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Increasing nitric oxide availability via ingestion of nitrate-rich beetroot juice improves vascular responsiveness in individuals with Alzheimer's Disease

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ABSTRACT

Poor vascular function and reduced nitric oxide (NO)-bioavailability have been recognized to be involved in aging and Alzheimer's Disease (AD). A non-pharmacological treatment that is gaining clinical interest in the context of vascular function is dietary inorganic nitrate (NO_3^-) supplementation which increases NO-bioavailability through the NO_3^- -nitrite (NO_2^-) - NO pathway. This treatment has been demonstrated to improve vascular function in several clinical populations, but no study has investigated the effects in individuals with AD. Therefore, changes in plasma NO_3^- and NO_2^- and vascular responsiveness (hyperemic response to single-passive leg movement (ΔPLM)) were measured in individuals with AD (n = 10, 76 ± 9 years), healthy elderly (OLD, n = 10, 75 ± 6 years), and young individuals (YN, n = 10, 25 ± 4 years) before (T0) and hourly for 4 h (T1, T2, T3, and T4) after ingestion of either NO_3^--rich beetroot juice (BR) or a placebo (PLA). No changes in NO_3^- and NO_2^-, nor ΔPLM were detected in any group following PLA intake. Plasma NO_3^- and NO_2^- increased significantly in all three groups at T1 ($p < 0.001$) and remained elevated for the rest of the trial. The same trend was found in ΔPLM, which significantly increased in all three groups over the time ($p < 0.001$). However, AD exhibited significantly lower ΔPLM values at any time point compared to YN ($p < 0.001$) and OLD ($p < 0.001$). These data suggest that AD-individuals included in this study were able to reduce NO_3^- to NO_2^- and to increase NO-mediated vascular responsiveness as non-AD-individuals. Other mechanisms, beyond NO-bioavailability, may be involved in vascular dysfunction in patients with AD. This research suggests that an acute administration of inorganic nitrate is not enough to revert chronically adapted vascular properties and completely restore vascular responsiveness in AD.

1. Introduction

Alzheimer's Disease (AD) is an irreversible and debilitating disorder characterized by progressive cognitive decline and accounts for nearly two thirds of all dementia cases in the older population [1]. Despite the well-known amyloid-cascade hypothesis pustulated in 1992 by Hardy and Higgins [2], according to which AD pathology is initiated by the deposition and accumulation of Amyloid-β (Aβ), it is becoming widely accepted that AD is instead a multifactorial and heterogenous disease resulting from the impact of several factors ultimately ending up in cerebral Aβ depositions and neuronal damage [3,4]. These factors include altered neuroendocrine and hormonal regulation, metabolic

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dysfunction, inflammatory response, and vascular dysfunction [3]. The underlying vascular physiology has received particular attention by the scientific community since it became evident that dysfunction of the cerebral vasculature and brain pathology interact in multiple ways [5–7]. Interestingly, changes of the cerebral blood flow have been proven to be triggered and driven by systemic vascular abnormalities that are part of normal aging [8]. Supporting this concept, a recent study by our research group demonstrated that cortical perfusion is linearly related with reduced peripheral circulation as well as reduced systemic vascular function in individuals with AD compared with healthy age-matched and young individuals [9]. Moreover, circulation impairment, already apparent in healthy elderly, is strongly associated with nitric oxide (NO, an ubiquitous signaling molecule that regulates a number of key biological processes, including vascular function) depletion which, in turn, is associated with aging and AD severity [9]. Indeed, individuals with the most severe cognitive impairment exhibit the poorest cortical perfusion, poorest peripheral and systemic circulation and the lowest NO-bioavailability [9]. These results highlight the key role of NO in vascular dysfunction resulting from aging and AD pathology and provide a clear rationale to investigate the therapeutic efficacy of interventions that increase NO-bioavailability in individuals with AD.

One such intervention is dietary inorganic nitrate supplementation which has been shown to consistently increase NO-bioavailability through the nitrate-nitrite-NO pathway [10,11]. Over the last decade, it has been demonstrated that supplementing diet with nitrate, for example nitrate-rich beetroot juice, a high nitrate-containing vegetable, exerts several beneficial effects in healthy volunteers as well as patients with uncontrolled hypertension, including the protection of the endothelium from stress-induced endothelial damage [12,13] and improved vascular function [14,15]. The production of NO from orally ingested inorganic nitrate relies on its conversion to nitrite by facultative bacteria present on the tongue [14,16,17]. The swallowing of this nitrite -rich saliva permits entry of nitrite into the circulation via the gastrointestinal system, and once within the circulation, nitrite can be converted to NO [18–20]. Nevertheless, not all research studies on older individuals confirms the positive effect of inorganic nitrate supplementation on vascular function [13,21]. The inconsistent responses to dietary nitrate might be a consequence of aging itself which has been shown to modulate of oral nitrate reductase activity [22,23] negatively affecting the ability to convert nitrate to NO. Despite this, no studies have investigated whether the nitrate-nitrite-NO pathway is further impacted in individuals with AD, and no studies has investigated whether nitrate supplementation can ameliorate the suppressed vascular function in this population.

