metabolic syndrome (Curtis et al., 2019; Johnson et al., 2015). Finally, McAnulty and colleagues reported a significant reduction in AIx, but not

Table 5
Salicylate serum concentrations registered following BB and C product intake.

salicylates	Time after ingestion					overtime absorption	
	baseline	1 h	1.5 h	2 h	4 h	p-value interaction timextreatment	p-value for time
Blueberry	0.14 ± 0.07	0.16 ± 0.10	0.19 ± 0.11	0.17 ± 0.12	0.14 ± 0.08	p = 0.079	p = 0.068
Control	0.16 ± 0.11	0.14 ± 0.10	0.14 ± 0.10	0.13 ± 0.11	0.13 ± 0.09		

Values are reported as uM concentration. Data are reported as mean ± standard deviation.

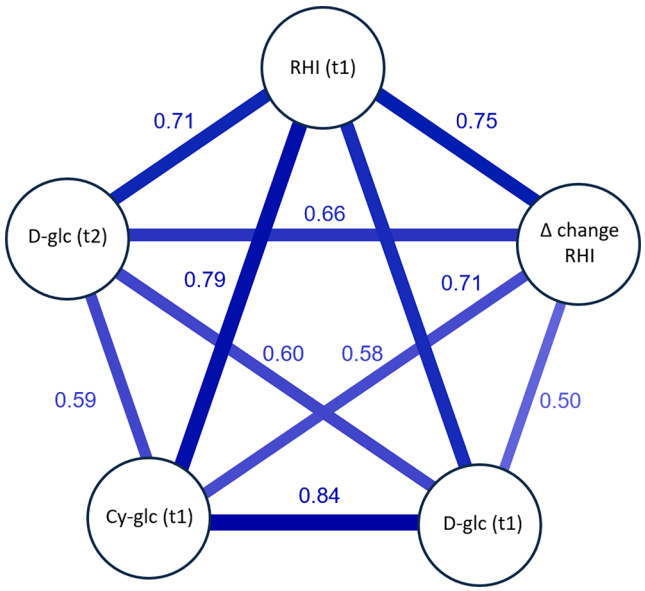

Fig. 5. Correlation between BB ACNs serum concentrations and RHI at 2 h or RHI deltas. Only correlations having $p < 0.01$ were considered.

in PWV following 6 weeks daily ingestion of whole blueberry powder (equivalent to 250 g fresh blueberries) in sedentary male and female adults (McAnulty et al., 2014). This difference among studies may depend on the different methodology used to assess vascular stiffness, type and dosage of blueberry products, way of administration (i.e., fruit mousse or freeze-dried powder), and length of intervention (acute versus chronic dietary intervention).

An improvement in vascular health should also reflect a positive effect on blood pressure. However, when considering the contribution of ACNs and/or blueberries, results from human studies are mixed (Vendrame et al., 2022; Vendrame & Klimis-Zacas, 2019). For example, Basu et al. (2010) reported a significant decrease in systolic and diastolic blood pressure following 8-week intervention with 480 mL blueberry drink (50 g freeze-dried powder, providing 742 mg ACN) in subjects with metabolic syndrome. Similar results were also documented by Johnson et al., Wood and coworkers, Basu and colleagues and Du et al., in different target populations (Basu et al., 2021; Du et al., 2019; Johnson et al., 2015; Wood et al., 2023). In our experimental conditions, we failed to document a positive modulation in blood pressure in this target population. The lack of effect could be due to the short duration of the intervention for inducing changes, but also to the normal levels of blood pressure due to the intake of medications by most of the subjects (11 out of 16 subjects). Several studies failed to observe a reduction in blood pressure both in short and long term interventions, and a recent systematic review concluded that there is insufficient evidence supporting the existence of a direct blood pressure lowering effect of blueberries, while there is stronger evidence for specific types of berries (i.e., chokeberries), possibly acting indirectly to normalize blood pressure in subjects that are already hypertensive (Vendrame et al., 2022).

The effect of blueberries on cardiometabolic markers such as lipids, glucose, and insulin has been evaluated in numerous dietary intervention studies (Bell et al., 2017; Palma et al., 2021; Stull, 2016). Also, for these markers, in particular for glucose response, the results are not univocal as reported in a recent systematic review and *meta*-analysis showing limited successful clinical interventions using dietary polyphenols, berry polyphenols and berries into glucose and insulin homeostasis (Rambaran et al., 2020; Venturi et al., 2023). In the present study, we documented an increase of glycemia and insulinemia at 1 h after blueberry intake, in comparison with control containing the same amount of sugars. It can be speculated that the peak of sugars absorption could have occurred within the first 1 h from the intake of the C product (data not available). Conversely, the presence of fiber and other bioactive components in the blueberry, could have induced a slow release and absorption of the sugars, thus postponing glucose and insulin response. However, at 2 h from the intake (in concomitance with the analysis of vascular function), the glucose and insulin levels were comparable between interventions. Curtis et al. also found that acute administration of 26 g of freeze-dried blueberry powder (equivalent to 150 g of fresh