The aim of this study, therefore, was to investigate the plasma nitrate and nitrite kinetics after a single dose of nitrate-rich beetroot juice and the consequent NO-dependent vascular responsiveness in individuals with AD compared to a group of non-demented, age-matched individuals and a group of young adults. Our starting hypothesis was that non-demented elderly and individuals with AD have lower nitrate and nitrite basal plasmatic concentration compared to young, as well as different plasmatic nitrate and nitrite kinetics after a single dose of nitrate-rich beetroot juice. Consequently, since these populations are characterized by a lower NO-bioavailability, we hypothesize an augmented vascular responsiveness in elderly and AD individuals compared to YG in response to an increase in nitrate and nitrite after a single dose of nitrate-rich beetroot juice.

2. Methods

Participants. Individuals with AD were recruited from the Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Italy and Mons. Mazzali Geriatric Institute, Mantua. Inclusion criteria were (a) age between 65 and 85 years; (b) clinical diagnosis of probable AD dementia, established according to the National Institute on Aging-Alzheimer's Association diagnostic guideline for AD [24]. Exclusion criteria were: (a) presence of any severe chronic disease (i.e.: COPD, Heart Failure, Diabetes), (b) presence of coagulation disorders, (c) inability to cooperate, (d) any known allergy or intolerance to supplementation compounds, (e) Mini Mental State Examination (MMSE) score< 15. At the time of the inclusion, MMSE was performed to evaluate the severity of the disease. A group of healthy, age-matched elderly controls (OLD) and a group of young (YN, 18–25 years old) subjects from the same geographical area of AD were also included in the study.

All experiments were conducted after informed and written consent was obtained from the participants and their relatives, in accordance with the Declaration of Helsinki, as part of a protocol approved by the Institutional Review Board of Val Padana, ASST Mantova e Cremona (Protocol number 33811/2020).

Study overview. The participants completed a placebo (PLA)-controlled, randomized, double-blind crossover study. Each participant completed two testing sessions prior to which they ingested a single dose of nitrate-rich beetroot juice (BR) [70 ml containing 5 mmol, or 400 mg of NO_3^-, (Beet It Sport, James White Drinks, Ipswich, UK)] or a nitrate-depleted PLA, matched in flavor, appearance, and packaging [70 ml with negligible content of NO_3^-]. This kind of nitrate-rich beetroot juice was chosen because it was already used in previous studies, well tolerated by subjects, and no side effects reported [25]. Participants arrived at the laboratory at 8 a.m. in a fasted state after which basal vascular assessments were performed and blood samples taken (T0). Immediately after the ingestion of the juice (either BR or PLA), a light breakfast was provided (toast with jam, cheese, and white bread, food with low-nitrate content [26]). Thereafter, vascular assessments and blood samples were repeated every hour for the following 4 h (T1, T2, T3 and T4). In between time points, participants were asked to rest and not to eat any other meal or snack. They were allowed to read books and journals, study, or any other resting activity, as well as use the toilet. During the study period, participants were required to abstain from using antibacterial mouthwashes and chewing gum, which are known to alter the oral bacteria responsible for the reduction of NO_3^- to NO_2^- [27]. YN group female subjects were tested at the follicular phase of the menstrual cycle.

Plasma nitrate and nitrite concentration. Measurements of NO_3^- and NO_2^- were conducted using ozone-based chemiluminescence [28]. For the measurement of plasma NO_3^-, vanadium reagent (24 mg of vanadium tri-chloride and 3 ml of 1 M Hydrochloric acid) and 100 μL of anti-foaming agent were placed into a customised glass purge vessel infused with nitrogen and heated to 95 °C. This purge vessel was connected to an NO analyser (Sievers NOA 280i, Analytix, UK). A standard curve was produced by injecting 25 μL of NO_3^- solutions (100 μM, 50 μM, 25 μM, 214 12.5 μM, and 6.25 μM) and a control sample containing deionised water. For the latter, the area under the curve (AUC) was subtracted from the NO3- solutions to account for NO_3^- in the water used for dilutions. Plasma samples were thawed in a water bath at 37 °C for 3 min and de-proteinised using zinc sulphate/sodium hydroxide solution (200 μL of plasma, 400 μL of zinc sulphate in deionised water at 10 % w/v and 400 μL of 0.5 M sodium hydroxide). The samples were then vortexed for 30 s before being spun at 4000 rpm for 5 min. Subsequently, 15–25 μL of the sample was injected into the purge vessel in duplicate. The concentration of NO cleaved during the reaction was then measured by the NO analyser. The AUC was calculated using Origin software (version 7) and divided by the slope gradient.

For the measurement of plasma NO_2^-, 2.5 ml glacial acetic acid, 0.5 ml of 18 Ω deionised water, 25 mg sodium iodide, and 100 μL of an anti-foaming agent were placed into the glass purge vessel and heated to 50 °C. A standard curve was produced by injecting 100 μL of NO_2^- solutions (1000 nM, 500 nM, 250 nM, 125 nM, and 62.5 nM) and a control sample of deionised water. The AUC for the latter was subtracted from the NO_2^- solutions to account for NO_2^- in the water used for dilutions. Following this, plasma samples were thawed in a water bath and 100 μL of the sample was injected into the purge vessel in duplicate. The NO_2^-

was determined via the AUC, as previously described.

Vascular responsiveness via single passive limb movement (PLM). Recent investigations have revealed that sPLM-induced hyperemia (Δpeak) is predominantly a consequence of NO-mediated vasodilation [29]. Also, the amplitude of the hyperemic response is positively related with vascular responsiveness [30]. Therefore, we have adopted this noninvasive and reliable method to determine vascular responsiveness. During the test, the subject rested in the upright-seated position for 10 min before the start of data collection and remained in this position. The sPLM protocol consisted of 30s of resting baseline femoral blood flow data collection, followed by 1 single passive knee flexion and extension with the same measure for the following 60s. sPLM was performed by a member of the research team, who moved the subject's lower leg through a 90° range of motion (180-90° knee joint angle). Blood mean velocity (Vmean) was analyzed with 1Hz resolution on the Doppler ultrasound system (GE Logiq-9) for 30s at rest and second by second for the 60s following the single passive movement. The resting arterial diameter and blood flow of the common femoral artery were determined for each participant. Arterial diameter was measured as the distance (mm) between the intima-lumen interfaces for the anterior and posterior walls in the common femoral artery. Blood flow was calculated using arterial diameter and blood velocity according to this formula:

$$\text{Blood Flow}\left(\text{ml}\cdot\text{min}^{-1}\right) = \text{Vmean}\cdot\pi\cdot(\text{vessel diameter}/2)^2\ 148\cdot 60$$

Δpeak was calculated subtracting basal blood flow to the peak blood flow reached during the sPLM test [31].

Statistical analysis and sample size calculation. All statistical analyses were performed with GraphPad Prism Version 8.4.3 (Graphpad software LLC., Boston, USA). Data are presented as mean ± SD, if not otherwise stated. First, normality was assessed by the Shapiro–Wilk test. A one-way (1x3) analysis of variance (ANOVA) was applied to individuals' characteristics to test the homogeneity of the groups before the study. A two-way ANOVA mixed-effect analysis (2x1) was applied to assess differences in plasma NO_3^- and NO_2^- and blood flow Δpeak. This model considered "Condition" (BR and PLA) and Time" (T0, T1, T2, T3, and T4) as within factors and "Group" as between factor. If a significant main effect or interaction was observed, post-hoc analysis was undertaken using multiple comparisons tests with Tukey's correction. The family-wise alpha level for significance was set at 0.05 (two-tails). The sample size has been calculated considering the study of McIlvenna et al. [32], and a sample size of 10 subjects per group has been chosen to guarantee a statistical power higher than 0.80 (type 1 error 0.05; two-tailed *t*-test).

3. Results

Subjects' characteristics. A total of 30 individuals were included in the study (AD = 10; OLD = 10; YN = 10). Subjects' characteristics are shown in Table 1. Other than for age, where AD and OLD were significantly older than YN, no between groups differences were found. For the participants with AD, diagnosis occurred 8 ± 3 years prior to this study, reporting a MMSE of 21 ± 3 out of 30 (where 30 is intended the maximum score and mostly preserved global cognitive functioning). No between groups differences were found in both systolic and diastolic blood pressure. Most of the OLD and AD were taking antihypertensive medications whereas the YN were not. Some of the OLD and AD were also taking gastro-protectors and statins. Individuals with AD were taking also cholinesterase inhibitors and benzodiazepines (Table 1).

Plasma nitrate and nitrite concentration. In the PLA trial, no Time and Group effects were found for NO_3^- and NO_2^- and there was no Group × Time interaction. (Fig. 1, panels A and B). In the BR trial, there was a significant effect of Time on NO_3^- ($p < 0.001$, $F = 164.4$) and NO_2^- ($p < 0.001$, $F = 21.63$). There were no Group effects or Time × Group interactions for either NO_3^- and NO_2^-. However, post-hoc analysis revealed

Table 1
Subjects' characteristics.