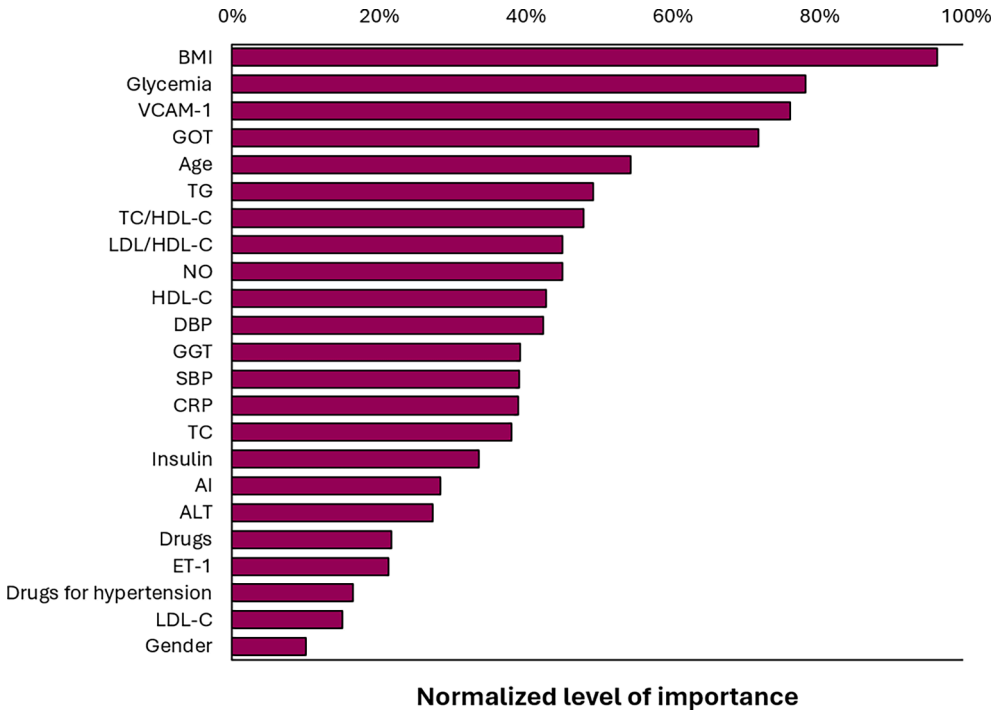

Fig. 6. Variable-importance plot for random forest regression models with RHI mean differences in blueberry intervention as the dependent variable and other collected variables (biochemical markers and demographics) as regressors.

blueberries) resulted in a higher glycemia in comparison with a sugar matched placebo, even if with a different study protocol (treatment administered following an energy-dense challenge meal) (Curtis et al., 2022). Some studies hypothesized that glucose and insulin could have a positive impact on vascular reactivity; in fact, insulin release may induce changes in microvascular vasomotion, promoting capillary recruitment and NO synthesis (Momin, 2006; Woerdeman et al., 2016). However, present data do not support this hypothesis since in concomitance with the improvement of vascular health (2 h) the levels of glucose and insulin were similar to baseline.

This study presents strengths and limitations. The main strength regards its rigorous scientific protocol, involving a crossover design able to reduce inter-individual variability, since subjects serves as their own control. Also, the transient nature of the effects of consuming one portion of blueberries, combined with a full week's washout period, should indeed be sufficient to avoid carryover effects. This method, along with other standardization procedures, ensures that any observed effects are attributable to the intervention itself rather than residual effects from prior consumption. In addition, the combination of different markers related to vascular function/inflammation, and dietary exposure (serum anthocyanins) can provide further insights for the identification of putative dietary components responsible for the beneficial effect observed. Finally, the blueberries used in this intervention were from the same batch of blueberry, grown and harvested in the same conditions, and individually quick-frozen after their transportation to the University facilities, to minimize the variability in their composition and bioactive profile.

Within limitations, one is represented by the difficulty of providing a placebo to the volunteers, and the lack of data on circulating metabolites that could have contributed to the general improvement in the vascular function. Another limitation may be the relatively small number of participants, which, although sufficient based on the calculated sample size, could affect the generalizability of the findings. However, the inclusion of older subjects presenting high inter-individual variability (common in this population group), together with the overall feasibility of this dietary intervention, can support the consumption of blueberries as a viable strategy to improve vascular function, once corroborated by additional *in vivo* evidence.

5. Conclusions

In conclusion, we documented that the consumption of a portion of blueberry can improve reactive hyperemia index, a marker of vascular function, in a group of older subjects. This improvement was positively associated with the serum ACNs supporting a possible contribution of these compounds in the modulation of vascular health. However, this effect does not appear to be mediated by a significant modulation of nitric oxide, endothelin-1, ICAM-1, VCAM-1, that could have in part explained the improvement in RHI. Thus, further studies are required, in the short and long term, to ascertain the actual mechanisms of action of blueberry bioactives in the context of vascular function and to identify the recommended dose to be consumed for the maintenance/improvement of vascular health in the older subjects.

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CRediT authorship contribution statement

Massimiliano Tucci: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Cristian Del Bo':** Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Daniela Martini:** Writing – review & editing, Supervision, Data curation, Conceptualization. **Simone Perna:** Writing – review & editing, Visualization, Data curation. **Mirko Marino:** Writing – review & editing, Formal analysis. **Marco Rendine:** Writing – review & editing, Formal analysis. **Claudio Gardana:** Validation, Methodology, Formal analysis. **Alberto Battezzati:** Writing – review & editing, Supervision, Resources. **Alessandro Leone:** Writing – review & editing, Data curation. **Simona Bertoli:** Writing – review & editing, Supervision. **Giancarlo Aldini:** Writing – review & editing, Resources, Data curation. **Patrizia Riso:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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