	YN (n = 10)	OLD (n = 10)	AD (n = 10)
M/F - n	3/7	3/7	2/8
Age - years	25 ± 4	75 ± 6 §	76 ± 7 §
Weight - kg	67 ± 10	69 ± 12	70 ± 9
Height - cm	161 ± 11	165 ± 8	162 ± 10
BMI - kg·m^2	25.8 ± 1.3	25.3 ± 2.6	26.6 ± 3.1
Years since diagnosis	n.a.	n.a.	8 ± 3
MMSE - (0-30)	n.a.	30 ± 0	21 ± 3
Arterial Blood Pressure			
Systolic - mmHg	125 ± 5	131 ± 10	135 ± 8 §
Diastolic - mmHg	82 ± 5	91 ± 8	94 ± 7 §
Pharmacological treatment			
Antihypertensive - n	0	7 §	8 §
Gastroprotectors - n	0	2 §	3 §
Statins - n	0	5 §	5 §
Cholinesterase inhibitors - n	0	0	10 §#
Benzodiazepines - n	0	0	5 §#

Note. § = $p < 0.05$ vs YN; # = $p \leq 0.05$ vs OLD.

that during the BR only at T0, AD exhibited lower NO_3^- compared to YN and OLD (vs YN = −106.1 ± 39.2 μM, $p = 0.020$; vs OLD –80.5 ± 57.7 μM, $p = 0.047$) (Fig. 1, panel C). No difference between YN and OLD were detected. No between groups differences in NO_2^- were found at T0 (Fig. 1, panel D). After BR intake all three groups exhibited a quick rise in NO_3^- with a significant difference compared to T0 at T1 (YN = +750.7 ± 183.2 μM, $p = 0.002$; OLD = + 797,5 ± 126,1 μM, $p < 0.001$; AD = +705.5 ± 194.3 μM, $p = 0.001$). No differences between T1, T2, T3, and T4 were found in any group, and no between groups differences were found at these timepoints (Fig. 1, panel C). For NO_2^-, no between group differences were found at T0, and at any other timepoint (Fig. 1, panel D). YN exhibited a significant increase in NO_2^- at T2 (+171.4 ± 32.6 nM, $p = 0.030$), and T3 (+158.4 ± 4.5 nM, $p = 0.002$), but not at T4 (138.9 ± 12.3 nM, $p = 0.059$). The same trend was found in OLD exhibiting a significant increase in NO_2^- at T2 (+166.1 ± 54.1 nM, $p = 0.003$), T3 (+189.9 ± 68.8 nM, $p = 0.003$), and T4 (+176.2 ± 58.7 nM, $p = 0.041$). AD also exhibited a rise in NO_2^- which became significant at T3 (+230.1 ± 30.1, $p = 0.044$) and T4 (+209.7 ± 19.9, $p = 0.045$) (Fig. 1, panel D).

Vascular responsiveness. In the PLA trial, there was a significant effect of time ($p = 0.035$, $F = 3.15$) and Group ($p = <0.001$, $F = 76.84$), but no Time × Group interaction. The post-hoc analysis revealed that during PLA session, no within group difference were found at any time point, Δpeak remained stable throughout the 4 h after BR or PLA. No differences between YN and OLD or between OLD and AD were detected at any time point. However, AD exhibited significantly lower Δpeak values than YN at every timepoint (T0 = −113.7 ± 20.2 ml·min^{-1}, $p = 0.005$; T1 = −139.7 ± 17.11 ml·min^{-1}, $p < 0.001$; T2 = −127.4 ± 23.82 ml·min^{-1}, $p < 0.001$; T3 = −167.1 ± 10.4 ml·min^{-1}, $p < 0.001$; T4, -128.4 ± 22.39 ml·min^{-1}, $p < 0.001$) (Fig. 2, panel A). In the BR trial, there was a significant effect of Time ($p < 0.001$, $F = 27.51$) and Group ($p < 0.001$, $F = 16.96$), but no Time × Group interaction. No differences between YN and OLD were detected at any timepoint except for T4 where OLD exhibited lower values than YN (−144.6 ± 44.5 ml·min^{-1}, $p = 0.017$) (Fig. 2, panel B). AD exhibited significantly lower Δpeak values than YN and OLD at most of the timepoints (T0: vs YN = −154.7 ± 37.2 ml·min^{-1}, $p = 0.005$; vs OLD = −68.1 ± 17.7 ml·min^{-1}, $p = 0.007$. T1: vs YN = −139.7 ± 41.3 ml·min^{-1}, $p = 0.019$; vs OLD = −62.9 ± 22.2 ml·min^{-1}, $p = 0.039$. T2: vs YN = −181.1 ± 48.1 ml·min^{-1}, $p = 0.0005$. T3: vs YN = −199.7 ± 11.4 ml·min^{-1}, $p = 0.001$. T4: vs YN = −191.2 ± 29.5 ml·min^{-1}, $p < 0.001$) (Fig. 2, panel B). After BR intake, all three groups exhibited a significant rise in Δpeak from T0 (Fig. 2, panel B). YN exhibited significant difference with T0 at T2 (+115.9 ± 51.2 ml·min^{-1}, $p = 0.011$), T3 (+146.8 ± 63.8 ml·min^{-1}, $p < 0.001$), and T4 (+178.7 ± 81.5 ml·min^{-1}, $p = 0.025$). No differences between any other timepoint

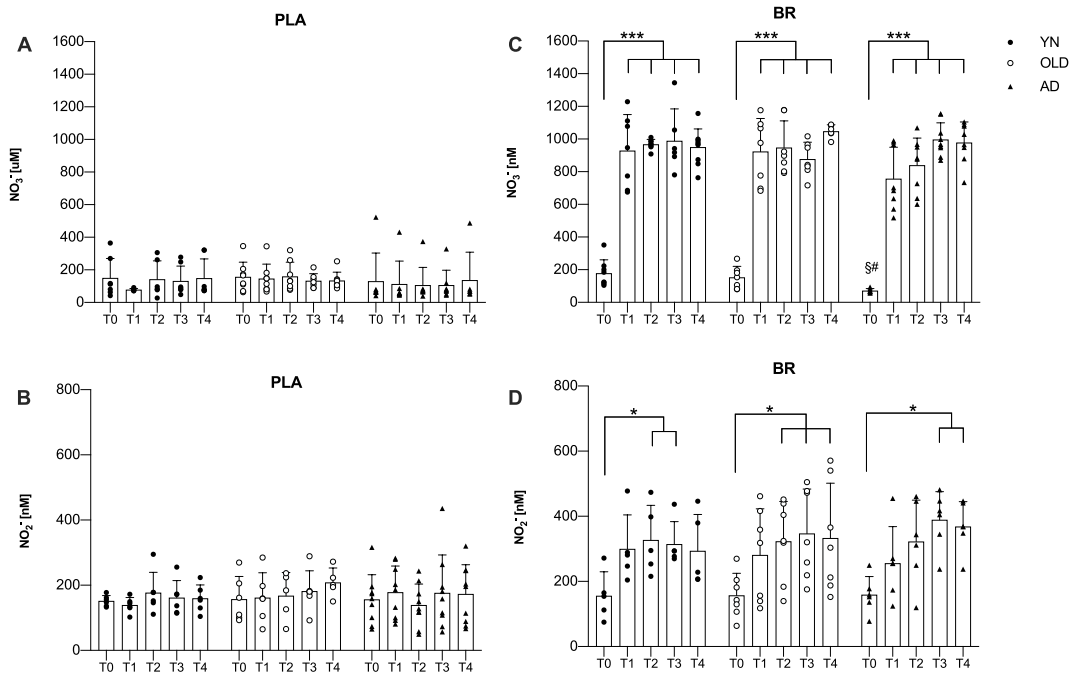

Fig. 1. Plasmatic nitrate (NO_3^-) and nitrite (NO_2^-) kinetincs of individuals with AD (AD), healthy elderly (OLD) and young (YN) controls in response to beetroot juice (BR) and placebo juice (PLA), measured before to (T0) and hourly for the following 4 h (T1, T2, T3 and T4). * = withing group differences; § = significantly different from YN, $p < 0.05$; # = significantly different from OLD, $p < 0.05$.

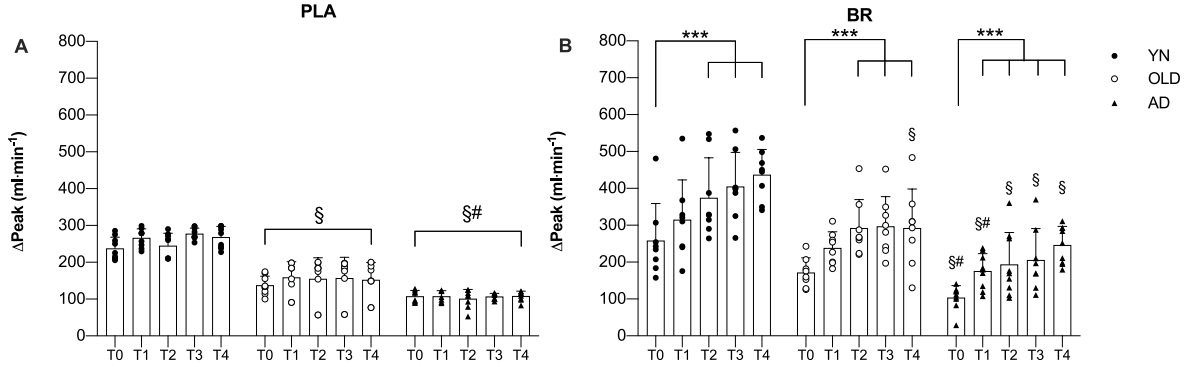

Fig. 2. Vascular responsiveness of individuals with AD (AD), healthy elderly (OLD) and young (YN) controls in response to beetroot juice (BR) and placebo juice (PLA), measured before to (T0) and hourly for the following 4 h (T1, T2, T3 and T4). * = withing group differences; § = significantly different from YN, $p < 0.05$; # = significantly different from OLD, $p < 0.05$.

were detected. OLD exhibited significant differences between T0 at T1 (+66.2 ± 21.1 ml·min^{-1}, p = 0.027), T2 (+120.2 ± 39.2 ml·min^{-1}, p = 0.006), T3 (+125.5 ± 51.7 ml·min^{-1}, p = 0.062), and T4 (+120.8 ± 41.9 ml·min^{-1}, p = 0.015). No differences between any other timepoint were detected. Similarly to OLD, AD also exhibited a significant difference with T0 at T1 (+71.7 ± 21.3 ml·min^{-1}, p = 0.007), T2 (+89.6 ± 31.2 ml·min^{-1}, p = 0.035), T3 (+101.8 ± 27.3 ml·min^{-1}, p = 0.044), and T4 (+142.3 ± 39.3 ml·min^{-1}, p=>0.001). No differences between any other timepoint were detected (Fig. 2, panel B). Table 2 shows the mean differences between T0 and the other timepoints in all three groups.

Table 2
Vascular responsiveness mean difference between T0 and the other timepoints in YN, OLD, and AD. These data represent the change in blood flow Δpeak in the 4 h following the BR intake compared to T0. All groups show significant increase in these values at every timepoint compared to T0, confirming the effect of BR on vascular responsiveness. However, no between groups difference were detected at any time point.

	YN			OLD			AD		
	Mean Diff.	95 % CI	*p-value*	Mean Diff.	95 % CI	*p-value*	Mean Diff.	95 % CI	*p-value*
T1 vs. T0	56.6	3.2 to 116.5	0.064	66.5	7.9 to 125.2	0.029	71.7	21.4 to 121.9	0.007
T2 vs. T0	115.9	30.2 to 201.6	0.011	120.2	39.1 to 201.2	0.007	89.6	6.1 to 173.2	0.035
T3 vs. T0	146.8	77.2 to 216.4	0.001	125.5	26.7 to 224.3	0.016	101.8	2.8 to 200.8	0.044
T4 vs.T0	178.7	24.3 to 333.2	0.025	120.8	6.1 to 247.7	0.062	142.3	78.4 to 206.2	<0.001

4. Discussion

To our knowledge this is the first study investigating plasma NO_3^- and NO_2^- kinetics and consequent NO-dependent vascular responsiveness, investigated by means of sPLM, following ingestion of nitrate-rich beetroot juice in individuals with AD compared to healthy elderly and young controls. Contrary to our hypothesis, healthy elderly and individuals with AD, did not demonstrate lower basal concentration and different NO_3^- and NO_2^- plasma kinetics in response to beetroot juice compared to YN, indicating that individuals included in this study were able to reduce nitrate to nitrite successfully. Moreover, vascular responsiveness, although consistently reduced in AD compared to OLD and YN, increased similarly in all three groups in the relative values (considered as the change from baseline measures) after the beetroot juice. These results did not support our second hypothesis, since a greater NO-dependent vascular responsiveness was expected in AD compared to YN and OLD, due to the previously reported reduction in NO-bioavailability in this population. These results suggest that other mechanisms, beyond solely NO-bioavailability, may be involved in vascular dysfunction in patients with AD.

Evidence that NO_3^- and NO_2^- plasma kinetics are not different in elderly and AD compared to young.

Previous studies on dietary nitrate have shown that consumption of nitrate supplements, such as concentrated beetroot juice, increases plasma NO_3^- and consequently NO_2^- [33–35]. However, these studies were conducted primarily to investigate the acute effect of nitrate ingestion on blood pressure in healthy individuals, individuals with hypertension, or exercise performance [33–35]. Miller et al., measured plasma levels of NO_3^- *and* NO_2^- prior to and hourly for 3 h after beetroot juice supplementation in healthy, normotensive elderly individuals showing a significant increase in NO_3^- *and* NO_2^-, peaking 1–2 h after the supplementation [36]. Stanaway et al., measured plasma NO_3^- *and* NO_2^- before to, and 2.5 and 3.5 h after a single beetroot juice oral ingestion in young and healthy elderly individuals showing a great increase in both molecules in both groups [37].

Our results confirm these previous findings on young and healthy elderly, considering both the amplitude and the timing of NO_3^- *and* NO_2^- increase, but also demonstrate the same trend in individuals with AD. Our study is the first to look at the changes in plasma NO_3^- *and* NO_2^- at multiple timepoints across these populations, investigating the effect of aging as well as the effect of a neurodegenerative disease, such as AD, on plasmatic nitrate kinetics. Our results show a significant increase of both NO_3^- *and* NO_2^- 1 h after the beetroot juice intake in all three groups. Moreover, no between groups differences were found after the juice intake at any timepoint, suggesting neither ageing nor AD limit the increase in plasmatic concentration of NO_3^- to NO_2^-. These results may suggest that the individuals with AD included in this study did not present significant microbiome dysbiosis, as showed in previous literature [38]. Indeed, oral and gut microbiome is known to have a primary role in nitrate metabolism [39], and if microbiome dysbiosis was present in these individuals, we would have found differences in plasmatic concentration of NO_3^- to NO_2^- after the beetroot juice intake.

4.1. Vascular responsiveness after a single dose of inorganic nitrate during aging and AD

In the last decades, dietary nitrate has emerged as a viable intervention to improve cardiovascular health by increasing NO-bioavailability [40–42]. Several studies have been conducted on different populations providing evidence about the effect of nitrate supplementation primarily on blood pressure [37,40,41,43–45], but also on circulation [46], vasodilation response and endothelial function [40–43]. Only few studies have investigated the acute effect of nitrate supplementation on vascular function. Somani et al. [42] investigated the effect of a single dose of dietary nitrate supplementation on endothelial ischemia-reperfusion (IR) in early postmenopausal women demonstrating that this intervention can minimize IR-induced macrovascular endothelial dysfunction in this population. However, no effect on resting macrovascular and microvascular function were detected [42]. Mattor et al. [43] investigated the acute effect of dietary nitrate by means of beetroot juice on endothelial function in hypertensive patients measuring cutaneous microvascular reactivity during post-occlusive reactive hyperemia. The acute beetroot juice intake resulted in an improvement of endothelial function in this population [43]. However, to our knowledge no studies have investigated whether NO-mediated vascular responsiveness after beetroot juice intake is dampened in individuals with AD compared to elderly adults and young. This study is the first to answer these questions and, furthermore, the first to investigate the kinetics of vascular responsiveness, measured by means of sPLM, after beetroot juice intake in different populations. It needs to be highlighted that PLM was chosen because of its response is mainly mediated by NO-bioavailability, differently from other vascular tests [47]. During this procedure the shear stress increases in the microcirculation due to the passive movement, stimulating NO-production by the endothelial cells [47]. Since the alternative nitrate-nitrite-NO pathway can overcome NO deficiency due to impairments in NOS function, we expected a rise in sPLM response after a single beetroot juice intake. The results of our study are partly in line with prior evidence showing the effect of nitrate-rich beetroot juice on vascular responsiveness in young, as well as in healthy elderly participants [10,48]. Most importantly, these effects also occur in individuals with AD. Although, as expected, AD exhibited a poorer vascular responsiveness compared to YN and OLD all timepoints, the amplitude and kinetics of Δpeak increase was similar between the three groups (Table 2), since no group × time interaction was found. These results indicate that, contrary to our expectation, both healthy elderly and individuals with AD included in this study have a functional nitrate-nitrite-NO pathway and can increase vascular responses after an acute dose of inorganic nitrate. The fact that the absolute Δpeak values remain lower in OLD compared to YN and in AD compared to OLD and YN suggests that other physiological mechanisms are potentially responsible of vascular dysfunction in aging and neurodegenerative disease [7,49]. Still, these results prove that the same dose of nitrate supplementation provides comparable vascular responses in young, healthy elderly, and individuals with AD. However, our results do not align with Walker et al. who did not find any increase in hyperemic response to PLM after nitrate intake [50]. This might be due to differences in the methodology. In our study, a single passive leg movement (sPLM) was conducted, whereas Walker et al. used a 5-min bout of passive leg movement. Thus, the hyperemic response may not be driven by NO-bioavailability alone as for sPLM, but it can involve additional mechanisms, including metabolic factors and changes in heart rate/blood pressure.

4.2. Physiological determinants of vascular responsiveness in individuals with AD

Despite the evidence that the individuals included in this study were able to reduce NO_3^- to NO_2^- and to increase NO-mediated vascular responsiveness, it still remains to be determined why the absolute vascular response to inorganic nitrate supplementation remains poorer in this population.This may be a consequence of the physiological age-related vascular changes, known to be exacerbated in individuals with AD [6,51]. Indeed, regular aging is accompanied by functional and structural changes of the endothelium and surrounding vessel layers that make up the vascular wall [51]. These changes are not sudden but rather built up over time, leading to increased arterial stiffness and endothelial dysfunction caused by several age-dependent factors [52]. Indeed, DNA damage and telomere attrition, epigenetic modifications, defects in protein processing, reduced nutrient sensing, mitochondrial dysfunction, and reduced stem cell availability all occur during the typical aging process [52]. As a consequence of these age-related dysfunctional

events, there is an increase in the inflammatory response and thickening of the vessel walls, with consequent progressive loss in the ability of the vessels to dilate properly in response to stimuli [52,53]. Furthermore, other predominant mechanisms mediating vascular dysfunction with advancing age are oxidative stress and chronic low-grade inflammation, both contributing to increasing vascular smooth muscle tone and stimulating changes to the extracellular matrix of the vessel walls, including the degradation of elastin fibers, the compensatory deposition of collagen conferring additional stiffening and further reducing NO-bioavailability [46]. All these processes are recognized to increase the risk of AD and be markedly present in this population [4–8,54]. Therefore, the acute administration of inorganic nitrate might be insufficient to overcome the dysfunctional structural and functional vascular properties and completely restore vascular responsiveness in healthy elderly and individuals with AD.

4.3. Clinical implications

Results of this study confirm that beetroot juice may elicit positive physiological responses concerning NO-bioavailability, which translate in an improved vascular responsiveness, also in individuals with AD. These might be precursor for further health-benefits which may increase the quality of life of these individuals, that may be potentiated by a longer time supplementation. However, too little is known about the effects of chronic supplementation and the potential toxicity of this strategy. For this reason, further studies focused on longer administration, as well as testing different nitrate concertation need to be run.

4.4. Limits of the study

Despite this is the first study investigating plasma NO_3^- and NO_2^- kinetics and consequent NO-dependent vascular responsiveness following ingestion of nitrate-rich beetroot juice in individuals with AD, there are several aspects that may limit the interpretation of the results. First, a small sample size and the fact that most of the individuals in AD group was under pharmacological therapy. There are not clear evidence about the effect of cholinesterase inhibitors and other medications on NO_3^-- $NO_2^- - NO$ pathway and NO-mediated vascular responsiveness. However, most of the AD-patients are commonly under this kind of pharmacological therapy and including only individuals without medication would not have been accurate. Second, vascular assessments are limited to sPLM and no other investigations were conducted. Nevertheless, this test was chosen because of its strong relation with NO bioavailability, differently from FMD which reflect NO-bioavailability only for a small part and it depends on many other physiological mechanisms. Third, this study is focused on the acute response, and the effect of a longer treatment is not investigated. However, based on the results reached by this experimental design, further studies focused on longer administration will be developed. Lastly, our study did not include evaluations or assessments that could help describe the mechanisms underlying our findings. Further studies focused on this aspect are needed to better understand the potential of this strategy.

5. Conclusions

The results of this study reveal that the nitrate and nitrite plasma concentration is not altered by aging or AD, since both healthy elderly and individuals with AD exhibited the same nitrate and nitrite kinetics compared to young individuals. Interestingly, although vascular responsiveness remained poorer in individuals with AD compared to the control groups, the amplitude and timing of the rise in vascular responsiveness was similar in young, healthy elderly, as well as individuals with AD. Indeed, the same dose of inorganic nitrate elicited the same vascular responsiveness in all three groups, despite AD showed significantly lower absolute values compared to the other groups. This may be a consequence of chronic age- and disease-induced vascular deterioration that cannot be reversed by a single dose of beetroot juice. Further studies on chronic exposure to inorganic nitrate are needed to investigate whether this non-pharmacological strategy can consistently ameliorate vascular function in this population, lower the cardiovascular risk factor and induce a positive effect on the pathology burden.

CRediT authorship contribution statement

Anna Pedrinolla: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Gianluigi Dorelli:** Writing – review & editing, Validation, Formal analysis, Data curation. **Simone Porcelli:** Writing – review & editing, Resources, Methodology. **Mia Burleigh:** Writing – review & editing, Methodology, Investigation, Data curation. **Martina Mendo:** Writing – review & editing, Methodology, Data curation. **Camilla Martignon:** Writing – review & editing, Formal analysis, Data curation. **Cristina Fonte:** Writing – review & editing, Formal analysis, Data curation. **Luca Giuseppe Dalle Carbonare:** Writing – review & editing, Supervision, Resources. **Chris Easton:** Writing – review & editing, Supervision, Resources, Formal analysis. **Ettore Muti:** Writing – review & editing, Resources, Formal analysis. **Federico Schena:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Massimo Venturelli:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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Data availability

Data will be made available on request.

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